Communicative & Integrative Biology 7, e28740; April 2014; © 2014 Landes Bioscience

The role of F-actin in modulating Clathrin-mediated endocytosis

Lessons from neurons in health and neuropsychiatric disorder

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Keywords: Clathrin-mediated endocytosis, F-actin, cytoskeleton, CPG2, SYNE1, schizophrenia, synaptic plasticity, internalization, AMPA receptor, bipolar disorder, synapse

Clathrin-mediated endocytosis is one of several mechanisms for retrieving transmembrane proteins from the cell surface. This key mechanism is highly conserved in evolution and is found in any eukaryotic cell from yeast to mammals. Studies from several model organisms have revealed that filamentous actin (F-actin) plays multiple distinct roles in shaping Clathrin-mediated endocytosis. Yet, despite the identification of numerous molecules at the interface between endocytic machinery and the cytoskeleton, our mechanistic understanding of how F-actin regulates endocytosis remains limited. Key insights come from neurons where vesicular release and internalization are critical to pre- and postsynaptic function. Recent evidence from human genetics puts postsynaptic organization, glutamate receptor trafficking, and F-actin remodeling in the spotlight as candidate mechanisms underlying neuropsychiatric disorders. Here I review recent findings that connect the F-actin cytoskeleton mechanistically to Clathrin-mediated endocytosis in the central nervous system, and discuss their potential involvement in conferring risk for neuropsychiatric disorder.

Clathrin-mediated endocytosis (CME) is one of several mechanisms for retrieving plasma membrane constituents and transmembrane proteins from the cell surface (reviewed in[1]). Transmembrane proteins targeted for internalization associate with components of the endocytic machinery, predominantly with subunits of Clathrin adaptor complexes such as AP2, AP180, or Clathrin assembly lymphoid myeloid leukemia (CALM). While AP180 is a neuronal specific adaptor complex predominantly found at presynaptic terminals, AP2 and CALM are ubiquitously expressed. Recent reports have described a novel role for AP180 and CALM in sorting of the synaptic vesicle SNARE protein synaptobrevin into endocytic compartments.[2,3] These mechanistic advances have been reviewed elsewhere.[4,5] The adaptor complex recruits Clathrin triskelion units which assemble into a characteristic three-dimensional structure, the Clathrin-coated pit. Pit formation is assisted by various proteins that induce or facilitate membrane curvature, such as endophilins or BAR-domain containing proteins. After pit maturation, the GTPase Dynamin assembles in a spiral arrangement around the neck of the invaginated structure and ultimately leads to severing of the Clathrin-coated vesicle (CCV) from the plasma membrane. Dynamin has long been regarded as a mechanoenzyme which provides the necessary mechanical force to pinch off the endocytic vesicle by neck constriction. However, recent biophysical approaches propose a mechanochemical model in which Dynamin acts to provide a rigid neck that holds the bulbous CCV in place. The resulting curvature at the neck-vesicle interface induces a self-enhancing interfacial force generated by splaying of membrane phospholipids which ultimately culminates in vesicle scission.[6,7] After scission, the Clathrin coat disintegrates and the assembled machinery is shed under the influence of uncoating factors such as synaptojanin (reviewed in[8]).

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Submitted: 03/11/2014; Revised: 04/01/2014; Accepted: 04/01/2014; Published Online: 04/03/2014
Citation: Loebrich S. The role of F-actin in modulating Clathrin-mediated endocytosis: Lessons from neurons in health and neuropsychiatric disorder. Communicative & Integrative Biology 2014; 7:e28740; http://dx.doi.org/10.4161/cib.28740

