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DISSERTATION

Synaptojanin and Endophilin Mediate Neck Formation During Ultrafast Endocytosis

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Neurons use and recycle synaptic vesicles continuously during synaptic transmission. The mechanisms of synaptic vesicle release and recycling have been a subject of much study. There are several known mechanisms of vesicle endocytosis, and recent work suggests that ultrafast endocytosis (UFE) occurs under physiologic conditions at a much faster timescale than previously thought. In this process, endocytosis of membrane occurs in a clathrin-independent manner on the order of 100ms in both C. elegans and mammalian mouse neurons. Following the retrieval of membrane, endocytosed vesicles fuse to an endosome, from which clathrin coated pits bud off, and regenerate synaptic vesicles within 30-300ms. In spite of its rapid time-scale, separation from the plasma membrane in UFE is dynamin-dependent and requires actin. The other proteins that are required to orchestrate the steps of UFE at its characteristic rapid speed are not yet known. Here we show that synaptojanin and endophilin play critical roles in allowing ultrafast endocytosis to achieve its rapid time-scale. Specifically, we show that endophilin and synaptojanin are each necessary for UFE to occur. In this manuscript, we go one step further to address these proteins' specific roles in this process. We show that UFE requires a functional 5-phosphatase domain but not Sac-1 domain of synaptojanin to occur at its characteristic rapid timescale and that endophilin is required for neck formation in UFE. We also show that endophilin A1 and A2 can both fill the endophilin requirement for UFE and reacidification. By using "flash-and-freeze" electron microscopy, we examine the trafficking of membranes in the absence or modification of endophilin and synaptojanin. Our fluorescence imaging experiments allow us to observe corresponding effects on protein trafficking and vesicle acidification. Taken together, our data are the first to suggest a specific role for synaptojanin and endophilin in UFE, and allow us to better understand this mechanism of vesicle recycling.

Während der synaptischen Übertragung werden kontinuierlich synaptische Vesikel verwendet und recycelt. Die dabei involvierten Mechanismen sind der Gegenstand vieler Untersuchungen und mehrere Mechanismen sind bereits bekannt. Jüngste Ergebnisse legen nahe, dass eine ultraschnelle Endozytose (UFE) unter physiologischen Bedingungen viel schneller auftritt als bisher angenommen. Bei diesem Prozess läuft die Endozytose in einer Clathrin-unabhängigen Weise innerhalb von grob 100ms ab, dies wurde sowohl in C. elegans und Säugetier-Mäuse-Neuronen gezeigt. Nach der Rückholung der Membran, fusionieren endozytierte Vesikel zu einem Endosom, vom dem sich Clathrin-beschichtete Pits ablösen und synaptische Vesikel innerhalb von 30-300ms regenerieren. Trotz des raschen Ablaufs dieses Prozesses ist die Trennung der Plasmamembran im Rahmen der UFE Dynamin-abhängig, und benötigt Aktin. Die anderen Proteine, die erforderlich sind, um die Schritte der UFE mit ihrer charakteristischen schnellen Geschwindigkeit zu orchestrieren, sind noch nicht bekannt. Wir zeigen, dass Synaptojanin und Endophilin einen entscheidenden Beitrag zu diesem raschen Ablauf der ultraschnellen Endozytose leisten. Insbesondere zeigen wir, dass sowohl Endophilin als auch Synaptojanin für das Auftreten von UFE notwendig sind. In diesem Manuskript gehen wir auf die spezifischen Rollen dieser Proteine im Prozess der UFE ein. Wir zeigen, dass UFE eine funktionelle 5-Phosphatase-Domäne, aber keine Sac-1-Domäne von Synaptojanin benötigt, um ultraschnelle Endozytose zu erlauben, und dass Endophilin für die Hals-bildung der Vesikel in UFE erforderlich ist. Wir zeigen auch, dass Endophilin A1 und A2 sowohl den Endophilin-Bedarf für UFE als auch für die Widerherstellung eines sauren Milieus innerhalb der Vesikel erfüllt. Mit der "Flash-and-Freeze" -Elektronenmikroskopie untersuchen wir den Austausch der Membranen in Abwesenheit, oder mit einer Modifikation, von Endophilin und Synaptojanin. Unsere Fluoreszenz-Bildgebungs-Experimente erlauben uns, entsprechende Auswirkungen auf den Proteinverkehr und die Ansäuerung der Vesikel zu beobachten. Unsere Daten weisen erstmals auf eine spezifische Rolle von Synaptojanin und Endophilin im Rahmen der UFE hin und erlauben ein besseres Verständnis der Mechanismen des Vesikel-recyclings.

