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Beyond Clathrin: Decoding the Mechanism of Ultrafast Endocytosis

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Endocytosis in nonneuronal cells requires gradual recruitment of proteins to endocytic sites for inducing membrane curvature and forming scaffolds around the neck of endocytic pits. This recruitment process is thought to be rate-limiting, requiring tens of seconds. In contrast, a form of endocytosis in neurons called ultrafast endocytosis is much faster, requiring only 100 ms. In this review, we compare the mechanisms of protein recruitment during clathrin-mediated endocytosis in nonneuronal cells and ultrafast endocytosis in neurons and discuss how endocytosis can complete within 100 ms. We then discuss the potential clinical relevance of this endocytic pathway.

clathrin-mediated endocytosis; neurological disorders and diseases; synaptic vesicle recycling; ultrafast endocytosis

Introduction

Neurons release neurotransmitter via exocytosis of synaptic vesicles at the specialized junctions called synapses. To maintain synaptic communication, synaptic vesicle membranes and proteins are retrieved from the plasma membrane after exocytosis and recycled to restore the number of synaptic vesicles at synapses. Several models have been described over the last 50 years, including clathrin-mediated endocytosis (CME) (1), kiss and run (2, 3), fast compensatory endocytosis (4), and activity-dependent bulk endocytosis (5–7).

A decade ago, through the use of optogenetic and electrical stimulation of neurons in combination with high-pressure freezing, another form of clathrin-independent endocytosis was discovered. This pathway occurs within 100 ms of vesicle fusion (8–10) and a second step that utilizes clathrin-dependent protein sorting at endosomes (11). This process separates a time-consuming step of synaptic vesicle recycling (cargo sorting and vesicle generation), potentially enabling synapses to maintain the surface area and thereby tension constant during the ongoing neuronal activity. This rapid mode of endocytosis, known as ultrafast endocytosis, is evident at physiological temperatures in *Caenorhabditis elegans* and in mouse hippocampal synapses (8, 9) and may be analogous to fast compensatory endocytosis in goldfish bipolar cells (4) or fast clathrin-independent endocytosis in mossy fiber boutons of rodent hippocampus and cerebellum (12).

Several factors distinguish ultrafast endocytosis from other mechanisms. First, unlike other mechanisms, clathrin is not involved at the plasma membrane. Ultrafast endocytosis can be induced by both a single action potential and moderate stimuli (10–100 action potentials at 20 Hz), although its capacity for vesicle proteins may be limited and the kinetics slowed with increased number of stimuli (13). Ultrafast endocytosis is clathrin independent regardless of the stimulation duration. By contrast, in the compensatory mechanism strong stimulation slows down the endocytosis, and this mechanism appears to be dependent on clathrin (4). Second, ultrafast endocytosis occurs exclusively in a region immediately peripheral to the active zone, which is where exocytosis occurs. Bulk endocytosis and clathrin-mediated endocytosis are thought to occur more randomly in the periactive zone. Third, a specific splice variant of dynamin 1 is necessary for the process, and this protein is unique to ultrafast endocytosis (14, 15). Thus, both spatial and molecular requirements distinguish this endocytic pathway from other mechanisms.

Besides the unique requirement of dynamin 1, ultrafast endocytosis uses typical endocytic proteins for clathrin-mediated endocytosis (CME), such as endophilin, synaptojanin, syndapin, epsin, and actin (9, 10, 14, 16, 17), albeit ultrafast endocytosis ($<$100 ms) is 100-fold faster than CME in typical nonneuronal cells (tens of seconds to minutes). Therefore, how these proteins can operate with such kinetics remains elusive. Here, we review the evidence for a noncanonical route of endocytic protein recruitment at synapses and unique molecular organization of synapses that accelerate endocytosis. We then discuss how defects in synaptic vesicle endocytic proteins can be associated with neurological disorders and diseases.

Kinetics and Molecular Mechanisms of Clathrin-Mediated Endocytosis

CME is involved in uptake of a wide range of cell surface proteins and lipids and is the most well-characterized endocytic pathway to date (for details, excellent reviews are available in Ref. 18). The timescale of synaptic vesicle retrieval via CME is 10–30 s, as determined by pHluorin assay (19, 20). In general, CME is mediated by clathrin, the AP2 adaptor complex, coat proteins (e.g., Eps15, FCHO1/2, and epsin), lipid-modifying enzymes (e.g., synaptojanin), membrane bending and scission proteins (e.g., dynamin, endophilin, and syndapin), and actin cytoskeletal proteins. These proteins are recruited from the cytoplasm to endocytic sites in a specific order and assembled on the plasma membrane to sort cargo proteins, induce membrane curvature formation, and sever the neck of endocytic pits (18, 21).

The process of CME is described as occurring in three stages: an early stage, an intermediate stage, and a late stage (22, 23) (FIGURE 1). Note, however, that these processes are continuous, and therefore these distinctions are somewhat arbitrary. In the early stage, endocytic sites are initiated; this process includes cargo sorting followed by membrane curvature generation. At 37°C, the early endocytic proteins such as Eps15 and FCHO1/2 coassemble at the plasma membrane to initiate endocytosis (24–27). These proteins can form molecular condensates at the plasma membrane to facilitate efficient initiation and recruit other early endocytic proteins (28–30).