Requirement for F-actin Dynamics in Clathrin-Mediated Endocytosis

Early observations in different model organisms, yeast and mammalian, have shown that the F-actin cytoskeleton plays a variety of roles in CME. Initial insight came from experiments using drugs that interfere with F-actin dynamics. For example, Latrunculin A or Cytochalasin D (alkaloids from *Latruncula sp.* and *Helminthosporium sp.*) act as potent inhibitors of actin polymerization. Conversely, Jasplakinolide or Phalloidin (alkaloids from *Jaspis splendens* and *Amanita phalloides*) stabilize F-actin filaments and prevent their turnover. Using a perforated cell assay, Fujimoto and colleagues investigated the effects of F-actin modulating drugs on transferrin internalization, an established cargo for CME in mammalian cells.[9] They found that Cytochalasin D and Latrunculin A significantly reduced transferrin internalization in A431 cells, but results varied depending on whether

the cells were kept in suspension or allowed to adhere to plates. The study obtained similar results for CHO cells under most but not all conditions, but reported differential outcomes testing the effects of Jasplakinolide in suspension vs. adhesive cultures. Although open to varied interpretation, these early experiments revealed a complex interplay of CME with the F-actin cytoskeleton in mammalian cells. Accumulating evidence suggests an indirect influence of the F-actin cytoskeleton on Clathrin coated pit dynamics. One such indirect way to regulate local CME could be via F-actin's role in regulating local membrane tension, a crucial factor for vesicle fission,[7] and a determinant for the requirement of F-actin in CME.[10]

However, the question of F-actin's point of action in direct regulation of CME was hotly debated even a decade ago.[11] One report found a major influence of the F-actin cytoskeleton on the dynamics of Clathrin-coated structures and on several stages of the endocytic cycle, including pit formation, splitting of Clathrin-coated structures, merging and coalescing, as well as constriction and internalization.[12] In this study, deeply invaginated Clathrin-coated structures accumulated upon inhibition of F-actin dynamics with Latrunculin or Jasplakinolide, hinting at a role for F-actin in the later stages of the endocytic cycle. Consistently, F-actin accumulations at endocytic sites were mostly observed immediately prior to scission.[12]

Merrifield and colleagues found otherwise when they marked endocytic sites in Swiss 3T3 cells by expression of Clathrin-dsRed and visualized them by Evanescent Field (EF) microscopy. They saw that internalization of Clathrin-coated pits is accompanied by recruitment of F-actin[13] and that the presence of fluorescently labeled Dynamin peaks shortly before scission, consistent with Dynamin's role in constriction of the vesicular neck. Conversely, peak F-actin signals were observed shortly after vesicle scission, supporting the notion that the major role of F-actin polymerization lies in post-scission events, such as propulsion of the newly endocytosed vesicle away from the plasma membrane. A later study used live cell imaging of pH-sensitive cargo to show that multiple CCVs are generated from the same Clathrin-coated pit in rapid succession.[14] In this study, scission of CCVs was found to coincide with local recruitment of cortactin-dsRed, and the efficiency of membrane scission was dependent on F-actin remodeling, consistent with a model of F-actin aiding membrane invagination by providing mechanical force.

A landmark study in yeast analyzing more than 60 genetic deletion mutants began to disentangle the complex requirement for F-actin in CME.[15] The authors analyzed protein dynamics at endocytic sites by imaging GFP-tagged endocytic proteins in combination with genetic ablations. Based on how application of Latrunculin A disrupted the spatial organization of different endocytic factors the authors proposed a modular design for the endocytic protein machinery, consisting of four groups of proteins that cooperate to fulfill the different functions required for completion of an endocytic event. According to their core components or functions these protein groups were named the "coat," "amphiphysin," "WASP/Myo," and "actin" module. Each of these modules is affected differentially by F-actin blockade: The "actin module" consists exclusively of components which all rely on F-actin for proper localization. Components of the "coat," "WASP/Myo" and "amphiphysin" modules are each affected differentially by blockade of F-actin. The "coat" and "amphiphysin" complexes can form without F-actin but need the cytoskeleton for movement and disassembly, consistent with a role for F-actin in recruiting coat disassembly factors such as synaptojanin.[16] The "WASP/Myo" module remains immotile at the plasma membrane and disassembles after F-actin polymerization has occurred. Interestingly, assembly of some components into this module is independent of F-actin, while disassembly depends on F-actin. Despite these conceptual advances, the use of yeast as a model to delineate the role of the F-actin cytoskeleton in mammalian CME has been somewhat problematic because the cytoskeletal contribution to endocytosis differs in both systems and findings from yeast do not necessarily translate into the mammalian system (reviewed in[17]). A recent study suggests that in yeast turgor pressure, the osmotic force counteracted by the yeast cell wall, is a key element in shaping the requirement for F-actin dynamics.[18] Mutant yeast strains with elevated turgor pressure show increased dwell times for endocytic proteins at sites of endocytosis. Conversely, reducing turgor pressure eliminated the strict need for F-actin remodeling in yeast CME. These observations are consistent with a model in which F-actin dynamics are needed in the invagination stage of endocytosis to provide the mechanical force necessary to overcome osmotic pressure in yeast, something not quite relevant to mammalian cells. Further insights on CME in yeast have recently been summarized elsewhere.[19] It is, however, tantalizing to speculate that a temporal and spatial assembly of different protein modules may play similar roles in mammalian CME. It is currently not known if during the life cycle of an endocytic vesicle these module components can connect temporarily and assemble into functional protein complexes. How assembly or disassembly of such higher-order structures would be regulated to mediate CME, and at which points in time and space they interface with the F-actin cytoskeleton is not clear. However, several points of contact have been documented linking the process of CME to F-actin via molecules that physically interact with both the cytoskeleton and components of the endocytic machinery.

