Genetic Analysis of Synaptogyrin Function in the Synaptic Vesicle Cycle

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ABSTRACT

Neuronal communication relies on the continual replenishment of synaptic vesicles that are primed for neurotransmitter release in response to action potentials. A vast array of proteins is required to mediate synaptic vesicle biogenesis, trafficking, docking, exocytosis, and endocytosis. Synaptogyrin and synaptophysin are abundant and evolutionarily conserved synaptic vesicles proteins that were identified over twenty years ago, yet their exact function in the synaptic vesicle cycle remains unknown. To further elucidate the role of these proteins, we have generated and characterized a *synaptogyrin* null mutant in *Drosophila*, whose genome encodes a single synaptogyrin isoform and lacks a synaptophysin homolog. Here we demonstrate that *Drosophila* synaptogyrin is abundantly expressed in neurons, where it localizes to the presynaptic terminal of the larval neuromuscular junction (NMJ). *Drosophila* lacking synaptogyrin are viable and fertile and have no overt deficits in motor function or courtship behavior. Ultrastructural analysis of mutant larvae revealed an increase in average synaptic vesicle diameter as well as enhanced variability in the size of synaptic vesicles. In addition, the resolution of endocytic cisternae into synaptic vesicles in response to robust exocytosis is defective in *synaptogyrin* mutants. While basal synaptic transmission at the larval NMJ is unaffected, *synaptogyrin* mutants do display increased facilitation during high-frequency stimulation, indicating that synaptic vesicle exocytosis is abnormally regulated during strong stimulation conditions. These results suggest that, while not required for neurotransmission, *Drosophila* synaptogyrin nevertheless modulates synaptic vesicle exo-endocytosis, especially during elevated rates of synaptic vesicle fusion.

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Chapter 1

Synaptogyrin, Synaptophysin, and The Synaptic Vesicle Cycle

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Introduction

One of the early debates in the field of neurobiology centered on the nature of synaptic transmission, namely whether communication between neurons was conducted via chemical or electrical means (reviewed in (Eccles, 1982)). Key discoveries, including the identification of a synaptic delay and the detection of synaptic inhibition using intracellular recordings, eventually demonstrated that chemical transmission occurs at a majority of synapses, while gap junction-mediated electrical transmission is less common (Kuffler, 1942b, a; Brock et al., 1952). It has since been established that chemical neurotransmitters are stored in synaptic vesicles and are released in discrete packets upon calcium entry into the presynaptic terminal. Although small in size and simple in appearance on electron micrographs, synaptic vesicles are complex organelles containing dozens of proteins that are responsible for the targeting, fusing, and recycling of these vesicles (reviewed in (Sudhof, 2004)). Among these proteins are synaptophysin and synaptogyrin, two evolutionarily related proteins that were identified as synaptic vesicle components over twenty years ago. Despite several decades of research and numerous hypotheses about their functions, exactly how synaptophysin and synaptogyrin participate in the synaptic vesicle cycle remains unknown.

The discovery of synaptic vesicles

Early evidence supporting the existence of synaptic vesicles came from seminal work by Bernard Katz and colleagues, who were studying synaptic transmission at the frog neuromuscular junction (NMJ). Fatt and Katz (1951, 1952) noticed the existence of small, spontaneous membrane depolarizations on the order of 0.5-1.0 mV that occurred

in the absence of nerve stimulation and did not require extracellular calcium. These events were subsequently named spontaneous miniature end-plate potentials (mEPPs), to distinguish them from the large stimulation-evoked responses known as end-plate potentials (EPPs). Interestingly, as the calcium concentration in the saline bath was reduced, the size of an EPP declined until its amplitude reached that of a mEPP (Fatt and Katz, 1952; Del Castillo and Katz, 1954b). At sufficiently low calcium levels, many stimuli completely failed to depolarize the muscle, while those stimuli that succeeded resulted in postsynaptic responses that were integral multiples of the average mEPP amplitude (Fatt and Katz, 1952; Del Castillo and Katz, 1954b). These observations were the foundation of the quantal hypothesis of synaptic transmission, namely that discrete packets (quanta) of neurotransmitter are released with a given probability in response to an action potential, with each quantum contributing about 0.5-1.0 mV to the total EPP amplitude (Del Castillo and Katz, 1954a, b).

Ultrastructural evidence in support of the quantal hypothesis arrived shortly thereafter with the discovery of synaptic vesicles through studies utilizing electron microscopy (De Robertis and Bennett, 1955; Palay and Palade, 1955). These structures were abundant, homogeneous, and situated at the presynaptic terminal, making them ideal candidates for the neurotransmitter-containing packets predicted by the quantal hypothesis (Fatt and Katz, 1952). Studies using freeze-fracture techniques in conjunction with electron microscopy were able to capture the extremely rapid release of synaptic vesicles and thereby correlate synaptic vesicle exocytosis with nerve stimulation (Heuser et al., 1974; Heuser et al., 1979). Moreover, the development of purification techniques to isolate presynaptic nerve terminals (synaptosomes) and

synaptic vesicles allowed for more in-depth analysis of these organelles and their protein and lipid components (De Robertis et al., 1962; Gray and Whittaker, 1962; Whittaker et al., 1964). Despite these technical advances, it took decades to isolate and clone the first integral synaptic vesicle protein – synaptophysin (Jahn et al., 1985; Wiedenmann and Franke, 1985; Buckley et al., 1987; Leube et al., 1987; Sudhof et al., 1987). Since the discovery of synaptophysin, dozens of proteins associated with synaptic vesicles have been identified and characterized, making the synaptic vesicle one of the best-described intracellular organelles (Jahn and Sudhof, 1994; Sudhof, 2004).

The synaptic vesicle cycle

Calcium-dependent exocytosis is a highly regulated process, requiring the coordination of a wide array of proteins. Synaptic vesicles must first traffic to specialized release sites known as active zones, where a subset of vesicles dock and are primed for exocytosis (reviewed in (Sudhof, 2004)). During docking and priming, the vesicular v-SNARE synaptobrevin interacts with the plasma membrane t-SNARE syntaxin and SNAP-25 to form the core SNARE complex (Sollner et al., 1993). The association of these proteins results in the formation of a tight, four-helix bundle that brings the vesicle and plasma membrane into close proximity and is thought to assist in overcoming the energetic requirements necessary for vesicle fusion (Hanson et al., 1997; Sutton et al., 1998; Sorensen et al., 2006; Li et al., 2007). However, unlike most other forms of intracellular vesicle trafficking, the formation of the synaptic vesicle SNARE complex does not automatically promote full membrane fusion. Rather, the complex is held in a partial, primed state of fusion until an action potential reaches the

presynaptic terminal and calcium entry occurs through voltage-gated calcium channels. Synaptotagmin, as the major calcium sensor in the presynaptic terminal, plays a critical role in coordinating calcium influx with synaptic vesicle fusion (Geppert et al., 1994; Fernandez-Chacon et al., 2001). Furthermore, the protein complexin associates with the intact SNARE complex (McMahon et al., 1995), and while its exact exocytic function is still being elucidated, complexin has been shown to both inhibit and facilitate synaptic vesicle fusion depending on the model organism and assay used (Reim et al., 2001; Giraudo et al., 2006; Huntwork and Littleton, 2007; Xue et al., 2007; Cho et al., 2010).

After synaptic vesicles have fused with the presynaptic plasma membrane in response to an action potential, synaptic vesicle proteins and lipids are retrieved through one of three endocytic pathways (Figure 1). First, they may be endocytosed by way of traditional clathrin-mediated endocytosis, often at locations somewhat removed from the sites of exocytosis (Heuser and Reese, 1973; Gad et al., 1998; Slepnev and De Camilli, 2000; Murthy and De Camilli, 2003). Second, synaptic vesicles and their components may be recycled intact through a mechanism called “kiss-and-run” endocytosis, which is proposed to result from the direct fission of a synaptic vesicle immediately following neurotransmitter release and prior to full collapse of the vesicle into the plasma membrane (Ceccarelli et al., 1973; Breckenridge and Almers, 1987; Fesce et al., 1994; Valtorta et al., 2001). Finally, multiple synaptic vesicles may bud from large endocytic cisternae that are formed through a mechanism known as bulk endocytosis (Richards et al., 2000; Evans and Cousin, 2007; Cousin, 2009). Regardless of the endocytic pathway used to regenerate a synaptic vesicle, once it has formed it

must be refilled with neurotransmitter, which is accomplished by various transporters that utilize an electrochemical gradient generated by a vacuolar proton pump (Maycox et al., 1988; Sudhof, 2004). After a synaptic vesicle has been refilled with neurotransmitter, it is ready to re-enter one of several synaptic vesicle pools, and this is discussed in more detail below.

Clathrin-mediated vs. kiss-and-run endocytosis

Efficient synaptic vesicle endocytosis is necessary to maintain a pool of synaptic vesicles that can continually sustain neurotransmission. Early ultrastructural analysis led to two alternative hypotheses as to the nature of synaptic vesicle retrieval. One hypothesis proposed by Heuser and Reese stated that synaptic vesicles fully collapse into the plasma membrane during neurotransmitter release and are later retrieved via clathrin-coated vesicles (1973). Ceccarelli and colleagues suggested a different mechanism in which synaptic vesicles directly reform following release without proceeding through a clathrin-coated intermediate (1973). This method of vesicle exo-endocytosis was later named "kiss-and-run" and proposes the formation of a narrow fusion pore that allows for transient neurotransmitter release and prevents significant mixing of membrane lipids during exocytosis (Fesce et al., 1994; Stevens and Williams, 2000). Kiss-and-run vesicle retrieval provides certain theoretical advantages over traditional clathrin-mediated endocytosis, namely that vesicles could recycle more quickly and they could retain a full complement of synaptic vesicle proteins and lipids without having to undergo any subsequent sorting steps. Some studies suggest a modified version of kiss-and-run called "kiss-and-stay," in which vesicles remain apposed to the plasma membrane and are therefore rapidly available for re-use once

they have been reloaded with neurotransmitters (Pyle et al., 2000; Stevens and Williams, 2000; Sudhof, 2000).

While evidence in support of clathrin-mediated endocytosis at synapses is convincing, the existence and relative contribution of kiss-and-run endocytosis in neurons remains under intense debate (Valtorta et al., 2001; He and Wu, 2007; Rizzoli and Jahn, 2007). This dispute has focused specifically on synaptic vesicles because there is widely accepted evidence in support of kiss-and-run endocytosis in large secretory cells such as mast and chromaffin cells. In these cell types, capacitance measurements and amperometric recordings can detect the rapid opening and closing of fusion pores, which can result in the partial discharge of secretory transmitters (Fernandez et al., 1984; Oberhauser and Fernandez, 1996; Ales et al., 1999; Wang et al., 2003). However, demonstrating the existence of kiss-and-run endocytosis at synapses is more difficult since it relies on indirect methods of observation that can lead to conflicting interpretations (He and Wu, 2007). For example, two groups examining mutations of the endocytic protein endophilin at the *Drosophila* neuromuscular junction observed the same electrophysiological phenomenon, namely that sustained synaptic transmission was dramatically inhibited (~80-90%) in the *endophilin* mutant, although a low level of synaptic vesicle release was maintained indefinitely (Verstreken et al., 2002; Dickman et al., 2005). One group (Verstreken et al., 2002) attributed this residual fusion to kiss-and-run endocytosis, while the other (Dickman et al., 2005) concluded that clathrin-mediated endocytosis was crippled but not entirely abolished in the *endophilin* mutant. Work in hippocampal cells suggests that clathrin-mediated endocytosis is the dominant endocytic pathway under mild stimulation conditions, as

the inhibition of this pathway using RNA interference against clathrin or the overexpression of a dominant-negative clathrin accessory protein (AP-180) resulted in a complete loss of endocytosis (Granseth et al., 2006). A more recent study used the FlAsH-FALI technique to photoinactivate clathrin and found that complete inactivation of clathrin-mediated endocytosis resulted in a total loss of synaptic vesicles, again arguing against a clathrin-independent version of kiss-and-run endocytosis (Heerssen et al., 2008). These studies suggest that, even if kiss-and-run endocytosis does occur under certain circumstances at these synapses, clathrin-mediated endocytosis is still the dominant synaptic vesicle retrieval mechanism under most physiological stimulation conditions (although see the discussion on bulk endocytosis below).

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis of synaptic vesicles is analogous to other ubiquitous forms of intracellular trafficking that involve clathrin-coated vesicles (Brodin et al., 2000). Heuser and Reese originally proposed that clathrin-mediated endocytosis of synaptic vesicles occurred at distinct sites from exocytosis (1973), and indeed, at certain synapses such as the *Drosophila* NMJ, proteins associated with clathrin-mediated endocytosis appear to concentrate in specialized “endocytic zones” that surround active zones (Gad et al., 1998; Roos and Kelly, 1999; Dunaevsky and Connor, 2000; Rizzoli and Jahn, 2007). Clathrin-mediated endocytosis is initiated when a clathrin triskelion interacts with adaptor proteins on the plasma membrane to form a protein lattice that covers the invaginating vesicle (Smith and Pearse, 1999). At the synapse, the assembling coat relies on adaptor-binding protein 2 (AP-2) to link clathrin to the membrane (Slepnev and De Camilli, 2000). AP-2 in turn associates with proteins

(such as synaptotagmin 1) that contain tyrosine-based targeting sequences and certain acidic phospholipids such as phosphoinositides, thereby linking clathrin coat formation with specific synaptic vesicle proteins and lipids (Gaidarov and Keen, 1999; Haucke and De Camilli, 1999; von Poser et al., 2000).

While purified clathrin and AP-2 alone can form cages that resemble clathrin coats, many accessory proteins help mediate clathrin assembly and disassembly and can thereby dramatically affect endocytosis at the synapse (reviewed in (Hirst and Robinson, 1998; Slepnev and De Camilli, 2000)). For example, synaptic vesicle size is regulated at least in part by the neuronal clathrin adaptor protein AP-180, as the loss of this protein in *Caenorhabditis elegans* or *Drosophila* causes an increase in synaptic vesicle size and heterogeneity (Zhang et al., 1998a; Nonet et al., 1999). Similarly, *in vitro* clathrin assembly assays result in smaller, more homogeneous vesicles in the presence of AP-180 (Ye and Lafer, 1995). Misregulation of synaptic vesicle size can directly impact synaptic transmission, as quantal size is larger and more variable in the *Drosophila* AP-180 mutant (also known as *lap*) (Zhang et al., 1998a). AP-180 appears to promote clathrin coat assembly through interactions with both clathrin and phosphoinositides (Ahle and Ungewickell, 1986; Hao et al., 1997), and may also play a role in targeting synaptobrevin, as this protein is mislocalized in the *C. elegans* knockout (Nonet et al., 1999).

Another important clathrin accessory protein is synaptojanin, a poly-phosphoinositide phosphatase that localizes to coated endocytic vesicles (McPherson et al., 1996; Haffner et al., 1997). Synaptojanin plays a critical role in controlling the phosphorylation state of phosphoinositides such as polyphosphatidylinositol-4,5-

bisphosphate (PI(4,5)P_2), a phospholipid that is thought to promote endocytosis via interactions with other endocytic proteins such as synaptotagmin, AP-2, AP-180, and clathrin (Takei and Haucke, 2001). Like AP-180, synaptojanin appears to directly or indirectly regulate synaptic vesicle size, as synaptic vesicle diameter is increased in the *Drosophila* mutant (Dickman et al., 2005). The loss of synaptojanin also leads to an accumulation of clathrin-coated vesicles, which suggests that synaptojanin promotes the uncoating of synaptic vesicles following endocytosis (Cremona et al., 1999; Harris et al., 2000; Verstreken et al., 2003).

Endophilin, which contains an N-terminal BAR (Bin/Amphiphysin/Rvs-homology) domain and a C-terminal SH3 domain, appears to play a key role in endocytosis (Ringstad et al., 1997; Schuske et al., 2003). The BAR domain forms a rigid, crescent shape that promotes membrane curvature and induces lipid tubulation *in vitro* (Farsad et al., 2001; Masuda et al., 2006). Endophilin was also hypothesized to promote membrane curvature by combining lysophosphatidic acid and acyl-CoA to create phosphatidic acid, which has a more conical shape and could induce asymmetry into the lipid bilayer (Schmidt et al., 1999). However, this lysophosphatidic acid acyl transferase activity was later demonstrated to be an artifact of purification (Gallop et al., 2005), so it appears as though endophilin's membrane-bending activity is merely structural in nature. Endophilin also binds synaptojanin and dynamin through its SH3 domain, and may therefore play a role in recruiting other endocytic proteins (Ringstad et al., 1997). The loss of endophilin results in phenotypes that resemble those of *synaptojanin* mutants and causes a severe depletion of synaptic vesicles as well as slow endocytic recovery after stimulation (Harris et al., 2000; Verstreken et al., 2002; Dickman et al.,

2005). Interestingly, overexpression of endophilin can partially rescue a *synaptojanin* hypomorphic mutant, which lends support to the idea that these proteins function together to promote synaptic vesicle uncoating (Verstreken et al., 2003).

The importance of dynamin in synaptic vesicle endocytosis was elegantly revealed in experiments utilizing a temperature-sensitive allele of the *Drosophila* dynamin homolog named *shibire* (Koenig and Ikeda, 1989). At the restrictive temperature, endocytosis ceases and *shibire* mutants gradually lose synaptic vesicles until evoked and spontaneous release arrest and the animals become paralyzed (Grigliatti et al., 1973; Koenig and Ikeda, 1983). Ultrastructural analysis at this point reveals a lack of synaptic vesicles and the existence of numerous membrane invaginations attached to the plasma membrane by a thin neck surrounded by an electron-dense substance (Koenig and Ikeda, 1989). When the endocytic block is released by a shift to the permissive temperature, the synaptic vesicle pool begins to recover, demonstrating that dynamin activity is essential for a late step in endocytosis. Although the precise mechanisms are unclear, dynamin appears to promote synaptic vesicle scission using the energy generated by GTP hydrolysis to alter its conformation and mechanically induce fission (Sweitzer and Hinshaw, 1998; Stowell et al., 1999; Roux et al., 2006; Bashkirov et al., 2008).

Bulk endocytosis

Bulk endocytosis differs from clathrin-mediated endocytosis in that it does not result in the formation of a single vesicle, but rather generates multiple vesicles through a two-step process (Richards et al., 2000; Cousin, 2009). First, large regions of the plasma membrane are internalized to form endocytic cisternae, which are large

intracellular structures that resemble vacuoles or endosomes (Miller and Heuser, 1984; Takei et al., 1996). Next, synaptic vesicles are regenerated from these cisternae, with at least some of these vesicles forming via clathrin-mediated endocytosis (Miller and Heuser, 1984; Takei et al., 1996; Gad et al., 1998; Richards et al., 2000). Bulk endocytosis provides a mechanism through which the endocytic capacity of a presynaptic terminal can be enhanced and is specifically triggered by stimuli that overwhelm the traditional clathrin-mediated pathway, which appears to have a limited capacity (Sankaranarayanan and Ryan, 2000; Clayton et al., 2008; Cousin, 2009). These stimuli can be both non-physiological, such as incubations with solutions containing 4-aminopyridine or high concentrations of potassium (Miller and Heuser, 1984; Takei et al., 1996; Akbergenova and Bykhovskaia, 2009), or more physiological such as an action potential tetanus (Paillart et al., 2003; Clayton et al., 2008). These forms of intense stimulation can generate endocytic cisternae within a few seconds (Miller and Heuser, 1984; Teng et al., 2007), while clathrin-mediated endocytosis has a time constant of ~14-15 seconds (Granseth et al., 2006). Furthermore, uptake experiments using the fluid-phase marker horseradish peroxidase (HRP) suggest that bulk endocytosis ceases almost immediately following intense stimulation, as very little HRP is internalized into cisternae if it is added after stimulation ends (Clayton et al., 2008). Bulk endocytosis therefore provides an extremely rapid and precise mechanism for reclaiming synaptic vesicle proteins and lipids from the plasma membrane following excessive exocytosis.

Compared to clathrin-mediated endocytosis, relatively little is known about the molecular mechanisms behind bulk endocytosis, including how it is initiated. One intriguing candidate for the activity-dependent endocytic trigger is the calcium-

dependent phosphatase calcineurin, which may link the elevated calcium levels induced by intense stimulation to the induction of bulk endocytosis (Cousin, 2009). Work in primary neuronal cultures has demonstrated that the inhibition of calcineurin dephosphorylation activity results in the arrest of bulk endocytosis (Evans and Cousin, 2007) and that calcineurin dephosphorylates dynamin under stimulation conditions that trigger bulk endocytosis (Clayton et al., 2009). Furthermore, calcineurin is not required for synaptic vesicle biogenesis during mild activity, suggesting that its function is specific for bulk endocytosis triggered by intense stimulation (Kumashiro et al., 2005; Evans and Cousin, 2007; Clayton and Cousin, 2008).

Synaptic vesicle pools

Aside from the small minority of vesicles that are morphologically docked at active zones, synaptic vesicles appear relatively indistinguishable by electron microscopy. Nevertheless, synaptic vesicles can be separated into groups based on their functional properties, most notably by their varying propensities to undergo exocytosis (reviewed in (Rizzoli and Betz, 2005)). The definitions and exact names vary, but in general synaptic vesicles can be broken down into three pools: the readily releasable pool (RRP), the recycling pool, and the reserve pool. The RRP comprises those synaptic vesicles that are released immediately upon stimulation with short action potential bursts (Elmqvist and Quastel, 1965; Delgado et al., 2000; Richards et al., 2003). This also is the pool thought to represent the synaptic vesicles docked at release sites (Schikorski and Stevens, 1997, 2001). The recycling pool is defined as the vesicles that are released and reclaimed by endocytosis under normal physiological stimulation conditions (Harata et al., 2001; Kuromi and Kidokoro, 2003; Richards et al., 2003),

while the reserve pool is only mobilized by intense or even non-physiological stimuli, such as prolonged high-frequency stimulation or the application of high-potassium solutions (Heuser and Reese, 1973; Delgado et al., 2000; Kuromi and Kidokoro, 2000; Richards et al., 2000; de Lange et al., 2003). In some preparations, including much of the work done at the *Drosophila* larval NMJ, the RRP and recycling pool are combined and referred to as the exo-endo cycling pool (Kuromi and Kidokoro, 1998, 2002, 2003). In general, the vast majority of synaptic vesicles at a synapse are part of the reserve pool, which can encompass ~80-90% of vesicles, while most of the remaining vesicles are found in the recycling pool (~15-20%), with only a small minority (~1-2%) making up the RRP (Rizzoli and Betz, 2005). At the *Drosophila* NMJ, analysis of synaptic vesicle release kinetics using the *shibire* dynamin mutant to block endocytosis similarly found that out of a total of ~84,000 quanta released during synaptic vesicle depletion, about 15-20% were part of the recycling pool and ~300 quanta made up the rapidly depleted RRP (Delgado et al., 2000).