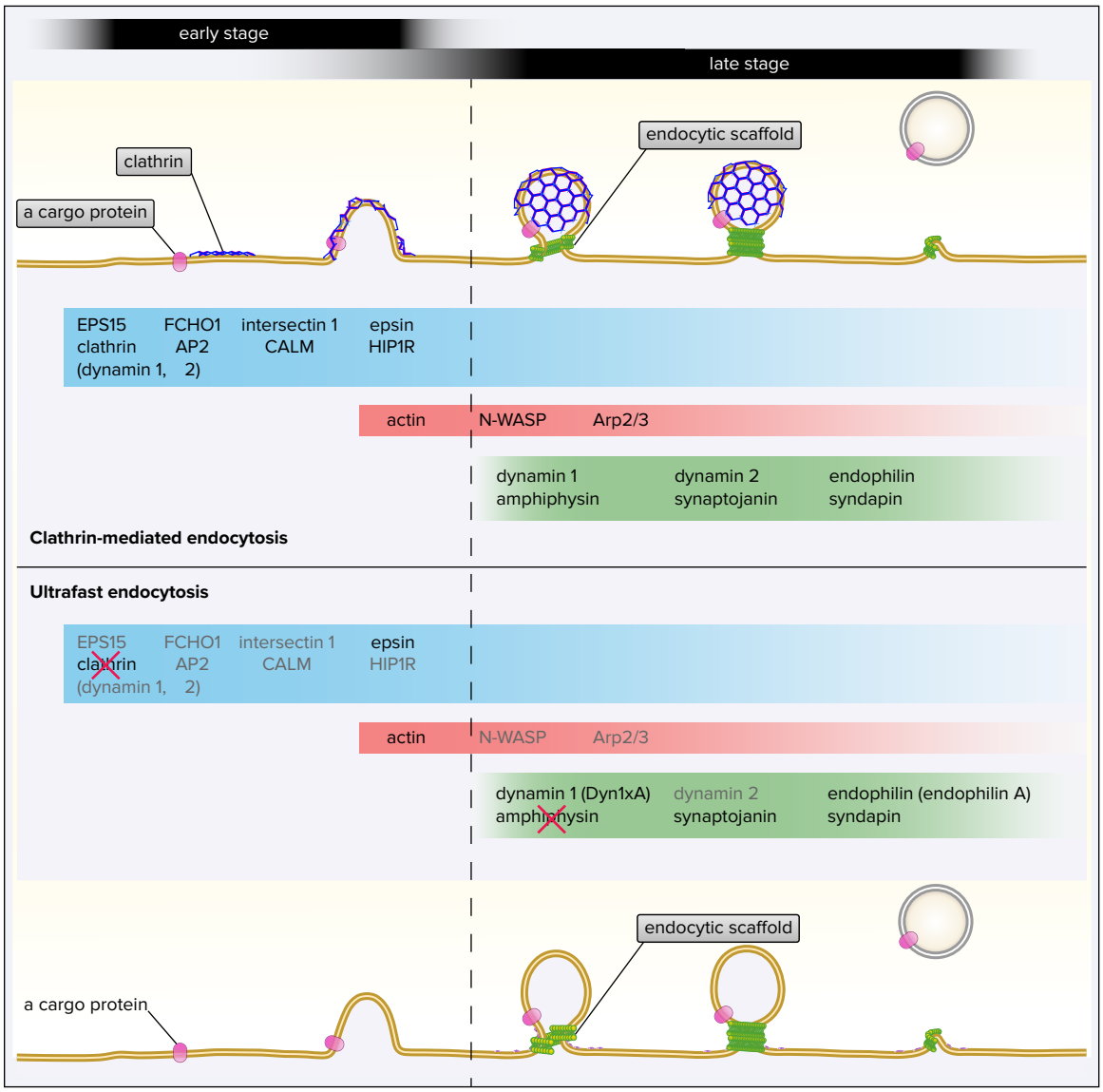

FIGURE 1. **Comparison of proteins participating in clathrin-mediated endocytosis or ultrafast endocytosis**
Top: clathrin-mediated endocytosis. *Bottom*: ultrafast endocytosis. Each panel shows the known early-stage (blue), cytoskeletal (red), and late-stage (green) proteins involved in each process. Gray font indicates proteins that have not been previously characterized. The red "X" indicates proteins that are not required for ultrafast endocytosis. For dynamin 1 and endophilin, specific splice variants that are involved in ultrafast endocytosis are listed in parentheses.

The initiation of endocytosis further requires AP2 for cargo engagement (31, 32), achieved by interaction of FCHO1/2 with AP2. Clathrin, actin polymerization, and the intermediate coat proteins then promote curvature generation (27, 33–38). The timing of their recruitment and initiation of endocytosis is variable (39, 40), but the whole initiation process takes at least tens of seconds (22, 33, 41).

In the transitional intermediate stage, endocytic membranes are sculpted by different sets of BAR (Bin/amphiphysin/Rvs) domain-containing proteins. Cryo-electron microscopy studies show that the local curvature of the BAR domain matches the curvature of the underlying membrane (42). At 37°C, the low-curvature early endocytic proteins FCHO1/2 induce or stabilize shallow membrane invagination (27). FCHO1/2 are dissociated at the beginning of the late stage. Maturation of shallow clathrin-coated pits toward the late stage is accompanied by the recruitment of highly curved N-BAR proteins, such as amphiphysin and endophilin (43), adding additional time to the whole process.

In the late stage, as endocytic pits are matured, vesicles are severed from the plasma membrane and clathrin coats removed. Fission of endocytic vesicles is led by scaffold proteins such as BAR domain-containing proteins endophilin (44, 45), amphiphysin (46, 47), and PACSIN (syndapin) (48, 49) and by GTPase dynamin proteins (50, 51). The BAR domain-containing proteins can assemble to form a rigid structure around the neck to stabilize or induce membrane curvature (21, 52). These proteins contain a Src Homology 3 (SH3) domain, which recognizes proline-rich motifs (PRMs, PxxP where x can be any amino acid) found on other endocytic proteins like dynamin and synaptojanin and recruit them to endocytic sites (21). Dynamin molecules assemble into a helix, which in turn causes constriction and pinching off of the membrane (50, 51, 53). Synaptojanin, recruited by endophilin A, contributes to the clathrin uncoating process after vesicle scission (54, 55). As in early-phase proteins, these late endocytic proteins are also recruited from the cytosol, requiring at least 10 s at 30°C (22, 56). Thus, CME is inherently slow.

Kinetics and Molecular Mechanisms of Ultrafast Endocytosis

Mechanisms of Ultrafast Endocytosis in the Early Stage

The early stage of ultrafast endocytosis is likely initiated by mechanical force alone and may not require cargo sorting and active recruitment of endocytic proteins. Ultrafast endocytosis initiates within 20–50 ms after exocytosis (8, 9, 11), and its initiation is tightly coupled to exocytosis of synaptic vesicles (57, 58) (FIGURE 2). In fact, addition of synaptic vesicle membrane through exocytosis is sufficient for initiation of ultrafast endocytosis without calcium signaling (16); in the absence of key exocytic protein UNC-13/MUNC13 calcium signaling is present, but both exocytosis and endocytosis do not take place (8, 9). The amount of internalized membrane also correlates with the amount of membrane from synaptic vesicles that have fused during exocytosis (8, 9), further indicating a tight coupling between the two processes.

Two potential models have been proposed for the exocytosis-endocytosis coupling: the tension-driven model and the membrane compression model. The tension-driven model suggests that reduction in membrane tension due to exocytosis propagates laterally and activates tension sensors for initiation of ultrafast endocytosis. With in vitro experiments done in giant unilamellar vesicles decorated by endophilin, Shi and Baumgart (59) show that reduction of membrane tension can activate membrane tubulation by endophilin within 2 s. Our recent work indeed shows that endophilin is prerecruited to the endocytic zone (15), raising a possibility that the tension reduction in the plasma membrane due to synaptic vesicle exocytosis can initiate endophilin-mediated fast endocytosis. In addition, increasing the membrane tension of calyx of Held nerve terminals (60) and retinal bipolar synapses (61) blocks endocytosis, suggesting that membrane tension is a key component of synaptic vesicle endocytosis (60). In further support of this model, a recent study by Orlando et al. (62) suggests that reduction of membrane tension by sucrose application causes synaptic vesicles to fuse and trigger the formation of endocytic intermediates at the active zone edge where ultrafast endocytosis normally takes place. However, it is worth noting that endocytosis in this case may be initiated by addition of vesicle membranes and proteins rather than the membrane tension. Similarly, several factors may be considered for the tension-driven mechanism, proposed by Shi and Baumgart (59). First, tension propagates faster than the timescale of ultrafast endocytosis (63, 64). Second, the propagation of tension is different in cellular membranes (65, 66). Finally, in the absence of all three isoforms of endophilin A, endocytosis initiates normally (10), suggesting that endophilin is not necessary for the initiation. Nonetheless, we cannot exclude the possibility that other BAR domain proteins are involved in the tension sensing. However, tension sensors for synaptic vesicle endocytosis have not been discovered to date. Thus, if reduction of the membrane tension is the trigger, alternative tension-sensitive proteins need to be discovered.

