Presynaptic dysfunction in neurodevelopmental disorders: Insights from the synaptic vesicle life cycle

Presynaptic dysfunction in neurodevelopmental disorders: Insights from the synaptic vesicle life cycle

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Abbreviations –ADBE - activity-dependent bulk endocytosis; AED - antiepileptic drug; AP-1 -adaptor protein complex 1; AP-2 -adaptor protein complex 2; AP180 - adaptor protein 180; ASD - Autism spectrum disorder; BDNF - brain-derived neurotrophic factor; BK channel - large conductance calcium-gated potassium channel; CYFIP - cytoplasmic FMRP-interacting protein; DYRK1A - dual-specificity tyrosine phosphorylation-regulated kinase 1A; EPSC – excitatory postsynaptic current; FMRP - fragile X mental retardation protein; FXS – fragile-X syndrome; GAP - GTPase activating protein; ID - intellectual disability; IPSC – inhibitory postsynaptic current; iTRAP - intrinsic trafficking partner; mEPSC – miniature excitatory postsynaptic current; mIPSC – miniature inhibitory postsynaptic current; NDD - neurodevelopmental disorders; NSF - NEM-sensitive factor; PRRT2 - proline-rich transmembrane protein 2; RIM1 - Rab3-interacting molecule 1; RRP - readily releasable pool; SCAMP5 - secretory carrier associated membrane protein 5; α-SNAP - α-soluble NSF-

attachment protein; SNARE - soluble NSF attachment protein receptor; STP – short-term plasticity; SV - synaptic vesicle; SV2A – synaptic vesicle protein 2A; Syt1 – synaptotagmin-1; TBC - Tre-2/Bub2/Cdc16; VAMP2 – vesicle-associated membrane protein 2; v-ATPase - vacuolar-type proton ATPase; VDCC – voltage-dependent calcium channel; WRC - Wave Regulatory Complex.

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Abstract

The activity-dependent fusion, retrieval and recycling of synaptic vesicles is essential for the maintenance of neurotransmission. Until relatively recently it was believed that most mutations in genes that were essential for this process would be incompatible with life, due to this fundamental role. However, an ever-expanding number of mutations in this very cohort of genes are being identified in individuals with neurodevelopmental disorders, including autism, intellectual disability and epilepsy. This article will summarise the current state of knowledge linking mutations in presynaptic genes to neurodevelopmental disorders by sequentially covering the various stages of the synaptic vesicle life cycle. It will also discuss how perturbations of specific stages within this recycling process could translate into human disease. Finally, it will also provide perspectives on the potential for future therapy that are targeted to presynaptic function.

Introduction

The evoked release of neurotransmitter in response to action potential invasion at the presynapse is an essential component of brain function. Neurotransmitter release is controlled by the recycling of synaptic vesicles (SVs), a process that comprises a series of intricate molecular events that are coupled to neuronal activity both temporally and spatially. Because of its critical importance in maintaining the fidelity of neurotransmission, the assumption was that individuals harbouring mutations within key SV recycling genes would not be identified. However strong evidence has emerged that, rather than being incompatible with life, mutations in the most essential of SV recycling genes precipitate a series of neurodevelopmental disorders (NDDs).

NDDs are a series of heterogeneous disorders that can be grouped by the presentation of abnormal brain development (Moretto *et al.* 2017, Krol & Feng 2017). This dysfunction manifests early in childhood and can lead to a spectrum of deficits, from specific limitations to global impairments across different functions, including social and adaptive behaviour (American Psychiatric & American Psychiatric Association 2013). Various complex genetic factors and epigenetic modifications are associated with NDDs, some of which are yet to be determined or fully understood (van Loo & Martens 2007, Sontheimer 2015). Although these disorders are common (approximately 1 in 30 live births, (Sontheimer 2015)), very little is known regarding their pathophysiology and therefore therapeutic options. NDDs can be subcategorised further based on clinical presentation into various categories, including intellectual disability (ID), autism spectrum disorder (ASD) and/or epilepsy among others, including attention deficit disorder and ataxia (American Psychiatric & American Psychiatric Association 2013, Mefford *et al.* 2012, American Psychiatric & American Psychiatric Association. Task Force on 1994, Bozzi *et al.* 2012). However, a great deal of comorbidity exists between the various disorders. For example, ID is present in 50-70 % of individuals with ASD (Mefford et al. 2012) and epilepsy is present in 4-38 % of individuals with ASD (Thomas *et al.* 2017).

Neurotransmitter release is stimulated by the activity-dependent influx of extracellular calcium into the presynapse via voltage-dependent calcium channels (VDCCs). This triggers the fusion of neurotransmitter-containing SVs (Sudhof 2012, Sudhof 2013, Jahn & Fasshauer 2012) (Figure 1). Prior to their fusion, SVs are filled with neurotransmitter via the action of specific transporters on their membrane. This filling is driven by a proton-motive force, generated by a vacuolar-type proton ATPase (v-type ATPase), rendering the interior of the SV acidic (Chanaday *et al.* 2019). After filling, SVs physically attach to a dense network of proteins at the active zone (Gundelfinger & Fejtova 2012) and are then rendered fusogenic via a priming reaction (Rizo 2018, Brunger *et al.* 2018).

After activity-dependent fusion, SV cargo and membrane are deposited in the presynaptic plasma membrane. They are subsequently retrieved via a series of discrete endocytosis modes including ultrafast endocytosis, clathrin-mediated endocytosis and activity-dependent bulk endocytosis (ADBE) (Chanaday et al. 2019) (Figure 1). These endocytosis modes are triggered by different patterns of neuronal activity and are essential to sustain the supply of SVs for neurotransmission. Newly generated SVs populate a series of different pools within the nerve terminal (Cheung *et al.* 2010, Granseth & Lagnado 2008, Watanabe *et al.* 2014). These are either the resting pool (which is refractory to action potential stimulation) or the recycling pool (which can be accessed by neuronal activity) (Chanaday et al. 2019, Kim & Ryan 2010). The recycling pool can be further subdivided into the readily releasable pool (RRP, SVs which are docked and primed at the active zone) and the reserve pool (which are only mobilised during intense periods of activity) (Chanaday et al. 2019) (Figure 1).

In this review, we summarise the current state of knowledge with respect to the links between NDDs and specific stages of the SV life cycle described above. In addition, we will discuss how disturbances in SV recycling at various steps may result in dysfunctional circuit activity and brain function. Finally, we will examine whether possible convergence points may provide therapeutic potential for some disorders.

Disruption of SV fusion events

The principal role of the presynapse is to ensure the synchronous release of neurotransmitter in response to neuronal activity. Central to this event is SV fusion, which is driven by SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptors) proteins via the assembly of the SNARE complex. A series of classical studies identified synaptobrevin-2 (also known as VAMP2 (vesicle-associated membrane protein 2), and referred to as this herein), syntaxin-1 and SNAP-25 as the minimal machinery required to fuse a SV (Weber *et al.* 1998) (Figure 2). VAMP2 is referred to as a vesicle-SNARE since it is resident on the SV membrane, whereas syntaxin-1 and SNAP-25, which are located on the plasma membrane, are target-SNAREs. All three proteins contain a SNARE motif (a short 65 amino acid coiled-coil structure) which allows them to interact progressively from their N-termini (Sorensen *et al.* 2006, Poirier *et al.* 1998, Sutton *et al.* 1998). This has the effect of bringing the SV and plasma membrane into close apposition and eventually mediating membrane fusion. Evidence is accumulating which links each component of the SNARE complex to NDDs.

v-ATPase

For SV fusion events to be physiologically relevant, SVs must be filled with neurotransmitter. Interestingly, the amount of neurotransmitter inside SVs can determine their release probability, with incompletely filled SVs being less fusogenic (Rost *et al.* 2015, Bodzeta *et al.* 2017). As stated above, the v-ATPase pumps protons into SVs to produce an electrochemical gradient that is utilised by neurotransmitter transporters to fill SVs (Gowrisankaran & Milosevic 2020).

The v-ATPase contains a V_1 cytosolic domain and a V_0 membrane-bound domain. The V_1 domain is responsible for ATP hydrolysis, which causes a conformational change leading to the rotation of the V_0 domain and proton translocation into the SV (Vasanthakumar & Rubinstein 2020). The pH gradient across the SV membrane determines the v-ATPase's state of assembly. V_1 and V_0 domains are assembled on non-acidified SVs, however the V_1 domain detaches from this complex when the SV is fully acidified and filled with neurotransmitter (Bodzeta et al. 2017). This process occurs upstream of SV docking and may act to facilitate the fusion of completely filled SVs (Bodzeta et al. 2017). The synaptic protein DmX-like protein 2 (DMXL2, also known as rabconnectin-3a) is thought to regulate this v-ATPase assembly and thus SV acidification (Gowrisankaran & Milosevic 2020).

Mutations in the various genes encoding both domains have been associated with multiple genetic congenital diseases that can present with neurological defects (Fischer *et al.* 2012, Kornak *et al.* 2008, Kortüm *et al.* 2015, Van Damme *et al.* 2017). More recently, four *de novo* mutations in *ATP6V1A* (D27R, D100Y, D349N and D349G) have been associated with developmental encephalopathies and epilepsy (Fassio *et al.* 2018) (Figure 3). *In silico* modelling predicted each mutation to perturb v-ATPase function. For example, overexpression of the D100Y mutation in HEK293T cells resulted in a loss of function effect due to increased degradation. Conversely, the D349G mutation led to an increased acidification of intracellular organelles, suggesting a gain of function effect.

DMXL2 is also a NDD risk gene, with copy number variations and *de novo* missense mutations observed in individuals with ASD (Iossifov *et al.* 2014, Krumm *et al.* 2015, Costain *et al.* 2019). Additionally, biallelic loss of function mutations in *DMXL2* are associated with Ohtahara syndrome, which is characterised by severe epileptic encephalopathy (Esposito *et al.* 2019) (Figure 3).

SNAP-25

Mutations in the *SNAP25* gene result in patients presenting with a combination of seizures, ID, severe speech delay, and cerebellar ataxia (Hamdan *et al.* 2017, Rohena *et al.* 2013, Shen *et al.* 2014,

Fukuda *et al.* 2018) (Figure 3). All identified mutations lie within the two SNARE motifs of SNAP-25, with four being missense and one nonsense (Hamdan et al. 2017). Two further missense mutations were found in exon 5 of SNAP-25b. These latter mutations are intriguing, since exon 5 can be alternatively spliced to generate either SNAP-25a or SNAP-25b (Bark 1993). These variants have different expression patterns during development, with SNAP-25a predominantly restricted to embryonic brain, and SNAP-25b expressed in adult (Bark *et al.* 1995). Furthermore, SNAP-25b-deficient mice with protected SNAP-25a expression demonstrate neurological hyperactivity, anxiety, learning deficits, and spontaneous seizures (Johansson *et al.* 2008). This suggests that the adult form of SNAP-25 is important for the correct development of brain circuitry.

SNAP-25 knockout mice die at birth due to respiratory failure; however heterozygotes are viable (Washbourne *et al.* 2002). Nevertheless, heterozygous mice display an abnormal electroencephalogram pattern and are more susceptible to kainate-induced seizures (Corradini *et al.* 2014). This suggests that at least some mutations may result in loss of function. However, some SNAP-25 mutations may be dominant. For example, the I67N mutation in the SNARE motif greatly reduced evoked release when expressed in secretory cells and interfered with in an *in vitro* liposome fusion assay (Shen et al. 2014). Supporting a dominant effect, a similar phenotype was observed in *blind-drunk* mice, which harbour a heterozygous SNAP-25 mutation (I67T, (Jeans *et al.* 2007)). These mice exhibit ataxia and impaired gait, and display profound defects in both mEPSC frequency and evoked release. This mutant has a two-fold increased affinity for syntaxin-1, suggesting its dominant phenotype is due to the formation of more stable SNAP-25/syntaxin-1 complexes (Jeans et al. 2007).