Introduction:

My dissertation project was to investigate the roles of proteins involved in endocytosis in the regulation of exocytosis through release probability. Because of its rapid time-scale, ultrafast endocytosis (UFE) provides a temporal opportunity for crosstalk between vesicle release and recycling that was not previously understood to be possible. To better understand this relationship, it was first important to understand which proteins were involved in ultrafast endocytosis. One of the aims of my thesis project was to understand the roles of classical endocytic proteins in ultrafast endocytosis. This dissertation describes the methods and contributions of my recent co-first author publication investigating the roles of synaptojanin and endophilin in ultrafast endocytosis.

Why study vesicle recycling?

The temporal fidelity of signal transmission between neurons is critical to the function of the nervous system. The precise timing of transmission between synapses allows circuits of neurons to integrate information and ultimately allows an organism to interact with the world. The rapid time from action potential to release of vesicles is the product of an array of molecular machines that coordinate vesicle release. For the nervous system to continue to function, the synaptic vesicles must be resupplied at a rate that keeps pace with neurotransmission. This regeneration occurs by endocytosis, in which the plasma membrane must curve inward to form a bud, which then pinches off from the membrane in a coordinated manner, creating a vesicle inside the cell[1]. An understanding of the recycling process is just as critical as an understanding of synaptic release if we are to understand how the nervous system functions in health and disease.

Endocytosis occurs through the dynamic interactions of a network of specialized proteins. Two such proteins are endophilin and synaptojanin. Endophilin is one of a class of proteins that contain a region called a BAR-domain that allows them to participate in the generation of membrane curvature at the neck of budding endocytic vesicles, and in multiple steps during the endocytosis process. Endophilin's SH3 domain allows it to interact with dynamin, mediating scission from the cell membrane[2,3], and with synaptojanin at the clathrin-uncoating step[3–6]. Synaptojanin is a phosphoinositide phosphatase that has been shown to participate in the coupling of the separation of a clathrin-coated bud from the plasma membrane to the uncoating of clathrin[3,7]. It has been shown that both synaptojanin and endophilin are critical for the uncoating of clathrin vesicles in both *C elegans* and mouse[8–10]. Not only do they function in the same pathway, experiments in *drosophila* and *C elegans* have demonstrated that synaptojanin and endophilin work cooperatively in the endocytosis process[5,6,11].

A new mechanism of vesicle recycling: ultrafast endocytosis

Early experiments to study endocytosis suggested three candidate mechanisms: clathrin-mediated endocytosis, bulk endocytosis, and an abbreviated "kiss-and-run" mechanism[12]. Subsequent studies have shown evidence for each of these three mechanisms under different conditions, although there is significant overlap in the molecular players involved.

Clathrin-mediated endocytosis occurs when a protein called clathrin forms a coat around vesicles that guides membrane curvature and facilitates the budding off of

vesicles from the plasma membrane[1,12]. More intense stimulation paradigms have also shown evidence of a dynamin 1 and 3 independent bulk endocytosis, which retrieves membrane in large vesicles in the periactive zone and regenerates vesicles from an endosomal structure without requirement of clathrin [13–16]. Kiss and run endocytosis has also been proposed as a model for endocytosis in which a vesicle fuses with the membrane transiently at the active zone but retains its structure, alleviating the need for the assembly of the clathrin coat to regenerate vesicles [17,18].