Another proposed mechanism suggests that flattening of fused synaptic vesicles exerts lateral compression in the plasma membrane against the membrane region enriched with cortical filamentous actin (F-actin) (16, 67, 68). In this model, the membrane tension is assumably needed to conserve the membrane area, rather than activating tension-sensitive proteins (16). The formation of the endocytic pit does not involve

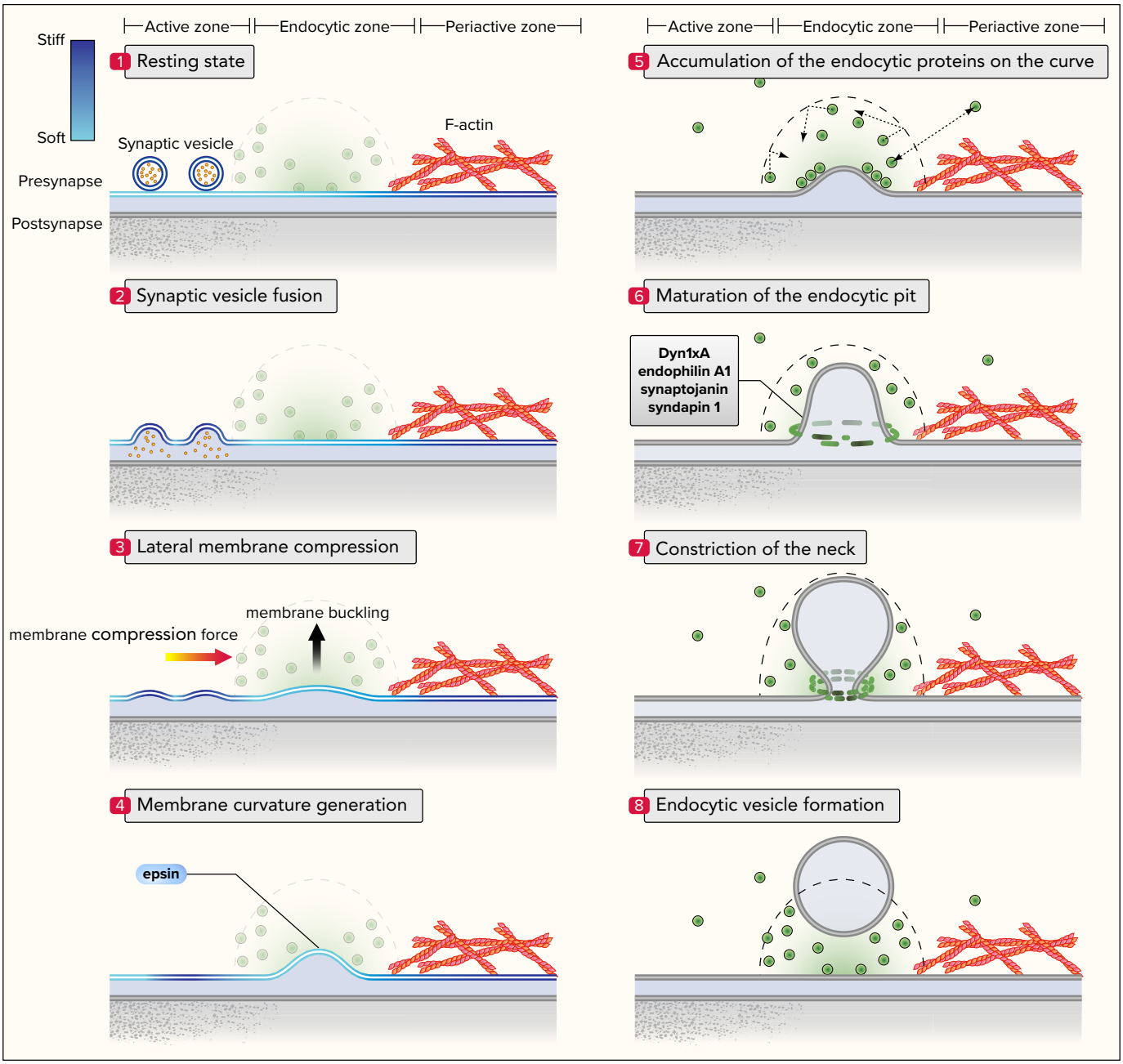

FIGURE 2. **A model of ultrafast endocytosis**
The schematic represents a working model of ultrafast endocytosis initiation. *1*) In the resting state, endocytic proteins accumulate around the edge of the active zone (the endocytic zone) and F-actin accumulates at the periphery of the endocytic zone (the periactive zone). The color gradient indicates membrane stiffness. Accumulation of F-actin increases the stiffness of the periactive zone membrane. *2*) Synaptic vesicles fuse to the plasma membrane within the active zone upon neuronal stimulation. *3*) The fused synaptic vesicle membrane exerts lateral membrane pressure to compress the plasma membrane against the stiff periactive zone membrane. *4*) The lateral membrane compression induces curvature formation. *5*) An endocytic BAR (Bin/amphiphysin/Rvs) domain-containing protein recognizes membrane curvature. *6*) Dyn1xA, endophilin A1, synaptojanin, and syndapin 1 coordinate to create a deep endocytic pit. *7*) Dyn1xA constricts the vesicle neck. *8*) The endocytic vesicle is generated within 100 ms of the synaptic vesicle exocytosis.

dynamic actin polymerization (9, 16). Consistent with this notion, stimulated emission depletion (STED) microscopy analysis shows that F-actin is organized in a ring, surrounding the active zone where exocytosis occurs, creating a gradient of membrane stiffness at synapses, with the active zone softer than the surrounding periactive zone. Thus, stiff synaptic vesicle fusion intermediates (69, 70) compress the active zone membrane laterally, and this compressive force is rectified at the edge of the active zone to form the initial membrane curvature due to the phenomenon akin to buckling instability (FIGURE 2). Furthermore, stabilizing F-actin with jasplakinolide, which blocks CME, does not interfere with ultrafast endocytosis (16), further lending support for this model that ultrafast does not require dynamic polymerization of actin. However, critical to this model is the measurement of membrane stiffness created from synaptic vesicle

fusion specifically at the active zone and endocytic zones, which is not possible with current tension sensor technology.