Syntaxin-1

Syntaxin-1 has two highly conserved isoforms, syntaxin-1A and syntaxin-1B. In agreement with this high degree of conservation, either can fully rescue function when the endogenous protein is depleted in neuronal culture (Zhou *et al.* 2013). Syntaxin-1 has two key domains, a Habc domain, which controls its “closed” or “open” conformation (more information below) and its SNARE motif. Syntaxin-1A knockout mice are viable, whereas expression of the “open” form of 1B in the 1A knockout background results in seizure activity within 2 weeks and premature death within 1-2 months (Gerber *et al.* 2008). Recent studies examining the genetic basis of fever-associated epilepsies have identified a wide range of nonsense and missense mutations in the *STX1B* gene (Schubert *et al.* 2014, Wolking *et al.* 2019, Epi *et al.* 2013, Vlaskamp *et al.* 2016) (Figure 3). The missense mutations were mainly in the Habc and SNARE motif, suggesting a deleterious effect on

syntaxin-1B function. However, no molecular interrogation of these mutations at the level of the SNARE complex or neurotransmitter release has yet been performed.

VAMP2

The cytoplasmic domain of VAMP2 consists of a short N-terminus and SNARE motif (Elferink *et al.* 1989, Archer *et al.* 1990). Loss of the *Vamp2* gene in mice results in an almost complete cessation of evoked release (Schoch *et al.* 2001), highlighting its essential role in neurotransmission. Homozygous *Vamp2* knockout mice die immediately after birth, whereas heterozygote mice are viable. However, these mice display delayed postnatal development, reduced anxiety-related behaviour and decreased baseline neurotransmission (Koo *et al.* 2015).

Mutations in the *VAMP2* gene have been reported in five unrelated individuals with ID and hypotonia (Salpietro *et al.* 2019) (Figure 3). All mutations were located within the SNARE motif and were either missense or nonsense. *In vitro* liposome fusion assays revealed that a S75P variant displayed reduced rate and extent of fusion compared to wild-type (Salpietro et al. 2019). Interestingly, the effect of this mutant was much more profound during munc-18-triggered fusion, which is closer to the biological context (see below) (Salpietro et al. 2019). This phenotype was retained when S75P was mixed in equal amounts with wild-type VAMP2 (to mimic the heterozygous condition), suggesting it acted in a dominant manner. In contrast, another mutant (E78A) had no effect on fusion, suggesting it may interfere with another aspect of VAMP2 function.

Disruption of calcium-triggered SV fusion

Synaptotagmin-1

The rates of SV fusion driven by the SNARE proteins alone *in vitro* are orders of magnitude too slow to mediate fast synaptic transmission (Weber et al. 1998). To ensure a high degree of synchrony, a series of molecules prepare the SNAREs to accelerate the process by coupling SV fusion to neuronal activity (Figure 2). Central to this is the calcium sensor synaptotagmin-1 (Syt-1). Syt-1 is an integral SV protein that binds calcium with low affinity via two C2 domains (C2A and C2B) (Perin *et al.* 1990). Homozygous deletion of the *Syt1* gene in mice results in death within 48 hours, however heterozygotes are viable (Geppert *et al.* 1994). Primary neuronal cultures from *Syt1* knockout mice display a profound reduction in synchronous release and a large increase in mEPSC frequency, suggesting that Syt-1 acts to limit SV fusion events in the absence of calcium, before triggering neurotransmitter release on calcium binding (Shao *et al.* 1997).

Subsequent studies revealed the molecular mechanism of Syt-1-mediated SV fusion. Firstly, calcium binding by negatively charged amino acid residues on both C2 domains neutralises their charge. This allows insertion of these loops into the plasma membrane (Chapman & Davis 1998) to aid fusion by deforming this membrane (Martens *et al.* 2007). Modulation of the calcium affinity of these loops has parallel effects on neurotransmitter release, indicating that Syt-1 is the major calcium sensor for SV fusion (Fernandez-Chacon *et al.* 2001, Rhee *et al.* 2005). Syt-1 also forms a direct association with the SNARE complex and a small protein called complexin in the absence of calcium, potentially explaining why Syt-1 can restrict spontaneous fusion events (Rizo 2018, Brunger et al. 2018).

A series of heterozygous mutations in the *SYT1* gene are responsible for Baker-Gordon syndrome, a NDD that includes ID and hypotonia (Baker *et al.* 2015, Baker *et al.* 2018, Cafiero *et al.* 2015) (Figure 3). All mutations clustered within the Syt-1 C2B domain, with calcium- and lipid-binding residues disproportionately represented. When these mutants were overexpressed in wild-type neuronal cultures (to reflect the heterozygous condition), all displayed reduced SV fusion kinetics, as expected for mutations in these residues (Baker et al. 2015, Baker et al. 2018). Interestingly, these defects could be ameliorated by elevating extracellular calcium (Baker et al. 2018), suggesting these dominant mutations were inefficient in coupling calcium influx to neurotransmitter release.

PRRT2

Mutations in the gene *PRRT2*, which encodes the protein proline-rich transmembrane protein 2 (PRRT2) is responsible for a number of NDDs (Valtorta *et al.* 2016, Chen *et al.* 2011) (Figure 3). PRRT2 is enriched at the presynapse (Valente *et al.* 2016, Lee *et al.* 2012, Liu *et al.* 2016), with its C-terminus forming an anchor that spans the plasma membrane (Rossi *et al.* 2016). Depletion of endogenous PRRT2 via shRNA or via constitutive knockout in mice results in a reduction in both synchronous neurotransmitter release and mEPSC frequency in autaptic neuronal cultures. This phenotype was suggested to be due to decreased release probability and calcium sensitivity (Valente et al. 2016, Valente *et al.* 2019). This does not appear to be a generalised defect however, since this phenotype was not observed in mice when *Prrt2* was conditionally deleted in cerebellar granule neurons (Tan *et al.* 2018). However, both mouse models displayed an increase in cerebellar short-term plasticity (STP), and a recapitulation of many of the symptoms seen in humans with these disorders, including paroxysmal dyskinesia and higher seizure propensity (Tan et al. 2018, Michetti *et al.* 2017, Valente et al. 2019).

PRRT2 mutations are distributed throughout the gene, with many giving rise to premature stop codons (Valtorta et al. 2016). This suggests that dysfunction is due to PRRT2 haploinsufficiency, a hypothesis supported by the face validity of the mouse models. How could reduced PRRT2 function result in this varied phenotype? An increase in SNARE complex formation and docked SVs was observed in the conditional *Prrt2* knockout mouse (Tan et al. 2018) with the latter phenotype also observed in both knockdown neurons (Valente et al. 2016) and the constitutive *Prrt2* knockout mouse (Valente et al. 2019). This increase in docked SVs most likely reflects impaired release probability (Valente et al. 2016) and suggests that PRRT2 acts at the level of the SNARE complex, possibly inhibiting its formation. Affinity purification assays revealed that PRRT2 interacts with Syt-1, with interactions also observed for SNAP-25 and VAMP2 (Valente et al. 2016). These interactions are reportedly weak, however the resulting stearic hindrance of PRRT2 interacting will all SNARE components may be sufficient to impede efficient SNARE complex assembly. Indeed, liposome fusion assays revealed PRRT2 was a key factor in limiting the density of primed SVs at the active zone by regulating trans-SNARE complex formation (Coleman *et al.* 2018). This agrees with the observed phenotypes of increased SV docking (Valente et al. 2019, Valente et al. 2016, Tan et al. 2018) and accelerated replenishment of the RRP resulting in synaptic facilitation (Tan et al. 2018, Michetti et al. 2017, Valente et al. 2019). In addition, increased intrinsic excitability contributes to network hyperactivity and instability in PRRT2 knockout brain slices (Fruscione *et al.* 2018, Valente et al. 2019).

Voltage-dependent calcium channels

An essential step in neurotransmitter release is the activity-dependent influx of calcium via voltage-dependent calcium channels (VDCCs). The Cav2 group of VDCCs comprise the Cav2.1, Cav2.2 and Cav2.3 channels that contain the pore-forming α1 subunit, auxiliary β subunits and α2δ subunits (Mochida 2019). The β subunit modifies channel kinetics and activation, whereas α2δ subunits promote trafficking of the channel complex and control release probability (Hoppa *et al.* 2012). Cav2.1 and Cav2.2 are highly expressed at the presynaptic active zone (Gundelfinger & Fejtova 2012), and perform a key role coupling neuronal activity to SV fusion (Takahashi & Momiyama 1993, Wheeler *et al.* 1994).

Loss of function mutations in *CACNA1A* and *CACNA1B* (the genes encoding Cav2.1 and Cav2.2 respectively) result in developmental and epileptic encephalopathy (Gorman *et al.* 2019, Jiang *et al.* 2019) (Figure 3). *De novo* missense mutations in *CACNA1A* result in decreased channel expression and reduced calcium current densities (Jiang et al. 2019). Conversely, gain of function *de novo*

missense mutations in *CACNA1A* result in increased channel opening (Jiang et al. 2019). Therefore, it is predicted that these mutations would affect the efficiency of neurotransmitter release, however these experiments have still to be performed. For a comprehensive review on calcium channel function and dysfunction in neurodevelopmental disorders please refer to Tagliatti et al (2020) in this issue.

Disruption of SV priming

Other proteins perform key roles in SNARE-dependent SV fusion in addition to Syt-1 and PRRT2. Two essential proteins are munc-18 and munc-13 (Figure 2). Knockout of either of the genes encoding these proteins in mice results in the complete cessation of SV fusion (Augustin *et al.* 1999, Verhage *et al.* 2000, Varoqueaux *et al.* 2002), highlighting their central role.

Munc-18 and munc-13 work together to coordinate and synchronise SNARE complex assembly. The start point is the closed munc-18/syntaxin-1 complex, where munc-18 binds to the closed conformation of syntaxin-1, stabilising it and restricting its entry into the SNARE complex (Rizo & Sudhof 2012, Jahn 2000). Munc-18 also binds to VAMP2. This interaction is relatively weak, however it is important, since its disruption impairs liposome fusion *in vitro* (Sitarska *et al.* 2017, Parisotto *et al.* 2014). Additionally, munc-18 binds to the SNARE complex after its assembly via the syntaxin-1 N-terminus, its Habc domain and the four-helix SNARE bundle (Deak *et al.* 2009, Rickman *et al.* 2007, Shen *et al.* 2007). Simultaneous to the action of munc-18, munc-13 opens syntaxin-1, while providing a stabilising link between the SV and plasma membrane (Lai *et al.* 2017). Critically, this arrangement allows the SNARE complex to become resistant to the action of NSF/α-SNAP (NEM-sensitive factor / α-soluble NSF-attachment protein, which can stochastically disassemble SNARE complexes), increasing the accuracy and synchronicity of assembly (Brunger et al. 2018, Rizo 2018). This latter step equates to the control of SV priming and thus the size and replenishment of the RRP (Yang *et al.* 2015, Rosenmund *et al.* 2002, Junge *et al.* 2004). A further key molecule is the active zone scaffolding protein RIM1 (Rab3-interacting molecule 1), which interacts with munc-13, VDCCs, RIM-binding protein and Rab3, among others, to facilitate SV exocytosis (Torres and Inestrosa, 2018).