Most recently, ultrafast endocytosis (UFE) has been shown at synapses at physiologic temperature as a mechanism for retrieving membrane in a dynamin and actin-dependent manner[19,20]. After endocytosis via UFE, large endosomal structures are formed, from which clathrin-coated buds regenerate synaptic vesicles on the order of seconds[19].

What do we know about UFE so far?

Prior to the publication discussed in this dissertation, ultrafast endocytosis had been observed, but its molecular mechanism and the proteins involved were not known. In UFE, endocytosis of “large endocytic vesicles” from the membrane occurs in a clathrin-independent manner on the order of 100ms. Following this, endocytosed vesicles fuse to an endosome, from which clathrin coated pits bud off, and regenerate synaptic vesicles by 6s after stimulation[19,21]. In spite of its rapid time-scale, scission from the plasma membrane in UFE is dynamin-dependent[21]. What sets this mechanism of endocytosis apart is the time scale over which it occurs and the location at which it occurs[21]. Experiments with low-noise capacitance measurements also support a fast pathway for clathrin-independent membrane

retrieval observed at physiologic temperature in cerebellar and hippocampal mossy fiber synapses[22]. While there are known proteins involved in these other mechanisms, the proteins required to orchestrate the steps of UFE at its characteristic rapid speed were not yet known.

Why endophilin and synaptojanin?

In this publication, we examined synaptojanin and endophilin specifically for their roles in UFE. As mentioned above, endophilin is a BAR containing protein that participates in endocytosis through its SH3 domain interaction with dynamin and the recruitment of synaptojanin [2,3,11]. In the absence of endophilin, endocytosis occurs more slowly and can only be rescued by constructs that contain a functional SH3 domain[3]. By recruiting synaptojanin, endophilin also plays a role in removing the clathrin coat from vesicles[3,11,23]. Two different phosphatase domains of synaptojanin have been shown to have different functions in the endocytic process, with the 5 phosphatase domain being more universally important for endocytosis, and the sac-1 phosphatase domain more important for endocytosis following short stimuli[24]. Work in mouse, *drosophila, and C elegans* has provided strong evidence that synaptojanin and endophilin are critical for the uncoating of clathrin vesicles, and that the two proteins must work cooperatively in this pathway.

Although endophilin and synaptojanin have established roles in other types of endocytosis, it is unclear what roles if any they would play in ultrafast endocytosis. Because synaptojanin and endophilin each play a role both at the plasma membrane and in clathrin uncoating in traditional endocytosis, they are particularly interesting candidates to investigate in the context of UFE, in which the steps of membrane

endocytosis and clathrin mediated vesicle bud formation are separated in both time and space[21].

Methodology

Overview:

Leading up to this work, the following the following timeline was established for ultrafast endocytosis following a stimulus: endocytic pits with a neck on the plasma membrane (75ms) progress into large vesicles at the edge of active zone (100ms). These vesicles then fuse into an endosome (1s), from which clathrin coated pits bud off (3s), and the clathrin coat falls off, ultimately regenerating uncoated synaptic vesicles by 6s after stimulation[19,21]. By manipulating temperature and pharmacology, we know that this process happens more readily at physiologic temperature, and that it depends upon both actin and dynamin[21]. What sets this mechanism of endocytosis apart from others is its time scale and location at the periactive zone[21]. In this project, we used the following general approach: neurons cultured from transgenic mouse lines for proteins of interest were infected or “rescued” by infection with lentivirus carrying a version of the protein with a targeted mutation. The “rescued” neurons were examined with “flash-and-freeze” electron microscopy[21], pH-sensitive imaging[25], and electrophysiology to understand the effect of protein absence or modification on ultrafast endocytosis.