Molecular Links Connecting F-actin and the Endocytic Machinery

Most proteins of the endocytic machinery are ubiquitous, although isoforms specific to pre- or postsynaptic compartments have been described for some, including Dynamins and Endophilins. Despite the fact that endocytosis is much more prevalent in the presynaptic compartment, some evidence concerning the contact points of CME with the F-actin cytoskeleton has been garnered from studies at the postsynaptic site.

In the presynaptic terminal, hundreds of synaptic vesicles undergo fusion and pursuant recycling through CME. A recent report has characterized a novel form for endocytosis at presynaptic terminals of hippocampal synapses. Using optogenetics in combination with flash-and-freeze electron microscopy, Watanabe et al. report a form of endocytosis that is 200-fold faster

than CME. This ultrafast endocytosis depends on Dynamin function and can be blocked with Latrunculin A, suggesting that a dynamic F-actin cytoskeleton is crucial for its progression.[20]

The F-actin cytoskeleton is a highly dynamic meshwork and hundreds of proteins have been shown to either bind to globular or filamentous actin. These myriad actin binding proteins regulate numerous functions such as filament nucleation, bundling, cross-linking, anchoring, turnover, and cargo transport to name a few. Which of these molecules mediate the functional interplay of the dynamic F-actin cytoskeleton with the process of CME? A linker function has been postulated for HIP1 (Huntingtin interacting protein 1) in combination with its close relative HIP1R (HIP12). HIP1 is considered an auxiliary member of the endocytic machinery since it binds directly to α-Adaptin and Clathrin heavy chain.[21] An in vitro structure-function analysis showed that HIP-1's interaction with Clathrin heavy chain is mediated by the coiled-coil domain in HIP1. The same study showed that HIP1 is present in CCVs purified from human brain extracts, and that HIP1 co-localizes with endocytosed cargo in cultured COS-1 cells, although only a subset of CCVs were positive for HIP1.[21] Its close relative HIP1R has been found to co-localize with actin and endocytic pits, and it binds to F-actin via its C-terminal Talin-like F-actin binding domain.[22] HIP1R does not seem to bind AP2 directly but it does bind to Clathrin[23] albeit with much lower affinity than HIP1. A later report showed that HIP1 and HIP1R directly bind to each other,[24] opening the possibility that these two proteins in conjunction could form a structural link between the endocytic machinery and the F-actin cytoskeleton. Manipulating the interaction between HIP1R and Clathrin, Boulant and colleagues demonstrated that HIP1R can mediate actin engagement in CME.[10] Under which circumstances the HIP1-HIP1R interaction couples the cytoskeleton to the endocytic process, and how this association is regulated, remains unknown.