Munc-13

Munc-13 is a large multi-domain protein, with three C2 domains, one C1 domain, a calmodulin-binding domain and a MUN domain (Brose *et al.* 1995). The MUN domain in particular is essential for munc-13 function, since it works in concert with munc-18 to open syntaxin-1 (Ma *et al.* 2011). A link between munc-13 dysfunction and NDDs came from a patient displaying microcephaly, cortical

hyperexcitability and myasthenia that was homozygous for a truncating mutation in the *UNC13A* gene (Figure 3). *In vitro* analysis of neuromuscular transmission revealed that excitatory postsynaptic potentials were almost absent, explaining why the patient died of respiratory failure (Engel *et al.* 2016). In agreement with an essential role, *Unc13a* knockout mice die at birth, displaying a dramatic decrease in spontaneous and evoked glutamate release (Augustin et al. 1999). A later study identified a patient harbouring a *de novo* missense mutation in munc13-1 located outside of any established functional domain (Lipstein *et al.* 2017). Expression of this mutant in knockout cultures revealed a gain of function phenotype, with increased probability of SV fusion and altered STP during low, but not high, frequency neurotransmission (Lipstein et al. 2017). The mechanism of this increase is not currently understood, but may reflect either enhanced calcium binding by the adjacent C2B domain (Shin *et al.* 2010) or direct regulation of calcium influx via interactions with VDCCs (Calloway *et al.* 2015).

Munc-18

Mutations in *STXBP1* (the gene that encodes munc-18) were first identified in patients with Ohtahara Syndrome (Saitsu *et al.* 2008) and afterwards in individuals with West Syndrome, atypical Rett's syndrome and Dravet's syndrome (Stamberger *et al.* 2016) (Figure 3). In fact, *de novo* mutations in *STXBP1* are among the most frequent causes of epilepsies and encephalopathies with most patients also having severe to profound intellectual disability and movement disorders (Stamberger et al. 2016). More than 85 pathological *STXBP1* variants are reported, with little genotypic-phenotypic correlation (Lanoue *et al.* 2019). This suggested that pathology primarily arises from a loss of function and *STXBP1* haploinsufficiency (Yamamoto *et al.* 2016). In support, many missense mutations are located in the internal hydrophobic core of the protein, decreasing its stability leading to degradation (Suri *et al.* 2017, Kovačević *et al.* 2018, Martin *et al.* 2014, Saitsu et al. 2008).

Human embryonic stem cells engineered to contain heterozygous *STXBP1* mutants display a marked reduction in both spontaneous and evoked neurotransmitter release (Patzke *et al.* 2015). Furthermore, expression of human mutants in *Stxbp1* heterozygous neurons results in greatly reduced total munc-18 levels, exceeding that expected from loss of mutant protein alone (Chai *et al.* 2016, Patzke et al. 2015, Kovačević et al. 2018, Guiberson *et al.* 2018). This is proposed to reflect a gain of function pathology that is associated with an aggregation of *STXBP1* mutants with remaining wild-type protein (Lanoue et al. 2019). When common pathological *STXBP1* variants were expressed in either *Stxbp1* knockout mouse neurons or were added to an *in vitro* fusion assay, impaired

neurotransmission or defective SNARE-dependent membrane fusion were respectively observed (Shen *et al.* 2015, Kovačević et al. 2018, Guiberson et al. 2018). Interestingly, when these mutants were expressed in the heterozygous context these parameters are normal (Kovačević et al. 2018). The recent identification of homozygous *STXBP1* gain of function mutations have added to the complexity (Lammertse *et al.* 2020). Individuals with these mutations have almost identical clinical features to those with *STXBP1* mutations that result in haploinsufficiency, even though they have opposite effects (increased release probability) at a cellular level.

In summary, it appears that *STXBP1* haploinsufficiency provides the best explanation of symptoms presented by patients, however more direct dominant effects of specific missense *STXBP1* mutants that display either altered binding to, or functional properties with, SNARE proteins, may also play a role (Saitsu et al. 2008, Yamashita *et al.* 2016, Shen et al. 2015). In agreement, a series of different heterozygous *Stxbp1* mouse models display impaired cognitive ability, anxiety-like behaviour and seizure phenotypes that can be controlled by widely used antiepileptic drugs (AEDs) (Kovačević et al. 2018).

RIM1

In humans, *de novo* frameshift insertions and deletions in *RIMS1* have been associated with autism spectrum disorder (Dong *et al.* 2014, Iossifov *et al.* 2012) (Figure 3). *RIMS1* codes for the active zone scaffolding protein RIM1, which is required for SV docking and priming (Figure 2). RIM1 binds the C2A domain of munc-13 via a zinc finger domain, and this munc-13-RIM1 heterodimer acts as a switch to facilitate SV fusion, which is restricted when munc-13 is in its homodimerized state (Deng *et al.* 2011a, Camacho *et al.* 2017). This munc-13-RIM1 interaction also optimises the function of munc-13 in SV priming (Camacho et al. 2017). RIM1 also plays an important role in anchoring VDCCs to release sites to facilitate fast synchronous neurotransmitter release (Torres & Inestrosa 2018). In doing so, it ensures the proximity of VDCCs and SVs by binding VDCCs either directly through its PDZ domain or indirectly via RIM binding-proteins and binding SVs via Rab3 (Hibino *et al.* 2002, Kaeser *et al.* 2011). The conditional deletion of all RIM isoforms greatly reduced neurotransmitter release via a combination of disrupted priming and delocalisation of VDCCs (Kaeser et al. 2011). Interestingly, these two functions could be rescued independently by the expression of either the RIM1 N-terminus or its PDZ domain respectively (Kaeser et al. 2011). Therefore, it is likely that the frameshift mutations identified in *RIMS1* will result in reduced neurotransmission, most likely via reduced SV priming, delocalisation of VDCCs or both.

Disruption of SV cargo selection and clustering

The accurate and efficient retrieval of SV cargo from the plasma membrane is essential for the maintenance of neurotransmission. One of the initial stages of this process is the directed clustering of SV cargo for retrieval by endocytosis (Figure 2). Key roles have been defined for the plasma membrane adaptor protein complex AP-2 (Jung & Haucke 2007) and also monomeric adaptor proteins, such as adaptor protein 180 (AP180) and stonin-2 which facilitate the retrieval of VAMP2 and Syt-1 respectively (Kononenko *et al.* 2013, Koo et al. 2015). No mutations in the genes encoding AP180 or stonin-2 have been linked to NDDs, however the AP180 homolog *PICALM* is a risk gene for Alzheimer's disease (Harold *et al.* 2009). An additional mechanism to select and cluster cargo is for SV proteins to interact with each other. SV cargo that perform this task are called intrinsic trafficking partners (iTRAPs) (Gordon & Cousin 2016). Two iTRAPs have been identified thus far, synaptophysin and synaptic vesicle protein 2A (SV2A), and dysfunction of both have been linked to NDDs.

AP-2

A heterozygous missense mutation in the μ2 subunit of AP-2 has been reported in four individuals displaying developmental delay and epilepsy (Helbig *et al.* 2019) (Figure 3). This mutation is located in a region predicted to allow AP-2 to interact with SV cargo. In agreement, expression of this variant in μ2 knockout astrocytes reduced transferrin uptake (Helbig et al. 2019). Heterozygous μ2 knockout mice have no obvious phenotype, whereas homozygous knockouts display early embryonic lethality (Mitsunari *et al.* 2005), suggesting this mutation is a gain of function.

Synaptophysin

Synaptophysin is a four transmembrane domain SV protein and the second most abundant cargo on SVs (Takamori *et al.* 2006, Wilhelm *et al.* 2014). It interacts with VAMP2, and this interaction is mutually exclusive to SNARE complex formation (Edelmann *et al.* 1995, Washbourne *et al.* 1995, Calakos & Scheller 1994). Synaptophysin performs an essential role in both clearing VAMP2 from release sites and facilitating VAMP2 retrieval by the endocytosis machinery (Gordon *et al.* 2011, Rajappa *et al.* 2016). Synaptophysin is encoded by the X-linked *SYP* gene, and a series of nonsense and missense mutations have been identified in individuals with X-linked ID and epilepsy (Tarpey *et al.* 2009, Harper *et al.* 2017) (Figure 3). In all cases, these mutants display a loss of function in their ability to accurately retrieve VAMP2 during endocytosis (Harper et al. 2017, Gordon & Cousin 2013). Synaptophysin knockout mice are viable, but display defects in cognitive function (Schmitt *et al.* 2009) in agreement with the individuals identified with mutations in the *SYP* gene.

SV2A

SV2 is encoded by three independent genes *SV2A, SV2B* and *SV2C.* SV2A interacts with Syt-1 in a phosphorylation-dependent manner (Pyle *et al.* 2000, Zhang *et al.* 2015), and coordinates Syt-1 trafficking during neuronal activity (Zhang et al. 2015, Yao *et al.* 2010, Kaempf *et al.* 2015). Loss of SV2A function precipitates seizure activity, since *Sv2a* knockout animals display severe seizures and die after 3 weeks (Crowder *et al.* 1999, Janz *et al.* 1999). These knockout animals also display greatly reduced levels of Syt-1, in agreement with the iTRAP role of SV2A (Yao et al. 2010). Furthermore, rats harbouring a spontaneous missense mutation in SV2A display increased seizure susceptibility and reduced Syt-1 expression (Tokudome *et al.* 2016a, Tokudome *et al.* 2016b). Finally, homozygous and heterozygous missense mutations in SV2A have been identified in individuals with intractable epilepsy (Serajee & Huq 2015, Wang *et al.* 2019) (Figure 3). These mutations reside in large cytoplasmic or lumenal loops, with potential adenine binding or trafficking functions respectively (Ciruelas *et al.* 2019). Interestingly, the R383Q mutant fails to rescue both Syt-1 expression and trafficking in SV2A-depleted neurons (Harper *et al.* 2020), suggesting defective iTRAP function is central to this disorder.

SCAMP5

One other potential molecule required for cargo clustering and active zone clearance is secretory carrier associated membrane protein 5 (SCAMP5). Mutations in the *SCAMP5* gene were recently identified in two unrelated individuals presenting with similar clinical phenotypes that included, ASD, ID and seizures (Hubert *et al.* 2020) (Figure 3). SCAMPs are integral SV membrane proteins with 4 transmembrane domains, similar to synaptophysin (Fernández-Chacón & Südhof 2000). Depletion of SCAMP5 in primary neuronal culture resulted in inefficient SV endocytosis, an effect that was exacerbated after intense stimulation (Zhao *et al.* 2014). This frequency-dependence appears to result from defective clearance of SV cargo from the active zone, resulting in short-term depression of neurotransmitter release (Park *et al.* 2018). Recently, whole exome sequencing identified a homozygous *SCAMP5* mutation (R91W) in a family with paediatric epilepsy and juvenile Parkinson's disease. R91W knock-in mice displayed early-onset epilepsy, and a 100 % seizure susceptibility to audiogenic stimuli. Electrophysiological recordings in cultured neurons from these mice displayed a significant increase in mEPSC frequency and evoked EPSC amplitude (Zhang *et al.* 2020). Biochemical studies revealed that the R91W SCAMP5 mutant is less stable and has a weaker interaction with Syt-1 than the wild-type protein. Interestingly, the R91W mutation falls within a conserved domain of SCAMP5 proposed to inhibit SV exocytosis (Guo *et al.* 2002), hinting to a second mechanism by which SCAMP5 dysfunction may contribute towards NDDs.