Flash and freeze:

The electron microscopy method used in this publication is called “flash-and-freeze” electron microscopy. Originally published in 2013[20], the technique uses a custom-built controller to precisely coordinate the timing of a flash of LED light with the

delivery of high pressure liquid nitrogen to a sample chamber. This allows a sample to be frozen at a very specific interval following exposure to light. When neurons are then infected with a virus expressing channelrhodopsin, neurons can be stimulated to release vesicles by a flash of light and then frozen at precise time intervals afterward. When samples from each freezing are then processed and examined under electron microscopy, they provide a series of snapshots of the vesicle dynamics following a stimulus. These snapshots can be examined in the presence or absence of each protein and each specific residue by manipulating the genetic background, and lentiviral transfection of the neurons in the sample.

Manipulation of Synaptojanin and Endophilin genes in neurons:

Here we use knockout transgenic mouse lines and lentiviral rescue experiments in mouse hippocampal neurons to examine the roles of synaptojanin and endophilin in ultrafast endocytosis. For synaptojanin experiments, synaptojanin 1 heterozygous mice[8] were crossed to generate offspring with wildtype and knockout phenotype. For endophilin experiments, a conditional triple knockout for the three endophilin genes was used with the following features: Endophilin A1 knockout, Endophilin A2 heterozygous, and Endophilin A3 cre-inducible conditional knockout[3]. Offspring of the desired genotypes were obtained at postnatal day 0 for synaptojanin and embryonic day 18 for endophilin. The hippocampi were dissected and primary neuronal cultures were created as previously described[26].

Lentiviral infection is an established method in our laboratory for expressing modified or rescue proteins in primary neuronal cultures. As previously published, lentiviral vectors were prepared in our laboratory and infection was performed at 48-72 hours

after plating primary neurons[26,27]. By combining the techniques of lentiviral infection with primary cultures from transgenic mice, we were able to add and subtract different residues of endophilin and synaptojanin to the neurons, and examine the effects of these manipulations in the early timepoints after stimulation. The rescue viruses combined with the genotypes of synaptojanin and endophilin neurons allowed us to examine four different groups for synaptojanin and five groups for endophilin. These groups are detailed in Table 1.

Table 1: Genotypes and experimental groups

Genotype	Virus	Outcome
Synaptojanin 1 het x Synaptojanin 1 het		
Synaptojanin 1 KO	Synaptojanin 1 full length	Complete Rescue
Synaptojanin 1 KO	Synaptojanin 1 5 phos dead	Rescued synaptojanin with defective 5 phosphatase residue
Synaptojanin 1 KO	Synaptojanin 1 Sac 1 dead	Rescued synaptojanin with defective Sac 1 residue
Synaptojanin 1 KO	Control virus	Complete KO
Synaptojanin 1 WT	Control virus	Complete WT synaptojanin function
Endophilin A1 KO, A2 het, A3 cKO x Endophilin A1 KO, A2 het, A3 cKO		
Endo A1 KO, A2 KO, A3 cKO	Cre	Endophilin A1, 2, 3 TKO
Endo A1 KO, A2 KO, A3 cKO	Control	Endophilin A1, 2 DKO
Endo A1 KO, A2 KO, A3 cKO	Endophilin A1	Endophilin A2 KO
Endo A1 KO, A2 WT, A3 cKO	Control	Endophilin A1 KO
Endo A1 KO, A2 WT, A3 cKO	Endophilin A1	Endophilin A1, 2, 3 Full rescue

The synaptojanin lentivirus constructs are based on the 145kD mouse synaptojanin transcript. Table 2 contains a list of viruses used along with a brief description. Except for the endophilin A1 virus, all viruses contained GFP or RFP, to facilitate identification of infected cells for electrophysiology and live cell experiments. The use of a visible marker further allowed monitoring of successful viral infection in the culture prior to experiments.