The use of styryl dyes such as FM1-43 and FM2-10 has greatly facilitated the study of synaptic vesicle pools and recycling dynamics. These dyes dramatically increase their quantum yield upon binding lipid membranes and therefore can be used to track the movement of vesicles and cisternae through the exo-endocytic cycle (Cochilla et al., 1999; Verstreken et al., 2008). Due to their slightly altered affinities for synaptic lipids, different FM dyes preferentially label distinct endocytic pathways – FM1-43 is taken up by both clathrin-mediated endocytosis and bulk endocytosis, while FM2-10 is largely excluded from endocytic cisternae and therefore mostly labels clathrin-mediated endocytosis (Richards et al., 2000; Evans and Cousin, 2007; Clayton

and Cousin, 2008; Clayton et al., 2008). A recent study in neuronal cultures compared the loading of these dyes under stimulation conditions that induced either clathrin-mediated or bulk endocytosis to investigate how different synaptic vesicle pools were replenished by these two endocytic pathways (Cheung et al., 2010). Intriguingly, clathrin-mediated endocytosis preferentially refilled the recycling pool, while synaptic vesicles generated by bulk endocytosis were targeted to the reserve pool (Figure 1). These findings corroborated previous studies from the frog neuromuscular junction, which found that synaptic vesicles internalized via bulk endocytosis were not immediately available for release, suggesting that they were sent to the reserve pool (Richards et al., 2000; Richards et al., 2004).

The SNARE complex and regulated synaptic vesicle fusion

Liposome fusion assays have suggested that synaptobrevin, SNAP 25, and syntaxin, the core components of the SNARE complex, are sufficient to bring about membrane fusion (Weber et al., 1998). It should be noted, however, that the *in vivo* relevance of these experiments is debated, in part because liposome fusion was inefficient, with lipid mixing taking minutes to hours, and the protein/lipid ratios on liposomes were exceptionally high (~750 copies of synaptobrevin per liposome)(Rizo et al., 2006). Further analysis found that the SNARE protein concentration and the method of liposome preparation were critical determinants of lipid-mixing efficiency (Chen et al., 2006). Strikingly, an assay utilizing single molecule fluorescence demonstrated that, while the formation of one SNARE complex is sufficient for liposome docking, fusion was rare even with an average of 12 SNARE complexes per liposome, a value well within the estimated range of complexes necessary for synaptic vesicle

fusion (Bowen et al., 2004; Montecucco et al., 2005). These and other observations make it clear that efficient *in vivo* synaptic vesicle exocytosis requires additional components beyond the SNARE complex.

Sec1/Munc18 (SM) proteins participate in a variety of intracellular trafficking events, and many appear to interact specifically with syntaxins (Gallwitz and Jahn, 2003). Mammalian Munc18-1 is essential for neurotransmission, as the loss of this protein results in a complete absence of both spontaneous and evoked synaptic vesicle fusion, even following treatment with α-latrotoxin that induces massive neurotransmitter release in control animals (Verhage et al., 2000). Strikingly, the loss of Munc18 affects exocytosis to an even greater extent than the loss of synaptobrevin, one of the core components of the SNARE complex, indicating that Munc18 plays a critical role in promoting vesicle release (Verhage et al., 2000). Munc18 binds syntaxin in what is known as the "closed" conformation, where an N-terminal region containing a three-helix bundle folds back onto the region of syntaxin that interacts with the other SNARE proteins, thereby preventing SNARE complex formation (Fernandez et al., 1998; Dulubova et al., 1999). It is currently unknown if Munc18 promotes fusion indirectly via its interactions with the SNARE complex or whether it participates in exocytosis directly.

Synaptotagmin and complexin

Fast, calcium-dependent exocytosis necessarily requires a mechanism to rapidly couple calcium entry through voltage-gated channels to the fusion of synaptic vesicles. Moreover, mechanisms must be in place to ensure that synaptic vesicles do not

indiscriminately fuse in the absence of calcium. Synaptotagmin and complexin are two proteins that play critical roles in orchestrating the timing of release and ensuring that vesicle fusion is tied to calcium influx. Synaptotagmin contains two cytoplasmic calcium-binding domains (C_2A and C_2B) that are homologous to the regulatory regions of protein kinase C and bind three and two calcium ions, respectively (Perin et al., 1990; Ubach et al., 1998; Fernandez et al., 2001). Calcium binding causes synaptotagmin to interact with negatively charged phospholipids, which provide additional coordination sites and thereby increase the apparent calcium affinity of the C_2 domains (Zhang et al., 1998b; Fernandez-Chacon et al., 2001). Interestingly, the C_2B domain may form a calcium-dependent bridge between synaptic vesicles and the plasma membrane, as it has been shown to simultaneously bind two membranes and bring them into close proximity (Arac et al., 2006). In addition to binding phospholipids, synaptotagmin also interacts with the SNARE complex, although this interaction is only partially dependent on calcium (Bennett et al., 1992; Chapman et al., 1995; Li et al., 1995). As expected, the loss of synaptotagmin severely affects calcium-dependent exocytosis: both *Drosophila* and mouse *synaptotagmin* mutants have a dramatic decline in the amplitude of evoked responses, while spontaneous release frequency is enhanced in the *Drosophila* mutant and the mouse *synaptotagmin 2* mutant (but not in the mouse *synaptotagmin 1* mutant) (Littleton et al., 1993; Geppert et al., 1994; Pang et al., 2006).

Complexins are small, cytosolic proteins containing an α-helical region that tightly binds a groove in the four-helix bundle of the assembled SNARE complex (McMahon et al., 1995; Bracher et al., 2002; Chen et al., 2002). This finding, along with the observation that complexin can inhibit liposome or cell-cell fusion, led to the

hypothesis that complexin acts as a fusion clamp by holding SNARE complexes in a hemifused state (Giraudo et al., 2006; Schaub et al., 2006). Additional evidence in support of this hypothesis came from analysis of a *Drosophila complexin* mutant, which displayed a dramatic increase in the frequency of spontaneous synaptic vesicle release (Huntwork and Littleton, 2007). However, conflicting results were observed in mice, as synapses in the *complexin* knockout mouse showed a decrease in spontaneous fusion (Reim et al., 2001; Xue et al., 2007; Xue et al., 2008), while knockdown using RNA interference caused an increase in spontaneous release (Maximov et al., 2009). Evoked neurotransmitter release is inhibited in both *Drosophila* and mouse knockouts, suggesting that complexin can also facilitate release (Reim et al., 2001; Huntwork and Littleton, 2007; Xue et al., 2008). The discrepancies in the data imply that complexin function varies between species, and indeed there appear to be differences between the specific isoforms (Cho et al., 2010). Cross-species rescue and overexpression experiments confirm that *Drosophila* complexin has a mostly inhibitory role, while murine complexin has a primarily facilitating effect (Xue et al., 2009). Furthermore, in-depth structure-function analysis has shown that different regions within a single complexin isoform can either promote or inhibit vesicle fusion (Xue et al., 2007; Xue et al., 2009). Therefore, it remains to be determined exactly how complexin regulates synaptic vesicle exocytosis through its interactions with the SNARE complex, as well as how synaptotagmin and complexin might work together to coordinate calcium-dependent fusion.

Molecular anatomy of synaptic vesicles

Much of our understanding of the synaptic vesicle cycle has come from the identification and characterization of the proteins located on synaptic vesicles. Because synaptic vesicles are homogeneous in size and density, they can readily be purified from brain homogenates. Recent proteomic studies have utilized a variety of gel electrophoresis techniques combined with mass spectrometry in an attempt to ascertain the full complement of synaptic vesicle proteins (Blondeau et al., 2004; Burre et al., 2006; Takamori et al., 2006). While their findings differ in terms of the exact number and identity of synaptic vesicle proteins, all of these analyses conclude that several hundred different proteins either reside on or associate with synaptic vesicles.

One of these studies (Takamori et al., 2006) calculated the protein stoichiometry for synaptic vesicles using quantitative Western blotting and found that the two most abundant proteins were synaptobrevin (~70 copies/vesicle) and synaptophysin (~32 copies/vesicle), while synaptogyrin had approximately two copies per vesicle. In terms of mass, however, synaptophysin is the most abundant, accounting for ~10% of total synaptic vesicle protein (Takamori et al., 2006). Analysis of single isolated synaptic vesicles using total internal reflection fluorescence (TIRF) microscopy gave different copy numbers per vesicle, most notably for synaptobrevin, with averages of about 10 synaptobrevins, 13 synaptophysins, and 7 synaptogyrins per vesicle (Mutch et al., 2011). Although the exact values vary between these two studies, it is clear that both synaptophysin and synaptobrevin are among the most abundant synaptic vesicle proteins.

Initial characterization of synaptophysin and synaptogyrin

Synaptophysin

Synaptophysin, initially named p38 due to its apparent molecular mass of 38 kDa, was among the first synaptic vesicle proteins to be discovered and characterized in detail (Jahn et al., 1985; Wiedenmann and Franke, 1985). The name synaptophysin comes from the combination of "synapse" and the Greek "φῦσα" (bubble or vesicle), and was given due to the protein's location on synaptic vesicles at the presynaptic terminal (Wiedenmann and Franke, 1985). Due to its ubiquitous presence on synaptic vesicles, synaptophysin has historically been used as a marker to measure synaptic density or diagnose neoplasms, among other uses (Valtorta et al., 2004). Synaptophysin is an N-glycosylated tetraspanning membrane protein with cytoplasmically located N- and C-termini (Figure 2) (Jahn et al., 1985; Wiedenmann and Franke, 1985; Leube et al., 1987; Sudhof et al., 1987). Synaptophysin can form noncovalent homomultimeric complexes ranging in size from dimers to hexamers, and its oligomerization state is not dependent on its C-terminus (Wiedenmann and Franke, 1985; Rehm et al., 1986; Thomas et al., 1988; Johnston and Sudhof, 1990; Pennuto et al., 2002; Arthur and Stowell, 2007). Each of synaptophysin's two luminal domains contains a disulfide bond that forms within each intravesicular loop and does not interact with neighboring synaptophysin subunits in a complex (Johnston and Sudhof, 1990). Synaptophysin's C-terminus has numerous glycine and proline residues and contains ten pentapeptide repeats, nine of which have a tyrosine residue (Sudhof et al., 1987). The large number of tyrosine residues suggests that synaptophysin can undergo phosphorylation, and indeed synaptophysin is a major target of pp60$^{c\text{-}src}$ tyrosine kinase, which is also associated with synaptic vesicles (Pang

et al., 1988; Barnekow et al., 1990; Linstedt et al., 1992). The interaction between synaptophysin and pp60$^{c\text{-}src}$ is enhanced following training with the Morris water maze spatial learning task, suggesting that synaptophysin function may be modulated during learning and memory (Zhao et al., 2000). Similarly, tyrosine phosphorylation of synaptophysin was increased in hippocampal brain slices that had undergone long-term potentiation, suggesting that synaptophysin may play a role in synaptic plasticity (Mullany and Lynch, 1998). Synaptophysin also appears to be regulated via serine phosphorylation, as it has been shown to be a calcium-dependent substrate of CaM kinase II (Rubenstein et al., 1993). This raises the possibility that synaptophysin function may be modulated in response to the calcium influx triggered by action potentials. While there is strong evidence to suggest that synaptophysin is phosphorylated under physiological conditions, the locations of the tyrosine and serine residues that undergo phosphorylation are currently unknown (Evans and Cousin, 2005).

Synaptogyrin

Synaptogyrin was originally identified as a 29 kDa protein located throughout the nervous system as well as endocrine cells that have regulated secretion (Baumert et al., 1990). Like synaptophysin, synaptogyrin received its name due to its presence on synaptic vesicles (the Greek "γῦϱος" means circle)(Stenius et al., 1995). Synaptogyrin also contains four transmembrane domains and has the same membrane orientation as synaptophysin (Stenius et al., 1995). Furthermore, like synaptophysin, synaptogyrin has several tyrosine residues in its C-terminus that are phosphorylated by pp60$^{c\text{-}src}$ (Figure 2) (Janz and Sudhof, 1998). When transfected into COS cells, synaptogyrin and

synaptophysin are both phosphorylated by $pp60^{c\text{-}src}$ and c-fyn, with synaptogyrin appearing to be the better target for phosphorylation by c-fyn (Janz et al., 1999). However, unlike synaptophysin, synaptogyrin is not glycosylated, and only its first intravesicular domain has a pair of conserved cysteine residues that potentially form a disulfide bond (Baumert et al., 1990; Stenius et al., 1995). The C-terminus of synaptogyrin is required for proper targeting of the protein to synapses in *C. elegans* and is also necessary for the correct localization of GFP-tagged rat synaptogyrin in cultured hippocampal neurons (Zhao and Nonet, 2001). Unlike synaptophysin, the quaternary structure of synaptogyrin has not been investigated (Hubner et al., 2002). Since relatively few studies have examined synaptogyrin, much less is known about the structure and function of synaptogyrin compared to synaptophysin.

Physins and gyrins: evolutionarily related MARVEL domain proteins

Once the amino acid sequences of synaptophysin and synaptogyrin were determined, it became apparent that these proteins were distantly related through evolution (Sudhof et al., 1987; Stenius et al., 1995). Moreover, each of these proteins became the founding member of two larger protein families: the physins and the gyrins (Hubner et al., 2002). In mammals, the physin family encompasses synaptophysin, synaptoporin (also called synaptophysin 2), pantophysin, and mitsugumin 29, while the synaptogyrin family consists of synaptogyrins 1-4 (synaptogyrin 2 is also referred to as cellugyrin). All of these proteins contain a conserved tetraspanning membrane motif called the MARVEL domain, for MAL and related proteins for vesicle trafficking and membrane link (Sanchez-Pulido et al., 2002). However, the composition and length of

the N- and C-termini of physins and gyrins varies significantly, even within a family (Hubner et al., 2002).

As the name suggests, MARVEL domain-containing proteins have been found to regulate membrane associations and intracellular trafficking. For example, the myelin and lymphocyte protein (MAL) and the related protein MAL2 mediate apical transport in polarized cells. MAL regulates the transport of cargo from the *trans*-Golgi network to the apical membrane in epithelial cells (Cheong et al., 1999; Puertollano and Alonso, 1999), while MAL2 participates in transcytosis in hepatocytes (de Marco et al., 2002). Both MAL and MAL2 are present in lipid rafts, raising the possibility that these proteins mediate apical transport by recruiting appropriate lipids and proteins through their transmembrane domains (Cheong et al., 1999; Puertollano and Alonso, 1999; de Marco et al., 2002). Interestingly, MAL (but not MAL2) has been shown to oligomerize and recruit apical proteins in a cholesterol-dependent manner (Magal et al., 2009; Ramnarayanan and Tuma, 2011). MAL's ability to promote and stabilize lipid rafts may lie in its relatively long transmembrane helices (23-25 amino acids), which may specifically recruit proteins and lipids destined for the apical membrane, since the apical surface is thicker than other cellular membranes (Magal et al., 2009; Ramnarayanan and Tuma, 2011). Plasmolipin and BENE, two other members of the MAL protein family, are also associated with lipid rafts (de Marco et al., 2001; Bosse et al., 2003). Plasmolipin is enriched in myelin and is linked to the pleiotropic disorder Bardet-Biedl syndrome (Hamacher et al., 2001), while BENE has been implicated in intracellular trafficking in endothelial cells (de Marco et al., 2001). BENE also interacts with caveolin, a protein known to organize lipid rafts during the formation of caveolae –

the plasma membrane invaginations involved in diverse processes such as cell signaling and lipid regulation (Parton and Simons, 2007).

A second family of MARVEL proteins comprising occludin, tricellulin, and the newly characterized protein marvelD3 are targeted to tight junctions, which are the structures that separate apical and basolateral membranes and form a barrier to prevent the movement of solutes between epithelial cell layers (Furuse et al., 1993; Tsukita et al., 2001; Raleigh et al., 2010). However, occludin is not required for the formation of functional tight junctions, since *occludin* mice, while they are slow to develop postnatally and have several histological phenotypes, are nevertheless able to form effective epithelial barriers (Saitou et al., 2000). Although the exact role of the occludin family is unknown, all three proteins are present in detergent-insoluble membrane microdomains that are enriched in cholesterol and sphingolipids, suggesting that they may play a role in organizing the lipid rafts around tight junctions (Nusrat et al., 2000; Raleigh et al., 2010). Interestingly, overexpression of chicken occludin in insect cells (which normally do not form tight junctions) causes the formation of intracellular multilamellar structures with connections resembling tight junctions, suggesting that occludin has the ability to induce membrane reorganization (Furuse et al., 1996; Furuse and Tsukita, 2006).

In *Drosophila*, the MARVEL protein Singles Bar is involved in regulating myoblast fusion during the formation of multinucleated muscles (Estrada et al., 2007; Abmayr et al., 2008). In the absence of Singles Bar, myoblasts adhere to one another but do not fuse, indicating that the protein is involved in a late step in the fusion process (Estrada et al., 2007). While the exact mechanism is unknown, Singles Bar is proposed to

mediate the fusion of dense-core vesicles with the plasma membrane to deliver components required to promote the final steps of myoblast fusion. No vertebrate homologs of Singles Bar have been identified, and currently no other MARVEL domain-containing homolog has been implicated in myoblast fusion (Estrada et al., 2007). However, caveolin-3 is required for myoblast fusion in mammals (Galbiati et al., 1999), raising the possibility that proper organization of membrane lipids is an essential step for promoting membrane fusion in both systems.

Taken together, these findings suggest that the MARVEL domain plays a general role in organizing membrane microdomains and controlling membrane apposition events. The ability of many MARVEL domain-containing proteins to manipulate the lipid composition and architecture of membranes may also underlie the function of gyrins and physins.

Synaptoporin, pantophysin, and mitsugumin 29

Synaptophysin and synaptoporin are more closely related to each other than to the other physins pantophysin and mitsugumin 29 (Hubner et al., 2002). Like synaptophysin, synaptoporin is enriched in the brain and neuroendocrine cells (Knaus et al., 1990), although its pattern of expression is more narrow than that of synaptophysin (Hubner et al., 2002). Pantophysin was initially believed to be ubiquitously expressed (Leube, 1994); however, although all major tissues contain pantophysin mRNA, its protein expression is heterogeneous in various cell types (Haass et al., 1996; Windoffer et al., 1999). On the other hand, mitsugumin 29 expression is limited to skeletal muscle, small intestine, and kidney (Shimuta et al., 1998; Komazaki et al., 1999). In skeletal muscle, mitsugumin 29 is enriched at the triad junction, a location

where changes in membrane potential are coupled to calcium release from the sarcoplasmic reticulum (Franzini-Armstrong and Jorgensen, 1994; Nishi et al., 1999). Knockout experiments in mice revealed that the loss of mitsugumin 29 causes morphological abnormalities in the membranes surrounding the triad junction, as well as increased fatigue and delayed recovery of certain muscle types, which may stem from altered intracellular calcium homeostasis (Nishi et al., 1999; Nagaraj et al., 2000; Komazaki et al., 2001).

Synaptogyrins 2-4

Cellugyrin, also known as synaptogyrin 2, is ubiquitously expressed, although its mRNA levels are lower in the brain relative to other tissues (Janz and Sudhof, 1998; Kedra et al., 1998). Synaptogyrin 3 mRNA is restricted to the brain and placenta (Kedra et al., 1998); however, synaptogyrin 3 expression in the brain is not as widespread as synaptogyrin 1 (Belizaire et al., 2004). Thus, both synaptophysin and synaptogyrin have a paralog with a more restricted neuronal expression pattern, suggesting that the functions of synaptoporin and synaptogyrin 3 may be more specialized. Synaptogyrin 4 expression has not yet been established (Hubner et al., 2002).

SCAMPs

Secretory carrier-associated membrane proteins (SCAMPs), like physins and gyrins, are tetraspanning membrane proteins with cytoplasmic N- and C-termini (Hubbard et al., 2000). While their membrane topology is similar, SCAMPs lack a MARVEL domain and share little sequence homology with physins or gyrins (Hubner et al., 2002; Sanchez-Pulido et al., 2002). All SCAMPs share a conserved domain that

encompasses the four transmembrane helices, and each has a highly conserved region located in the cytoplasmic loop between the second and third transmembrane domains called the E peptide (Hubbard et al., 2000). SCAMPs were originally identified as components of secretory vesicles in exocrine glands (Cameron et al., 1986; Brand et al., 1991; Laurie et al., 1993). However, most SCAMPs have since been shown to have a much broader expression pattern and are not restricted to synaptic vesicles (Singleton et al., 1997; Fernandez-Chacon and Sudhof, 2000; Hubner et al., 2002). Unlike gyrins and physins, SCAMP homologs are also found in plants, with the *Arabidopsis* genome encoding four SCAMP homologs, all of which are closely related to one another (Fernandez-Chacon and Sudhof, 2000). Mammals have five SCAMP isoforms, three of which (SCAMPs 1-3) have multiple NPF repeats in their N-termini, while SCAMPs 4 and 5 lack this domain (Fernandez-Chacon and Sudhof, 2000). Interestingly, SCAMP 5 was only found in the brain where it is highly enriched on synaptic vesicles, suggesting that its function is specific to neurons, unlike the other more ubiquitous SCAMPs that likely have a more general cellular function (Fernandez-Chacon and Sudhof, 2000). The NPF domain has been identified as a binding site for proteins containing an Eps15 homology (EH) domain (de Beer et al., 1998; Paoluzi et al., 1998), a domain that has been implicated in clathrin-mediated endocytosis and vesicle budding from the *trans*-Golgi complex (Tebar et al., 1996; Chi et al., 2008). SCAMPs also are thought to function in endocytosis in multiple cell types by recruiting EH domain-containing proteins such as intersectin (Fernandez-Chacon et al., 2000), a protein that also has been implicated in endocytosis (Hussain et al., 1999).