The donutlike organization of F-actin at the periphery of the active zone, typically the site at which endocytosis occurs, has been observed in vertebrate and invertebrate presynapses (71–75). These studies suggest that actin nucleation and assembly are important for synaptic vesicle endocytosis (13, 62). In *Drosophila* neuromuscular junctions, Nwk/FCHSD2 and Dap160/intersectin are accumulated around active zones and regulate actin patches for synaptic vesicle endocytosis (71). In mammalian central synapses, mDia1/3, which promotes nucleation of linear actin polymers, is needed for rapid synaptic vesicle endocytosis (13, 76), implying active polymerization of actin during endocytosis. However, whether mDia1/3-mediated actin polymerization is involved in membrane curvature formation or maintenance of the donutlike F-actin pool at the periactive zone remains elusive. It is worth noting that the donutlike F-actin organization requires rapid turnover of F-actin, which can be observed in latrunculin-treated cells, where F-actin is depleted rapidly within 2 min (16). Collectively, further investigation of the role of active actin polymerization in ultrafast endocytosis is needed.

Regardless of the models, several proteins are identified as early factors. One such factor is the clathrin/AP2 binding partner epsin (16, 77), despite the fact that ultrafast endocytosis does not require clathrin and AP2 at the plasma membrane (11, 78). Genetic knockdown (KD) of epsin prevents the initial membrane curvature formation (16). The finding that epsin is involved in membrane bending via amphipathic helix insertion (79) or steric crowding (34) likely suggests its active role in ultrafast endocytosis. However, it is unclear whether epsin actively sculpts membranes. In epsin KD, F-actin organization at the periactive zone is disrupted (16), resulting in failure of ultrafast endocytosis by lateral membrane compression. Thus, the role of epsin may be passive. Further studies are needed to test essential domains of epsin in membrane bending and actin binding and involvement of other early factors such as Eps15 and FCHO1 to determine their roles in ultrafast endocytosis. In addition, ultrafast endocytosis occurs independently of clathrin (8–12, 14). Similarly, knockout of AP2 does not affect membrane retrieval during clathrin-independent endocytosis (13, 78). Nonetheless, actin and its associated proteins have an important role in the early stage of synaptic vesicle endocytosis.

Mechanisms of Ultrafast Endocytosis in the Late Stage

Regardless of the models for the early phase, endocytic pits eventually mature into vesicles and must be cleaved from the plasma membrane by late endocytic proteins. In fact, several studies suggest essential roles of late endocytic proteins such as dynamin, endophilin, or syndapin in synaptic vesicle endocytosis (8–10, 14, 80). One enigma is the kinetics of their recruitment. These proteins are typically recruited from the cytoplasm after endocytosis initiates, requiring $>$10 s.

How can these late endocytic proteins mediate ultrafast endocytosis? Recent studies suggest that late endocytic proteins are prerecruited at endocytic zones in synapses (14, 80) (FIGURE 2). An alternative splice variant of dynamin 1, Dyn1xA, forms molecular condensates with syndapin 1, a brain-specific isoform of Pacsin (81), via a weak hydrophobic interaction mediated by Dyn1xA proline-rich region (PRR) and the SH3 domain of syndapin 1. Given that Dyn1xA molecules in condensates are at their critical concentration and their diffusion is 10-fold slower (14), this assembly allows polymerization of scaffolds around the neck of endocytic pits at the enhanced kinetics. Our theoretical calculations suggest that only $\sim$28–39 ms is needed to polymerize dynamin around the neck of endocytic pits (14), sufficiently rapid for ultrafast endocytosis (9). Therefore, prerecruitment of late endocytic proteins at the endocytic zone accelerates synaptic vesicle endocytosis in the late phase.

Interestingly, another splice variant of dynamin 1, Dyn1xB, only differs by a few amino acids at the COOH terminus (xA is 13 amino acids longer), but it cannot mediate ultrafast endocytosis, suggesting that the key to untangling the enigma may lie within the amino acid sequence unique to Dyn1xA. This region contains three proline-rich motifs (PxxP) in addition to the seven shared between the two variants. Our recent study suggests that this COOH-terminal tail enhances binding of endophilin A1, and this interaction confers the specificity for ultrafast endocytosis (15). In fact, mutations that disrupt this interaction cause endocytic proteins to be diffuse in the cytoplasm (14, 15), slowing down ultrafast endocytosis by 100-fold. Thus, ultrafast kinetics of the late phase is generated through prerecruited endocytic proteins.

Intermediate Stage of Ultrafast Endocytosis

Unlike the early and late stages of ultrafast endocytosis, little is known about the mechanisms underlying the transition from the early to the late stage. As mentioned above, the F-BAR protein syndapin 1 is accumulated at the endocytic zone. Its F-BAR domain has a short amphipathic “wedge” loop that can be inserted into the lipid bilayer to potentially bend the membrane and aid in pit maturation (48, 82). However, mutations in the wedge loop disrupt the condensate formation at endocytic zones but do not affect pit maturation (14), suggesting that the wedge loop is dispensable for the initial membrane curvature. However, the requirement for the F-BAR domain remains to be tested. Unlike

other membrane curvature proteins, the F-BAR domain of syndapin 1 is flexible and almost flattens out on membranes, but it can resume the "banana" shape on curved membranes (42, 83). This conformational change may explain the rapid transition of its function from preaccumulation of Dyn1xA and endophilin A to membrane sculpting. This possibility should be tested by mutating the charged amino acids on F-BAR (48). In addition, other F-BAR proteins like FCHO1/2 or N-BAR proteins like endophilin, but not amphiphysin 1 (15), may also participate in induction and stabilization of the shallow curvature at the endocytic zone. Dynamin itself may also participate in membrane bending in addition to its role in the membrane fission, like it does during CME (84). In fact, dynamin 1 knockout in neurons causes a mild delay in the transition from shallow to deeper pits during ultrafast endocytosis (15). Expression of Dyn1xB in Dyn1,3 double knockout (DKO) leads to a complete failure in the pit maturation: shallow endocytic pits are stalled on the plasma membrane, further supporting the active participation of Dyn1xA in the pit maturation. Thus, the intermediate stage is likely mediated by BAR domain-containing proteins and dynamin, but further studies are needed to elucidate the mechanisms.