Table 2: Virus List

BL 347	Channel Rhodopsin, see [19]
BL 116	Synaptophysin pHluorin, see [27]
BL 829	Full length synaptojanin 145
BL 844	Synaptojanin 145 D730A (Sac-1 dead)
BL 845	Synaptojanin 145 D730A (5-phosphatase dead)
BL 140	Control- GFP only
BL 150	Cre (for cre-inducible endophilin 3 conditional knockout)
HL 203	Endophilin A1 for rescue of Endophilin A1 knockout

pH-sensitive Fluorescent Imaging:

Flash-and-freeze electron microscopy allows us to observe events at the nerve terminal in a time-window following exocytosis that we have not been able to visualize before. However, electron microscopy provides a static image of a dynamic process. To examine the roles of synaptojanin and endophilin in living cells, we used an imaging technique that relies on a pH-sensitive fluorescent protein on the inside of the synaptic vesicle [25]. Since the synaptic vesicle is an acidified environment, the fluorophore is exposed to acidic pH (and therefore quenched) until that vesicle is exocytosed, and it is exposed to neutral pH, able to shine, and able to be detected by microscopy. The pH-sensitive protein in our system is conjugated to the synaptic vesicle protein, synaptophysin, and neurons are infected with a lentivirus that allows this protein to be expressed. By expressing the pH-sensitive indicator in knockout and rescued neurons, and subjecting them to field stimulation, we were able to observe the effects of these mutants on endocytosis in real time, complementing our electron microscopy data.

Temperature control:

Previous work has shown that ultrafast endocytosis occurs preferentially at near-physiologic temperatures (35-37C)[21]. Accordingly, our fluorescence imaging and electrophysiology experiments were also performed at near-physiologic temperature to favor UFE-compatible conditions. Temperature control was maintained via the extracellular solution flow-delivery system, using a custom-built flow-pipe wrapped with copper wire and connected to a commercially available temperature controller[28]. Temperature during experiments was verified by spot-checking bath temperature throughout experiments.

Whole-cell voltage clamp:

Neurons from endophilin and synaptojanin knockout mice as well as their rescues were plated as autaptic cultures in parallel with imaging experiments. The fact that the lack of functional endocytic proteins has repercussions for synaptic transmission is well documented. When the endocytic protein dynamin is disrupted in a living fly, continued stimulation results in the ultimate failure of exocytosis leading to paralysis[29]. Neurons lacking all three isoforms of the membrane-curving protein endophilin show markedly reduced spontaneous and evoked activity[3]. In neurons lacking the *drosophila* homolog of the clathrin adaptor protein AP180, repeated stimulation led to the complete depletion of the end junctional potential in 5 minutes[30].

We also performed electrophysiologic recordings of knockout and rescued neurons were recorded shown in supplementary figures S2 (Synaptojanin) and S3 (endophilin) of the publication. These recordings are consistent with previously

published electrophysiology data from these and related transgenic lines, and illustrate that the neurons measured in imaging experiments are indeed functional and consistent with existing literature.

Significant Results

Our publication provides the first insight into the molecular players in UFE. We show that endophilin and synaptojanin are required for UFE to occur. Some of the specific results detailed in our publication are highlighted below.

First, we show that UFE requires synaptojanin-1 and endophilin to achieve its native time course. In the synaptojanin knockout neurons, endocytic pits formed at around 100 ms after stimulus, at almost the same rate as in wildtype neurons, however, they rarely resolved into large endosomes (Fig 1c,d) the next step in the process. Furthermore, the synaptojanin 1 knockout neurons have shallower endocytic pits at 100ms (Fig 1e), suggesting that the ultrafast endocytosis process is stalled. The fact that these neurons still have a buildup of clathrin-coated vesicles at rest (Fig 1h), suggests that UFE is delayed, but not abolished.