SCAMPs also have been proposed to regulate exocytosis, as overexpression of the E peptide in both mast cells and PC12 cells inhibits the release of granules and dense-core vesicles, respectively (Guo et al., 2002; Liu et al., 2002). SCAMP1-deficient mice are viable and fertile and do not appear to have major deficits in exocytosis or endocytosis (Fernandez-Chacon et al., 1999). However, capacitance recordings from mast cells revealed an increase in the frequency of transient fusion events, suggesting that SCAMP1 may function to stabilize a fusion pore and/or regulate endocytosis following fusion (Fernandez-Chacon et al., 1999). Interestingly, the SCAMP cytoplasmic loop (the E peptide) and the two cytoplasmic segments bordering transmembrane domains 1 and 4 are amphiphilic, raising the possibility that these regions might interact with lipid bilayers to affect fusion pore dynamics (Hubbard et al., 2000).

Potential functions of synaptophysin and synaptogyrin

Regulation of exocytosis

The observation that synaptophysin forms a multimeric complex has lead to speculation that it might form an ion channel or proteinaceous fusion pore that could allow for rapid neurotransmitter release during exocytosis (Sudhof et al., 1987; Valtorta et al., 2004). Ultrastructural analysis of purified synaptophysin revealed rosette-like particles with a mean diameter of approximately 8 nm (Thomas et al., 1988), while single-particle three-dimensional reconstruction identified a hexameric structure with an outer diameter of about 7 nm and an inner diameter of approximately 3 nm (Arthur and Stowell, 2007). When synaptophysin complexes were reconstituted into planar lipid bilayers, they displayed voltage-sensitive channel activity with an average conductance of 150 picosiemens (Thomas et al., 1988), a value similar to that of gap

junctions (Young et al., 1987). These reconstituted channels appear to be highly selective for potassium ions (Gincel and Shoshan-Barmatz, 2002), although whether synaptophysin has the ability to conduct ions *in vivo* remains to be determined.

The hypothesis that synaptophysin creates a fusion pore or an ion channel is enhanced by the observation that synaptophysin and the gap junction protein connexin share a similar membrane topology and overall structure (Leube, 1995; Arthur and Stowell, 2007). Connexin's third transmembrane domain is predicted to line the pore of gap junctions (Skerrett et al., 2002), and while connexin and synaptophysin have little global sequence homology, their third transmembrane domains are much more similar, raising the possibility that synaptophysin's third transmembrane domain may, by analogy, line the fusion pore (Arthur and Stowell, 2007). Furthermore, experiments utilizing fluorescence resonance energy transfer (FRET) of CFP- and YFP-tagged synaptophysin molecules found that synaptophysin oligomers dissociate upon synaptic vesicle exocytosis, which may represent the disassembly of a fusion pore upon synaptic vesicle integration into the plasma membrane (Pennuto et al., 2002).

While the idea of synaptophysin forming a fusion pore analogous to gap junctions is intriguing, there are several lines of evidence that argue against this possibility. First, there is debate as to which transmembrane domain of connexin lines the pore of gap junctions. Several groups favor the third transmembrane domain as the major pore-lining helix (Skerrett et al., 2002; Fleishman et al., 2004), while other models predict the first transmembrane domain (Zhou et al., 1997; Kronengold et al., 2003; Maeda et al., 2009). Since there is little sequence homology between connexin and synaptophysin outside of the third transmembrane domain, the similarities

between the two proteins are lessened if the first transmembrane helix of connexin lines the pore. Second, gap junctions are formed by the association of two connexin hemichannels located on the plasma membranes of adjacent cells, while a putative synaptophysin fusion pore would connect synaptic vesicles with the plasma membrane. Since both connexin and synaptophysin have cytoplasmic N- and C-termini, the interaction between connexons is mediated by their extracellular loops, while a presumed association between synaptophysin oligomers would occur through their cytoplasmic domains (Sudhof and Jahn, 1991). In other words, the orientation of the hemichannels would be reversed. Finally, if synaptophysin interacts with another binding partner located on the plasma membrane to form a fusion pore, such a protein has not yet been definitively identified (Valtorta et al., 2004). A synaptophysin binding partner named physophilin was initially a candidate for the plasma membrane component of the fusion pore (Thomas and Betz, 1990). However, physophilin was later shown to be a subunit (V_o) of the vacuolar H^+ ATPase, a complex enriched on synaptic vesicles that seems unlikely to be the major plasma membrane component of the fusion pore (Siebert et al., 1994; Galli et al., 1996; Carrion-Vazquez et al., 1998).

In spite of the evidence against synaptophysin forming a proteinaceous fusion pore analogous to gap junctions, it remains possible that synaptophysin may promote fusion through alternative mechanisms. Indeed, additional support implicating synaptophysin in regulating calcium-dependent synaptic vesicle exocytosis has come from overexpression studies using *Xenopus* oocytes. Upon injection with total rat cerebellar mRNA, *Xenopus* oocytes become capable of secreting glutamate in a calcium-dependent manner (Alder et al., 1992b). When either synaptophysin antisense

oligonucleotides or synaptophysin antibodies are co-injected, this glutamate release is substantially inhibited (Alder et al., 1992b). Furthermore, overexpression of synaptophysin increases both spontaneous and evoked neurotransmitter responses in cultured *Xenopus* motor neurons (Alder et al., 1995), while the injection of synaptophysin antibodies inhibits release (Alder et al., 1992a). Taken together, these results suggest that synaptophysin promotes calcium-dependent neurotransmitter fusion.

However, another group reported that overexpression of synaptophysin or synaptogyrin inhibited calcium-dependent release in neuroendocrine PC12 cells engineered to secrete human growth hormone (Sugita et al., 1999). This same study found that synaptogyrins 1-3 all inhibited secretion to a greater extent than synaptophysin and determined that the C-terminus of gyrins was largely disposable for this inhibition. It should be noted, however, that human growth hormone in PC12 cells is stored in and released from large dense-core vesicles rather than traditional synaptic vesicles (Lowe et al., 1988). While secretion via dense-core vesicles shares many similarities with synaptic vesicle exocytosis (e.g., it is triggered by calcium and inhibited by tetanus toxin), it is unclear whether synaptophysin and synaptogyrin normally localize to dense-core vesicles, raising uncertainty about whether the effects of their overexpression in PC12 cells is physiologically relevant (Wiedenmann and Franke, 1985; Navone et al., 1986; Baumert et al., 1990; Sugita et al., 1999).

How might synaptophysin and/or synaptogyrin regulate synaptic vesicle exocytosis? One appealing possibility comes from the observation that synaptophysin interacts with synaptobrevin, a critical component of the SNARE complex and the only

SNARE component found on synaptic vesicles (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995). The interaction between synaptophysin and synaptobrevin is lost when synaptophysin's disulfide bonds are reduced, which causes synaptophysin to lose its oligomeric state (Johnston and Sudhof, 1990). Since synaptophysin complexes dissociate upon exocytosis (Pennuto et al., 2002), this implies that the synaptophysin/synaptobrevin interaction is also lost following synaptic vesicle fusion. Furthermore, the interaction between synaptobrevin and synaptophysin precludes the association of synaptobrevin with SNAP-25 and syntaxin (Edelmann et al., 1995), which raises the possibility that synaptophysin may regulate the availability of synaptobrevin in a manner similar to the syntaxin/Munc18 interaction (Hata et al., 1993). The availability of syntaxin to interact with other SNARE components is controlled by Munc18, which appears to mediate a conformational switch in syntaxin that allows it to interact with the other SNAREs (Dulubova et al., 1999). Similarly, synaptophysin may regulate SNARE complex assembly and fusion by controlling the availability of synaptobrevin.

Overexpression and knockout studies in cultured neurons suggest that synaptophysin may play a role in targeting synaptobrevin to synaptic vesicles. When synaptobrevin is overexpressed only in the presence of endogenous synaptophysin, a portion of synaptobrevin is mislocalized away from synapses (Pennuto et al., 2003; Gordon et al., 2011). However, co-transfection of exogenous synaptophysin along with synaptobrevin restored the correct localization of synaptobrevin. Similar results were obtained in *synaptophysin* knockout cultures, with both endogenous and exogenous synaptobrevin showing an increased presence on the plasma membrane in the absence

of synaptophysin (Gordon et al., 2011). The synaptobrevin mislocalization appears to be due to a failure to retrieve the protein from the plasma membrane via endocytosis, as the levels of synaptobrevin on the plasma membrane after robust stimulation remain elevated in the *synaptophysin* mutant cultures compared to controls. The promotion of synaptic vesicle protein sorting by synaptophysin appears to be specific for synaptobrevin, because co-transfection of synaptotagmin and synaptophysin cannot rescue synaptotagmin mislocalization (Pennuto et al., 2003).

Regulation of endocytosis

Additional lines of evidence suggest that gyrins and physins may play a role in endocytosis and/or vesicle biogenesis. Synaptophysin has been shown to bind cholesterol, and the depletion of cholesterol in PC12 cells results in a preferential decrease in the formation of synaptic-like microvesicles (SLMVs) but not an overall decline in endocytosis (Thiele et al., 2000). Cholesterol is enriched in synaptic vesicles (Breckenridge et al., 1973; Takamori et al., 2006), and it has been proposed that synaptophysin's multimeric oligomerization may recruit cholesterol to promote the formation of highly curved membranes (Thiele et al., 2000). Cellugyrin has also been shown to induce the formation of SLMVs in PC12 cells (Belfort et al., 2005) and to promote the targeting of synaptophysin to SLMVs (Belfort and Kandror, 2003). Interestingly, the hydrophilic cytoplasmic regions of cellugyrin do not appear to be important for vesicle biogenesis. Instead, certain hydrophobic stretches near the transmembrane domains significantly influence cellugyrin's ability to promote the formation of small vesicles, potentially by directly influencing membrane curvature (Belfort et al., 2005). Transfection of synaptogyrin also results in increased targeting of

synaptophysin to small vesicles (Belfort and Kandror, 2003), although the enhanced synaptophysin targeting appears to be limited to PC12 cells (Belfort et al., 2005).

Further evidence implicating synaptophysin in vesicle formation comes from the observation that the C-terminus of synaptophysin interacts with dynamin in a calcium-dependent manner, with half-maximal binding occurring around 150 μM (Daly and Ziff, 2002). Dynamin plays a critical role in endocytosis by utilizing GTPase activity to constrict the neck of a budding vesicle and induce membrane scission (Hinshaw, 2000). Blocking the interaction of dynamin and synaptophysin by injecting the C-terminus of synaptophysin fused to glutathione S-transferase (GST) results in a depletion of the overall synaptic vesicle pool in the squid giant synapse (Daly et al., 2000). At the same time, the number of clathrin-coated vesicles increases, suggesting that clathrin-mediated endocytosis is not blocked (Daly et al., 2000). Interestingly, the interaction between synaptophysin and dynamin occurs at calcium concentrations seen in microdomains near active zones (~200-300 μM), where voltage-gated calcium channels are enriched (Pumplin et al., 1981; Llinas et al., 1992; Heidelberger et al., 1994; Daly and Ziff, 2002). Taken together, these results suggest that the synaptophysin-dynamin interaction promotes a clathrin-independent form of endocytosis (potentially a version of kiss-and-run endocytosis) that specifically occurs near sites of exocytosis where calcium concentrations are elevated. These findings also imply that the inhibition of this version of endocytosis results in a compensatory enhancement of clathrin-mediated endocytosis (Daly et al., 2000; Daly and Ziff, 2002).

A recent study provided additional support for a role of synaptophysin in synaptic vesicle recycling through the examination of endocytic kinetics in dissociated

hippocampal neurons from *synaptophysin* mutant mice (Kwon and Chapman, 2011). During sustained stimulation, the knockout cultures were slower to internalize synaptic vesicle proteins tagged with a pH-sensitive GFP reporter. However, endocytosis was unaltered during mild stimulation, suggesting that synaptophysin is only required for efficient endocytosis during intense stimulation. Interestingly, synaptophysin promotes endocytosis both during and after stimulation, but the C-terminus of synaptophysin is required only for synaptic vesicle retrieval during the stimulus and not for endocytosis that occurs once the stimulus ceases (Kwon and Chapman, 2011). These findings suggest that distinct synaptophysin domains regulate different endocytic processes.

Synaptophysin and synaptogyrin knockouts

Although synaptophysin and synaptogyrin are evolutionarily conserved and abundant synaptic vesicle proteins, the loss of these proteins results in relatively mild phenotypes. Both synaptophysin and synaptogyrin knockout mice are viable and fertile, as is the double mutant (Eshkind and Leube, 1995; McMahon et al., 1996; Janz et al., 1999). Mice lacking synaptophysin initially were reported to have slightly lower levels of synaptobrevin (McMahon et al., 1996). However, subsequent analysis of the synaptophysin/synaptogyrin double knockout revealed no significant changes in synaptobrevin expression or in the expression levels a wide variety of other synaptic vesicle proteins (Janz et al., 1999). Intriguingly, *synaptophysin* mutants exhibit a decrease in synaptic vesicle density and an increase in the number of clathrin-coated vesicles specifically in photoreceptor cells in the outer plexiform layer of the retina – a region that lacks synaptoporin (McMahon et al., 1996; Spiwoks-Becker et al., 2001). Moreover, the rod photoreceptors in knockout animals have altered synaptic vesicle

morphology, with many vesicles misshapen or unusually flat (Spiwoks-Becker et al., 2001). On the other hand, synaptic vesicle morphology appears normal in a cerebellar region that also contains little to no synaptoporin (Fykse et al., 1993; Eshkind and Leube, 1995). Many of the phenotypes observed in the retinas of *synaptophysin* mutant animals were exacerbated during periods of high activity (i.e., when animals were not exposed to light), indicating that synaptophysin's function may be specific to certain high-intensity stimulation conditions.

Electrophysiological analysis of synaptophysin-deficient mice found no significant changes in excitatory postsynaptic current (EPSC) amplitude, mEPSC frequency, or release probability. However, there was a slight (less than 10%) increase in quantal size (McMahon et al., 1996). Although synaptic vesicle morphology was altered in the retinal cells of the knockout mice, electroretinography revealed no significant differences compared to controls, suggesting that the defects seen by electron microscopy do not severely impair vision (Spiwoks-Becker et al., 2001; Schmitt et al., 2009). Further analysis revealed that *synaptophysin* mutant mice have no significant changes in paired-pulse facilitation (PPF), post-tetanic potentiation (PTP), or long-term potentiation (LTP)(Janz et al., 1999). Moreover, synaptogyrin-deficient mice have only a slight decrease in PTP, with no changes in other forms of synaptic plasticity. Interestingly, mice lacking both synaptophysin and synaptogyrin display defects in all of these types of short- and long-term synaptic plasticity, as well as a delay in the recovery from synaptic depression, indicating that there is functional redundancy between these two proteins (Janz et al., 1999). Anatomically, double mutant mice have normal brain architecture, suggesting that these proteins are not required to establish

gross neuronal patterning during development (Janz et al., 1999). Behaviorally, synaptophysin-deficient mice display normal levels of locomotor activity; however, they were more exploratory in an enriched open field and displayed reduced novel object recognition (Schmitt et al., 2009). The knockout mice also exhibited reduced performance in spatial learning and memory as assayed by the Morris water maze hidden platform test (Schmitt et al., 2009). Interestingly, synaptophysin has also been implicated in human cognitive ability, as large-scale chromosomal sequencing has identified several variants at the *synaptophysin* locus in subjects with X-linked mental retardation (Tarpey et al., 2009). However, to date no detailed behavioral assays have been performed on synaptogyrin knockout mice or synaptophysin/synaptogyrin double knockouts.

As has been previously discussed, analysis of neuronal physin and gyrin function in mammals is complicated by the presence of multiple isoforms of both proteins in the brain. *C. elegans*, on the other hand, has one synaptophysin homolog and one synaptogyrin homolog, thereby allowing issues of redundancy between physins and gyrins to be more easily ascertained. Somewhat surprisingly, the initial analysis of a *C. elegans* synaptophysin/synaptogyrin/SCAMP triple knockout also revealed no significant changes in neuronal morphology, synaptic transmission, or behavior aside from a slight increase in the number of clathrin-coated synaptic vesicles (Spiwoks-Becker et al., 2001; Abraham et al., 2006). Closer examination of the *C. elegans synaptogyrin* single mutant identified slight changes in sensitivity to certain drugs affecting GABAergic and cholinergic neurons, subtle alterations in motility, and a slight decrease in synaptobrevin targeting to synapses (Abraham et al., 2011). Interestingly,

synaptophysin expression in *C. elegans* appears to be largely restricted to muscle cells in the pharynx and anal sphincter (Abraham et al., 2006). However, synaptogyrin is expressed in almost all neurons and colocalizes with other synaptic vesicle proteins (Nonet, 1999; Zhao and Nonet, 2001; Abraham et al., 2011), indicating that synaptogyrin is likely to be the predominant synaptic vesicle MARVEL protein in nematodes.

Interestingly, an increase in clathrin-coated vesicles is observed in both the *C. elegans* triple knockout and in mouse retinal photoreceptors that lack both synaptophysin and synaptoporin (Spiwoks-Becker et al., 2001; Abraham et al., 2006). As previously mentioned, inhibition of the dynamin-synaptophysin interaction also resulted in an increase in clathrin-coated vesicles, which was presumably compensating for the disruption of a clathrin-independent endocytic pathway mediated by synaptophysin (Daly et al., 2000; Daly and Ziff, 2002). Several of the defects observed in knockout mice, namely the delay in recovery from synaptic depression in the synaptophysin/synaptogyrin double knockout and the synaptic vesicle morphological abnormalities in retinal cells lacking synaptophysin, point to deficiencies in synaptic vesicle endocytosis (Janz et al., 1999; Spiwoks-Becker et al., 2001). Intriguingly, in *C. elegans*, the loss of synaptogyrin enhanced the phenotypes observed in *synaptojanin*, *endophilin*, and *synaptotagmin* mutants, all of which are regulators of clathrin-mediated endocytosis (Song and Zinsmaier, 2003; Shupliakov, 2009; Abraham et al., 2011). If synaptophysin and/or synaptogyrin do play a role in regulating synaptic vesicle regeneration via a clathrin-independent mechanism, it appears as though clathrin-mediated endocytosis may be partially compensating for the loss of those proteins.

Under conditions of increased neuronal activity (e.g., in dark-adapted retinal rod photoreceptors), the clathrin-mediated endocytosis pathway would be insufficient, thereby increasing the phenotypic severity. However, as previously discussed, the existence of such a clathrin-independent endocytic mechanism is debated, and clathrin-mediated endocytosis appears to account for the vast majority of synaptic vesicle endocytosis at most synapses (Granseth et al., 2006; Heerssen et al., 2008).

Synaptogyrin: a schizophrenia susceptibility gene?

Schizophrenia is a complex mental disorder with a wide array of manifestations including hallucinations, delusions, and decreased emotional expression (Andreasen, 1995). While the exact cause of schizophrenia is unknown, there is significant evidence suggesting that there is a strong genetic component to the disorder with multiple loci contributing to disease susceptibility (Sullivan et al., 2003; Gejman et al., 2010). Several lines of evidence have implicated synaptogyrin as a candidate schizophrenia susceptibility gene. The chromosomal locus encoding human synaptogyrin 1 (22q11-13) has been linked with schizophrenia in a variety of genetic studies (Coon et al., 1994; Pulver et al., 1994; Gill et al., 1996; Verma et al., 2005; Bassett and Chow, 2008). Microarray analysis also identified synaptogyrin as one of the downregulated genes in a subset of schizophrenic patients whose prefrontal cortices were examined postmortem (Mirnics et al., 2000). Furthermore, several families in India and China with a history of schizophrenia and/or bipolar disorder have been found to have mutations within the *synaptogyrin 1* locus (Verma et al., 2004; Cheng and Chen, 2007), and an association study in Italy identified several novel polymorphisms within the *synaptogyrin* locus, including a mutation that eliminates a potential serine phosphorylation site

(Iatropoulos et al., 2009). While definitive evidence linking synaptogyrin mutations to schizophrenia predisposition remains to be determined, synaptogyrin remains an intriguing candidate due to the changes in synaptic plasticity seen in knockout mice (Janz et al., 1999).

The *Drosophila* larval NMJ as a model of synaptic development and function

The neuromuscular junction (NMJ) of the *Drosophila* third instar larva has long been used as a system for studying synaptic transmission due to its easy accessibility, precise innervation patterns, and relative simplicity (Keshishian et al., 1996; Hoang and Chiba, 2001). Moreover, most of the key genes involved in synapse development, synaptic vesicle exo-endocytosis, and intracellular trafficking are conserved in the *Drosophila* genome (Littleton, 2000; Lloyd et al., 2000). This system also provides access to a variety of genetic tools, most notably the GAL4/UAS system, which allows for spatially- and temporally-targeted transgene expression (Brand and Perrimon, 1993; McGuire et al., 2004). Furthermore, the larval NMJ is similar to vertebrate central synapses in that it is glutamatergic, has graded responses to stimulation, and displays several forms of synaptic plasticity, including facilitation and post-tetanic potentiation (Jan and Jan, 1976; Zhong and Wu, 1991; Broadie et al., 1997). These factors, combined with a short life cycle and easy maintenance, make *Drosophila* extremely amenable to study the genetic components of neurotransmission.