Cargo Sorting in Ultrafast Endocytosis

Cargo sorting is an essential component of endocytosis and typically mediated by clathrin-associated proteins in CME. Similarly, synaptic vesicle proteins are sorted by clathrin adaptor protein complexes like AP2, AP180, and CALM [for more details, refer to recent reviews (85–87)]. However, how vesicle proteins are internalized during ultrafast endocytosis remains a mystery. The conundrum lies in the kinetics of protein diffusion and vesicle retrieval by ultrafast endocytosis. Based on pHluorin assays, the amounts of vesicle proteins exocytosed and endocytosed are equal, meaning that those exocytosed need to be immediately retrieved. If all recently exocytosed vesicle proteins are internalized by ultrafast endocytosis, these proteins must travel to the endocytic zone, which is ~100–200 nm away, in ~50 ms. Certainly, diffusion of proteins is fast enough for this translocation, but a direct path to the endocytic zone may be needed if these proteins were to be incorporated into ultrafast endocytic pits. Such mechanisms may not exist or still need to be discovered.

The most plausible explanation thus far is that a pool of vesicle proteins left on the plasma membrane from previous rounds of exocytosis is preferentially retrieved, as demonstrated for synaptotagmin-1. Previous studies suggest that endocytosed synaptotagmin-1 molecules are different from the ones exocytosed: those endocytosed are preaccumulated at the periactive zone before stimulation (88). This surface pool is referred to as a readily retrievable pool (88). Likewise, a significant fraction of synaptobrevin-2 molecules is present on the plasma membrane and confined to the endocytic zone by adaptor proteins AP180/CALM (89). Several other vesicle proteins are also available on the plasma membrane, and this pool of proteins is likely exchanged with recently exocytosed ones during ongoing neuronal activities.

One potential exception to this pool exchange hypothesis is vesicular glutamate transporter 1 (vGlut1) (10) because its amount on the surface is negligible (90). Interestingly, vGlut1 is internalized with similar kinetics as other vesicle proteins (e.g., synaptophysin, synaptobrevin-2, v-ATPase, and synatptotagmin-1) (91), indicating that vGlut1 molecules from recently exocytosed vesicles are likely incorporated into endocytic vesicles. However, as mentioned above, simple diffusional spread of vGlut1 molecules from exocytic sites may not be fast enough unless directed, questioning whether they are internalized via ultrafast endocytosis or alternative routes like clathrin-mediated endocytosis. Based on our recent studies (14, 15), the former is the most likely scenario. Several mutations on Dyn1xA slow down the kinetics of membrane internalization measured by time-resolved electron microscopy because of mislocalization of Dyn1xA, but the same mutations also slow down the kinetics of vGlut1-pHluorin uptake to the same degree, suggesting that the same endocytic mechanism is likely at play for recycling of vGlut1. Thus, these proteins may be directed to the endocytic zone by yet undiscovered mechanisms. Interestingly, vGlut1 interacts with endophilin A1 (92), an essential endocytic protein in ultrafast endocytosis, and understanding this interaction may give us a hint in untangling this mystery. Another possibility is that an internal reservoir of vGlut1 in endosomes may act as the exchange pool. This scenario is plausible given that endosomal sorting is clathrin dependent (11) and the ultimate molecular composition of synaptic vesicles is likely determined at endosomes. Consistent with this notion, in the absence of functional AP-2 complexes, synaptic vesicles are not regenerated from endosomes (11, 78, 93, 94). However, adaptor proteins likely have additional roles at the plasma membrane for sequestering vesicle proteins at endocytic zones, and cargoes may not be loaded into endocytic vesicles and ultimately delivered to endosomes, leading to the secondary defect in endosomal sorting. Thus, further definition of the roles of each endocytic protein is needed to fully elucidate the mechanisms of protein sorting at synapses.

Association of Neurological Disorders with Synaptic Vesicle Endocytosis

Mutations or dysregulation of genes encoding endocytic proteins are associated with neurological disorders (87, 95–97). Many studies have been conducted to elucidate the roles of each protein in neuronal

physiology and associated diseases (FIGURE 3). Here, we discuss key ultrafast endocytosis proteins in the context of neurological disorders.

Alzheimer's Disease

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases and is characterized by cognitive impairment with progressive memory loss (98). Cognitive impairment in AD is pathologically correlated with synapse loss (99). Before the synapse loss, synapse dysfunction is observed as the earliest AD phenotype and involves extracellular or intracellular accumulations of amyloid-β (Aβ) (100) (FIGURE 3*A*). Aβ is produced by sequential amyloidogenic processing (β-secretase- and γ-secretase-dependent cleavage) of the integral type I membrane glycoprotein amyloid precursor protein (APP) (101–103). These sequential cleavage processes are regulated by endocytic pathways. In fact, well-characterized endocytic proteins are identified as AD risk factors or AD-associated proteins. These include clathrin adaptors *AP2A1/A2* (encodes the α-subunit of the clathrin adaptor AP2) (104) and phosphatidylinositol-binding clathrin assembly protein (*PICALM/CALM*) (105), the N-BAR domain-containing protein bridging integrator protein 1 (*BIN1*) (106, 107), and dynamin 1.

How do these proteins function at synapses? As discussed above, PICALM/CALM and AP-2 are likely involved in protein sorting at either the plasma membrane or endosomes for synaptic vesicle recycling. In addition to these functions, they may be involved in APP processing. APP is efficiently retrieved from the plasma membrane (108, 109) through COOH-terminal NPTY motif (110, 111). Internalized APP encounters β-secretase 1 (BACE1) and γ-secretases in an acidic compartment, likely endosomes, for production of Aβ (112–116). In addition to the endosomal accumulation, Aβ is released upon synaptic activity (117, 118) and accumulates extracellularly (100). These Aβ molecules are reinternalized and accumulate inside endocytic vesicles (119). This process may require PICALM/CALM (120). Interestingly, disruption of the GTPase dynamin 1 results in extracellular accumulation of Aβ due to either the surface accumulation of BACE1 and APP (121, 122) or reduced uptake of Aβ. Given that synaptic activity promotes APP endocytosis and BACE1-mediated Aβ production (117) and that Dyn1xA, a splice variant of dynamin 1, specifically mediates ultrafast endocytosis, APP and BACE1 may also be a part of the readily retrievable pool, organized by the clathrin adaptor proteins. Therefore, defining the localization and physiological functions of these proteins is of importance.

BIN1 is implicated in the endosomal function. *BIN1* encodes multiple splice variants, of which a neuronal specific splice variant lacks exon 11 that encodes a phosphoinositide binding domain (123). In AD brain tissues, the expression of this BIN1 variant is reduced while the ubiquitous isoform that additionally lacks exons 13–16 encoding the AP-2 binding CLAP domain is increased (124, 125). However, the expression level may depend on the types of AD. Nonetheless, *BIN1* knockdown causes BACE1 accumulation in endosomes and increased Aβ production (126). Similarly, the paralog of BIN1, amphiphysin 1, is involved in endosomal budding (15) but dispensable for ultrafast endocytosis. Further investigation is necessary to understand the roles of BIN1 in synaptic vesicle endocytosis and APP processing.