We also examined the role for the 5-phosphatase and sac-1 phosphatase residues of synaptojanin, by rescuing the synaptojanin 1 knockout neurons with wildtype synaptojanin and the two mutants. To track the fate of vesicles taken up immediately following the stimulus, we applied cationized ferritin to the extracellular solution during the stimulus (Fig 2). In the full-length synaptojanin and sac-1 mutated versions of synaptojanin, ferritin was found in endosomes and large endocytic vesicles at 1s after stimulation (Fig 2 b, f, h). At 1 second after stimulation, neurons

rescued with the synaptojanin with a defective 5 phosphatase domain of synaptojanin-1, showed most of the ferritin in endocytic pits at 1 second, (Fig 2 i,j). Since endocytic pits are an earlier step in UFE, we show that the 5-phosphatase domain is critical for prompt endocytic neck formation in ultrafast endocytosis.

Interestingly, a buildup of clathrin-coated vesicles (Fig 2e-h) was observed in the neurons rescued with synaptojanin without a functional sac 1 or 5-phosphatase domain. This suggests that the phosphatase activity at both residues is required for clathrin uncoating of vesicles after they bud off of the endosome. The sac-1 requirement may also be secondary to the 5-phosphatase requirement due to the way in which phosphate groups need to be removed from PI(4,5)P. Interestingly, the same clathrin-coated vesicle phenotype was observed in endophilin DKO and TKO neurons (Fig 5j), suggesting that both proteins may be required for vesicle uncoating.

In the endophilin knockouts, we showed that neurons lacking a single endophilin isoform had the same number of endocytic structures internalized at 100 ms as in the wildtype (Fig 4 a-d), while neurons lacking both endophilin 1 and 2, or all three endophilins, did not show any structures that had endocytosed and separated from the membrane at 100ms (Fig 4e-h). From these results, we suggest that endophilin 1 and 2 can each mediate the role of endophilin during UFE, and that endophilin 3 is not a critical player in this process. For the Endophilin 1,2 DKO and Endophilin 1,2,3 TKO mutants, the resolution of endocytic pits was delayed past 1-3 seconds (Fig 5B,C, E,F). In keeping with endophilin's function in other forms of endocytosis in

endocytic neck formation, the necks of the endocytic pits in Endo DKO and TKO neurons were wider than in controls (Fig 5B, C).

Fluorescence imaging was used in our experiments to correlate membrane movement via ultrafast endocytosis with protein movement. It has been shown in prior work with ultrafast endocytosis that short trains of action potentials provoke ultrafast endocytosis in multiple rounds[19]. In our pHluorin experiments, we used short trains of action potentials, first 20AP at 10Hz and ultimately 75AP at 10Hz to increase the signal to noise ratio (Fig 3a-d). We observed a slowing of the decay of the pHluorin signal in synaptojanin 1 mutants that agrees with previously published studies [24]. We observed that fluorescence quenching was significantly slowed in the synaptojanin knockout neurons and neurons rescued with synaptojanin with a defective 5-phosphatase (Fig 3e,g). We also showed that in the presence of defective Sac 1 phosphatase but normal 5 phosphatase, the rate of fluorescence decay was somewhat improved (Fig 3f, i). This may result from the preserved ultrafast endocytosis observed in our electron microscopy experiments. The defect in reacidification at 30 seconds (Fig 3i) correlates with a deficit in clathrin uncoating (Fig 3j), and may reflect a connection between these two processes.

Our experiments using pHluorin in the endophilin mutant and rescued neurons underscore the extent to which endophilin A1 and A2 may substitute for each other (Fig 6a, 6b), as they are both able to support normal kinetics of reacidification. The double knockout and triple knockout of endophilin, however, had significantly impaired reacidification (Fig 6 f,g), again correlating with the buildup in number of

clathrin coated vesicles observed in our electron microscopy experiments, suggesting a potential connection between reacidification and clathrin uncoating.