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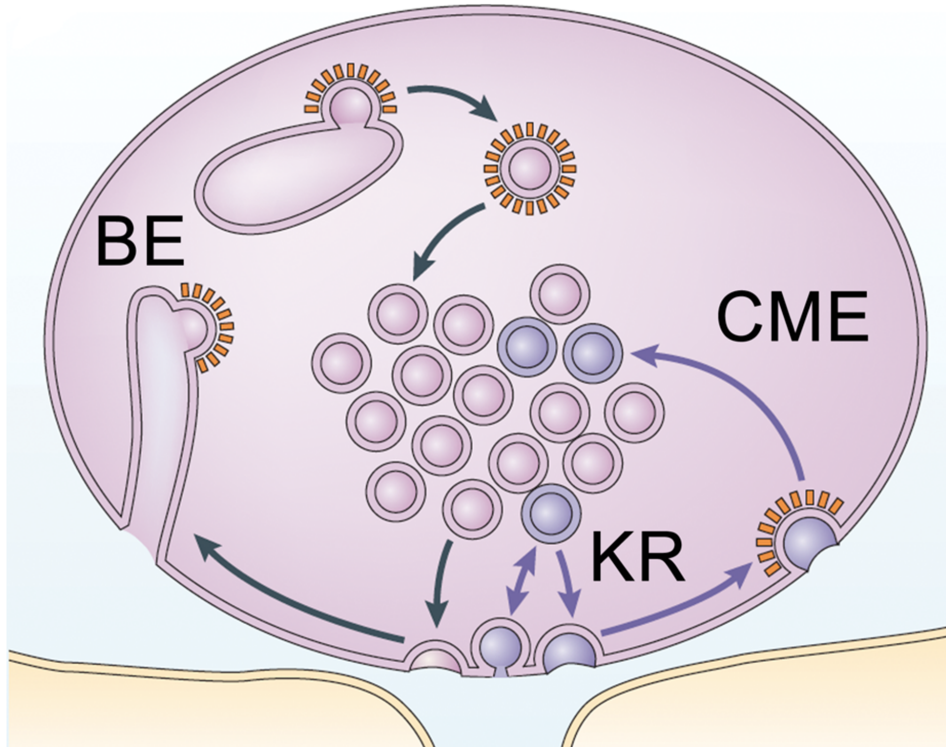

Figure 1. Endocytosis at the presynaptic terminal. Three main endocytic routes for synaptic vesicle recycling have been described. Pink vesicles indicate the reserve pool, while blue vesicles signify the recycling pool. Clathrin-mediated endocytosis (CME) usually occurs at a site somewhat removed from the active zone and involves the formation of a clathrin coat. During kiss-and-run endocytosis (KR), neurotransmitters are released through a fusion pore that subsequently closes to directly regenerate a synaptic vesicle. Both kiss-and-run endocytosis and clathrin-mediated endocytosis are proposed to replenish the recycling pool. Bulk endocytosis (BE) occurs during intense neuronal stimulation and involves the invagination of large regions of the plasma membrane. Synaptic vesicles then bud from internalized endocytic cisternae and are preferentially targeted to the reserve pool. Image adapted from (Rizzoli and Betz, 2005).

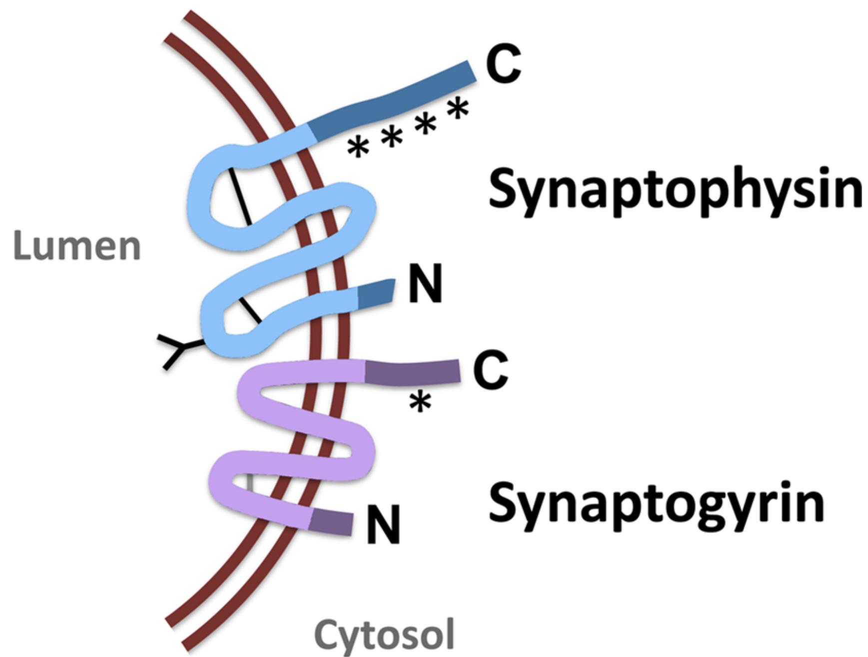

Figure 2. Structure of synaptophysin and synaptogyrin. Synaptophysin and synaptogyrin are tetraspanning integral membrane proteins with cytoplasmic N- and C-termini. Lighter shading indicates the MARVEL transmembrane domains. Both proteins contain tyrosine phosphorylation sites (asterisks), although the exact number and positions of these sites are unknown. Synaptophysin has two disulfide bonds between its intravesicular loops (black lines) and is N-glycosylated. Synaptogyrin contains two conserved cysteine residues within its first intravesicular loop, although it is unknown whether these residues form a disulfide bond *in vivo*. Figure based on (Fernandez-Chacon and Sudhof, 1999).

Chapter 2

Characterization of the *Drosophila* Synaptogyrin Homolog

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Robin Stevens performed the majority of the work described in this chapter. Dina Volfson generated the UAS-SCAMP-RFP flies.

Introduction

Sustained neurotransmission depends on the continual renewal of a pool of synaptic vesicles that are ready to respond to action potential propagation into the presynaptic nerve terminal (reviewed in (Sudhof, 2004)). Following exocytosis, synaptic vesicle proteins and lipids must be reclaimed via endocytosis and refilled with neurotransmitter. Synaptic vesicles must then traffic to the plasma membrane, where they dock at active zones and are primed for subsequent rounds of fusion. All steps of the synaptic vesicle cycle require the coordination of hundreds of proteins, many of which are uniquely targeted to synaptic vesicles. Much of our knowledge about neurotransmission and synaptic vesicle recycling has come from reductionist approaches that identify proteins enriched in the brain and then characterize their function by generating mutants, performing inhibition or overexpression studies, or conducting other *in vivo* or *in vitro* assays. Synaptophysin and synaptogyrin were among the first synaptic vesicle proteins to be discovered over twenty years ago (Jahn et al., 1985; Wiedenmann and Franke, 1985; Baumert et al., 1990; Stenius et al., 1995). Despite decades of research, the role of these proteins in the synaptic vesicle cycle remains elusive.

Synaptogyrin and synaptophysin are evolutionarily related members of the MARVEL domain family, which defines a group of tetraspanning membrane proteins involved in processes such as vesicle trafficking and membrane apposition events (Hubner et al., 2002; Sanchez-Pulido et al., 2002). These two proteins are abundantly expressed on synaptic vesicles, with synaptophysin alone constituting approximately 10% of synaptic vesicle protein by mass in rats (Takamori et al., 2006). Due to their

enrichment on synaptic vesicles, it is likely that these proteins play a functional or regulatory role in synaptic vesicle trafficking, release, or retrieval. Indeed, work in a variety of systems has implicated synaptogyrin and synaptophysin in synaptic vesicle exocytosis, endocytosis, and vesicle biogenesis, suggesting that these proteins may regulate multiple aspects of the synaptic vesicle cycle.

Synaptophysin can oligomerize into homomultimers that may form ion channels or proteinaceous fusion pores (Rehm et al., 1986; Thomas et al., 1988; Gincel and Shoshan-Barmatz, 2002; Arthur and Stowell, 2007). Synaptophysin also has been implicated in regulating synaptic vesicle exocytosis, perhaps via its interaction with synaptobrevin/VAMP2, which prevents the association of synaptobrevin with other members of the SNARE complex (Edelmann et al., 1995; Washbourne et al., 1995). Whether synaptophysin and synaptogyrin promote or inhibit synaptic vesicle release is unclear, as studies using *Xenopus* oocytes and cultured *Xenopus* motor neurons concluded that synaptophysin positively regulates exocytosis (Alder et al., 1992b; Alder et al., 1992a; Alder et al., 1995), while work using PC12 cells engineered to secrete human growth hormone suggested that synaptogyrin and synaptophysin inhibit fusion (Sugita et al., 1999).

Additional studies suggest that synaptophysin and synaptogyrin family members (physins and gyrins, respectively) may play a role in shaping lipid membranes and promoting vesicle biogenesis. Synaptophysin binds cholesterol, potentially promoting the formation of high-curvature membranes required for vesicle biogenesis (Thiele et al., 2000). Moreover, cellugyrin, a non-neuronal paralog of synaptogyrin, can increase the formation of synaptic-like microvesicles (SLMVs) in neuroendocrine cells (Belfort et

al., 2005). Synaptophysin may also participate in synaptic vesicle recycling through a calcium-dependent interaction with dynamin, as the inhibition of this interaction results in a smaller synaptic vesicle pool following high-frequency stimulation (Daly et al., 2000; Daly and Ziff, 2002). Similarly, a recent study in cultured hippocampal neurons revealed that the kinetics of endocytosis are slowed during periods of sustained stimulation in the absence of synaptophysin (Kwon and Chapman, 2011).

Although synaptophysin and synaptogyrin are evolutionarily conserved and abundant proteins, their absence results in relatively mild phenotypes. Synaptophysin/ synaptogyrin double knockout mice are viable and fertile with normal basal neurotransmission, but they display defects in several forms of synaptic plasticity, including paired-pulse facilitation (PPF) and long-term potentiation (LTP) (McMahon et al., 1996; Janz et al., 1999). Similarly, the initial characterization of a *C. elegans* triple knockout of synaptophysin, synaptogyrin, and the tetraspanning membrane protein SCAMP also revealed no detectable morphological, electrophysiological, or behavioral phenotypes aside from a slight increase in the number of clathrin-coated synaptic vesicles (Abraham et al., 2006). In-depth analysis of the *C. elegans synaptogyrin* single mutant identified slightly altered motility and changes in sensitivity to drugs affecting GABAergic and cholinergic neurons (Abraham et al., 2011).

The genetic analysis of synaptophysin and synaptogyrin in mammals, while potentially more relevant to human neurophysiology, is complicated by the presence of four physin and four gyrin paralogs. The synaptophysin paralog synaptoporin and the synaptogyrin paralog synaptogyrin 3 are enriched in the brain, and therefore may be able to substitute for the loss of either synaptophysin or synaptogyrin (Knaus et al.,

1990; Belizaire et al., 2004). Indeed, a synaptophysin knockout mouse had altered synaptic vesicle density and vesicle size in photoreceptors in the outer plexiform layer of the retina, a region that lacks synaptoporin (McMahon et al., 1996; Spiwoks-Becker et al., 2001). Similarly, synaptogyrin and synaptophysin are to some extent functionally redundant, as electrophysiological defects in PPF and LTP were only observed in the double knockout mice (however, a slight decrease in post-tetanic potentiation was observed synaptogyrin-deficient mice) (Janz et al., 1999). We therefore sought to investigate synaptogyrin function in *Drosophila*, which lacks a synaptophysin homolog and has only one synaptogyrin isoform. This approach allows us to simplify genetic analysis and avoid compensation by other gyrins and physins and simultaneously provides us with access to a well-characterized, stereotypic synaptic structure – the *Drosophila* larval neuromuscular junction (NMJ).

Our initial characterization of *Drosophila* synaptogyrin revealed that it localizes presynaptically at the larval NMJ and is abundantly expressed in the central nervous system of larvae and adult flies. We generated a *synaptogyrin* knockout and found that mutant animals are viable, fertile, and behaviorally normal, indicating that synaptogyrin is not an essential protein in *Drosophila*.

Results

Evolutionary analysis of synaptogyrin and synaptophysin

Synaptogyrin and synaptophysin have been established as evolutionarily conserved proteins (Hubner et al., 2002; Abraham et al., 2006). We were interested in further examining the evolutionary emergence of these proteins in relation to other synaptic vesicle components. While many synaptic vesicle proteins including SNAREs, V-ATPase, and Rab3 are ancient eukaryotic proteins (Srivastava et al., 2010), key proteins involved in regulated calcium-dependent exocytosis such as synaptotagmin 1 and complexin appeared more recently in evolution. Synaptotagmin and complexin homologs are absent in plants and fungi but both are present in the placozoan *Trichoplax adhaerens* – a simple multicellular organism that lacks a nervous system but has several organized cell layers (Srivastava et al., 2008; Barber et al., 2009). A protein BLAST search revealed that synaptogyrin and synaptophysin homologs are also found in *T. adhaerens*, indicating they also evolved before the emergence of defined synapses. Interestingly, we also identified a putative synaptogyrin homolog in the unicellular choanoflagellate *Monosiga brevicollis* with 33% identity to human synaptogyrin 1; however, we were unable to find a homolog in the yeast *Saccharomyces cerevisiae* (Table 1) (King et al., 2008). Our BLAST search did not reveal a *M. brevicollis* synaptophysin homolog, raising the possibility that all gyrin and physin family members are descended from an ancestral protein that was more similar to synaptogyrin than synaptophysin. Since choanoflagellates are thought to be the closest extant unicellular relative of metazoans (King et al., 2008), the presence of a synaptogyrin homolog in *M. brevicollis* but not in yeast suggests a unique role for

synaptogyrin in early metazoan evolution. Interestingly, the sea anemone *Nematostella vectensis* does not have a synaptogyrin homolog, but it does have at least one synaptophysin homolog, suggesting either that some metazoans may not require both proteins or that the functions of physins and gyrins are interchangeable (Putnam et al., 2007).

We also searched for homologs of several other MARVEL domain-containing proteins and found that some, including MAL (myelin and lymphocyte protein), MYADM (myeloid-associated differentiation marker gene), and occludin (a component of tight junctions), appear to be vertebrate-specific proteins. This is unsurprising given that vertebrate myelin and the myelin-like sheaths found in some invertebrates appear to have arisen through convergent evolution (Hartline and Colman, 2007). Similarly, invertebrates do not have tight junctions, but rather have analogous structures known as septate junctions (Furuse and Tsukita, 2006). We also examined the CMTM family (CKLF-like MARVEL transmembrane domain-containing family, where CKLF stands for chemokine-like factor), a novel group of proteins with a highly conserved member named CMTM4 that appears to be involved in regulating the cell cycle and cellular growth (Plate et al., 2010). Interestingly, CMTM4 homologs are found in *Drosophila*, *C. elegans*, *N*. *vectensis*, and potentially in *T. adhaerens*, although this putative homolog has only three predicted transmembrane domains. Finally, we investigated the evolutionary conservation of the secretory carrier-associated membrane proteins (SCAMPs), which lack the MARVEL domain but have the same membrane topology (Hubbard et al., 2000). A SCAMP homolog was found in every organism we examined with the exception of yeast. Interestingly, SCAMP homologs are also found in the plant kingdom, which

suggests that they perform a more ubiquitous role in multicellular organisms (Fernandez-Chacon and Sudhof, 2000).

Protein sequence alignment of representative synaptogyrin homologs confirmed the previously reported observation that these proteins share the highest degree of homology within their transmembrane regions and that there is a substantial amount of variability in the composition and length of the N- and C-termini (Figure 1) (Hubner et al., 2002). Unlike synaptogyrin homologs from other species, the putative *M. brevicollis* synaptogyrin lacks the two conserved cysteine residues located between the first two transmembrane domains that may form a disulfide bond within the first luminal loop (Figure 1). Interestingly, the *M. brevicollis* synaptogyrin homolog is almost entirely comprised of the MARVEL domain, with its short N- and C-termini each predicted to be less than 15 amino acids in length. Therefore, it is likely that the MARVEL domain itself has an important cellular function and that the N- and C-termini of gyrins and physins may have elaborated and adapted over the course of evolution to perform additional or more specialized tasks in other organisms.

The *Drosophila* genome encodes a single synaptogyrin homolog (CG10808) that is 42% identical to human synaptogyrin 1 and shares the same predicted membrane topology as other gyrin family members. Unlike many other invertebrates, including *C. elegans* and the red flour beetle *Tribolium castaneum*, the *Drosophila* genome lacks a synaptophysin homolog. However, the genome does encode other MARVEL-domain containing proteins, including a CMTM4 homolog (CG15211) and Singles Bar (CG13011), a protein involved in myoblast fusion (Estrada et al., 2007). The lack of a synaptophysin homolog in *Drosophila* is somewhat surprising given its high degree of

conservation across species. In *C. elegans*, however, synaptophysin expression is largely restricted to muscle cells in the pharynx and anal sphincter (Abraham et al., 2006), while synaptogyrin is expressed in most neurons (Nonet, 1999; Abraham et al., 2011), indicating that synaptogyrin is likely to be the predominant synaptic vesicle MARVEL protein in nematodes.

Drosophila **synaptogyrin is present in neurons and targeted to synaptic vesicles**

To our knowledge, synaptogyrin expression has not yet been characterized in *Drosophila*. Therefore, we examined the localization of endogenous synaptogyrin protein by generating antisera against the recombinant C-terminus of *Drosophila* synaptogyrin. A Western blot of *Drosophila* adult head extracts reveals a single major band at an observed molecular weight of approximately 29 kDa (Figure 2A). To examine the subcellular distribution of synaptogyrin, we performed velocity gradient fractionation experiments using 10-30% sucrose gradients. Synaptogyrin co-migrated with other known synaptic vesicle proteins, including synaptotagmin 1, cysteine string protein (CSP), and synaptobrevin, suggesting that *Drosophila* synaptogyrin is enriched on synaptic vesicles (Figure 2B).

As expected for a presumptive synaptic vesicle protein, immunohistochemistry of third instar *Drosophila* larvae results in abundant punctate synaptogyrin expression throughout the brain and ventral nerve cord (Figure 3). Similarly, immunohisto-chemistry of adult brains reveals profuse synaptogyrin protein expression in the glomeruli of the antennal lobes as well as in the optic lobes (Figure 4). Lower levels of punctate synaptogyrin immunofluorescence are found throughout the adult brain. Synaptogyrin also localizes to the neuromuscular junctions of third instar larvae where

it colocalizes with synaptotagmin 1 (Figure 5A) and partially overlaps with the active zone marker bruchpilot (nc82) (Figure 5B-C). Synaptogyrin staining often is absent in the center of boutons, which reflects the tendency for synaptic vesicles to concentrate on the periphery of boutons as observed via electron microscopy (Atwood et al., 1993). Taken together, these results indicate that, like other synaptogyrin homologs, *Drosophila* synaptogyrin is a neuronal synaptic vesicle protein.

Next, we utilized the GAL4/UAS system to compare the neuronal localization of GFP-tagged *Drosophila* synaptogyrin (gyrin-GFP) and RFP-tagged *Drosophila* SCAMP (SCAMP-RFP) driven by the pan-neuronal driver *elav^{c155}-GAL4* (Brand and Perrimon, 1993). SCAMP-RFP and gyrin-GFP extensively colocalize at the larval NMJ (Figure 6A), but show a more limited degree of overlap in the larval ventral nerve cord, with gyrin-GFP generally displaying a more restricted expression pattern (Figure 6B). In the large secretory cells of the salivary glands, SCAMP-RFP and gyrin-GFP also display a significant amount of colocalization (Figure 6C). However, gyrin-GFP fluorescence is absent from many large SCAMP-RFP-positive puncta, suggesting that *Drosophila* SCAMP and synaptogyrin have different localization patterns. While these experiments do not indicate the endogenous expression pattern of *Drosophila* SCAMP, they do suggest that synaptogyrin and SCAMP are to some extent targeted to different subcellular compartments.

Generation of a *Drosophila synaptogyrin* mutant

We next sought to investigate the function of *Drosophila* synaptogyrin by creating and characterizing a *synaptogyrin* null mutant. We isolated two independent partial deletions of the *synaptogyrin* genomic locus via imprecise excision of a P-

element insertion located approximately 500 bp upstream of the synaptogyrin translation start site. The first deletion, *gyr*^{1}, extends 2.5 kb into the *synaptogyrin* locus and removes the first two exons and a portion of the third exon, while the second deletion, *gyr*^{2}, is a smaller 1.7 kb deletion that removes the first exon (Figure 7A). A precise excision line, *gyr*^{PE}, was chosen to serve as the control for genetic background in all experiments. The extent of the deletions and the precise excision event were confirmed by PCR and sequencing (Figure 7B). A Western blot of protein extracts revealed the complete absence of synaptogyrin immunoreactivity in both *gyr*^{1} and *gyr*^{2} animals (Figure 7C). Similarly, synaptogyrin antibody staining was absent at the neuromuscular junction in *gyr* third instar larvae (Figure 7D). The antibody raised against synaptogyrin targets the C-terminus of the protein, and since the coding sequence of this region was left intact in both deletion lines, it is unlikely that a truncated version of the protein from a downstream start site is being produced. We therefore conclude that *gyr*^{1} and *gyr*^{2} are likely to be null mutations. Unless otherwise noted, *gyr*^{1} animals were used in all experiments.

In agreement with nematode and mouse knockouts, *Drosophila gyr* animals are viable, fertile, and appear behaviorally normal. We examined the protein levels of a variety of synaptic proteins in adult head extracts and found that there were no dramatic alterations in protein expression (Figure 8A). Since synaptophysin influences synaptobrevin localization in cultured neurons (Pennuto et al., 2003; Gordon et al., 2011) and synaptogyrin promotes the targeting of synaptophysin to microvesicles in PC12 cells (Belfort and Kandror, 2003), we examined whether the loss of synaptogyrin affected the localization of synaptobrevin or synaptotagmin 1 in *Drosophila*. Both

synaptotagmin and synaptobrevin are correctly targeted to boutons at the larval NMJ in *gyr* mutants, with no apparent protein mislocalization to axons or inter-bouton regions (Figure 8B). Therefore, we conclude that the loss of synaptogyrin does not dramatically affect the expression or localization of synaptotagmin and synaptobrevin. However, it remains possible that other synaptic proteins are mislocalized in *gyr* animals.