Parkinson's Disease

Parkinson's disease (PD) is another common neurodegenerative disease (127). In PD pathology, dopaminergic neurons degenerate primarily in the substantia nigra, a region that projects to the striatum (128, 129). As in AD, presynaptic dysfunction is the earliest characteristic of PD (130–135) before axon degeneration (136–139). Although PD is primarily sporadic, a small population of patients have genetic variants associated with endocytic pathways at synapses, including variants in *SNCA* (encoding α-synuclein) (140, 141), *LRRK2* (encoding leucine-rich repeat kinase 2) (142–144), *DNAJC6* (encoding DnaJ heat shock protein family member 6, or auxilin) (145, 146), and *SYNJ1* (encoding synaptojanin 1) (147, 148) (FIGURE 3*B*).

These proteins are reported to function in several steps of the synaptic vesicle cycle from exocytosis to protein sorting. Under physiological conditions, α-synuclein forms soluble monomers or tetramers (149) and is involved in synaptic vesicle exocytosis and endocytosis (150), potentially through curvature formation or sensing mechanisms (151–153). In PD, its structure adapts a pathological β-sheet conformation that recruits additional monomers to form amyloid fibrils (154), ultimately leading to the formation of aggregates called Lewy bodies, a hallmark of PD pathology (155). However, the molecular mechanisms of how such accumulations cause synaptic dysfunction remain unclear. Overexpression of α-synuclein to the degree observed in PD neurons disrupts synaptic vesicle recycling and inhibits neurotransmitter release at hippocampal and dopaminergic neurons, despite the absence of inclusions or oligomers (156). Similarly, elevated monomeric or dimeric α-synuclein levels inhibit synaptic vesicle endocytosis (153, 157). With accumulation of clathrin-coated vesicles in synapses (151, 153, 157), it has been suggested that synuclein may be involved in clathrin-mediated endocytosis, although contribution of CME to synaptic vesicle recycling in mammalian central synapses is currently debated (17, 158).

Clathrin-uncoating phenotypes are also observed in other PD-associated mutations in *DNAJC6* and *SYNJ1*. Auxilin acts as a cochaperone to support the Hsc70-