Dynamins, too, have several indirect points of contact with the actin cytoskeleton. An early study identified Dynamin 1 as an interactor of Profilin II, but not Profilin I, in mouse brain extracts.[25] Profilin II is almost exclusively expressed in brain and Dynamin 1 is considered the major presynaptic dynamin in neurons, involved in rapid synaptic vesicle recycling. Likewise, the proline-rich domain of Dynamin 2 has been found to mediate interaction with the SH3 domain of the actin-binding protein Cortactin.[26] In cultured fibroblasts that have been stimulated with PDGF Dynamin 2 becomes prevalent at membrane ruffles and lamellipodia, regions with increased membrane turnover and hence elevated requirements for efficient CME. This localization depends on the interaction with Cortactin, as it can be disrupted by expression of a Cortactin that lacks the SH3 domain (or a Dynamin 2 lacking the proline rich domain), leading to morphological changes in cell shape, likely due to increased actin stress fibers.[26] Kessels and colleagues also identified Dynamin from mouse brain as an interactor of the F-actin binding protein Abp1 in blot-overlay assays.[27] Biochemical analysis showed that this interaction is mediated by the SH3 domain of Abp1. Overexpression of the SH3 domain in COS-7 cells blocked endocytosis of labeled transferrin; however, the blockade could be overcome by co-expression of Dynamin, showing that this interaction has direct relevance to CME. While Abp1 seems to be expressed in cultured hippocampal neurons it remains unclear if the interaction with Dynamin is relevant to endocytic processes at the synapse. Dynamin also interacts with the multi-adaptor protein Intersectin, a RhoGEF that can lead to activation of Cdc42[28] and has been reported to bind to Epsin and Amphiphysin, but also N-WASP suggesting that Intersectin can directly influence F-actin dynamics (recently reviewed in[29]). Intersectin's role is best understood in the presynaptic terminal of neuronal synapses where it targets Dynamin to the periactive zone to balance membrane retrieval at times of high-frequency activity.[30] However, how these interactions are regulated is not currently known.

The presynaptic neuronal Dynamin I also associates with sorting nexin 9 (SNX9), which is capable of binding to N-WASP.[31] SNX9 localizes to Clathrin-coated pits and stimulates N-WASP-mediated actin assembly,[32] a finding that has implicated SNX9 in coupling actin dynamics to membrane remodeling, although its role in endocytosis is mostly attributed to its interaction with Dynamin. Interestingly, the presence of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) increases SNX9-mediated actin polymerization,[32] and PI(4,5)P2 formation from precursor phospholipids is required for local enrichment of SNX9 at late-stage endocytic intermediates.[33] Together, these findings suggest that the concerted interplay of phospholipid metabolism and SNX9 localization/activity can regulate F-actin polymerization to shape CME.

The activity-regulated gene *Arc/Arg3.1* has also been reported to bind to Dynamin 2, as well as Endophilin 3.[34] The Arc protein binds to Dynamin and Endophilin through distinct parts and induces relocation of either protein to early endosomes when expressed in HeLa cells. At least the interaction of Arc with Endophilin 2 or 3 appears to be regulated, because Arc shows greater affinity for Endophilin fragments that are missing the N-terminal BAR domain, suggesting that Endophilins are auto-inhibited to bind Arc. In neurons, expression of Arc leads to increased AMPA-type glutamate receptor internalization, a function that depends on its association with both Endophilin and Dynamin.[34] Although Arc has been shown to associate with F-actin in vitro,[35] little evidence has been reported to link Arc's potential association with the neuronal cytoskeleton to the receptor internalization phenotype. Using local infusion of *antisense* oligodeoxynucleotides directed against the *Arc/Arg3.1* message, Messaoudi and colleagues found that knockdown of *Arc/Arg3.1* mRNA after LTP induction in the dentate gyrus resulted in reversal of LTP,[36] in line with earlier reports demonstrating that *Arc/Arg3.1* expression is necessary for the expression of L-LTP and memory consolidation.[37] Knockdown of Arc/Arg3.1 message also led to increased dephosphoryation of cofilin and loss of filamentous actin at synapses, an effect that could be blocked by the F-actin stabilizing drug Jasplakinolide.[36] This data suggests that Arc's association with the cytoskeleton may be important for F-actin stabilization, spine growth and the expression of LTP, and may not be directly linked to its role in receptor internalization.