We then asked whether the loss of synaptogyrin might affect the development or overall health of *gyr* mutants. When cultured in parallel, *gyr* mutants and controls proceed through the larval instar stages, pupate, and eclose at a similar rate, indicating no developmental delay (data not shown). We assayed the viability of *gyr* animals at 25°C by generating lifespan curves and quantifying the T_{50} (age at which 50% of the animals have died) for control (*gyr^{PE}*) and *gyr* adult males and females, as well as for synaptogyrin rescues (*elav^{c155}-GAL4*; *gyr*; *UAS-myc-gyrin*/+) and *gyr* with the GAL4 driver alone (*elav^{c155}-GAL4*; *gyr*). The T_{50} values were nearly identical for control (males = 71 days, females = 63 days) and *gyr* (males = 71 days, females = 62 days), indicating that *gyr* does not cause a decrease in lifespan (Figure 8C). The presence of the *elav^{c155}-GAL4* driver slightly increased life expectancy, with both driver alone (males = 78 days, females = 67 days) and rescue animals (males = 79 days, females = 73 days) having a higher T_{50} than control and *gyr* animals. This is most likely due to differences in genetic backgrounds, as the *elav^{c155}-GAL4* lines are in the *white^{+}* background, while control and *gyr* animals are *white^{-}*. Nevertheless, we conclude that synaptogyrin does not significantly impact the viability, fertility, or lifespan of *Drosophila*.

Behavioral analysis of *gyr* mutants reveals no significant deficits in climbing ability or courtship

Although synaptogyrin-deficient animals do not display any noticeable motor defects or a decrease in life expectancy, we wondered whether *gyr* mutants might have more subtle changes in locomotion or behavior. We therefore performed a climbing assay to determine whether *gyr* mutants were impaired in their basic motor function. *Drosophila* adults normally display a strong negative geotactic response (i.e., they climb upward) if disturbed (Erlenmeyer-Kimling and Hirsch, 1961; Desroches et al., 2010). Climbing ability can be impaired by a wide variety of factors, including advanced age (Gargano et al., 2005), exposure to certain metals (Bonilla-Ramirez et al., 2011), or overexpression of α-synuclein in a *Drosophila* model of Parkinson's disease (Feany and Bender, 2000). We compared the climbing ability of two-day-old and two-week-old *gyr* and control adult males by gently knocking the flies to the bottom of a vial and measuring the fraction of flies that had climbed 3 cm over 10 s intervals (Figure 9). Control and mutant animals performed similarly at both ages tested ($p > 0.05$, Student's t-test), indicating that there is no dramatic change in locomotor function up to two weeks of age in *gyr* mutants. Although we did not explicitly test climbing ability beyond two weeks of age, during the lifespan analysis we did not observe significant differences between control and *gyr* animals with respect to their ability to walk or climb, suggesting that the loss of synaptogyrin does not severely impact motor coordination in aged animals.

To further analyze the behavior of *gyr* mutants we turned to the well-characterized *Drosophila* male courtship ritual. During courtship a male performs a

series of innate, stereotyped behaviors including tracking a female, tapping her with his forelimbs, extending and vibrating a wing to produce a species-specific wing song, licking the female's genitalia, and bending his abdomen to attempt copulation (Hall, 1994; Greenspan and Ferveur, 2000). These behaviors require the use of multiple sensory modalities as the male integrates visual, auditory, gustatory, and olfactory cues to successfully court a receptive conspecific female. When a naïve male is exposed to an unreceptive courtship target (e.g., a recently mated female or another male), he will learn to suppress courtship behavior upon subsequent encounters (Siegel and Hall, 1979; Vaias et al., 1993; Siwicki and Ladewski, 2003). This courtship suppression, also known as courtship conditioning, can last for hours to days depending on the training protocol and therefore provides a method of assaying short- and long-term learning and memory in *Drosophila* (Siegel and Hall, 1979; McBride et al., 1999).

We examined courtship conditioning in *gyr* mutant and control animals using a training protocol in which virgin males are paired with a previously mated female in a food vial for five hours (McBride et al., 1999). During this training period the males court on-and-off and therefore experience multiple rounds of rejection, which is thought to mimic the spaced training required to establish long-term memory in several *Drosophila* learning paradigms (Tully et al., 1994; Keleman et al., 2007). One day following training, the courtship behavior of trained males is compared to age-matched naïve males to determine the extent of courtship suppression (Figure 10A). Courtship levels are quantified using the courtship index (CI), which is defined as the fraction of time a male spends engaged in courtship behaviors over the course of ten minutes or until successful copulation.

Naïve *gyr* animals have a similar CI compared to naïve control males, and visually their overall courtship behavior appears normal (Figure 10B; control = 0.68 ± 0.05; *gyr* = 0.73 ± 0.05, $p > 0.05$, Student's t-test). A majority of mutant males successfully copulated during the ten-minute observation period (10 of 17), indicating that *gyr* males are not considerably impaired in their ability to complete the courtship ritual (13 of 17 control males copulated). Furthermore, both *gyr* and control males that underwent training with a mated female displayed a similar amount of courtship suppression (indicated by a decrease in the CI) one day following training, signifying that *gyr* animals are also capable of learning (Figure 10B; control = 0.40 ± 0.08; *gyr* = 0.47 ± 0.08, $p > 0.05$, Student's t-test). We also quantified the average time to courtship initiation and the average time to copulation (for those copulations occurring in less than ten minutes) and found no significant differences between control and *gyr* males (Figure 10 C-D; $p > 0.05$, Student's t-test). However, males of both genotypes that had undergone training with a previously mated female took a significantly longer time to initiate courtship with the tester female compared to naïve males (Figure 10 C; $p < 0.05$, two-way ANOVA). This is unsurprising given that training induced an overall decrease in courtship behavior, therefore trained males would be expected to take longer to begin courting.

Taken together, these results reveal no significant differences in courtship behavior or the ability of *gyr* mutants to form long-lasting (one day) courtship memory. While we cannot rule out defects in other behavioral or learning paradigms, synaptogyrin does not appear to be required for locomotion or courtship, two essential *Drosophila* behaviors.

Discussion

Drosophila synaptogyrin is a synaptic vesicle protein

In mammals, synaptogyrin and synaptophysin are highly enriched on synaptic vesicles, with little to no expression outside of neuronal or neuroendocrine cells (Wiedenmann and Franke, 1985; Baumert et al., 1990). However, both proteins have ubiquitously expressed non-neuronal homologs (cellugyrin and pantophysin), while the synaptophysin homolog mitsugumin 29 is only expressed in skeletal muscle, kidney, and the small intestine (Shimuta et al., 1998; Komazaki et al., 1999; Hubner et al., 2002). Protein BLAST searches confirmed that *Drosophila* synaptogyrin is similar in sequence to mammalian synaptogyrin 1, but this does not guarantee that *Drosophila* synaptogyrin is also a synaptic vesicle protein. Indeed, the expression pattern of the *C. elegans* synaptophysin homolog is restricted to certain muscle cells (Abraham et al., 2006), which implies that synaptophysin function is more limited in nematodes.

Our work suggests that *Drosophila* synaptogyrin is a true neuronal homolog as it is broadly expressed throughout the nervous system, including at the neuromuscular junction where it colocalizes with the synaptic vesicle protein synaptotagmin 1. However, we did not investigate whether synaptogyrin is restricted to certain neuronal cell types or whether it is expressed pan-neuronally. Only faint, nonspecific staining is observed in the muscle, suggesting that synaptogyrin is not expressed postsynaptically at the NMJ. Nevertheless, we cannot rule out that synaptogyrin expression is also postsynaptic in the central nervous system, or that it is present in non-neuronal cell types. However, synaptogyrin mRNA expression levels as described by FlyAtlas (http://flyatlas.org) confirm that synaptogyrin is enriched 10-26 times in the brain

relative to the whole fly, with little to no synaptogyrin mRNA present in non-neuronal tissues (Chintapalli et al., 2007).

Drosophila synaptogyrin comigrates with other known synaptic vesicle proteins in sucrose density gradients, suggesting that it localizes to synaptic vesicles. The extent to which synaptogyrin is targeted to other intracellular membranes is currently unknown, although we expect that some fraction of synaptogyrin is found on endosomes and at the plasma membrane as a result of synaptic vesicle exo-endocytosis and normal intracellular trafficking. Neuronal coexpression of tagged synaptogyrin and SCAMP transgenes revealed a high amount of colocalization at the larval NMJ and ventral nerve cord, suggesting that *Drosophila* SCAMP, like several mammalian SCAMPs, is also targeted to synaptic vesicles (Fernandez-Chacon and Sudhof, 2000; Hubner et al., 2002). However, SCAMP-RFP also was found in many regions that lacked gyrin-GFP, indicating that it may associate with a greater range of subcellular structures and therefore may play a more general role in intracellular trafficking. The endogenous expression pattern of *Drosophila* SCAMP is currently unknown, but its mRNA appears to be broadly expressed throughout the animal (http://flyatlas.org), suggesting that its function is not limited to neurons.

The loss of synaptogyrin does not significantly impact certain adult behaviors

The behavioral assays we undertook suggest that *gyr* adult flies are highly coordinated and that males are capable of effective courtship behavior. Although we cannot rule out subtle courtship defects such as alterations in wing song vibration, *gyr* males were capable of performing all aspects of the courtship ritual. The finding that *gyr* males are able to remember prior courtship encounters for at least one day suggests

that synaptogyrin is not required for all types of learning and memory in flies. However, it is currently unknown whether *gyr* mutants have defects in retaining memories for periods beyond one day. To examine this possibility, we attempted the same courtship conditioning assay with a four-day interval between training and testing, but we only observed a slight, non-significant drop in the CI of trained males compared to naïve males in both control and *gyr* animals. Changes to our experimental protocol, such as testing a larger number of males or using an immobilized female (which also strongly inhibits courtship), might allow us to determine whether the loss of synaptogyrin impacts long-term memory beyond one day (Siegel and Hall, 1979). Similarly, other behavioral assays for testing long-term memory could be employed, such as the classical conditioning paradigm in which olfactory cues are paired with a shock (Tully and Quinn, 1985; Tully et al., 1994). Work in mice has implicated synaptophysin in certain aspects of learning and memory including spatial learning and object novelty recognition (Schmitt et al., 2009). However, synaptogyrin's impact on learning and memory in mice has not been investigated.

Synaptogyrin is not required for viability, fertility, or basic motor function

Our results indicate that synaptogyrin is not essential for viability, fertility, basic motor function, or courtship in *Drosophila*. This is consistent with knockout studies in other species that have also identified relatively mild phenotypes due to the loss of synaptogyrin and/or synaptophysin. Functional redundancy between synaptogyrin and synaptophysin has been documented in mice (Janz et al., 1999). Therefore, it is possible that the lack of severe phenotypes in flies, mice, or nematodes is due to compensation by some as yet unidentified protein or proteins. SCAMPs, although

lacking a MARVEL domain, have historically been linked with physins and gyrins because they share the same transmembrane topology and some SCAMP paralogs are located on synaptic vesicles (Brand et al., 1991; Fernandez-Chacon and Sudhof, 2000; Hubner et al., 2002). Therefore, one or more SCAMPs potentially could compensate for the loss of gyrins and physins. However, the *C. elegans* synaptogyrin/synaptophysin/ SCAMP triple knockout demonstrates that the absence of all of these protein families does not result in a dramatic neuronal phenotype (Abraham et al., 2006). Since mice have four physins, four gyrins, and five SCAMPs, it is impractical to resolve this issue using the murine model. Even if one were to focus only on the proteins highly enriched in neurons, the total would still be six genes – synaptophysin, synaptoporin, synaptogyrins 1 and 3, and SCAMPs 1 and 5 (Grabs et al., 1994; Kedra et al., 1998; Fernandez-Chacon and Sudhof, 2000; Belizaire et al., 2004). *Drosophila* has only a single SCAMP homolog and is therefore amenable to genetic analysis to examine issues of redundancy in future studies, although the work in *C. elegans* suggests it is unlikely that a *Drosophila* synaptogyrin/SCAMP double knockout would have severe neuronal impairments.

Methods

Homology searches and protein alignment

NCBI BLAST (blastp) was used to identify potential homologs, which were confirmed using a reciprocal blastp search (http://blast.ncbi.nlm.nih.gov). TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM) was used to verify that the prospective homologs had four transmembrane domains in the correct orientation (cytoplasmic N- and C-termini). The synaptogyrin protein alignment was performed using the T-Coffee algorithm (http://tcoffee.org) with default settings (Notredame et al., 2000; Di Tommaso et al., 2011). CLC DNA Workbench 4.0 was used to visualize the alignment.

GenBank accession numbers used in the analysis are as follows:

Synaptogyrin family: *Drosophila melanogaster*, AAF58329; *Homo sapiens* (synaptogyrin 1a), EAW60323; *Mus musculus* (synaptogyrin 1a), AAI38729; *Danio rerio*, AAI65917; *Tribolium castaneum*, EFA07832; *Caenorhabditis elegans*, AAC27798; *Trichoplax adhaerens*, EDV27716; *Monosiga brevicollis*, EDQ85723

Synaptophysin family: *H. sapiens*, AAB92358; *M. musculus*, EDL33904; *D. rerio*, CAQ13974; *T. castaneum*, EFA10995; *C. elegans*, AAB92070; *N. vectensis*, EDO44392 and EDO43817; *T. adhaerens*, EDV29003

CMTM (CKLF-like MARVEL transmembrane domain-containing) family: *H. sapiens* (CMTM4 isoform 1), AAN73845; *M. musculus* (CMTM4), AAN73437; *D. rerio* (CMTM4), AAH76315; *D. melanogaster*, AAF47955; *T. castaneum*, NCBI Reference Sequence XP_972350; *C. elegans*, AAB52340 (the exact protein length is disputed, see also AAV58868); *N. vectensis*, EDO45986; *T. adhaerens*, EDV25996

Occludin family: *H. sapiens*, AAC50451; *M. musculus*, AAC52515; *D. rerio*, AAH49304

MAL family: *H. sapiens*, AAA36196; *M. musculus*, AAH06826; *D. rerio*, AAH93153

MYADM family: *H. sapiens*, AAH13995; *M. musculus*, AAH56355; *D. rerio*, CAQ13223

SCAMP family: *H. sapiens* (SCAMP 1), AAH15065; *M. musculus* (SCAMP 1), AAH34283; *D. rerio* (SCAMP 1), AAH65684; *D. melanogaster*, AAF64492; *T. castaneum*, EFA11500; *C. elegans*, AAF36687; *N. vectensis*, EDO31962; *T. adhaerens*, EDV26892; *M. brevicollis*, EDQ84443

Fly stocks and transgenics

Flies were cultured using standard media and techniques at room temperature (~22°C) unless otherwise noted. The GAL4/UAS system was utilized to drive neuronal expression of selected transgenes (Brand and Perrimon, 1993). The UAS-synaptogyrin-GFP (gyrin-GFP) and UAS-SCAMP-RFP (SCAMP-RFP) constructs were subcloned into pUAST vectors using standard subcloning techniques and were injected into *white* (w^{1118}) embryos. The UAS-myc-gyrin construct used for rescue experiments was subcloned into a modified pValum vector with an N-terminal myc tag (Cho et al., 2010). This construct was injected into the strain *yv;;attP2*, which contains a site for targeted transgene insertion on the third chromosome (Markstein et al., 2008; Ni et al., 2008). The pan-neuronal driver $elav^{c155}$*-GAL4* was used to express the transgenes in neurons. Embryo injections were performed at Duke University Model Systems Genomics (Durham, NC) and Genetic Services, Inc. (Cambridge, MA).

PCR primers were as follows:

gyrin-GFP 5’: 5’ – CGG AAT TCG GCG CCG GCG ATG AGT TCA C – 3’

gyrin-GFP 3’: 5’ – CTA CTT ATG CGG CCG CAT AGG TGG GCT GCT GGT ACT – 3’

myc-gyrin 5’: 5’ – CGC ATA TGG ACA TGC TCA ACC AGA TAC TC – 3’

myc-gyrin 3’: 5’ – GCT CTA GAC AAG TGT GGT AAT TCC TTA ATA G – 3’

Generation of a *synaptogyrin* null mutant

Two independent partial deletions of the *synaptogyrin* locus were generated by imprecise excision of a P-element (P{lacW}l(2)SH0644^{SH0644}) located approximately 500 bp upstream of the synaptogyrin translation start site in the first exon of the gene. This P-element insertion is listed as lethal on FlyBase (http://www.flybase.org). However, we were able to obtain viable and fertile homozygous animals, which suggests that either an unrelated lethal mutation was lost or a suppressor emerged. Approximately 200 *white*$^-$ excision events were screened by PCR, two of which resulted in deletions that extended into the *synaptogyrin* locus beyond the translation start site. The first, gyr^1, is a 2.5 kb deletion that removes the first two exons and part of the third exon; the second, gyr^2, is a 1.7 kb deletion that removes the first exon. A precise excision line isolated from the screen, gyr^{PE}, was confirmed to be a precise excision via sequencing and was used as a control for genetic background in all experiments. To confirm that the deletions in gyr^1 and gyr^2 were indeed null mutations, we performed Western blots on protein extracts from these lines as well as from *white* (w^{1118}) controls and gyr^{PE}. The polyclonal antibody raised against the synaptogyrin protein targets the C-terminus, and this coding region was left intact in both alleles. Synaptogyrin immunoreactivity was completely absent in gyr^1 and gyr^2 animals, indicating that a downstream start site is not producing a truncated version of the protein. gyr^1 and gyr^2 are in the *white* (w^{1118}) background unless otherwise indicated.

PCR primers used for the screen were as follows:

Across the P-element insertion site:

Primer 1: 5' – GTC CAT GGT GAT GAT GGG TCT CTG ATG – 3'

Primer 2: 5' – CAA TAT ATC TTG GGA GCT CTG CTG – 3'

Sequencing primers for deletions:

Primer 1: 5' – CGG AGG AGC AAG TGT GGT AAT TC – 3'

Primer 2: 5' – CAA TAT ATC TTG GGA GCT CTG CTG – 3'

Synaptogyrin antibody generation

The C-terminal fragment of *Drosophila* synaptogyrin encoding amino acids 181-241 (gyrin-cterm) was amplified from a *Drosophila* cDNA library and subcloned into the pGEX-4T-1 vector (GE Healthcare) using the EcoRI and NotI restriction sites. Recombinant GST-gyrin-cterm was expressed and processed in *E. coli* (BL21) using standard protocols. The fusion protein was purified from cell lysates using Glutathione Sepharose 4B (Amersham Biosciences) and was then used to immunize rabbits to generate polyclonal antibodies (Invitrogen). The synaptogyrin antibody serum recognizes a single major band at approximately 29 kDa on Western blots.

PCR primers for cloning into the pGEX4T-1 vector were as follows:

gyrin-cterm 5': 5' – CGG AAT TCG GCG CCG GCG ATG AGT TCA C – 3'

gyrin-cterm 3': 5' – CTA GTT ATG CGG CCG CAT AGG TGG GCT GCT GGT ACT – 3'

Immunohistochemistry

Wandering third instar larvae or pharate adults were dissected in calcium-free HL3.1 saline (70 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 4 mM $MgCl_2$, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES, pH 7.2) and fixed for 45 minutes in HL3.1 containing 4%

formaldehyde. Fixations using the synaptogyrin antibody (α-Gyr) were fixed for 5 minutes in ice-cold 100% methanol. Following several washes in phosphate-buffered saline (PBS) and PBST (PBS with 0.1% Tween 20), larvae were incubated with primary antibodies in PBST overnight at 4°C. After additional washes, larvae were incubated in secondary antibodies in PBST for four hours at room temperature, washed, and mounted in 70% glycerol. The dilutions for primary antibodies were: synaptogyrin, 1:500; nc82/bruchpilot, 1:100; synaptotagmin 1 (monoclonal), 1:200 (3H2 2D7, from K. Zinn); synaptotagmin 1 (polyclonal), 1:500 (Littleton et al., 1993); and synaptobrevin, 1:500. The nc82 antibody developed by Erich Buchner was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Secondary antibodies obtained from Jackson ImmunoResearch were used at a dilution of 1:250 and include Cy2-conjugated goat anti-rabbit, Cy3-conjugated goat anti-rabbit, and Rhodamine Red-conjugated donkey anti-mouse. Goat α-HRP antibodies conjugated to either fluorescein isothiocyanate (FITC) or Rhodamine Red were added as indicated with secondary antibodies and were used at a concentration of 1:10,000 or 1:500, respectively. Images were acquired using confocal microscopy (Axoplan 2; Carl Zeiss MicroImaging, Inc.) using PASCAL software (Carl Zeiss) with 40x, 63x, or 100x oil-immersion lenses.

Density gradient centrifugation

The density gradient experiments were based on (van de Goor et al., 1995) and (Adolfsen et al., 2004). Buffer A (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM

$MgCl_2$) was used as the lysis buffer and to make the sucrose gradients. Approximately 5 mL of adult flies of were collected on ice, flash frozen in liquid nitrogen, and vortexed vigorously to dislodge the heads from the bodies. Heads were collected using a pre-cooled sieve and homogenized on ice with a Dounce homogenizer in 1.5 mL buffer A with protease inhibitors (1 mM PMSF; 2 μg/mL aprotinin; 1 μg/mL leupeptin; 1 μg/mL pepstatin A; 1 mM EDTA). Post-nuclear homogenates were then loaded onto 10-30% sucrose gradients with a 50% sucrose pad and centrifuged in an SW 41 Ti rotor (Beckman Coulter) at 36,000 rpm for 4.5 hours at 4°C. Twenty 600 μL fractions were collected from the top of the gradient and were mixed with an equal volume of 2X SDS sample buffer prior to analysis by Western blot. Primary antibody concentrations were: synaptogyrin, 1:2,000; CSP, 1:500 (Zinsmaier et al., 1994); synaptotagmin 1 (DSyt2), 1:500 (Littleton et al., 1993); synaptobrevin, 1:1,000 (van de Goor et al., 1995); tubulin, 1:60,000; complexin, 1:2,000 (Huntwork and Littleton, 2007); HRS, 1:20,000 (Lloyd et al., 2002); syntaxin, 1:1,000 (8C3). The monoclonal antibody against syntaxin (8C3) developed by Seymour Benzer was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The secondary antibodies IRDye 800-conjugated goat anti-mouse (LI-COR Biosciences), IRDye 800-conjugated goat anti-rabbit (LI-COR Biosciences), and IRDye 800-conjugated goat anti-guinea pig (Rockland Immunochemicals, Inc.) were used at a dilution of 1:5,000. Membranes were visualized using the LI-COR Odyssey Imaging System (LI-COR Biosciences).