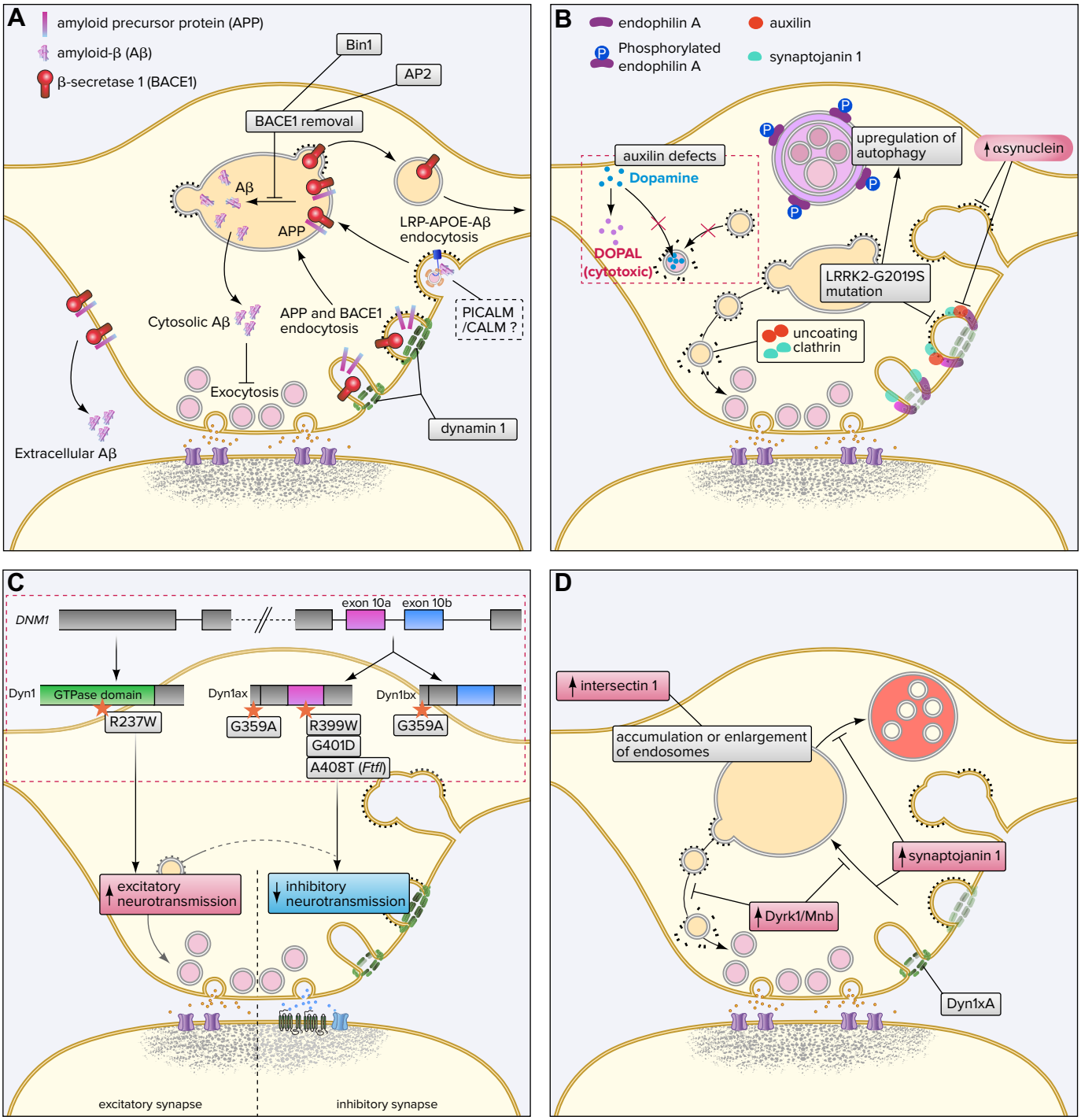

FIGURE 3. **Models of synaptic dysfunction in different neuronal disorders**
The schematic shows several models of synaptic vesicle endocytic pathways related to Alzheimer's disease (*A*), Parkinson's disease (*B*), epilepsy (*C*), and Down syndrome (*D*). *A*: mutations in endocytic proteins such as AP2 and BIN1 are associated with Alzheimer's disease. Mutations in AP2 or BIN1 cause accumulation of β-secretase 1 (BACE1), thus generating cytotoxic amyloid-β (Aβ) within synaptic endosomes via processing of amyloid precursor protein (APP). Aβ leaks into the cytoplasm and inhibits synaptic vesicle exocytosis. Mutations in phosphatidylinositol binding clathrin assembly protein (PICALM/CALM) alter clathrin-mediated endocytosis. This endocytic pathway internalizes extracellularly generated Aβ. Dynamin 1 is also potentially involved in the Alzheimer's disease phenotype via endocytic trafficking of APP and BACE. *B*: Parkinson's disease involves the abnormal accumulation and mutation of endocytic proteins such as α-synuclein, auxilin, leucine-rich repeat kinase 2 (LRRK2), and synaptojanin 1. Accumulation of pathological α-synuclein blocks synaptic vesicle endocytic pathways and leads to synaptic dysfunction. Mutation or deletion of auxilin causes accumulation of cytotoxic 3,4-dihydroxyphenylacetaldehyde (DOPAL) in dopamine neurons. The LRRK2-G2019S mutation causes relocalization of endophilin A from the plasma membrane to autophagosomes, resulting in neurodegeneration. Synaptojanin 1 plays a role in synaptic vesicle endocytosis and clathrin uncoating. A Parkinson's disease-associated mutation in synaptojanin 1 leads to degeneration of dopamine neurons. *C*: epilepsy-associated mutations within the GTPase domain or exon 10a of dynamin 1 disrupt the balance between excitatory and inhibitory output and lead to a seizure phenotype. Red stars indicate the locations of epileptic mutations. *D*: Down syndrome is caused by triplication of human chromosome 21 (chr 21). chr 21 encodes several endocytic proteins, including synaptojanin 1, intersectin 1, and dual-specificity tyrosine kinase 1A/minibrain kinase (Dyrk1/Mnb). Overexpression of these proteins leads to the accumulation or enlargement of synaptic endosomes. These abnormal endosomes accumulate old or damaged synaptic vesicle proteins, thus causing synaptic dysfunction.

dependent uncoating of clathrin-coated vesicles (159, 160). SYNJ1 aids in uncoating by its SAC1 domain activity, which is important for dephosphorylation of phosphatidylinositol 3′-phosphate (PI3P) and phosphatidylinositol 4′-phosphate (PI4P); this domain is mutated in PD (161). This uncoating process is required for the reacidification of synaptic vesicles (162, 163) and neurotransmitter reuptake (164), and thus this uncoating defect would likely lead to reduced dopamine release. In addition, impaired dopamine uptake may cause accumulation of oxidized dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL) in the cytoplasm, which in turn could trigger neurotoxic effects on dopaminergic neurons like aggregating α-synuclein (165) and accumulating ubiquitinated proteins (166), oxidative stress, and mitochondrial damage (167, 168). Such cytotoxicity may account for the degeneration of the dopaminergic synapses (169–171).

Another defect may stem from imbalance in protein homeostasis through the endocytic pathway. LRRK2 is a large, ∼290-kDa protein that contains GTPase, a kinase enzyme, and protein-protein interaction domains, including a leucine-rich repeat (LRR) and a WD40 repeat (172). Several PD-associated mutations in LRRK2 are thought to contribute to PD by impairing membrane trafficking processes, including endocytosis, endosomal sorting, and protein degradation (173–176). The most frequent mutation is LRRK2 G2019S, which induces kinase hyperactivity (177, 178). This mutation triggers a reduction in dopamine release, but it is not associated with degeneration of nigrostriatal terminals (179). Although the molecular mechanism of the LRRK2 G2019S-associated dopaminergic synapse defect is unclear, defects in synaptic vesicle recycling may be due to hyperphosphorylation of endocytic proteins. For example, LRRK2 phosphorylates the proline-rich domain (PRD) of synaptojanin 1, resulting in disruption of endophilin A1 interactions (180) and clathrin uncoating (54, 55). Another example reported in *Drosophila* neuromuscular junctions shows that expression of LRRK2 G2019S or the phosphomimetic mutation of endophilin A S75, the residue phosphorylated by LRRK2, causes dissociation of endophilin A from the plasma membrane and disruption of synaptic vesicle endocytosis (181). The phosphomimetic endophilin A S75 instead plays a role in autophagosome formation (182), as do other PD-associated endophilin mutations (183). Importantly, both the phosphomimetic and phosphodeficient forms of endophilin A S75 cause dopaminergic neuron degeneration (182), suggesting that the phosphorylation/dephosphorylation cycle of endophilin A1 is essential for synaptic function. Since synaptojanin and endophilin A1 are both essential for ultrafast endocytosis (10), LRRK2-mediated phosphorylation may act as a switch for their roles in ultrafast endocytosis and other functions. However, this hypothesis needs to be tested thoroughly. Moreover, the functions of other domains of LRRK2 are poorly defined at this stage, warranting further investigation.

Epilepsy

Epilepsy affects more than 65 million people worldwide and is characterized by recurrent unprovoked seizures (184, 185). Hyperexcitability and excessive synchronization of cerebral neurons are the main neurobiological features (186). An established mechanism of epilepsy is the imbalance between excitation and inhibition in neuronal circuits (187). Such an imbalance can be caused not only by enhanced neurotransmitter release but also by abnormal synaptic vesicle recycling. In fact, mutations in DNM1 (188–191) are a well-known cause of epileptic seizures (FIGURE 3C). The dynamin 1 binding partners syndapin 1 (192) and amphiphysin 1 (193) also cause seizure phenotypes in murine models. A spontaneously occurring mutation known as "fitful" ($Dnm1^{Ftfl}/Dyn1^{Ftfl}$) was the first seizure-associated mutation of Dnm1 identified in a murine idiopathic epilepsy model (188). This mutation is caused by an alanine-to-threonine substitution at amino acid 408 within the middle domain of Dyn1, which is an evolutionarily conserved residue in both vertebrates and invertebrates. Notably, the sequence encoding this residue is present only in exon 10a, one of two alternative splice exons of the *Dnm1* transcripts. *Dnm1* contains either exon 10a (*Dnm1aX/Dyn1aX*) or exon 10 b (*Dnm1bX/Dyn1bX*). Expression of *Dnm1aX* increases during development (embryonic day 17.5 to postnatal day 14), whereas *Dnm1bX* expression remains constant or slightly decreases. By contrast, *Dnm1bX* is upregulated in $Dnm1^{Ftfl}$ homozygous mice without changing the total Dnm1 mRNA or protein levels (188, 194). The $Dnm1^{Ftfl}$ variant is unable to efficiently form oligomeric structures, needed for vesicle scission, and stalls endocytosis in a dominant-negative manner. Consistently, heterozygous mice ($Dnm1^{Ftfl/+}$) show a less severe seizure phenotype than homozygous mice ($Dnm1^{Ftfl/Ftfl}$). Importantly, neither *Dnm1* heterozygous null mice ($Dnm1^{null/+}$) nor homozygous null mice ($Dnm1^{null/null}$) have seizures (195). Therefore, the Ftfl mutation is a gain-of-function mutation. Cortical neurons in $Dnm1^{Ftfl/Ftfl}$ mice show faster depression of inhibitory postsynaptic current (IPSC) amplitude (188), suggesting that hyperexcitability can be explained by altered inhibitory inputs. The *DNM1* G359A mutation found in epileptic encephalopathy patients is also in the middle domain (196). Cells with this mutation exhibit an endocytic defect like that in the murine $Dnm1^{Ftfl}$ mutant. Other patient DNM1 mutations also affect exon 10a (189). The c.1197−8G>A mutation is located just upstream of exon 10a and creates a new cryptic acceptor splice site. This leads to an in-frame insertion of two amino acids at the beginning of exon 10a-encoded region. The c.1195A>T mutation is located at the end of exon 9. It results in an R399W missense