Disruption of SV endocytosis

The rapid and efficient reformation of SVs after cargo clustering is key to sustaining presynaptic performance. Various modes of endocytosis are proposed to reform SVs, including ultrafast, clathrin-mediated and ADBE (Chanaday et al. 2019). A series of mutations in key endocytosis genes have suggested that dysfunctional SV retrieval may be another stage of the SV life cycle where NDDs originate (Figure 2).

Dynamin-1

Dynamin-1 is essential for all forms of SV endocytosis (Clayton *et al.* 2009, Kononenko *et al.* 2014, Watanabe *et al.* 2013, van der Bliek *et al.* 1993). It is a large GTPase that is recruited to the neck of budding SVs to mediate membrane fission (Sweitzer & Hinshaw 1998, Takei *et al.* 1995). Critical to this essential role is the GTP-independent assembly of dynamin-1 into helical "collars" around the vesicle neck (Shnyrova *et al.* 2013). This assembly is mediated by the stalk domain (Ramachandran *et al.* 2007), which promotes the formation of dynamin-1 helices (Morlot & Roux 2013). Dynamin-1 then exploits mechanical force generated via GTP hydrolysis to induce constriction of the neck of the nascent SV resulting in fission (Dar *et al.* 2015, Roux *et al.* 2006).

Dnm1 knockout mice are viable and do not display obvious defects at birth; however, offspring die within 2 weeks (Ferguson *et al.* 2007). In contrast, heterozygous *Dnm1* knockout mice are viable and fertile with no obvious defects. *Dnm1* knockout neurons display greatly reduced endocytic capacity (Ferguson et al. 2007), with the remaining endocytosis provided via functional redundancy with the closely related dynamin-3. Importantly *Dnm1,3* double knockout mice die within a few hours of birth and cultured neurons display a profound SV endocytosis impairment when compared to *Dnm1* knockout neurons (Raimondi *et al.* 2011).

Large-scale exome sequencing studies revealed *de novo* mutations in the *DNM1* gene to be an important risk factor for NDDs (Euro *et al.* 2014, von Spiczak *et al.* 2017). These mutations result in epileptic encephalopathies, including infantile spasms, frequently progressing to Lennox-Gastaut syndrome (Epi et al. 2013) (Figure 3). All identified *de novo* missense mutations in the *DNM1* gene cluster either within the GTPase or middle domains (Euro et al. 2014, von Spiczak et al. 2017, Epi et al. 2013, Deciphering Developmental Disorders 2015, Deciphering Developmental Disorders 2017, Lazzara *et al.* 2018, Kolnikova *et al.* 2018). There is one exception that was found in identical twins who have a mutation in the lipid-binding pleckstrin homology domain (Brereton *et al.* 2018).

Interestingly, these twins displayed delayed development, ID and ASD, but not epileptic encephalopathy.

Structural modelling suggests that the *DNM1* mutations have dominant negative effects that impair either GTP binding, GTPase activity or self-assembly, leading to impaired SV endocytosis (Euro et al. 2014, von Spiczak et al. 2017). In agreement, expression of three independent missense mutations in the GTPase or middle domain of dynamin-1 all disrupted clathrin-mediated endocytosis in non-neuronal cells (Dhindsa *et al.* 2015). In addition, the middle domain mutant displayed defective self-assembly. The correct self-assembly function of this domain is critical for both GTPase activity and membrane fission (Hinshaw & Schmid 1995, Warnock *et al.* 1996). Interestingly, a spontaneous mutation in the mouse *Dnm1* gene also results in seizure activity. Mice with heterozygous expression of this mutant allele (*Ftfl*, A408T) displayed recurrent generalised tonic-clonic seizures, whereas those that were homozygous had lethal seizures by 3 weeks (Boumil *et al.* 2010). Mechanistically, this mutation results in defective self-assembly and impaired endocytosis when overexpressed in fibroblast cells (Boumil et al. 2010).

Clathrin

The coat protein clathrin is essential for all forms of SV reformation during endocytosis, whether it is at the plasma membrane or endosome (Kononenko et al. 2014, Watanabe et al. 2014, Granseth *et al.* 2006). The basic clathrin unit is a triskelion made up of three light and heavy chains which provide structure for SV formation and help to drive curvature (Robinson 2015). Constitutive deletion of either the light or heavy chain is lethal in both invertebrates and vertebrates (Royle 2006). However, acute inactivation of either gene in *Drosophila* results in depletion of SVs and an accumulation of large endosome-like structures in nerve terminals, suggesting an arrest of SV generation at both the plasma membrane and endosomes (Heerssen *et al.* 2008, Kasprowicz *et al.* 2008). A number of missense, frameshift and nonsense mutations in the *CLTC* gene are present in individuals with ID and epilepsy (DeMari *et al.* 2016, Hamdan et al. 2017, Lelieveld *et al.* 2016) (Figure 3). However, the impact these mutations on clathrin function has still to be determined.

Disruption of ADBE

ADBE is only triggered during intense neuronal activity (Clayton *et al.* 2008), and is a two-stage process. Firstly, large endosomes are formed direct from the plasma membrane, followed by the generation of SVs in an adaptor- and clathrin-dependent process (Cheung & Cousin 2012,

Kononenko et al. 2014) (Figure 2). The molecular mechanism of ADBE is still being determined, meaning few essential genes are known (Kokotos & Cousin 2015).

AP-1

The adaptor complex AP-1 is essential for SV reformation at bulk endosomes (Cheung & Cousin 2012) and mutations in the gene encoding the σ1B subunit (*AP1S2*) are responsible for a specific type of X-linked ID, called Pettigrew Syndrome (Pettigrew *et al.* 1991) (Figure 3). These mutations were either splice site, or nonsense mutations, and were all predicted to be highly pathogenic (Tarpey *et al.* 2006, Cacciagli *et al.* 2014, Huo *et al.* 2019). Deletion of the *Ap1s2* gene in mice results in an activity-dependent accumulation of bulk endosomes, with a concomitant decrease in SV number and recycling pool replenishment, in agreement with its key role in SV generation via ADBE (Glyvuk *et al.* 2010). The mice also display hypoactivity, impaired motor skills and reduced spatial memory (Glyvuk et al. 2010).

Rab11

The small GTPase Rab11 is a positive regulator of ADBE (Kokotos *et al.* 2018). Several missense mutations in the *RAB11A* gene were identified in individuals with either epilepsy or abnormal electroencephalogram activity (Hamdan et al. 2017) (Figure 3). Interestingly, deletion of the *Rab11A* gene in mice results in embryonic lethality, whereas brain-specific deletion had no overt effects on survival or gross brain structure (Sobajima *et al.* 2014).

TBC1D24

A potential link between dysfunctional presynaptic endosome trafficking and NDDs was identified via mutations in the *TBC1D24* gene (Falace *et al.* 2010, Corbett *et al.* 2010). *TBC1D24* mutations were detected in individuals with epilepsy and DOORS syndrome, which displays five main features—deafness, onychodystrophy, osteodystrophy, ID, and seizures (Campeau *et al.* 2014) (Figure 3). In agreement with TBC1D24 playing a key role in disorder pathogenesis, mice that are homozygous for either a truncating or missense mutation associated with infantile epilepsy in *Tbc1d24* display tonic-clonic or spontaneous seizures respectively, and early lethality (Tona *et al.* 2019, Lin *et al.* 2020). Furthermore, neurons from mice haploinsufficient for *Tbc1d24* display reduced mEPSC frequency, impaired SV endocytosis and an enlargement of presynaptic endosomes (Finelli *et al.* 2019).

Clues to the mechanism underlying *TBC1D24* dysfunction come from the *Drosophila* orthologue, *skywalker*. *Skywalker,* identified as a Rab GTPase activating protein (GAP), restricts SV cargo

trafficking through presynaptic endosomes by acting on Rab35 (Uytterhoeven *et al.* 2011). Rab35 directs SV cargo for degradation via the ESCRT pathway (Sheehan *et al.* 2016), therefore *skywalker* loss of function mutants display increased SV protein degradation via the endosome-lysosome pathway (Fernandes *et al.* 2014). This increased endosome-lysosome flux positively affects presynaptic function, and *skywalker* mutants display enhanced neurotransmission and a larger RRP (Uytterhoeven et al. 2011). TBC1D24 also negatively regulates another small GTPase, Arf6, which controls RRP size via modulation of endocytosis modes (Tagliatti *et al.* 2016). The TBC1D24-Arf6 interaction, which is also important for neurite growth (Falace *et al.* 2014, Aprile *et al.* 2019), has relevance for NDDs, since *TBC1D24* patient mutations disrupt Arf6 binding (Falace et al. 2010).

The Tre-2/Bub2/Cdc16 (TBC) domain usually has GAP activity, however both *skywalker* and human *TBC1D24* lack key residues required for efficient GTP hydrolysis (Pan *et al.* 2006), suggesting GAP dysfunction is not responsible for this phenotype. The crystal structure of the *skywalker* TBC domain revealed a cationic pocket that directly binds to phosphoinositides phosphorylated at the (4,5) position (Fischer *et al.* 2016). Interestingly, prevalent patient mutations disrupt phosphoinositide-binding to this pocket. Furthermore, *skywalker* deficient in $PI(4,5)P_2$ binding could not fully rescue SV endocytosis defects and results in seizure-like activity when expressed in flies (Fischer et al. 2016). Therefore, rather than canonical GAP activity, the dysfunction in the lipid binding ability of the TBC domain of TBC1D24 appears to be central to the deficits in presynaptic function observed in models of the human disease.

Disruption of SV pools

Synapsins

SV endocytosis modes replenish specific pools within the nerve terminal (Cheung et al. 2010, Granseth & Lagnado 2008, Watanabe et al. 2014). The reserve pool is maintained by the synapsins, via interactions with both SVs and the actin cytoskeleton (Benfenati *et al.* 1993, Benfenati *et al.* 1989, Bahler *et al.* 1989, Krabben *et al.* 2011) (Figure 2). SVs are mobilised from the reserve pool via the activity-dependent phosphorylation and dephosphorylation of specific synapsin residues by kinases such as calmodulin-dependent kinase II (Torri Tarelli *et al.* 1992), PKA (Hosaka *et al.* 1999) and cyclin-dependent kinase 5 (Verstegen *et al.* 2014) and phosphatases such as calcineurin (Jovanovic *et al.* 2001). Synapsins also facilitate SV clustering via interactions with α-synuclein and VAMP2 (Atias *et al.* 2019, Sun *et al.* 2019) and through phase separation via intrinsically disordered C-terminal regions (Milovanovic *et al.* 2018).

The synapsin family is composed of 3 distinct genes *SYN1*, *SYN2* and *SYN3,* which can be alternatively spliced (Giovedi *et al.* 2014). The synapsins are not essential for SV recycling, since neurotransmission is mostly unaltered in triple knockout mice (Gitler *et al.* 2004, Orenbuch *et al.* 2012). However, they perform differential roles at either glutamatergic or GABAergic synapses. Synapsins have no role in excitatory basal transmission. Synapsin-2a does however control reserve pool size in addition to the number, distribution and mobility of SVs at glutamatergic synapses (Orenbuch et al. 2012, Gitler *et al.* 2008). In contrast, inhibitory nerve terminals of *Syn* triple knockout mice display altered basal neurotransmission and quantal content (Gitler et al. 2004). This defect was primarily due to the absence of synapsin-1, since *Syn1* knockout neurons also displayed a decreased RRP at inhibitory synapses in addition to slowed SV recycling (Baldelli *et al.* 2007). Furthermore, synapsin-2 knockout neurons exhibit increased synchronous release and reduced asynchronous release at inhibitory synapses. The control of asynchronous GABA release by synapsin-2 was mediated via interactions with Cav2.1 VDCCs (Medrihan *et al.* 2013). Interestingly, synapsin-1 synchronises GABA release in parvalbumin interneurons (Forte *et al.* 2019), confirming that different synapsin isoforms can perform discrete roles at specific synapses.