Western blots

Western blots were performed using standard laboratory procedures. Nitrocellulose membranes were blocked for 30 minutes at room temperature in four parts PBS to one part Rockland Blocking Buffer (Rockland Immunochemicals, Inc.). Primary antibody incubation was done overnight at 4°C in a solution containing four parts PBST (PBS with 0.1% Tween 20) to one part Rockland Blocking Buffer. Primary antibody concentrations were: synaptogyrin, 1:20,000; synaptobrevin, 1:5,000 (van de Goor et al., 1995); SNAP 25, 1:1,000; Rab 3, 1:500; complexin, 1:5,000 (Huntwork and Littleton, 2007); CSP, 1:200 (Zinsmaier et al., 1994); syntaxin, 1:1,000 (8C3); synaptotagmin 1 (DSyt2), 1:10,000 (Littleton et al., 1993); Dlg, 1:1,000 (4F3); α-SNAP, 1:1,000 (Babcock et al., 2004); ROP, 1:5,000 (4F8); arginine kinase, 1:10,000. The monoclonal antibodies against syntaxin (8C3), Dlg (4F3), and ROP (4F8) developed by Seymour Benzer, Corey Goodman, and Gerald Rubin, respectively, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The secondary antibodies IRDye 800-conjugated goat anti-mouse (LI-COR Biosciences) and IRDye 800-conjugated goat anti-rabbit (LI-COR Biosciences) were used at a dilution of 1:10,000. Visualization and quantification were performed using the LI-COR Odyssey Imaging System (LI-COR Biosciences).

Lifespan analysis

Approximately 200 males and 200 females of each genotype were collected shortly after eclosion, separated by sex, and grouped into 15 flies per vial. All flies were maintained at 25°C. The number of dead flies was recorded daily, and flies were

transferred to new vials three times per week. Flies lost or injured during transfer were removed from analysis. The totals at the end of the experiment were (female, male): *gyr*^{PE}: 213, 196; *gyr*^{1}: 211, 202; c155 control: 202, 182; rescue: 186, 190. c155 control genotype = *elav*^{c155}*-GAL4*/(*Y* or *w*); *gyr*^{1}; rescue genotype = *elav*^{c155}*-GAL4*/(*Y* or *w*); *gyr*^{1}; *UAS-myc-gyrin*/+

Climbing assay

Males were collected shortly after eclosion and grouped with ten males per vial (four vials per genotype for a total of forty males per genotype) at 25°C. Flies were tested at two days and two weeks post-eclosion and were transferred to new vials every 2-3 days to avoid bacterial growth. On testing days, flies were transferred to empty vials with a line drawn 3 cm above the bottom of the vial. One control vial and one *gyr* vial were tested simultaneously. After allowing the flies to acclimatize for two minutes, the vials were gently banged 5-6 times to knock the flies to the bottom of the vial, and the flies were given one minute to climb per trial. This process was repeated for a total of four trials for each pair of vials. Testing was videotaped, and the number of flies that had crossed the line was recorded for each trial. The four trials per vial were averaged, and then the vial averages were combined to give the genotype average.

Courtship assays

Courtship conditioning assays were based on (Keleman et al., 2007) and (McBride et al., 1999). All flies were raised, trained, and tested at 25°C. The *gyr* mutant and precise excision flies were generated in the *white*$^{-}$ background, which is known to alter courtship and learning (Campbell and Nash, 2001; Diegelmann et al., 2006).

Therefore, *gyr* strains were crossed into the *white*$^{+}$ background for courtship assays. Virgin males were collected shortly after eclosion, isolated in single food vials, and aged 3-4 days in a 12 hr:12 hr light-dark cycle (both training and testing were performed during the light phase). Virgin Canton-S females were aged 3-5 days in groups of 10-15 flies per vial. Canton-S females used for training were mated with Canton-S males (at least three days old) for ~20 hours the day before training and were removed approximately one hour prior to training.

For the training phase, randomly chosen males were either paired with a pre-mated female in a food vial for five hours (trained), or placed in a food vial without a female for five hours (naïve). The cotton plug at the top of the food vial was pushed down to ~1 cm above the food surface to increase the frequency of contact between the male and female. Males were transferred to fresh food vials after training and were kept in isolation until testing. For the testing phase, individual males were paired with virgin Canton-S females in a plastic mating chamber 1 cm in diameter for ten minutes. All tests were videotaped and manually scored for the courtship index, which is defined as the percentage of time a male engages in courtship behaviors during ten minutes or until copulation. Analysis was done blind to the genotype and experimental condition.

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Table 1. Evolutionary conservation of selected MARVEL domain and SCAMP proteins.

	Vertebrates			Arthropods		Nematodes	Cnidarians	Placozoa	Choano-flagellates	Fungi
	H.s.	M.m.	D.r.	D.m.	T.c.	C.e.	N.v.	T.a.	M.b.	S.c.
Gyrin	+	+	+	+	+	+	-[1]	+	+	-
Physin	+	+	+	-[2]	+	+	+	+	-	-
CMTM	+	+	+	+	+	+	+	?[3]	-	-
Occludin	+	+	+	-	-	-	-	-	-	-
MAL	+	+	+	-	-	-	-	-	-	-
MYADM	+	+	+	-	-	-	-	-	-	-
SCAMP	+	+	+	+	+	+	+	+	+	-

+ = homolog; - = no homolog

Notes:

1 N.v. has no gyrin homolog, but it does have two physin-like homologs

2 No sequenced *Drosophila* species has a physin homolog

3 BLAST using mouse CMTM 4 identified a potential T.a. homolog with a partial MARVEL domain, but this protein has only three predicted transmembrane domains

Abbreviations: H.s. = *Homo sapiens*; M.m. = *Mus musculus*; D.r. = *Danio rerio*; D.m. = *Drosophila melanogaster*; T.c. = *Tribolium castaneum*; C.e. = *Caenorhabditis elegans*; N.v. = *Nematostella vectensis*; T.a. = *Trichoplax adhaerens*; M.b. = *Monosiga brevicollis*; S.c. = *Saccharomyces cerevisiae*

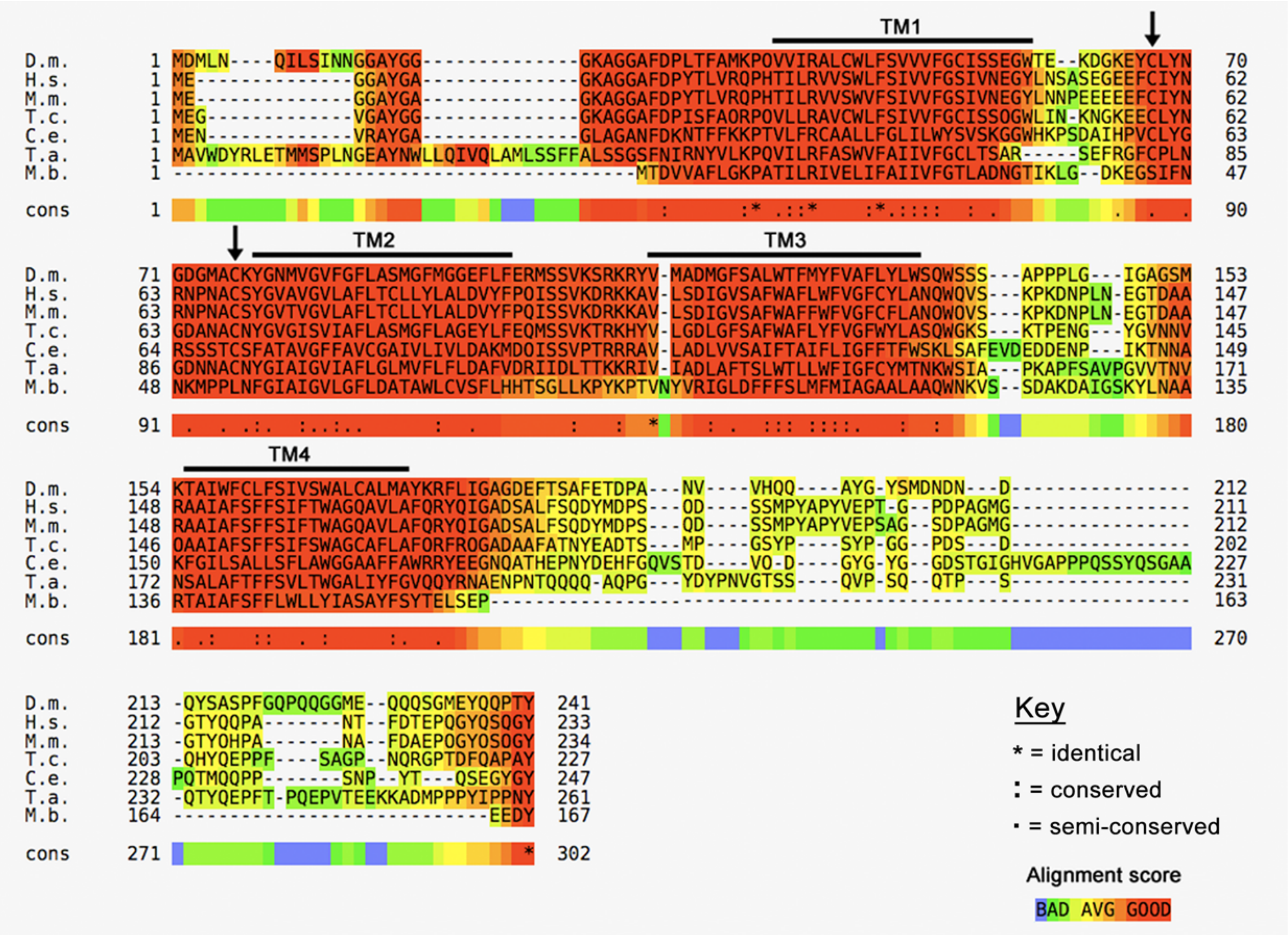

TM1
TM2
TM3
TM4
D.m.
H.s.
M.m.
T.c.
C.e.
T.a.
M.b.
cons
Key
* = identical
: = conserved
. = semi-conserved
Alignment score
BAD AVG GOOD

Figure 1. Protein sequence alignment of synaptogyrin homologs. An alignment of synaptogyrin homologs was generated using the T-coffee algorithm (http://tcoffee.org) with default parameters (Notredame et al., 2000). The T-coffee algorithm assigns a score based on the quality of the alignment, with red regions indicating a good alignment and blue signifying a region with poor alignment. Transmembrane (TM) domains are indicated for *Drosophila* synaptogyrin as determined by the trans-membrane region predictor TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM). Arrows designate the two conserved cysteine residues between the first two transmembrane helices. Sequence abbreviations: D.m., *Drosophila melanogaster*; H.s., *Homo sapiens*; M.m., *Mus musculus*; T.c., *Tribolium castaneum*; C.e., *Caenorhabditis elegans*; T.a., *Trichoplax adhaerens*; M.b., *Monosiga brevicollis*. GenBank accession numbers are listed in Methods.

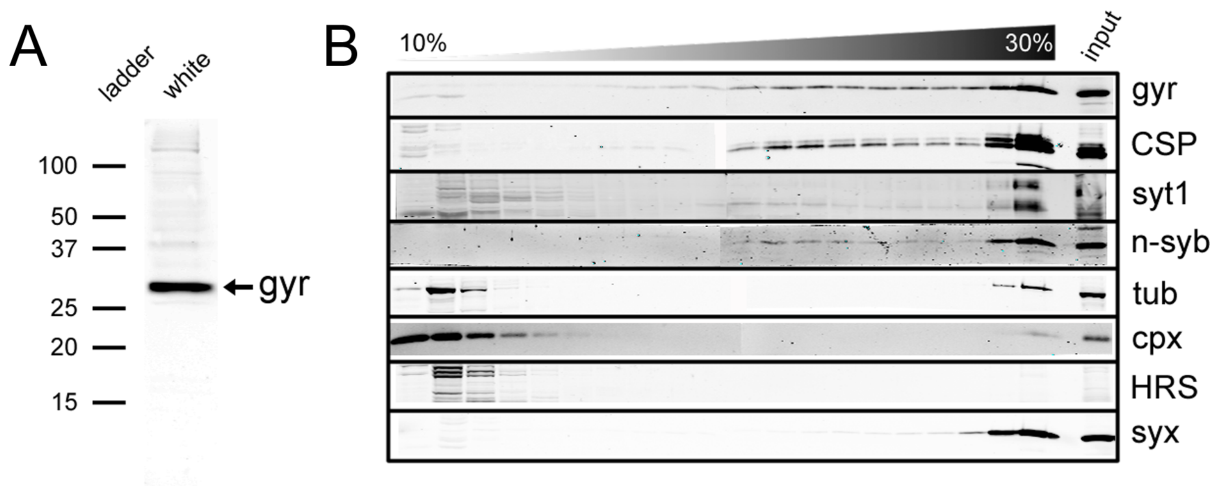


Figure 2. Subcellular localization of *Drosophila* synaptogyrin. **A)** A polyclonal synaptogyrin antibody recognizes a single major band at approximately 29 kDa in adult head homogenates. **B)** Post-nuclear head homogenates were separated using a 10-30% sucrose density gradient. Fractions were analyzed by Western blot using antibodies for various subcellular markers. Synaptotagmin 1 (syt1), synaptobrevin (n-syb) and cysteine string protein (CSP) were used as synaptic vesicle fraction markers. Antisera against HRS were used to identify fractions containing endosomes (left-most fractions), while syntaxin (syx) antibodies were used to mark the plasma membrane (right-most fractions). Fractions were also immunostained with complexin (cpx) and tubulin (tub) antisera. Synaptogyrin (gyr) primarily co-migrates with other synaptic vesicle proteins.

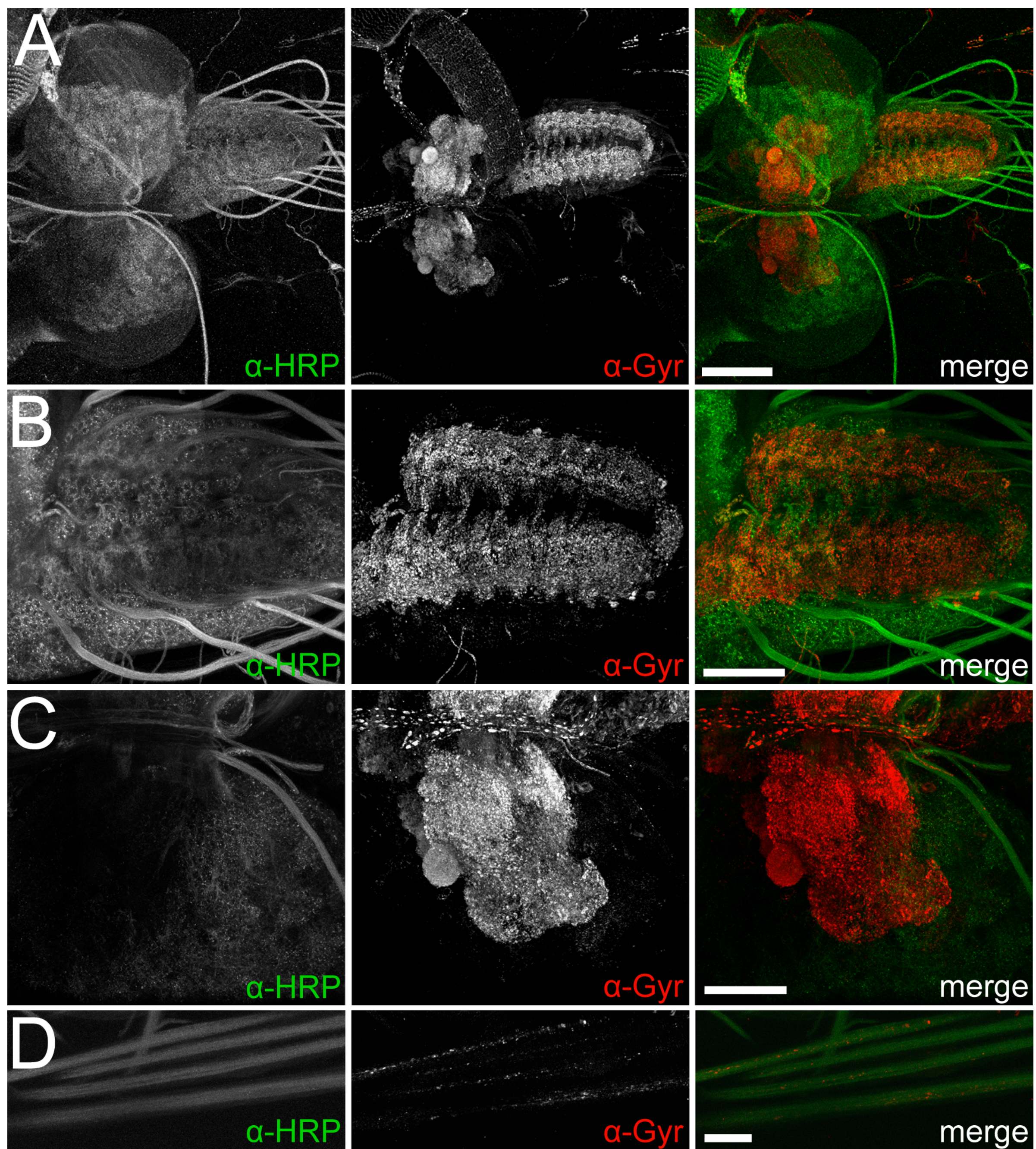
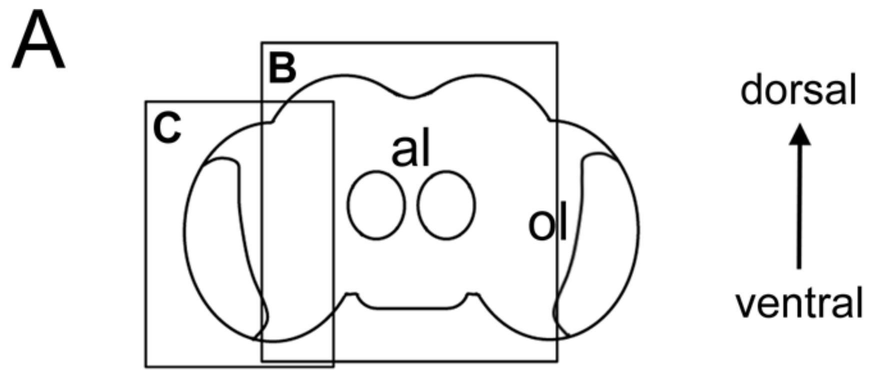

A
α-HRP
α-Gyr
merge
B
α-HRP
α-Gyr
merge
C
α-HRP
α-Gyr
merge
D
α-HRP
α-Gyr
merge

Figure 3. Synaptogyrin protein expression in larvae. **A)** Immunohistochemistry with antisera against the neuronal membrane marker horseradish peroxidase (HRP, green) and synaptogyrin (Gyr, red) indicates that synaptogyrin is broadly expressed throughout the central nervous system in third instar larvae. **B)** A higher magnification image of (A) reveals punctate synaptogyrin staining along the ventral nerve cord. **C)** One lobe of the larval brain from (A) at higher magnification. **D)** Synaptogyrin-positive puncta are also present in motorneuron axons and likely mark synaptic vesicles trafficking to the neuromuscular junction. Scale bars: A = 100 μm; B and C = 50 μm; D = 20 μm.


A
B
C
al
ol
dorsal
ventral

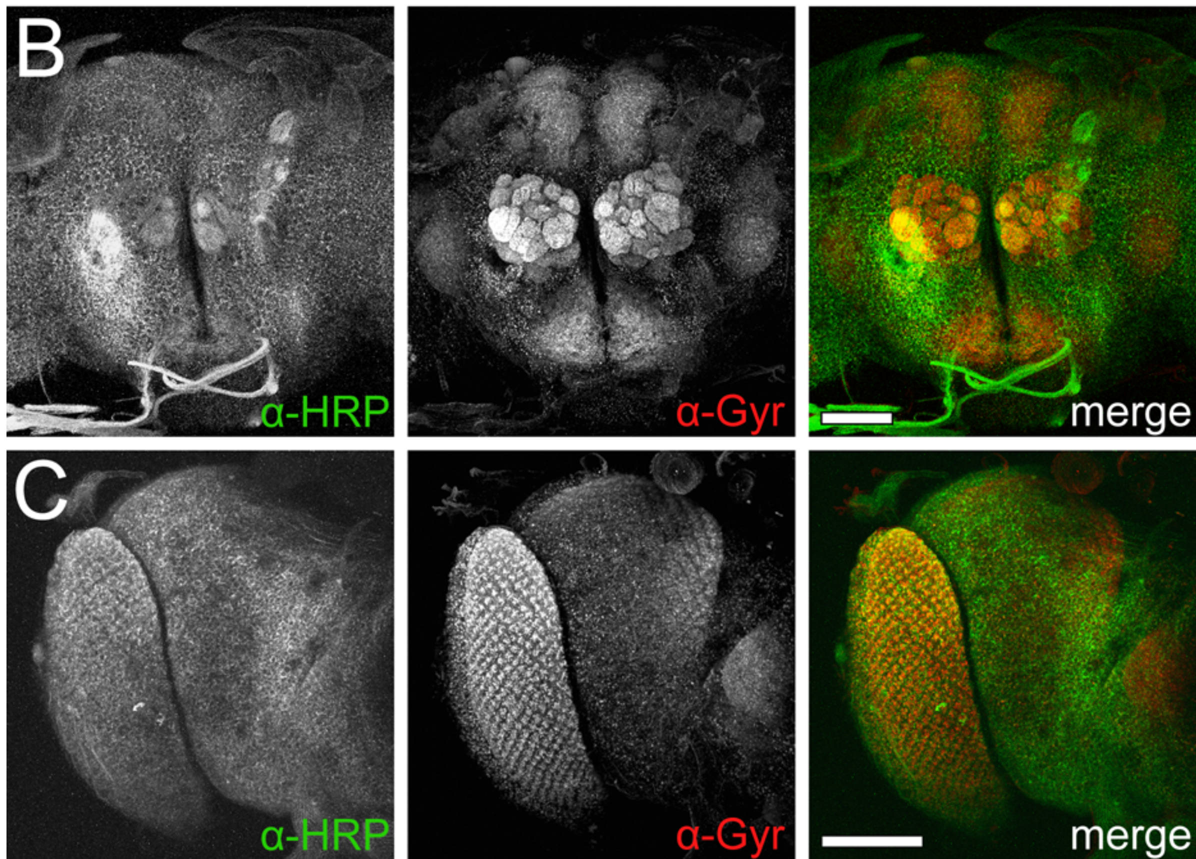

B
α-HRP
α-Gyr
merge
C
α-HRP
α-Gyr
merge

Figure 4. Synaptogyrin protein expression in the adult brain. **A)** A schematic of the adult brain (anterior view) with the regions imaged in (B) and (C) indicated by boxes. The antennal lobes (al) and optic lobes (ol) are highlighted since these regions have high levels of synaptogyrin protein expression. **B)** Synaptogyrin immunostaining (Gyr, red) is located throughout the central brain regions with particularly high expression found in the glomeruli of the antennal lobes. Horseradish peroxidase staining (HRP, green) labels neuronal membranes. **C)** Synaptogyrin protein expression is abundant in the optic lobes of adult flies. Scale bars = 50 μm.