The information gained in this study about the roles of synaptojanin and endophilin represents a new level of detail in our understanding of ultrafast endocytosis and clathrin-independent modes of endocytosis.

Clinical applications: Synaptic vesicle endocytosis and pathology

The intuitive clinical relevance of this work is that to understand pathophysiology of the brain, we must first understand normal physiology. Beyond this, synaptojanin and endophilin have also been explored specifically as mediators of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.

Synaptojanin 1 has been investigated for its connections to neurologic diseases because it is critical for survival and it is enriched in the brain, specifically at synapses[8]. It is an important player in Down's Syndrome, Alzheimer's Disease, and Parkinson's Disease. The role for synaptojanin 1 in Down's Syndrome and Alzheimer's disease is hypothesized to center around the 5-phosphatase activity of synaptojanin 1, which is important for hydrolyzing $PI(4,5)P_2$ to $PI(4)P$[31–33]. Interestingly, this same 5-phosphatase activity is required for efficient ultrafast endocytosis to occur in our experiments.

In patients with Down's Syndrome, levels of synaptojanin 1 are increased, likely because synaptojanin is present on the part of chromosome 21 that is present in triplicate[31]. In a mouse model of Down's Syndrome, it was shown that synaptojanin 1 levels are elevated, and that a reduction in copy number of synaptojanin 1 (and

therefore decrease in synaptojanin 1 levels) was sufficient to rescue the behavioral phenotype observed in these mice[33].

In Alzheimer's disease, the putative role for synaptojanin 1 is not as a disease causing protein so much as a therapeutic target[32]. Here too, the $PI(4,5)P_2$ level seems to correlate inversely with Aβ42 load as well as for some of the presenilin associated defects in ion channel conductance observed in cellular models[32]. Because of the significance of $PI(4,5)P_2$, the focus of synaptojanin 1 as a therapeutic target would be on the inhibition of the 5 phosphatase domain. Indeed, it was shown that expression of the 5 phosphatase domain of synaptojanin 1 increased Aβ42 levels in vitro[32].

The role for synaptojanin 1 in Parkinson's Disease, on the other hand, centers around the Sac1 domain and may indeed involve synaptic vesicle endocytosis process as a whole. Mutations of synaptojanin 1 have been found in several familial cases of Parkinson's Disease in three families with the same point mutation in the Sac1 domain. Each patient was homozygous for the mutation, and present with an atypical early onset Parkinson's Disease that shares several common features[34].

More recently, endophilin A1 has been identified by GWAS meta-analysis as a "risk-gene" for Parkinson's Disease, broadening attention from synaptojanin to synaptic vesicle endocytosis as a whole[35] as a mechanism of pathophysiology of neurodegenerative disease. The putative mechanism is that by slowing the process of synaptic vesicle endocytosis, vesicle contents build up in the synaptic terminal, which can be toxic to neurons[36]. Interestingly, work with mice heterozygous for

synaptojanin 1 has suggested that dopaminergic neurons in the heterozygous state are at increased risk for degeneration compared with glutamatergic neurons.

In conclusion, there is evidence to suggest that synaptojanin may indeed be involved in the pathogenesis of several neurologic diseases. The new data regarding the role of endophilin in regulating Parkinson's risk may suggest a mechanism for the possible involvement of endocytosis in PD pathogenesis. The precise mechanism of endocytosis that increases the risk of PD is still unknown, and these findings underscore the need for further investigation.

Further scientific work:

To explore the timecourse of ultrafast endocytosis and its component proteins, we used a light-stimulus with LED stimulation of channel rhodopsin. Although we have good control experiments[24] to support that our LED stimulus predictably triggers synaptic vesicle release, this is clearly not a physiologic stimulus. Neurons are connected through action potentials and are designed to transmit and integrate electrical signals. Future work will need to examine synaptic vesicle release and recycling in a short timescale with an electrical stimulus rather than a physiologic one.