Deletion of either *Syn1*, *Syn2* or all *Syn* genes in mice result in seizure activity and ASD-like behaviours (reviewed in (Cesca *et al.* 2010, Fassio *et al.* 2011b, Mirza & Zahid 2018)). In agreement, human mutations in either *SYN1* or *SYN2* lead to either ASD, epilepsy and/or ID, with no clustering around specific domains (Corradi *et al.* 2014, Nguyen *et al.* 2015, Peron *et al.* 2018) (Figure 3). *SYN1* mutations are X-linked and only affect males, suggesting they are loss of function mutations. The first mutation was identified in a family where almost all affected males displayed seizures (Garcia *et al.* 2004). This mutation resulted in nonsense mediated decay, indicating that these males were effectively *SYN1* null (Giannandrea *et al.* 2013). Other missense mutations and nonsense mutations were subsequently identified with differentially segregating ASD and epilepsy. When these mutant proteins were expressed in *Syn1* knockout neurons, some displayed defective or slowed targeting to nerve terminals and all failed to rescue SV reserve pool size (Fassio *et al.* 2011a, Tang *et al.* 2015b). Another missense mutation linked to X-linked ID displayed an enhanced ability to cluster SVs and increased mEPSC frequency (Guarnieri *et al.* 2017). This may be a gain of function mutation, since it is localised within the amphipathic lipid packing sensor motif, which senses membrane curvature and aids the association of synapsins to SVs (Krabben et al. 2011).

Neurodevelopmental risk genes and links to the SV life cycle

As outlined above, an ever-increasing number of genes with key roles in SV recycling are being linked to NDDs. In addition to these genes are genes with established causal roles (Sahin & Sur 2015). Interestingly, the study of these mutations in their natural context is beginning to reveal a number of presynaptic phenotypes. The genes with the most convincing evidence for this are described below.

FMRP

Fragile X Syndrome (FXS) is one of the most common monogenic causes of ID and ASD, accounting for 5 % of all cases (Mefford et al. 2012), with 10-20 % of cases co-morbid with epilepsy (Berry-Kravis 2002) (Figure 3). FXS is caused by the loss of the *FMR1* gene product, fragile X mental retardation protein (FMRP) (Pieretti *et al.* 1991). Most cases of FXS are caused by a CGG trinucleotide expansion in the 5' untranslated region of the *FMR1* gene, leading to repression of transcription (Bell *et al.* 1991, Verkerk *et al.* 1991). The major role of FMRP is the repression of protein translation of a subset of mRNAs downstream of Group1 metabotropic glutamate receptors, via polyribosome stalling (Darnell *et al.* 2011). Loss of FMRP results in increased translation of several presynaptic and postsynaptic proteins (Dolen & Bear 2009, Darnell & Klann 2013, Osterweil *et al.* 2010).

Evidence for a role of FMRP in SV recycling is emerging. *Fmr1* knockout neurons display a small acceleration in SV turnover and larger SV pools compared to wild-type littermate controls (Deng *et al.* 2011b). Furthermore, depletion of endogenous FMRP in dorsal root ganglion neurons enhanced SV exocytosis during high frequency stimulation (Ferron *et al.* 2014). Both defects are proposed to be due to non-canonical roles of FMRP. For example, FMRP controls action potential duration by directly binding to large conductance calcium-gated potassium channels (BK channels) and modulating their gating (Deng *et al.* 2013, Myrick *et al.* 2015). Therefore, FMRP loss results in excessive action potential broadening, culminating in increased calcium influx and synaptic transmission (Deng et al. 2013). FMRP is also proposed to control calcium influx by regulating the localization and density of Cav2.2 VDCCs via targeting them for proteosomal degradation (Ferron et al. 2014, Ferron *et al.* 2020).

CYFIP1

Copy number variations in the *CYFIP1* (cytoplasmic FMRP-interacting protein) gene have been strongly associated with ASD, ID and epilepsy in numerous studies (Doornbos *et al.* 2009, Picinelli *et al.* 2016, Pinto *et al.* 2014, Vanlerberghe *et al.* 2015, Oguro-Ando *et al.* 2014, Davenport *et al.* 2019) (Figure 3). In support, heterozygous *Cyfip1* rodents display autism-associated altered synaptic

structure and function and behavioural deficits (Bozdagi *et al.* 2012, Domínguez-Iturza *et al.* 2019, Silva *et al.* 2019, Pathania *et al.* 2014).

CYFIP1 has two highly conserved and distinct mechanisms of action. First, it represses cap-dependent translation of FMRP target mRNAs when complexed with FMRP (Napoli *et al.* 2008). Second, it is as a critical component of the Wave Regulatory Complex (WRC), which promotes actin polymerisation and branching (Chen *et al.* 2010). This second function appears to regulate presynaptic function, since an increase in the size of the recycling SV pool in either *Cyfip1*$^{+/-}$ or CYFIP1 knockdown primary neuronal cultures could be rescued by wild-type CYFIP1, but not by a mutant with ablated binding to the WRC (Hsiao *et al.* 2016). In a more recent study, ex vivo slice recordings in the somatosensory cortex revealed a lack of short-term synaptic depression in *Cyfip1*$^{+/-}$ mice compared to wild-type controls further indicating altered presynaptic function (Domínguez-Iturza et al., 2019).

BK channels

BK channels play an important role in maintaining the cell membrane resting potential by opening in response to calcium influx and membrane depolarization. This opening results in a rapid afterhyperpolarization following an action potential, which arrests calcium influx and neurotransmission by deactivating VDCCs (Griguoli *et al.* 2016). Thus, BK channels can modulate neurotransmitter release by controlling action potential shape through their opening and closing. The pore of the channel is formed by four α subunits, encoded by *KCNMA1,* and additional modulatory β and γ subunits control channel gating and pharmaceutical sensitivity (Griguoli et al. 2016, N'Gouemo 2011, Yan & Aldrich 2012). Perhaps unsurprisingly, due to its role, the BK channel and its activity has been linked to several forms of epilepsy. Interestingly, both gain of function mutations (Du *et al.* 2005, Moldenhauer *et al.* 2020) and mutations leading to haploinsufficiency (Laumonnier *et al.* 2006) result in NDDs and epilepsy (Figure 3), further highlighting the importance of this channel's bidirectional regulatory role in neurotransmitter release.

DYRK1A

DYRK1A (dual-specificity tyrosine phosphorylation-regulated kinase 1A) is a serine/threonine kinase implicated in the regulation of cellular processes involved in brain development and function. The gene encoding *DYRK1A* is located in the Down syndrome critical region of chromosome 21. *DYRK1A* is classed as a high-risk gene for ASD/ID with loss of function mutations leading to *DYRK1A*-related ID syndrome (van Bon *et al.* 1993, Wright *et al.* 2015, Deciphering Developmental Disorders 2015,

Deciphering Developmental Disorders 2017) (Figure 3). Gene dosage appears to be important, since *DYRK1A* triplication also leads to ID, microcephaly and distinct craniofacial features (Ahmed et al., 2005). Several *de novo* recurrent mutations in *DYRK1A* have been identified including nonsense, frameshift and splice site mutations (Deciphering Developmental Disorders 2017). Many missense mutations cluster in the protein kinase domain, which are predicted to disrupt catalytic activity, whereas mutations elsewhere are predicted to disrupt protein stability (Evers *et al.* 2017, Widowati *et al.* 2018). While homozygous deletion of the gene in mice is lethal, *Dyrk1a* haploinsufficient mice display small but correctly formed brains (Fotaki *et al.* 2002). These mice recapitulate other human phenotypes, including deficits in hippocampal-based learning, impaired sociability and increased susceptibility to hyperthermia-induced seizures (Fotaki et al. 2002, Arque *et al.* 2008, Raveau *et al.* 2018).

In vitro experiments identified key endocytic proteins as DYRK1A substrates, including dynamin-1, amphiphysin-1, AP180, α-adaptins, β-adaptins and synaptojanin. Phosphorylation of these substrates resulted in decreased interactions with their binding partners and was proposed to dissociate the endocytic protein complex on clathrin-coated SVs (Adayev *et al.* 2006, Chen-Hwang *et al.* 2002, Huang *et al.* 2004, Murakami *et al.* 2006, Murakami *et al.* 2009, Murakami *et al.* 2012). Interestingly, studies in *Drosophila* revealed that synaptojanin was the only *in vivo* substrate for the DYRK1A orthologue, *Mnb*. *Mnb* phosphorylation of synaptojanin on residue Ser-1029 inhibited its interaction with endophilin and increased its lipid phosphatase activity (Chen *et al.* 2014, Geng *et al.* 2016). Hypomorphic *Mnb* flies displayed an exacerbated rundown in synaptic transmission during high frequency stimulation and reduced uptake of lipophilic dyes, both indicative of reduced SV endocytosis. Overexpression of synaptojanin rescued these defects, suggesting the principal role of *Mnb* is the control of SV endocytosis via synaptojanin phosphorylation (Chen et al. 2014). Further studies using phospho-mutants revealed that Ser-1029 phosphorylation controls different modes of SV endocytosis, potentially via the reciprocal control of its interactions and phosphatase activity (Geng et al. 2016). In mammalian neurons that overexpress the human *DYRK1A* gene, SV endocytosis is significantly slowed during mild stimulation (Kim *et al.* 2010). However, the mechanism for this slowing has still to be determined.

<u>*Neurexins*</u>

Neurexins are presynaptic cell adhesion molecules that stabilise synapses via trans-synaptic interactions with postsynaptic partners. They are encoded by three genes (*NRXN1-3*), which can undergo extensive alternative splicing (Ushkaryov *et al.* 1992, Ushkaryov & Südhof 1993, Ushkaryov

et al. 1994, Missler & Südhof 1998). Initial studies identified an association between copy number variations and single nucleotide polymorphisms in *NRXN1* and ASD (Feng *et al.* 2006, Kim *et al.* 2008, Consortium *et al.* 2007, Sanders *et al.* 2011). After this, a genetic risk of all *NRXN* genes was established, albeit with *NRXN1* being most prevalent (Gauthier *et al.* 2011, Glessner *et al.* 2009, Stessman *et al.* 2017, Vaags *et al.* 2012) (Figure 3).

Deletion of all three *Nrxn* genes in mice result in lethality within the first day of life. Using neocortical slice culture and acute brainstem slices, triple knockout *Nrxn* mice displayed greatly reduced spontaneous and evoked neurotransmission due to reduced VDCC-mediated calcium influx (Missler *et al.* 2003). These functions were restored by expression of the *Nrxn1* gene product α-neurexin (Zhang *et al.* 2005). However more recent studies using conditional knockout *Nrxn* mice demonstrated that the complete loss of all neurexins produced pleiotropic effects dependent on synapse type (Chen *et al.* 2017). This suggests that neurexins perform distinct roles at specific synapses, rather than a uniform role across different synapses (Chen et al., 2017). However, at the calyx of Held synapse neurexins are essential for evoked neurotransmission, since their conditional deletion decoupled calcium influx from neurotransmitter release, due to loss of active zone VDCC clustering (Luo *et al.* 2020).