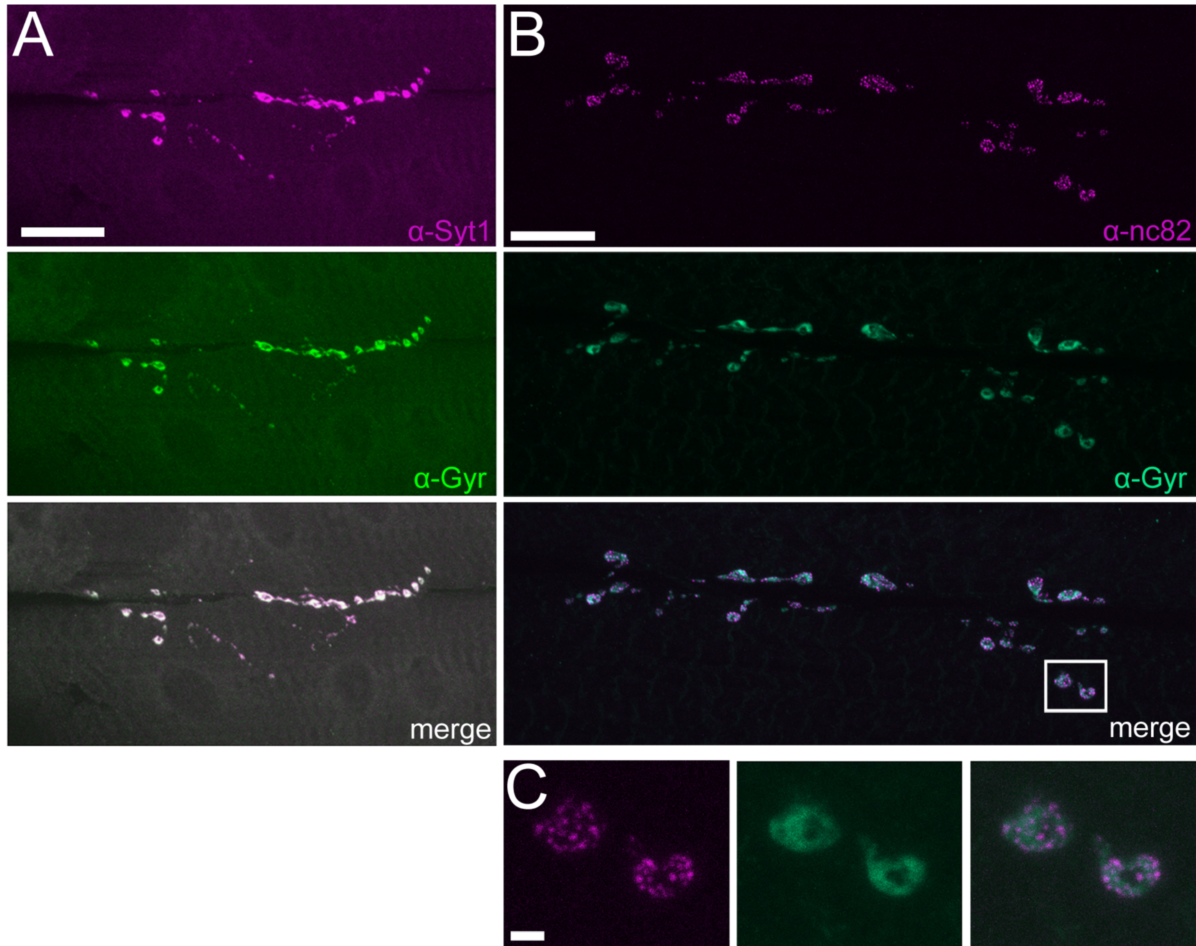


Figure 5. Synaptogyrin protein localization at larval NMJs. **A)** Synaptogyrin (Gyr, green) is expressed presynaptically at the larval NMJ where it colocalizes with synaptotagmin 1 (Syt1, magenta). **B)** Synaptogyrin is not confined to active zones, which are indicated by nc82 staining (magenta). **C)** A magnified view of the region indicated in (B) showing synaptogyrin's localization within boutons. Scale bars: A = 25 µm; B = 20 µm; C = 2 µm.

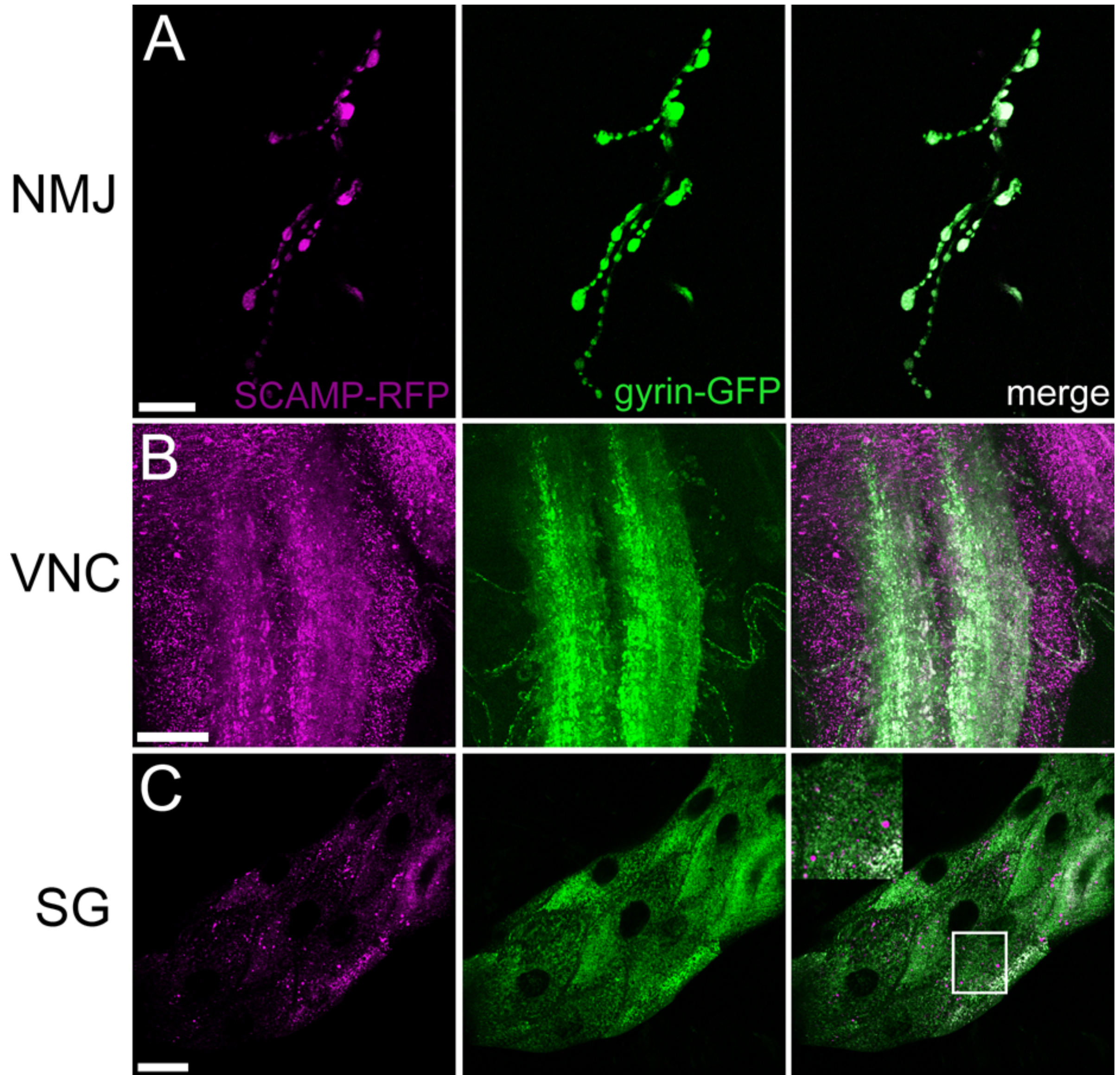
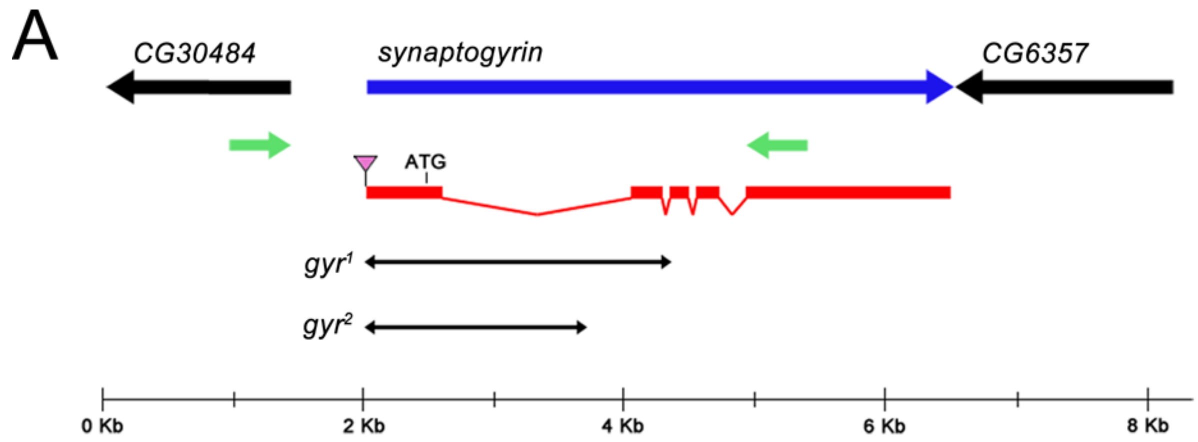
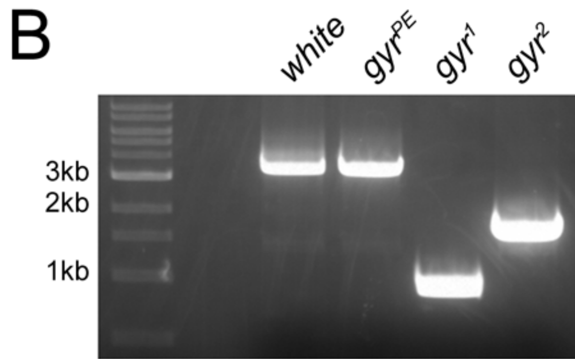

A
NMJ
SCAMP-RFP
gyrin-GFP
merge
B
VNC
C
SG

Figure 6. Gyrin-GFP and SCAMP-RFP partially colocalize in the nervous system. **A)** SCAMP-RFP (magenta) and gyrin-GFP (green) almost completely colocalize at the third instar larval NMJ when driven with a pan-neuronal GAL4 driver, suggesting that SCAMP is also targeted to synaptic vesicles. Both transgenes are enriched in boutons and are largely absent from the regions between boutons. **B)** SCAMP-RFP and gyrin-GFP have punctate staining along the larval ventral nerve cord (VNC), although gyrin-GFP localization is more restricted, especially in the lateral regions of the VNC. **C)** Cells in the salivary gland (SG) also express the SCAMP and gyrin transgenes. While there is some degree of overlap, SCAMP-RFP and gyrin-GFP also appear to be trafficked to different subcellular compartments. Note that gyrin-GFP is excluded from many large SCAMP-RFP-positive puncta (see inset). Scale bars: A = 20 μm; B and C = 50 μm.


A
CG30484
synaptogyrin
CG6357
ATG
gyr1
gyr2
0 Kb
2 Kb
4 Kb
6 Kb
8 Kb


B
white
gyrPE
gyr1
gyr2
3kb
2kb
1kb

C
gyr1
gyr2
gyrPE
white
α-gyrin
α-cpx

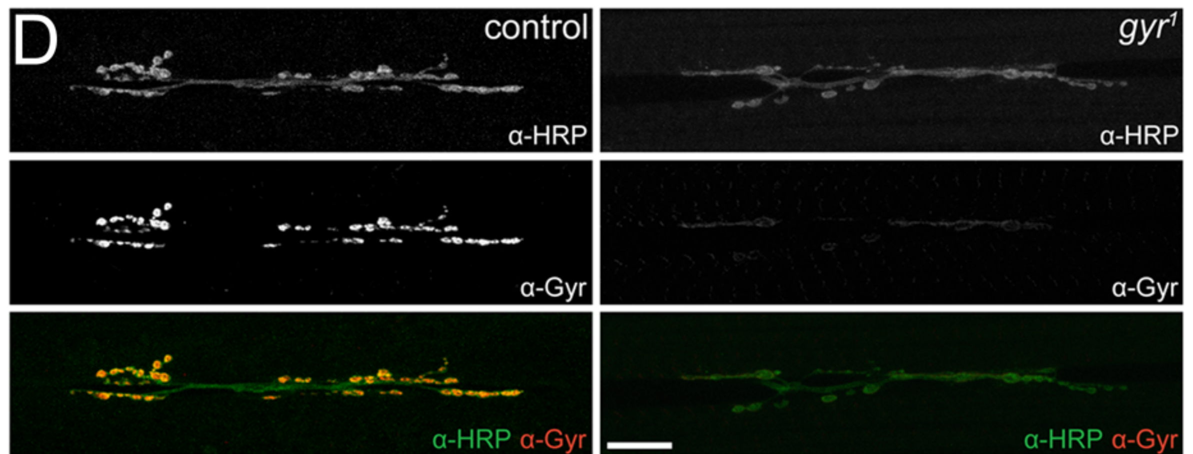
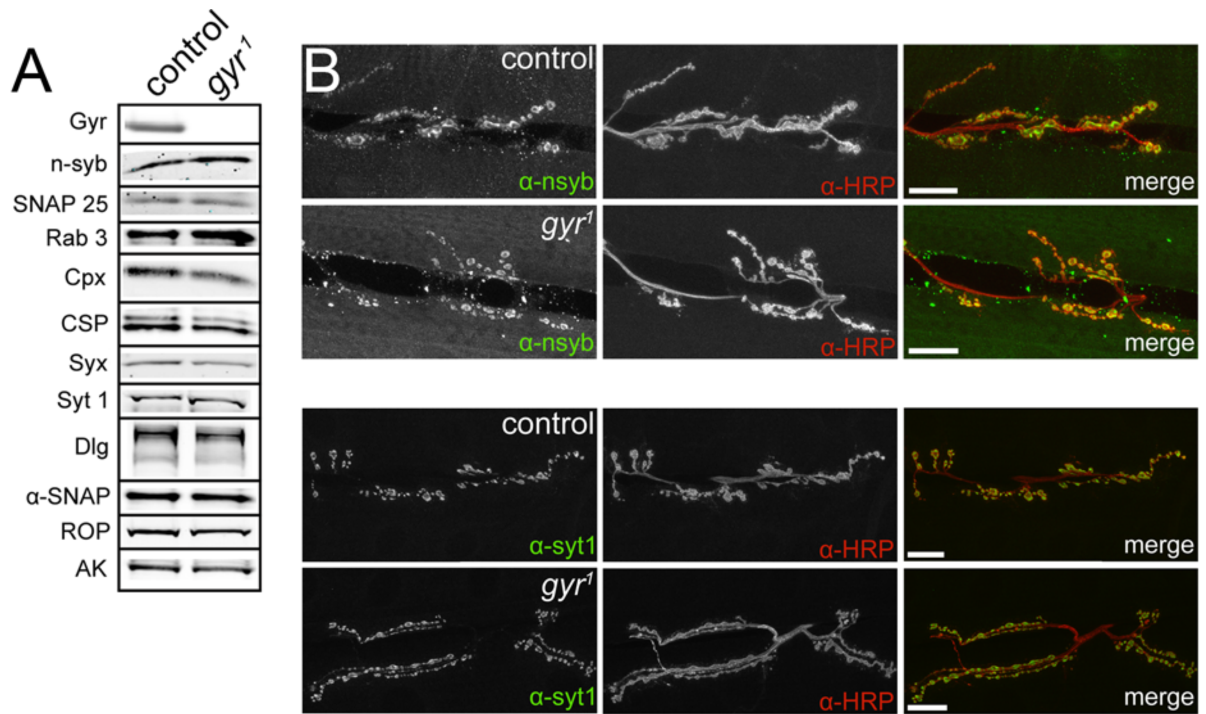

D
control
gyr1
α-HRP
α-Gyr
α-HRP α-Gyr

Figure 7. Generation of a *Drosophila synaptogyrin* (*gyr*) mutant. **A)** The *synaptogyrin* locus is diagrammed with the two neighboring genes (CG30484 and CG6357). The location of the P-element used for the excision screen is indicated by the pink triangle. Two separate deletions were isolated and are indicated by black lines. The green arrows mark the locations for the PCR primers used to determine the extent of each deletion. **B)** PCR products amplifying the region between the green arrows in (A) from genomic DNA isolated from *white* control, gyr^{PE}, gyr^{1}, and gyr^{2} adult flies. This region is approximately 3.5 kb in wild-type flies (lacking the P-element), while the gyr^{1} and gyr^{2} PCR products are ~1 kb and ~1.8 kb, respectively. Therefore, the gyr^{1} excision event is a ~2.5 kb deletion, while gyr^{2} is a ~1.7 kb deletion. PCR products were sequenced to confirm that gyr^{PE} is indeed a precise excision and to verify that the deletions do not extend outside of the *synaptogyrin* locus. **C)** Synaptogyrin protein expression levels from homogenates made from *white*, gyr^{PE}, gyr^{1}, and gyr^{2} adult heads were determined by Western blot analysis. Immunostaining for complexin (cpx) was used as a loading control. Synaptogyrin immunoreactivity is absent in gyr^{1} and gyr^{2}, and since the synaptogyrin polyclonal antibody was raised against a region of the protein downstream of both deletions, it is unlikely that a truncated version of synaptogyrin is being produced. We therefore conclude that gyr^{1} and gyr^{2} are most likely null mutations. **D)** Immunohistochemistry at third instar larval NMJs confirms that synaptogyrin (red) is absent in the gyr^{1} mutant but not in the control (gyr^{PE}). Synaptic varicosities were identified using α-HRP antibodies (green).


A
control
gyr1
Gyr
n-syb
SNAP 25
Rab 3
Cpx
CSP
Syx
Syt 1
Dlg
α-SNAP
ROP
AK
B
control
α-nsyb
α-HRP
merge
gyr1
α-nsyb
α-HRP
merge
control
α-syt1
α-HRP
merge
gyr1
α-syt1
α-HRP
merge

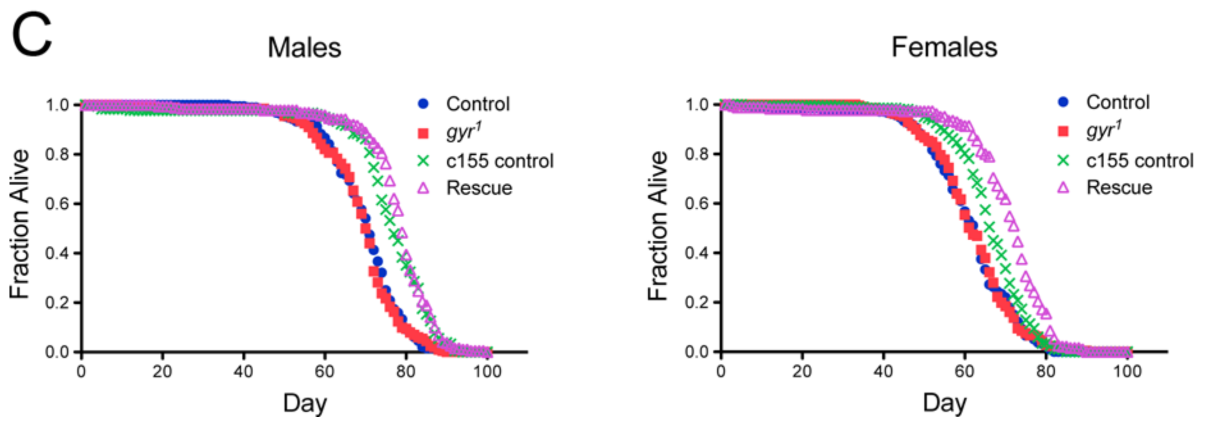

C
Males
Females
Fraction Alive
Day
Control
gyr1
c155 control
Rescue
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100

Figure 8. Examination of protein expression levels, synaptic vesicle protein localization, and viability in *gyr* mutants. **A)** The expression levels of a variety of synaptic proteins are unchanged in *gyr* head extracts relative to controls. Proteins analyzed include synaptobrevin (n-syb), SNAP 25, Rab 3, complexin (Cpx), cysteine string protein (CSP), syntaxin (Syx), synaptotagmin 1 (Syt 1), Discs large (Dlg), soluble N-ethylmaleimide-sensitive fusion protein (NSF)-attachment protein (α-SNAP), sec1/unc-18 (ROP), and arginine kinase (AK). **B)** The localization of synaptobrevin (upper panels) and synaptotagmin 1 (lower panels) is similar in *gyr* mutants compared to controls. Immunostaining of both proteins (green) is almost entirely restricted to synaptic varicosities with little trafficking to regions between boutons or along the axon. Axonal membranes are visualized with α-HRP antibodies (red). **C)** Both *gyr* mutant males (left) and females (right) have a similar lifespan compared to controls (gyr^{PE}). c155 control ($elav^{c155}$-*GAL4*; *gyr*) flies have a slightly increased lifespan, and female rescue flies ($elav^{c155}$-*GAL4/w*; *gyr*; *UAS-myc-gyrin*/+) live somewhat longer than c155 control females. The T_{50} (age at which 50% of the flies have died) in days for each sex and genotype were: males: control = 71, *gyr* = 71, c155 control = 78, rescue = 79; females: control = 63, *gyr* = 62, c155 control = 67, rescue = 73.