mutation when exon 10a is spliced in, but produces a nonsense mutation if exon 10b is used instead. The middle domain residue R399 is required for dynamin tetramer formation (197), and this structure is required for stable dynamin oligomer assembly on the membrane in vitro. Therefore, endocytic defect may be due to abnormal formation of the oligomer. Nonetheless, it is unclear why most of the DNM1 epilepsy-associated mutations are found in exon 10a but not in 10 b. Further studies are needed to test the function of these alternative exons.

A recent study also demonstrated that an epilepsy-associated mutation in the DNM1 GTPase domain (R237A) (196) affects neurotransmission. In contrast to the previous models, heterozygous R237A ($Dnm1^{R237A/+}$) mice have altered excitatory neurotransmission that does not affect inhibitory postsynaptic currents (198). Compared to wild-type controls, $Dnm1^{R237A/+}$ neurons show a greater amplitude of evoked excitatory postsynaptic currents. Those excitatory neurons also show an increase in release probability. However, this increase cannot be sustained during short-term plasticity, most likely because of a defect in synaptic vesicle endocytosis (198). It remains unclear how different types of mutations can preferentially affect synaptic vesicle recycling in inhibitory synapses or excitatory synapses. Further studies are required to identify the molecular mechanisms that determine the synaptic type specificity of epilepsy-associated mutant functions and to discover how different endocytic pathways contribute to epileptic phenotypes.

Down Syndrome

Down syndrome (DS) is the most common genetic cause of intellectual disability. It arises from a partial or complete triplication of human chromosome 21 (chr 21) (199). As a result, proteins encoded by chr 21 are overexpressed to some degree. chr 21 contains several genes involved in endocytic pathways, including *SYNJ1*, *ITSN1,* and *DYRK1A* (200). Consistently, the accumulation of abnormal endosomes is reported in cells from patients with DS (201, 202) (FIGURE 3*D*). As discussed, synaptojanin 1 may function at the plasma membrane as well as endosomes by controlling the phosphoinositide (PIP) composition of these compartments. Defects in endocytic pathways may arise from misregulation of PIP signaling. *ITSN1* encoding intersectin 1 may also contribute to the defects by misregulation of actin dynamics since it can interact with Cdc42 and activate N-WASP and Arp2/3 (203, 204). *DYRK1A* encodes the dual-specificity tyrosine kinase 1A (Dyrk1), which is also called the minibrain kinase (Mnb). Dyrk1/Mnb phosphorylates the consensus target sequences RPX(S/T)P or RX(S/T)P (205). Its substrates include endocytic proteins like AP180, the α- and β-adaptins from the AP-2 complex (206), synaptojanin 1 (207), and dynamin 1 (208). Dyrk1/Mnb-dependent phosphorylation of these proteins is necessary for *1*) clathrin coat disassembly by releasing AP180 and AP-2 from clathrin-coated vesicles (206), *2*) enhancing the 5′-phosphatase activity of synaptojanin 1 (209), and *3*) inactivation of Dyn1xA by redistributing them to the cytoplasm from the endocytic zones. Dynamin 1 is phosphorylated by Dyrk1/Mnb at S857 (208), which is only present in the Dyn1xA splice variant. Phosphomimetic mutations in S857 and a neighboring phosphorylation site at S851 impair ultrafast endocytosis (80), suggesting that this phosphorylation event acts as a negative regulation of Dyn1xA function at synapses. Currently, it is uncertain how Dyrk1/Mnb balances between the negative and positive regulations of Dyn1xA and synaptojanin 1, respectively. Ultimately, we need to understand how these changes lead to synaptic dysfunction in DS.

Conclusions and Perspectives

Over the last 10 years, the discovery of ultrafast endocytosis and its mechanisms has transformed our understanding of synaptic vesicle recycling. Although molecular machines are shared between CME and ultrafast endocytosis, their specific localization patterns determine the kinetics of endocytosis. For CME, endocytic proteins are in the cytoplasm and recruited to the endocytic sites after the initiation of endocytosis, making the process slow but allowing flexibility in locations. By contrast, these proteins are preaccumulated at the endocytic zone for ultrafast endocytosis, limiting the locations but enhancing the kinetics. Importantly, neuron-specific isoforms of endocytic proteins are essential for this localization.

As discussed throughout this review, many unanswered questions remain. In particular, answering pathophysiological questions likely requires consideration of ultrafast endocytosis. In the ultrafast endocytic pathway, synaptic vesicles are ultimately generated from endosomes, not from the plasma membrane. Interestingly, despite the requirement of additional membrane fusion and fission events, synaptic vesicles can be generated within 10 s, faster than the vesicle generation via clathrin-mediated endocytosis (∼30 s) (19, 20). Thus, further investigation of endosomal protein sorting and budding is warranted. Ultimately, basic understanding of ultrafast endocytosis gained largely by studying mouse hippocampal synapses must be evaluated in other types of synapses and other secretory cells (60, 210) and with different stimulation paradigms that alter internal calcium dynamics (4, 211–214) or induce neuronal plasticity. In addition, it is crucial to investigate how endocytic zones form during development (215). Although ultrafast endocytosis does not require clathrin (9), it is undoubtedly essential for synaptic vesicle recycling at the level of endosomal resolution (11, 78) or potentially other processes (216), and thus further refining its role is an important question. Finally, several other clathrin-independent endocytic mechanisms

including fast compensatory endocytosis and activity-dependent bulk endocytosis are also thought to participate in synaptic vesicle recycling (4, 159, 214). At a glance, these different mechanisms appear to share the same molecular players and may be distinguished by the semantics, but certain differences do exist such as the potential reliance of dynamin isoforms, dedicated cargoes, and clathrin. Thus, it would be essential to reevaluate their molecular mechanisms based on the discussed mechanisms of ultrafast endocytosis. Advancing our basic understanding of the synaptic vesicle cycle holds the potential for clinical intervention of devastating neurological disorders. ■

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