A new mechanistic link between CME and the F-actin cytoskeleton has recently been reported in the *Candidate plasticity*

gene 2 (*Cpg2*). *Cpg2*, a late response gene upregulated in the nervous system after elevated neuronal activity, has long been known to regulate glutamate receptor internalization in the postsynaptic compartment.[38] The CPG2 protein binds to F-actin through two coiled-coil regions in its C-terminus both in vitro and in vivo.[39] This interaction is critical for CPG2's localization to dendritic spines, and in particular to the endocytic zone, a specialized region for postsynaptic glutamate receptor internalization.[39,40] CPG2 binds F-actin in response to phosphorylation by PKA at two critical sites, S890 and S913, in the F-actin association domain. The phosphorylation status of these sites is instructive to CPG2 protein function: a phospho-incompenent double alanine mutant that abolishes binding to F-actin shows decreased receptor internalization reminiscent of overall CPG2 ablation by small hairpin RNAs.[39] These data show that structural and functional linking of the F-actin cytoskeleton to the endocytic machinery can be regulated by second messenger signaling, and that sensor molecules like CPG2 can integrate upstream signaling information to couple (or uncouple) both systems. Interestingly, knockdown of CPG2 leads to an accumulation of CCVs in the postsynaptic compartment,[38] suggesting that the interaction with the F-actin cytoskeleton is relevant to the later stages in the endocytic cycle, perhaps propulsion of the vesicle away from the plasma membrane and recruitment of uncoating factors such as synaptojanin. Clearly, further mechanistic insight can be gained from studying CPG2 and its endocytic machinery binding partners.

Taken together, recent advances in the field indicate that the F-actin cytoskeleton interacts with the process of CME in the nervous system in numerous ways, both in pre- and postsynaptic specializations. Given that the regulated display of glutamate receptors on the postsynaptic surface membrane is key for adjusting synaptic strength in several paradigms of synaptic plasticity, understanding the mechanics of this multifaceted interaction could prove crucial in decoding the underpinnings of central nervous system disorders that may have synaptic defects at their core.

Association of Postsynaptic Organization, Glutamate Receptor Trafficking and F-actin Remodeling with Neuropsychiatric Disorders

Neuropsychiatric disorders, including Autism Spectrum Disorders (ASDs), Bipolar Disorder (BD), and Schizophrenia (SCZ) have been known for a long time to be influenced by genetic risk factors without following Mendelian patterns of heritability. They are often co-morbid with each other or with other conditions, such as intellectual disability.[41-43] Rather than being caused by a few highly penetrant mutations, neuropsychiatric disorders appear to be associated with subtle defects in numerous genes, each conferring a small increase in disease risk. Recent advances in large scale exome sequencing and genome-wide association studies (GWAS) have identified numerous loci associated with elevated disease risk.[44-46] In addition, de novo mutations, predominantly large copy number variations (CNVs), have been identified as genetic risk factors. Although our understanding of the mechanisms underlying pathophysiology is rudimentary at best, groups of genes are beginning to emerge from these large scale human genetics studies. A significant body of evidence is pointing toward gene networks that regulate the biological processes of postsynaptic organization, glutamate receptor internalization and F-actin remodeling.