As mentioned above, some studies have suggested that dopaminergic neurons are particularly susceptible to the damage caused by the disruption of functioning endophilin and synaptojanin. The experiments in this publication are all performed with neuronal cultures from hippocampus enriched in glutamatergic neurons. Further experiments will be required to determine the roles of synaptojanin, endophilin and

indeed ultrafast endocytosis in general in different brain regions or subtypes of neurons. Although we have made progress in identifying the molecular players, the mechanism of ultrafast endocytosis is still not fully understood. Further work to manipulate binding partners of synaptojanin and endophilin as well as modifying and quantifying PI(4,5)P2 directly would also make important further steps for this work.

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Detailed Declaration of Contribution:

Lauren Mamer (doctoral candidate) had the following share in the following publication:

Publication: Watanabe, S*, Mamer, LE*, Raychaudhuri, S, Luvsanjav, D, Eisen, J, Trimbuch, T, Söhl-Kielczynski, B, Fenske, P, Milosevic, I, Rosenmund, C. "Synaptojanin and Endophilin Mediate Neck Formation during Ultrafast Endocytosis." Neuron **98**, 1184-1197 (2018).

Design of the experiments:

The experiments were designed by Watanabe, S, Mamer, L, Rosenmund, C, and Jorgensen, E.

Performance of the experiments:

Culture preparation from timed breeding: Mamer, L and Fenske, P (Fig:1-6, S1-5)
Fluorescence imaging experiments: Mamer, L (Fig 3, Fig 6, S2)
Flash and freeze experiments: Watanabe, S, Söhl-Kielczynski, B, Mamer, L, Fenske, P (Fig 1, 2, 4, 5, S1-5)
Electrophysiology: Mamer, L (Fig S2, S3)

Analysis of the experiments:

pHluorin imaging: Mamer, L (Fig 3, Fig 6, S2, S3)
Electrophysiology: Mamer, L (Fig S2, S3)

Writing and editing the publication:

The publication was written and edited by: Watanabe, S, Mamer, L, Jorgensen, E, and Rosenmund, C.

Signature, date, and stamp of supervising university professor:

Christian Rosenmund

Signature, date, of doctoral candidate:

Lauren Mamer

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15	ANNALS OF NEUROLOGY	37,259	10.250	0.053000
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17	REVIEWS	24,279	8.037	0.048000
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19	NEURAL NETWORKS	10,086	7.197	0.015000
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29	METABOLISM	19,450	6.045	0.028000
30	JOURNAL OF NEUROSCIENCE	176,159	5.971	0.266000
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34	Neurotherapeutics	3,973	5.719	0.009000
35	PAIN	36,132	5.559	0.038000
36	NEUROIMAGE	92,719	5.426	0.152000
37	Acta Neuropathologica Communications	2,326	5.414	0.012000
38	JOURNAL OF PSYCHIATRY & NEUROSCIENCE	2,989	5.365	0.005000
39	Multiple Sclerosis Journal	10,675	5.280	0.022000
40	NEUROBIOLOGY OF DISEASE	16,259	5.227	0.031000
41	Journal of Neuroinflammation	9,761	5.193	0.025000
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43	SLEEP	20,547	5.135	0.026000
44	MOLECULAR NEUROBIOLOGY	10,183	5.076	0.023000
45	NEUROENDOCRINOLOGY	4,670	5.024	0.005000
46	Alzheimers Research & Therapy	2,192	5.015	0.008000

Publication:

Please see published work:

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Schotten, S, Meijer, M, Walter, AM, Huson, V, **Mamer, L**, Kalogreades, L, ter Veer, M, Ruiter, M, Brose, N, Rosenmund, C, Sørensen, JB, Verhage, M, Cornelisse, LN. “Additive effects on the energy barrier for synaptic vesicle fusion cause supralinear effects on the vesicle fusion rate.”Elife **4**, e05531, 2015.

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