From dysregulated SV recycling to neurodevelopmental disorders

The breadth and depth of mutations in SV recycling genes identified in NDDs over the past 15 years suggest presynaptic dysfunction is one of the principal drivers for these conditions. However, a key question remains – how do defects in genes that are fundamental for accurate and efficient neurotransmitter release result in a spectrum of NDDs? Furthermore, is there convergence of dysfunction around specific aspects of the SV life cycle that would allow researchers to predict potential outcomes? It is clear from the range of SV recycling genes affected that there is not one specific event that when dysregulated culminates in NDDs. Therefore, how could dysfunctional SV recycling lead to altered neurotransmission, circuit activity and ultimately these disorders? We propose a series of different scenarios below (Figure 4).

Altered short-term plasticity

STP (depression or facilitation of neurotransmission during an action potential train), is essential for a series of higher brain functions such as working memory, network oscillations and the computation of motor, somatosensory and auditory inputs (Nadim *et al.* 1999, Nadim & Manor 2000, Abbott *et al.* 1997, Mongillo *et al.* 2008). Its transient nature provides a highly flexible and

dynamic code to prolong, modulate or create instability within specific circuits (Mongillo et al. 2008, Abbott & Regehr 2004). Perturbation of clathrin-mediated endocytosis or ADBE can modulate STP, by interfering with cargo clearance from the active zone (Hua *et al.* 2013, Park et al. 2018) diverting SV cargo via a slower endocytosis mode (Smillie *et al.* 2013, Clayton *et al.* 2010) or disrupting the replenishment of specific SV pools (Granseth & Lagnado 2008, Cheung et al. 2010, Watanabe et al. 2014, Junge et al. 2004). Since mutations in genes that control all of these processes have been identified in NDDs, this is a potentially attractive mechanism via which circuit dysregulation could occur.

Alterations in the synchrony of neurotransmitter release may also result in altered STP, since interventions that either increase or decrease this parameter affect information processing (Atluri & Regehr 1998, Manseau *et al.* 2010). In agreement, a number of key gene products required for synchronous release are mutated in NDDs, such as Syt-1 and VAMP2 (Baker et al. 2018, Salpietro et al. 2019). Furthermore, close links are emerging between ADBE and asynchronous release (Raingo *et al.* 2012, Evstratova *et al.* 2014, Li *et al.* 2017). Since asynchronous release is also a key element in information processing (Evstratova et al. 2014, Manseau et al. 2010, Rozov *et al.* 2019) any disruption in ADBE may result in altered STP. Therefore, dysfunctional STP may be a major convergence point for presynaptic dysfunction in NDDs.

Cell-specific dysfunction

Another convergence point may be disrupted "excitatory / inhibitory balance". This relatively general term has been widely used in the context of NDDs (Gao & Penzes 2015, Nelson & Valakh 2015), however, it does not directly address how, why or where any potential imbalance originates. This is particularly pertinent for presynaptic dysfunction, since previous studies have revealed no quantitative differences in the type, or expression, of SV proteins between glutamatergic and GABAergic neurons (Boyken *et al.* 2013, Gronborg *et al.* 2010). Nevertheless, some differences in the release of glutamate and GABA have been identified. For example, microdialysis studies in the amygdala and hippocampus demonstrated decreased GABA, but not glutamate release, in rats with a mutation in SV2A that results in increased seizure vulnerability (Tokudome et al. 2016b, Tokudome et al. 2016a). Furthermore, divergent effects on excitatory and inhibitory neurotransmission were observed in autaptic cultures from *Prrt2* knockout mice in response to either single action potentials or trains (Valente et al. 2019). One family of genes that do have differential effects on excitatory and inhibitory transmission are the synapsins (Fassio et al. 2011b). For example, overexpression of a truncating mutation of *SYN1* in *Syn1* knockout mouse neurons resulted in relatively similar effects on

baseline neurotransmission in glutamatergic and GABAergic neurons, but resulted in divergent effects in STP (Lignani *et al.* 2013). Furthermore, mEPSC, but not mIPSC, frequency was increased on expression of a *SYN* missense mutation linked to X-linked ID in *Syn1* knockout mouse neurons (Guarnieri et al. 2017). Finally, circuit destabilisation may result from *SYN1* or *SYN2* mutations, since synapsin-1 and synapsin-2 reciprocally control synchronous and asynchronous release in GABAergic neurons (Medrihan et al. 2013, Forte et al. 2019). Therefore, while differential effects on excitatory and inhibitory neurotransmission may explain altered circuit and brain function in specific cases, this does not appear to be widespread.

The specific synaptic partner of the presynapse may also contribute to dysfunction. For example, loss of presynaptic FMRP results in a decreased probability of neurotransmitter release with inhibitory synaptic partners, but not excitatory (Patel *et al.* 2013). Altered presynaptic function may differ across specific brain regions, potentially resulting in long-range connectivity deficits. For example, presynaptic neurexin-3 is selectively required to maintain AMPA receptor levels and postsynaptic integrity in the hippocampus, while at GABAergic synapses in the olfactory bulb it controls presynaptic release probability (Aoto *et al.* 2015). Therefore, disease-associated presynaptic proteins may form distinct functions across different cell types or brain regions, providing a potential explanation for circuit deficits observed in NDDs.

Different activity patterns

If no obvious alteration in either the expression or functional effect of genes mutated in NDDs is observed between excitatory and inhibitory neurons, how else could an imbalance be generated? One potential explanation is the firing patterns of individual neurons within a specific circuit. In this scenario, most neurons that carry a specific mutation do not display altered neurotransmitter release, with a phenotype only revealed by a particular pattern of neuronal activity. For example, if neurons carry a mutation that results in dysfunctional ADBE, only neurons that fire at high frequency would have their function impacted. Interestingly, a proteomic study that examined the composition of bulk endosomes revealed a number of proteins encoded by genes linked to ID and ASDs (such as FMRP, CYFIP2 and NUFIP2 (Kokotos et al. 2018)). This links these genes to ADBE and consequently implicates this endocytosis mode in potential circuit dysfunction.

An underlying defect revealed by specific patterns of activity is not the only potential mechanism via which activity-dependent deficits can occur. For example, since inhibitory interneurons tend to fire at higher frequencies (Bartos *et al.* 2007), dysfunctional SV retrieval may disproportionately impact

these neurons. For example, one might predict that *DNM1* mutations may result in exaggerated effects in GABAergic neurons, since the demand for SV endocytosis to sustain neurotransmission will be greater. This agrees with studies in primary cultures from *Dnm1* knockout mice, with more severe endocytosis phenotypes observed in GABAergic neurons when compared to glutamatergic (Hayashi *et al.* 2008). Furthermore, neurons that display elevated patterns of activity may be susceptible to iTRAP dysfunction, since their SV composition will be altered. As an illustration, repeated action potential trains in *Syp* knockout neurons resulted in depletion of VAMP2 from SVs and reduced neurotransmitter release (Kokotos *et al.* 2019).

Different developmental trajectory

A series of plastic changes occur early in development of the mammalian brain, resulting in the establishment of synapses and circuits (Harlow *et al.* 2010). This "critical period" is driven by specific patterns of neuronal activity; therefore dysregulated neurotransmission could result in altered neuronal circuits and brain function (Krol & Feng 2017). Mutations in presynaptic adhesion molecules may result in the establishment of different circuitry. For example, the neurexin family has greater than 1000 different variants that interact with a range of different postsynaptic adhesion molecules (Missler & Südhof 1998). This has the potential to generate a huge amount of synaptic diversity, which may underlie a molecular code for synapse and circuit development (Südhof, 2017). Disruption of this intricate coding could very conceivably result in altered brain function.

Rather than altering, "hardwired" neuronal circuitry, presynaptic dysfunction may also contribute to an altered developmental trajectory, resulting in a shift in the "critical period" (Harlow et al. 2010). The presynapse has a stereotypical developmental pattern. This includes the appearance of specific SV pools (Mozhayeva *et al.* 2002, Rose *et al.* 2013), a transition from spontaneous to evoked release (Andreae *et al.* 2012), the coupling of activity-dependent calcium influx to SV exocytosis and endocytosis (Smillie *et al.* 2005, Yamashita *et al.* 2010, Midorikawa *et al.* 2014), and the speed and mode of endocytosis used to retrieve SVs (Shetty *et al.* 2013, Rose et al. 2013). Therefore, a small shift in the expression of key presynaptic proteins may be sufficient to alter circuit activity during this period. In support, hippocampal nerve terminals in the *Fmr1* knockout mouse display an "immature" phenotype when examined by electron microscopy, with altered expression of a subset of presynaptic proteins (Klemmer *et al.* 2011). Additionally, FMRP expression peaks in brain within the first postnatal week (Till *et al.* 2015, Gholizadeh *et al.* 2015), and *Fmr1* knockout mice display differentially expressed synaptic proteins compared to wild-type during peak synaptogenesis, including increases in all three SNARE proteins (Tang *et al.* 2015a). This increase is due to enhanced

protein translation, and interestingly this divergence in synaptic protein expression is absent in adulthood.

One other possibility for the delay or acceleration of the critical period by specific mutations is an age-dependent requirement for a specific gene within this time window. For example, hippocampal slices from *Cyfip1*$^{+/-}$ mice display increased release probability at P10, however this effect is absent by P21 (Hsiao et al. 2016). Therefore, efficient SV recycling may be required during a period where synapses and circuits are being established and, if this process is disrupted, it may lead to NDDs.

Altered proteostasis

In contrast to dysfunctional SV recycling being the primary cause of NDDs, it may also occur as a secondary consequence of a more global deficit. For example, defects in local synaptic protein synthesis have been identified in monogenic models of NDDs such as FXS, Angelman syndrome and Tuberous Sclerosis (Louros & Osterweil 2016). Indeed, altered function of key postsynaptic protein synthesis regulatory pathways such as mammalian target of rapamycin and mGluR signalling are considered to be a point of pathogenic convergence for many NDDs (Costa-Mattioli & Monteggia 2013). Dysregulation of protein translation at the presynapse may also occur in NDDs, since nerve terminals were recently demonstrated to contain both ribosomes and mRNA (Hafner *et al.* 2019). This study revealed that a high level of translation occurs at the presynapse (including many SV recycling proteins), which can be regulated in a cell-specific manner by distinct signalling cascades. Furthermore, approximately one third of the presynaptic transcriptome are targets of FMRP (Darnell et al. 2011). Therefore, dysfunctional SV recycling may be a secondary consequence of disrupted proteostasis, which may either exacerbate or compensate for the initial insult.

Therapeutic intervention at the presynapse: current treatments and potential for the future

Current therapeutic strategies

The paucity of current therapeutic interventions to either control or correct NDDs is widely acknowledged. This may partly be related to both the targets chosen (Gurkan & Hagerman 2012, Wetmore & Garner 2010) and the design of clinical trials (Berry-Kravis *et al.* 2018). To date, the postsynapse has been the principal focus for these interventions, however many of the drugs tested also display presynaptic efficacy in preclinical models, suggesting their effect on neurotransmitter release may be central to their therapeutic potential.

The $GABA_B$ receptor agonist arbaclofen rescues synaptic abnormalities such as increased spine density and dysregulated protein synthesis (Henderson *et al.* 2012), in addition to a number of behavioural deficits (Silverman *et al.* 2015, Sinclair *et al.* 2017) in mouse models of FXS. $GABA_B$ receptor activation limits calcium influx via NMDA receptors at the postsynapse, by activating potassium channels to restrict depolarization (Chalifoux & Carter 2011). Presynaptic $GABA_B$ receptors, when activated, reduce calcium influx to limit neurotransmitter release via two different mechanisms. Firstly, via activation of potassium channels, which results in action potential shunting, and secondly, via direct effects on VDCCs (Chalifoux & Carter 2011). Therefore, arbaclofen has potential to control both pre- and postsynaptic function. However, when translated to clinical trials, it was unsuccessful in improving primary outcomes (Veenstra-VanderWeele *et al.* 2017, Berry-Kravis *et al.* 2017).