A recent study identified small de novo mutations (on the scale of single base pairs) in 623 SCZ trios and revealed that synaptic genes are significantly more affected by missense mutations than would be statistically expected.[47] In particular two sets of genes, named the NMDA receptor and Arc complexes, showed increased association with SCZ mutations, in line with earlier studies that also pointed toward synaptic genes implicated in SCZ[48] and BD.[49] Postsynaptic glutamate receptor trafficking in particular has moved into the focus of interest because several independent lines of evidence have implicated components of the protein machinery that regulates AMPA- and NMDA-receptors at the synapse. Lithium, the main-line treatment for BD, affects AMPA receptor trafficking,[50] and SNPs in *GRIA2*, which encodes the GluA2 subunit of AMPARs, have been associated with time to recurrence of mood episodes in BD patients on lithium.[51] Recently, the postsynaptic scaffold protein *SHANK* has been implicated in neuropsychiatric disease. In humans, de novo mutations in *SHANK2* have been associated with ASDs.[52] In addition, *SHANK3* mutant mice show deficits in social interaction and repetitive grooming, reminiscent of behavioral patterns seen in obsessive compulsive disorder (OCD) and ASDs.[53] A recent report used small interfering RNAs (siRNA) to knock down expression of Shank3 in cultured cortical rat neurons and found a significant reduction in NMDA receptor-mediated synaptic currents.[54] Interestingly, the effects of Shank3 knockdown on NMDAR currents were abolished in the presence of Phalloidin, and occluded when cells were treated with Latrunculin. The same study found that F-actin clusters were reduced in response to Shank3 knockdown and that pharmacological manipulation of the Rac1/PAK signaling cascade could mimic or occlude the effects on NMDAR currents. Together, these results suggest a mechanistic link in which the F-actin cytoskeleton regulates synaptic NMDA receptor currents through Shank. Similarly, another report used transgenic mice to model the effects of Shank3 gene duplication.[55] These transgenic mice exhibit manic-like behaviors reminiscent of some patients with a large duplication in chromosomal region 22q13, the area which – among other genes – harbors *SHANK3* in humans. The same study identified the Arp2/3 complex as a direct interactor of Shank3 and found increased F-actin levels in Shank3 transgenic mice, consistent with a role for Shank3 in regulating postsynaptic actin. An earlier study characterized both de novo and inherited mutations in Shank3 that are associated with ASD, and found that a truncating frame-shift mutation which leads to a premature STOP codon also affects dendritic spine morphology and synaptic transmission in mature cultured neurons.[56] Another de novo mutation, Q321R, leads to aberrant actin accumulation and spine morphology, further implicating the actin cytoskeleton in neuropsychiatric disorder.

In a meta-analysis of GWAS data for BD, markers in human *SYNE1* showed genome-wide statistically significant disease association ($P < 4x10^{-8}$),[46] a finding recently replicated in an independent sample.[57] The *SYNE1* gene has also been associated

with a severe de novo mutation identified by exome sequencing in sporadic cases of ASDs.[58] In addition, Yu and colleagues have found biallelic mutations in *SYNE1* by whole exome sequencing in consanguineous ASD.[59] *SYNE1* is a large gene with numerous annotated transcripts. In rats, it gives rise to various isoforms of Nesprin, Enaptin, and CPG2. The latter is crucial at the functional interface of glutamate receptor internalization with the F-actin cytoskeleton where it regulates AMPAR CME by integrating PKA signaling in spine heads.[39] Interestingly, the BD association signal spans, almost exclusively, parts of the human *SYNE1* gene that are homologous to the region encoding CPG2 in rat. Whether the autism-linked mutations fall within CPG2 transcripts, awaits mapping of the human *SYNE1/CPG2* locus and identification of full-length human *CPG2* transcripts.

Conclusions

In the past decade it has become increasingly clear that the process of CME is assisted at various stages by a dynamic F-actin cytoskeleton. Pioneering work in yeast has shaped our understanding of protein modules which depend differentially on a dynamic F-actin cytoskeleton. Because many endocytic factors in yeast have mammalian homologs, findings from yeast can help inform research in mammalian systems, where several steps in the CCV life cycle are assisted by actin polymerization or physical coupling to F-actin filaments. In neurons, where CME plays a critical role at both pre- and postsynaptic differentiations, second messenger signaling by PKA can directly influence postsynaptic glutamate receptor internalization by regulating the connection to the actin cytoskeleton via the *Syne1* product CPG2.

Interestingly, the human *SYNE1* gene has emerged as a genetic risk locus for BD and ASDs. Several other genes encoding key players at the synapse are also associated with neuropsychiatric disorders, pointing to the common underlying theme of postsynaptic organization, receptor internalization and F-actin remodeling. The challenge of the next decade will be to translate findings from human genetics into quantifiable effects on biological mechanisms, and to see if the disease association of these emerging pathways will stand the test of time.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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