Direct opening of potassium channels by agonists have also shown potential as therapeutic intervention. For example, the BK channel opener BMS-204352 rescued abnormal spine morphology and seizure behaviour in *Fmr1* knockout mice (Hebert *et al.* 2014). While the therapeutic effect of this drug was assigned to its postsynaptic role, BK channels also limit neurotransmitter release via the control of action potential width (see above) (Griguoli et al. 2016). In support of a therapeutic presynaptic role, loss of FMRP limits BK channel activity, resulting in action potential broadening and increased neurotransmission (Deng et al. 2013). A different potassium channel, the Kv7 channel, controls action potential initiation and propagation, thus controlling downstream neurotransmitter release (Greene & Hoshi 2017). The Kv7 channel agonist retigabine/ezogabine has been employed as an effective antiepileptic treatment in humans, but was discontinued due to undesirable side effects (Brickel *et al.* 2019). Finally, many current AEDs inhibit either voltage-dependent sodium channels or VDCCs to prevent repetitive firing, reduce calcium influx and limit neurotransmitter release (Abou-Khalil 2019). Thus, it is likely that there is a presynaptic component to most of these successful interventions.

A number of signalling cascades are being trialled as targets for NDD therapy. For example, the endocannabinoid system is disrupted in a number of genetic and environmental models (Zamberletti *et al.* 2017). Endocannabinoids are retrograde neurotransmitters that act presynaptically by activating CB1 receptors, which inhibit VDCCs and thus neurotransmitter release (Katona & Freund 2008). Furthermore, studies in conditional β-neurexin knockout mice revealed that postsynaptic generation of endocannabinoids is tonically inhibited by this neurexin isoform (Anderson *et al.*

2015). Therefore the current interest in the use of cannabinol in NDDs (Cross & Cock 2019) may have a molecular locus at the presynapse.

Future potential

Aside from the current therapies being trialled, the revelation that dysfunction in SV recycling underlies a series of NDDs has initiated a focus on the presynapse as a target for intervention. With many of these disorders it is unclear how much can be potentially corrected and how much is already "hardwired". In other words, do NDDs result from a dysfunction in neuronal development or neuronal maintenance or both? These are key questions that are critical to address, especially if the most direct route of correction – gene therapy is to be successful. Progress in this field is accelerating at a tremendous rate and will be covered in detail by another review in this issue (Turner *et al.* 2020). However, approaches to either replace the mutant gene with a wild-type copy either via re-expression (Mendell *et al.* 2017), base / prime editing (Anzalone *et al.* 2019, Komor *et al.* 2016) or, for X-linked disorders, reactivation of the wild-type allele (Bhatnagar *et al.* 2014) offer highly promising scope for future therapy. The rescue of a lethal phenotype in a mouse model of Rett syndrome (Guy *et al.* 2007), suggests that in some instances, there is therapeutic potential in delivering virus after neuronal development is complete.

As stated above, gene therapy is the most direct therapy for NDDs with a genetic cause. However, in many cases, it may not be appropriate, such as when circuits are already established or when overexpression of a specific gene may reproduce a similar effect to haploinsufficiency, such as with *DYRK1A*. Therefore, could the presynapse be an indirect target for therapy? One approach would be to reverse the immediate consequence of the original mutation. For example, increasing the levels of the $PI(4,5)P_2$ within the presynapse rescued SV endocytosis defects and seizure activity in *Drosophila* expressing *skywalker* mutations, suggesting that pharmacologically increasing phosphoinositide production is a therapeutic strategy for *TBC1D24*-related disorders (Fischer et al., 2016). Similarly, enhancing calcium influx at the presynapse by elevation of extracellular calcium reversed the reduced rate of SV fusion caused by the expression of *SYT1* patient mutations in neurons (Baker et al. 2018). Finding a mechanism to increase calcium influx in brain may be problematic; however a similar approach via increased neuronal excitability has been used to successfully treat peripheral disorders caused by *SYT2* mutations (Herrmann *et al.* 2014, Whittaker *et al.* 2015).

Many presynaptic molecules and SV recycling events are tightly controlled by protein phosphorylation. Therefore, the regulation of presynaptic signalling events and specifically protein kinases may provide an excellent target for future drug development (Chico *et al.* 2009). One example of this are neurotrophic factors such as brain-derived neurotrophic factor (BDNF), which has been targeted for treatment potential (Reim & Schmeisser 2017). BDNF levels are reduced in newborns who later develop ASDs (Skogstrand *et al.* 2019), whereas they are raised overall in individuals with ASD/ID compared to typically developing controls (Saghazadeh & Rezaei 2017). BDNF has a number of presynaptic roles, mostly mediated via signalling through TrkB receptors. For example, BDNF facilitates neurotransmission via either MAP kinase-dependent phosphorylation of synapsin-1 (Valente *et al.* 2012, Jovanovic *et al.* 2000), or via inhibition of glycogen synthase kinase 3-dependent dynamin-1 phosphorylation, resulting in reduced ADBE (Smillie et al. 2013). Therefore, targeting the phosphorylation status of presynaptic molecules is a promising approach for drug development, however the challenge will be ensuring specificity of action.

Finally, rather than addressing the outcomes of a mutation at a molecular level, or where there is no obvious genetic cause, NDDs could be treated via compensatory effects at the level of presynaptic processes. For example, in disorders of elevated excitability, targeting specific neurons or circuits in a use-dependent manner may be fruitful. One example is the leading AED levetiracetam, which binds SV2A (Lynch *et al.* 2004). A number of studies have suggested that the interaction with SV2A provides a use-dependent entry route for the drug, since levetiracetam interaction sites are located on the lumenal face of SV2A (Lee *et al.* 2015, Correa-Basurto *et al.* 2015). In agreement, the ability of levetiracetam to modulate neurotransmission is accelerated when SV recycling is triggered during its initial application (Meehan *et al.* 2011, Meehan *et al.* 2012). This provides the conceptually appealing prospect of targeting drugs to the lumenal domains of different SV proteins as a mechanism to deliver a payload in a use-dependent manner.

Conclusions

It is now established that in specific cases and circumstances, presynaptic dysfunction is central to a number of NDDs. The types of dysfunction, and number of NDDs that this dysfunction contributes to will continue to grow over time. From reviewing the literature, it is apparent that disruption of most aspects of SV recycling can result in NDDs, which makes the task of predicting where future mutations in NDD genes may occur more problematic. However, this also offers hope, since interventions that facilitate efficient SV recycling may have wider therapeutic potential. Therefore, the challenge for the future is not to simply continue to collect new mutations and link them to

specific disorders, but to focus efforts into determining where dysfunction occurs, whether this can be corrected, and/or whether these interventions can be translated across a wider spectrum of NDDs. This approach should be enabled by the fact that most nonsense and missense mutations result in a loss of function (however, notable gain of function mutations do exist, as found for *MUNC13*, *STXBP1* and *DNM1*). A positive outcome of this approach will provide a key validation for fundamental research in translational studies and most importantly, a generation of novel therapies for a series of debilitating disorders.

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Conflict of interest

The authors declare no conflict of interest.

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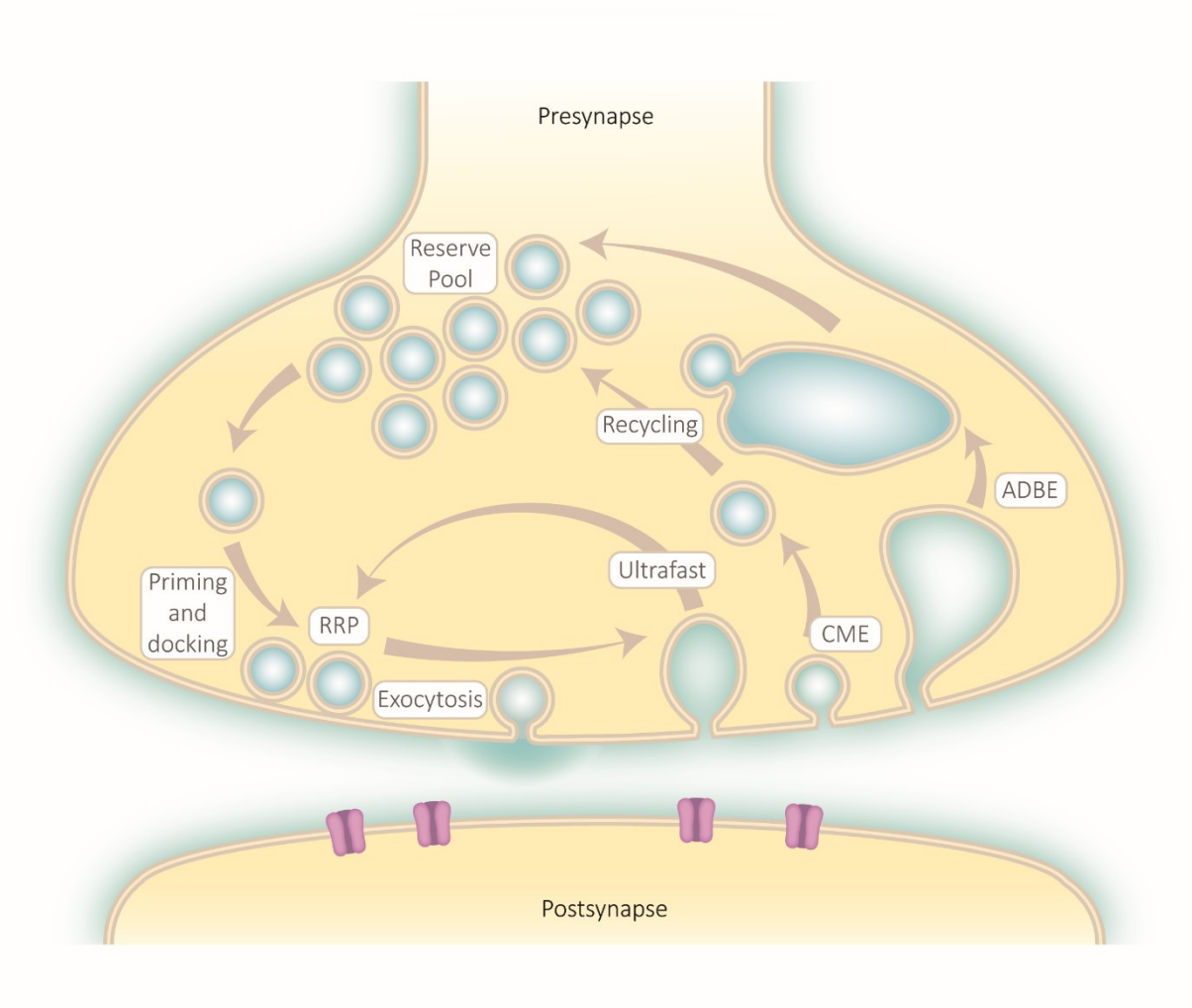

Figure 1: *The SV life cycle***.** Synaptic vesicles (SVs) are mobilised from the reserve pool by action potential stimulation. SV docking and priming at the active zone defines the readily releasable pool (RRP) which is triggered to fuse during action potential stimulation (exocytosis) resulting in neurotransmitter release. SVs are then reformed by one of several methods of endocytosis. Ultrafast endocytosis occurs at the periactive zone and retrieves vesicles that rapidly fuse with synaptic endosomes from which SVs form in a clathrin-dependent manner. Clathrin-mediated endocytosis (CME) generates SVs direct from the plasma membrane. At high levels of neuronal activity, activity-dependent bulk endocytosis (ADBE) is triggered, which retrieves large areas of membrane generating bulk endosomes, from which SVs regenerate in a clathrin-dependent process. SVs are then recycled back to their respective SV pools ready for further rounds of neuronal activity.

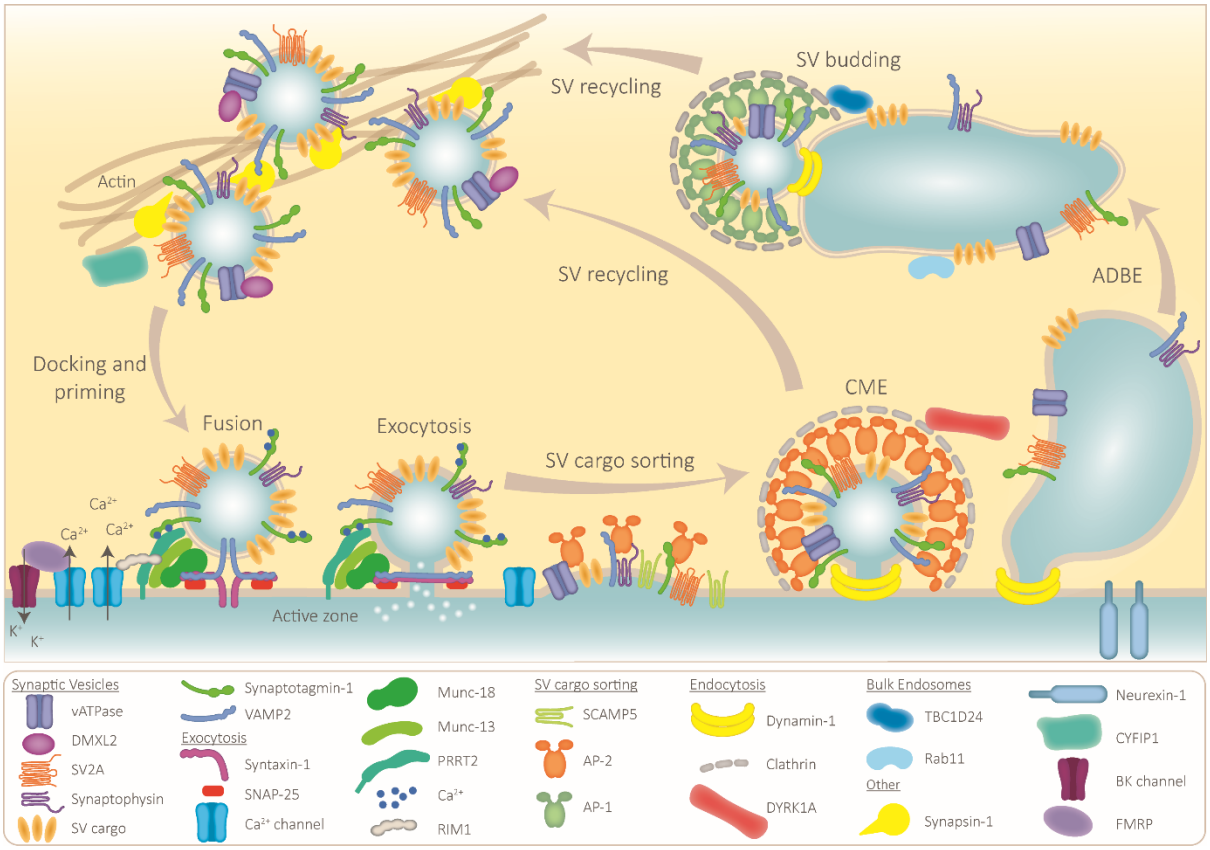

Figure 2: *Proteins associated with NDDs and the SV life cycle.* This more detailed view of the SV cycle summarises the presynaptic proteins associated with NDDs discussed in this review and at what stage of the cycle they are most likely to be involved.

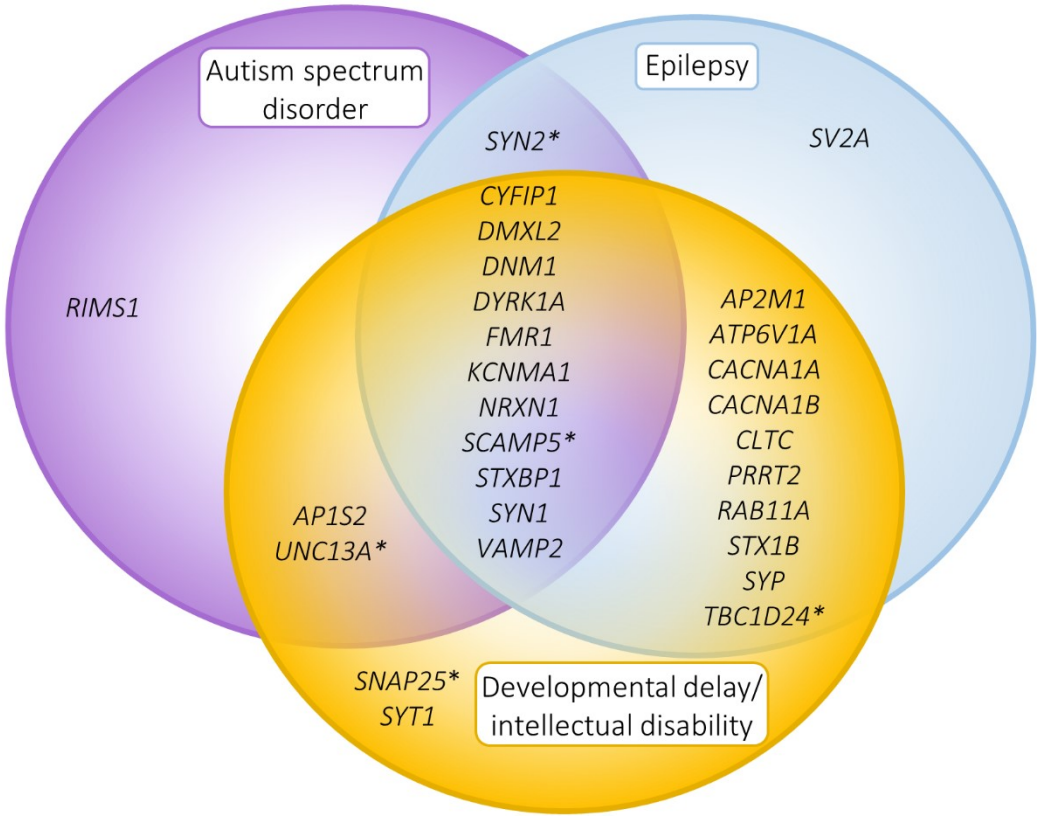

Figure 3: *SV life cycle genes categorized by NDD***.** The mutations in the SV life cycle genes discussed in this review can be categorized based on their clinical presentation into three disorders: autism spectrum disorder, epilepsy and developmental delay/intellectual disability. As observed in the overlapping portions of the diagram, different mutations in the same gene can lead to either single or multiple disorders highlighting the comorbidity between autism spectrum disorder, epilepsy and intellectual disability. Asterisks (*) indicate that mutations in these genes can be associated with seizures without clinically diagnosed epilepsy.

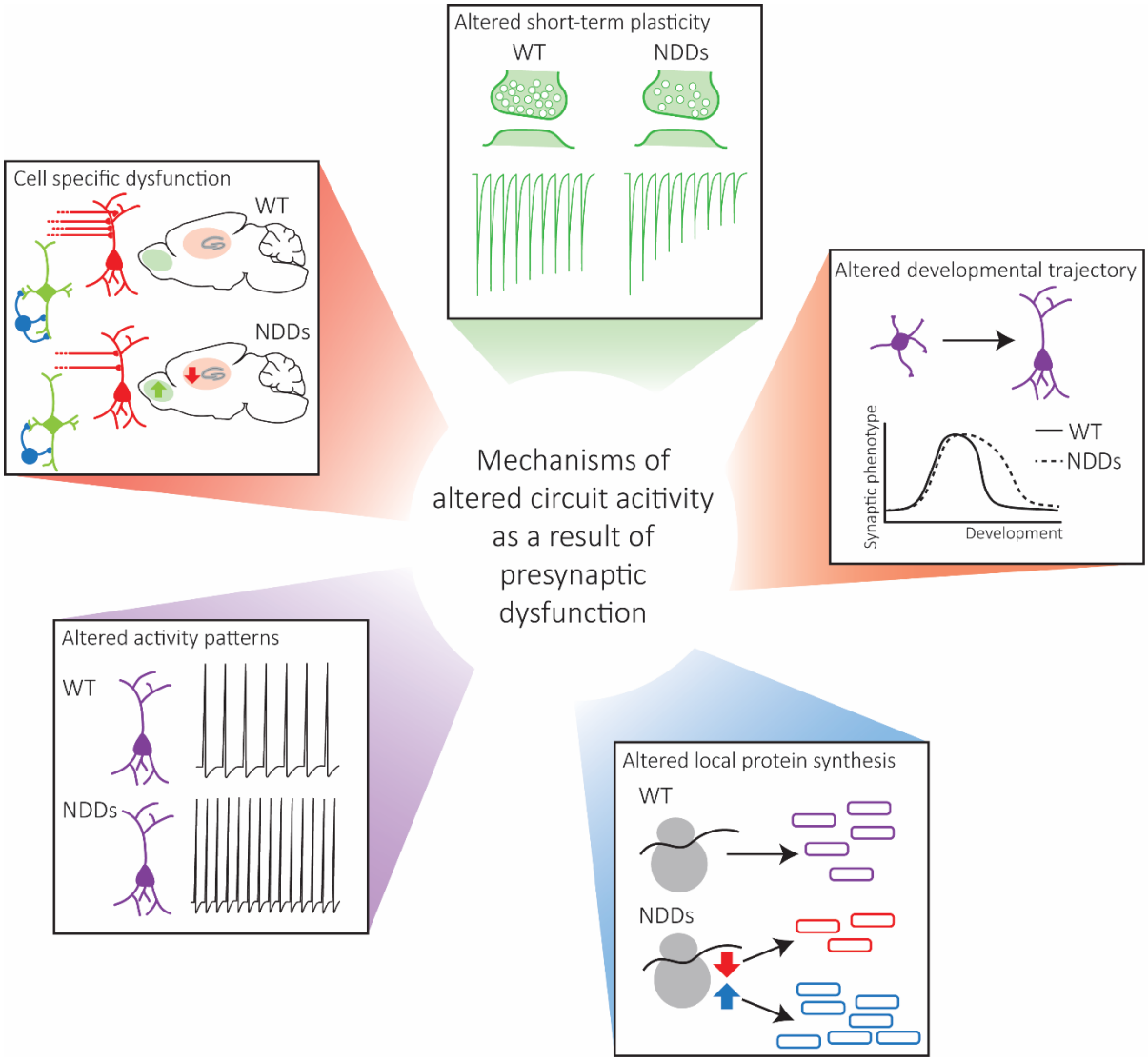

Figure 4: *Putative mechanisms of altered circuit activity due to presynaptic dysfunction***.** A schematic representation of five proposed scenarios whereby presynaptic dysfunction may result in altered neurotransmission, circuit activity and ultimately NDDs. In each scheme a wild-type (WT) and a neurodevelopmental disorder (NDD) condition are depicted.