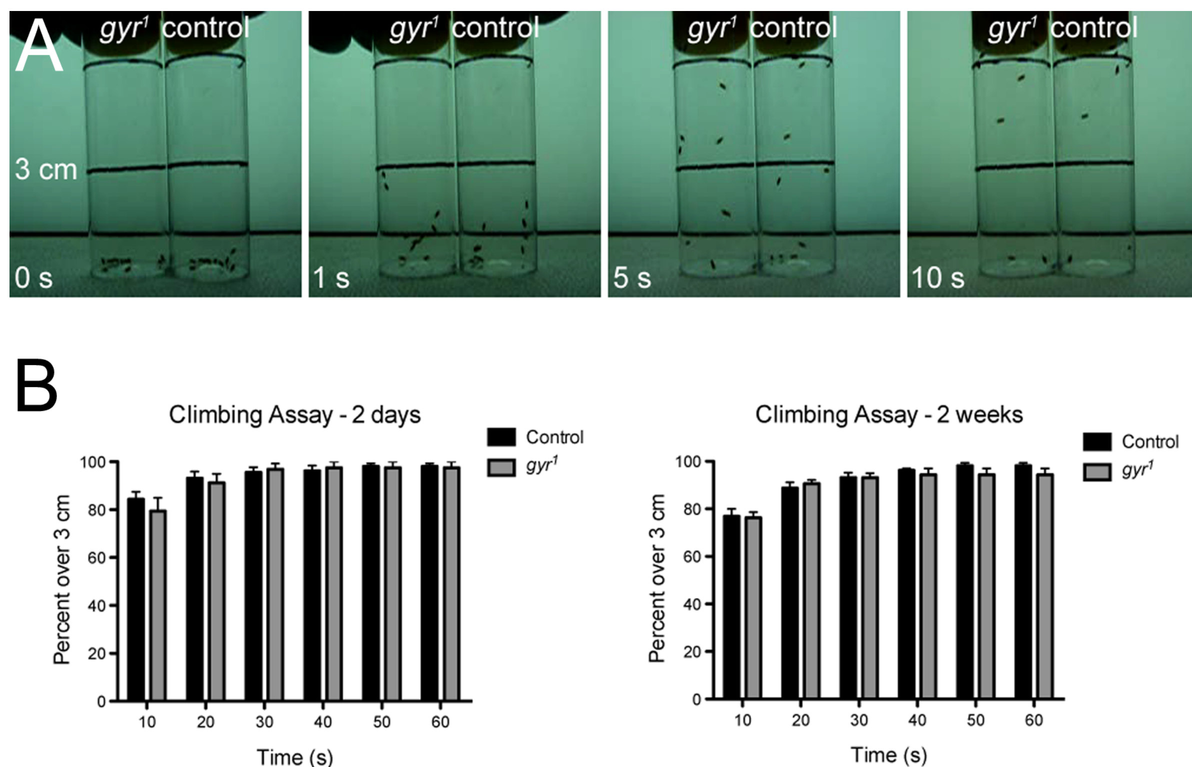
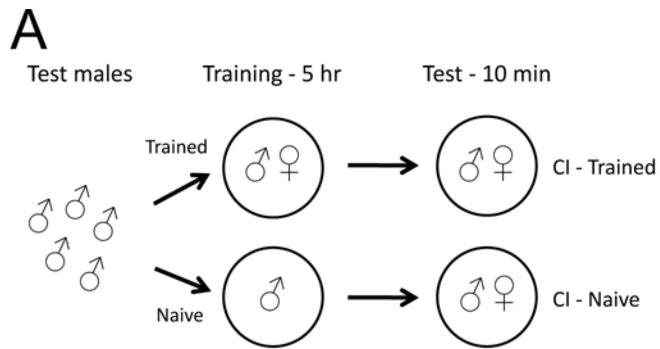
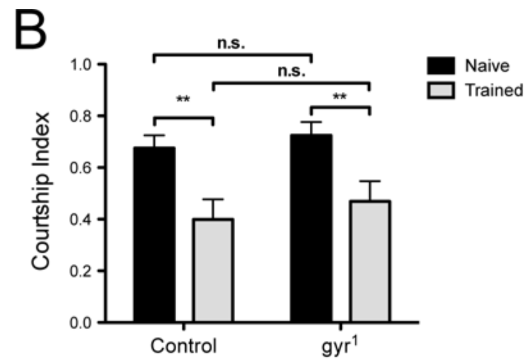
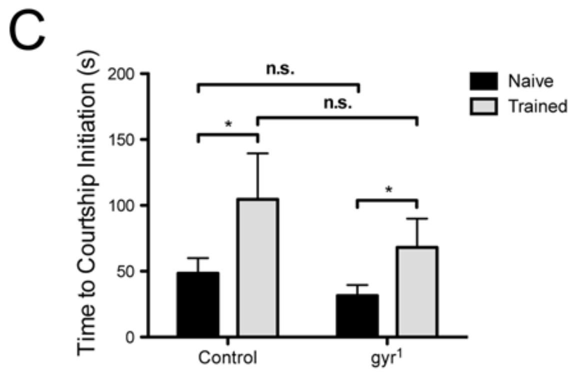


Figure 9. Locomotor ability as measured by a climbing assay is not significantly different in *gyr* mutants. **A)** Images captured from a video of a climbing assay trial. The vial on the left contains *gyr* mutant flies, while the vial on the right contains control flies. The numbers on the bottom left of the panels indicate the time in seconds after the flies were gently knocked to the bottom of the vials. After ten seconds most flies have crossed the black line drawn 3 cm above the bottom of the vial. **B)** Quantification of climbing assays. A total of forty males were analyzed in groups of ten, with four trials for each group. The percentage of flies that had crossed the 3 cm line at each time point (up to one minute in ten-second intervals) is not significantly different between *gyr* mutants and controls at any point ($p > 0.05$, Student's t-test). Similar results were seen using flies aged two days (left) and aged two weeks (right).


A
Test males
Training - 5 hr
Test - 10 min
Trained
Naive
CI - Trained
CI - Naive


B
Naive
Trained
Courtship Index
n.s.
n.s.
**
**
1.0
0.8
0.6
0.4
0.2
0.0
Control
gyr1


C
Naive
Trained
Time to Courtship Initiation (s)
n.s.
n.s.
*
*
200
150
100
50
0
Control
gyr1

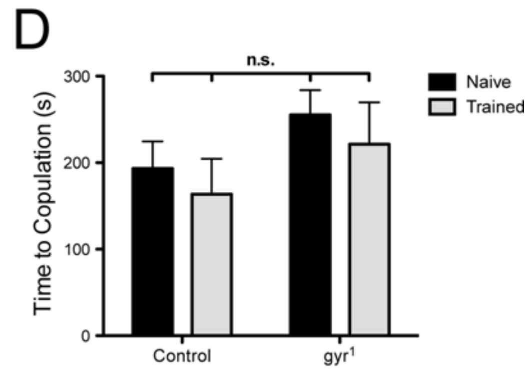

D
Naive
Trained
Time to Copulation (s)
n.s.
300
200
100
0
Control
gyr1

Figure 10. Analysis of courtship behavior in *gyr* mutant males. **A)** Schematic of the assay used to assess courtship conditioning. Males were kept in isolation after eclosion and between training and testing. During the training phase, half the males were paired with a previously mated female and the other half were sham trained for five hours. The following day males were paired with a new female for ten minutes and the courtship index (CI) was determined. **B)** CIs for *gyr* and control males under naïve and trained conditions. There is no statistically significant difference in performance between *gyr* and control males under either training condition ($p > 0.05$, Student's t-test). However, trained males of both genotypes display reduced courtship relative to naïve males (control, $p = 0.005$; *gyr*, $p = 0.009$, Student's t-test). Average CIs ± SEM: control, naïve = 0.68 ± 0.050, n = 17; control, trained = 0.40 ± 0.078, n = 17; *gyr*, naïve = 0.73 ± 0.051, n = 17; *gyr*, trained = 0.47 ± 0.078, n = 15. **C)** Analysis by two-way ANOVA reveals that trained males of both genotypes have a delay in courtship initiation relative to naïve males ($p < 0.05$). However, there is no significant difference in time to courtship initiation between control and mutant flies under either training condition ($p > 0.05$, Student's t-test). Average time to initiation (in seconds) ± SEM: control, naïve = 48.4 ± 11.6, n = 17; control, trained = 104.6 ± 34.9, n = 17; *gyr*, naïve = 31.5 ± 8.01, n = 17; *gyr*, trained = 68.1 ± 21.8, n = 15. **D)** For the males that successfully copulated during the ten-minute observation period there was no statistically significant difference in time to copulation (from the introduction of the female) between control and *gyr* males under both naïve and trained conditions ($p > 0.05$, Student's t-test). Average time to copulation (in seconds) ± SEM: control, naïve = 193.3 ± 31.3, n = 13; control, trained = 163.7 ± 40.7, n = 6; *gyr*, naïve = 255.3 ± 28.5, n = 10; *gyr*, trained = 222.4 ± 48.4, n = 8.

Chapter 3

Analysis of Synaptogyrin's Role in Synaptic Vesicle Exo-endocytosis

Robin J. Stevens[1], Yulia Akbergenova[1], Ramon Jorquera[1], and J. Troy Littleton[1]

[1] The Picower Institute for Learning and Memory, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

Robin Stevens performed the majority of the work described in this chapter. Yulia Akbergenova performed the electron microscopy. Ramon Jorquera performed the voltage clamp electrophysiology.

Introduction

The lack of severe neuronal phenotypes in the mouse, *C. elegans*, and *Drosophila* synaptophysin and synaptogyrin knockouts is somewhat surprising given the degree of evolutionary conservation of these proteins as well as their relatively high expression levels on synaptic vesicles (Hubner et al., 2002; Takamori et al., 2006; Mutch et al., 2011). In mice, there is clearly some degree of redundancy between synaptophysin and synaptogyrin (Janz et al., 1999), and potentially between synaptophysin and synaptoporin as well (Spiwoks-Becker et al., 2001). Furthermore, synaptogyrin 3 may compensate for the loss of these proteins, as it is highly enriched in the brain and targeted to synaptic vesicles (Belizaire et al., 2004). However, the absence of all physin and gyrin isoforms in *C. elegans* does not dramatically affect basal synaptic function or animal behavior (Abraham et al., 2006). Nevertheless, there is a growing body of evidence suggesting that these proteins, while not strictly required for exocytosis or endocytosis, are able to modulate certain aspects of synaptic vesicle release and recycling.

In particular, it appears as though synaptophysin and/or synaptogyrin may participate in a clathrin-independent version of synaptic vesicle endocytosis. An increase in clathrin-coated vesicles was observed in the *C. elegans* synaptophysin/ synaptogyrin/SCAMP triple knockout (Abraham et al., 2006), in retinal rod photoreceptor cells that lacked both synaptophysin and synaptoporin (Spiwoks-Becker et al., 2001), and in the squid giant synapse when the interaction between synaptophysin and dynamin was inhibited (Daly et al., 2000). Similarly, in the *C. elegans* synaptogyrin single knockout, synthetic phenotypes were observed with

endophilin, synaptojanin, and synaptotagmin, all of which participate in clathrin-mediated endocytosis (Song and Zinsmaier, 2003; Shupliakov, 2009; Abraham et al., 2011). Moreover, synaptobrevin, a known binding partner of synaptophysin (Edelmann et al., 1995), has been implicated in a rapid version of endocytosis that may not rely on clathrin-coated vesicles (Deak et al., 2004). Taken together, these results suggest that synaptogyrin and/or synaptophysin may regulate a separate endocytic pathway that is independent of clathrin-mediated endocytosis, e.g., kiss-and-run endocytosis. This raises the possibility that the lack of severe phenotypes in the synaptophysin and synaptogyrin knockouts is due to compensation by alternative endocytic mechanisms and implies that significant defects in synaptic vesicle recycling may only occur under conditions in which clathrin-mediated endocytosis is insufficient to replenish synaptic vesicle pools.

Analysis of the synaptophysin/synaptogyrin double knockout mouse revealed a delay in the recovery from synaptic depression induced by high-frequency stimulation, again suggesting that these proteins regulate synaptic vesicle recycling kinetics (Janz et al., 1999). However, these mice have no alterations in their evoked synaptic responses or in the release of glutamate from synaptosomes. On the other hand, the double knockout displayed deficits in paired-pulse facilitation (PPF), post-tetanic potentiation (PTP), and long-term potentiation (LTP), indicating that synaptophysin and synaptogyrin serve a functional role in synaptic plasticity (Janz et al., 1999). The observation that synaptophysin phosphorylation is enhanced in hippocampal brain slices that have undergone LTP lends additional support to the hypothesis that these proteins can modulate synaptic transmission (Mullany and Lynch, 1998).

We sought to further investigate the role of synaptogyrin in the regulation of synaptic vesicle exo-endocytosis through the generation and characterization of a *Drosophila synaptogyrin* mutant. Our initial analysis revealed no obvious behavioral phenotypes, despite the fact that *Drosophila* has no other gyrins or physins that could compensate for the absence of synaptogyrin. The loss of synaptogyrin did not dramatically affect protein expression levels of other synaptic proteins, nor did it result in overt changes in the localization of synaptotagmin or synaptobrevin, two essential synaptic vesicle proteins. Furthermore, the absence of synaptogyrin did not impact the viability or behavior of *gyr* animals as determined by several different assays. We therefore proceeded to perform a more in-depth analysis to search for alterations in synaptic function similar to those seen in the mouse and nematode knockouts. While gross synaptic morphology is unaffected in *gyr* mutants, we discovered changes in synaptic vesicle size regulation as well as alterations in the synaptic vesicle exo-endocytic cycle that are manifest under enhanced stimulation conditions.

Results

gyr mutants have normal synaptic growth and bouton morphology

As *Drosophila* proceed through the three larval stages, the surface area of the body wall muscles grows approximately 100-fold, and synaptic innervation at the NMJ increases in parallel through the addition of new boutons to maintain proper muscle depolarization. Development of the NMJ requires coordination between the synapse and muscle and involves several trans-synaptic signaling cascades including the Wnt and transforming growth factor-β (TGFβ) pathways (reviewed in (Collins and DiAntonio, 2007)). Disruptions in these growth signaling pathways can lead to synaptic undergrowth, synaptic overgrowth, or defects in establishing correct synaptic architecture. Connections between the motorneuron and muscle are also highly dependent on synaptic activity, as alterations in the levels of synaptic transmission brought about by a variety of factors can dramatically influence the extent of synaptic innervation (Budnik et al., 1990). For example, increased neuronal activity induced using either temperature-sensitive seizure mutants (Guan et al., 2005) or enhanced larval locomotion (Sigrist et al., 2003) can result in overgrowth of synaptic varicosities at the NMJ. Furthermore, many *Drosophila* endocytic and membrane recycling mutants, including *rab11*, *endophilin*, *synaptojanin,* and *dap160*, display an increase in supernumerary or "satellite" boutons (Koh et al., 2004; Dickman et al., 2006; Khodosh et al., 2006). Therefore, analysis of synaptic bouton morphology not only can provide insights into possible developmental defects, but also can hint at changes in synaptic activity or function.

When we examined the NMJs of third instar larvae in *gyr* mutants, we discovered that overall synaptic morphology is normal with no obvious synaptic overgrowth, undergrowth, or alterations in branching. The total number of synaptic boutons at muscle 6/7 is unchanged in both *gyr*^{1} and *gyr*^{2} mutant larvae compared to controls (Figure 1A-B; $p = 0.48$, one-way ANOVA). There also is no significant difference in muscle size, which is consistent with our previous observation that *gyr* mutants do not have developmental delays (Figure 1C; $p = 0.20$, one-way ANOVA). Furthermore, we did not observe an increase in satellite boutons or notice any dramatic changes in bouton size or shape (data not shown).

Two separate motorneurons innervate muscles 6 and 7, and while both use glutamate as their excitatory neurotransmitter, their boutons are morphologically distinct (Johansen et al., 1989; Atwood et al., 1993). Type Ib (big) boutons from the RP3 neuron are larger and are surrounded by a thicker subsynaptic reticulum (SSR) than type Is (small) boutons from the 6/7b neuron (Atwood et al., 1993; Keshishian et al., 1993). Type Ib and Is boutons can easily be distinguished by immunostaining for the protein Discs large (Dlg), a postsynaptic structural protein associated with the membrane infoldings of the SSR (Lahey et al., 1994). Since type Ib boutons are associated with a much more elaborate SSR, Dlg staining is significantly more intense at type Ib boutons. When we examined *gyr* third instar larvae, we found no significant difference in the number of type Ib or type Is boutons (Figure 1D-E; $p > 0.1$, one-way ANOVA), again suggesting that synaptogyrin does not play a developmental role at the larval NMJ. Similarly, the number of active zones (the sites from which synaptic vesicles are released) is also unchanged in *gyr* mutants compared to controls (Figure 2A-B; p =

0.61, Student's t-test). Active zone size and spacing also appear to be unaffected in *gyr* mutants, and since the average number of boutons and active zones is comparable in *gyr* mutants and controls, the average number of active zones per bouton is almost identical (Figure 2C; control = 7.47 active zones/bouton; *gyr* = 7.62 active zones/bouton). We therefore conclude that the loss of synaptogyrin in *Drosophila* does not significantly alter synaptic growth or influence the number of synaptic vesicle release sites.

Ultrastructural analysis of *gyr* larvae reveals alterations in synaptic vesicle diameter and density

Although bouton architecture in *gyr* mutants appears normal at the level of light microscopy, we performed electron microscopy at the NMJs of third instar larvae to determine whether *gyr* mutants have defects in ultrastructural synaptic morphology at type Ib boutons. We were surprised to discover that boutons at the larval NMJ in *gyr* animals display variable changes in synaptic vesicle diameter and density. In control boutons, synaptic vesicles are uniform in size and tightly clustered around the periphery of the bouton (Figure 3A-B). Similarly, some *gyr* boutons appear indistinguishable from controls with respect to synaptic vesicle diameter and density (compare Figures 3A and 3E). However, other *gyr* boutons have a noticeable decrease in synaptic vesicle density (Figure 3F) and/or an increase in the number of large synaptic vesicles, the largest of which may very well be endosomes or endocytic cisternae (Figure 3C-D). This dramatic increase in the variability of synaptic vesicle diameter in certain *gyr* boutons is illustrated in Figure 3D (compare to Figure 3B). While a fraction of *gyr* boutons have a marked decrease in synaptic vesicle density,

overall there is not a statistically significant difference in the density of synaptic vesicles per μm^2 when all *gyr* boutons are included in the analysis (Figure 4A; $p = 0.19$, Student's t-test). However, there is a statistically significant increase in the mean synaptic vesicle diameter in *gyr* boutons compared to controls (Figure 4B; control = 42.91 ± 0.49, *gyr* = 45.33 ± 0.82; $p = 0.02$, Student's t-test). There also is an overall shift in the distribution of synaptic vesicle diameter towards larger values with an increased amount of variability in diameter, which suggests that synaptogyrin directly or indirectly regulates synaptic vesicle size (Figure 4C).

As previously mentioned, muscle 6/7 is innervated by two bouton subtypes, type Ib and Is. Type Is boutons are reported to have a larger mean synaptic vesicle diameter than type Ib boutons (Karunanithi et al., 2002), which led us to question whether the increase in average synaptic vesicle diameter observed in certain *gyr* mutant boutons could be due to the inadvertent selection of type Is boutons instead of type Ib. We had previously determined that *gyr* mutants have no significant difference in the number of type Ib or Is boutons at the NMJ (Figure 1D-E), nevertheless we re-examined the electron micrographs to confirm that the *gyr* boutons we analyzed had the thick SSRs characteristic of type Ib boutons. Furthermore, there was no significant difference in the average bouton area of the micrographs we sampled, making it unlikely that the *gyr* bouton analysis was biased towards smaller boutons (average bouton area in μm^2 ± SEM: control = 3.21 ± 0.33, n = 24; *gyr* = 3.35 ± 0.36, n = 24; $p = 0.76$, Student's t-test).

If the smaller *gyr* boutons were indeed type Is, we might expect that there would be a linear correlation between average synaptic vesicle diameter and the area of a

bouton, with smaller boutons having a larger mean vesicle diameter. However, we found only a slight, non-significant trend in the *gyr* boutons analyzed (Figure 4D; r^2 = 0.065, p = 0.23, Pearson correlation). As expected, there also was no correlation between bouton size and average synaptic vesicle diameter in control boutons (Figure 4D; r^2 = 0.003, p = 0.79, Pearson correlation). Type Is boutons are also reported to have fewer mitochondria than type Ib boutons (Atwood et al., 1993), and we found no statistically significant difference between *gyr* mutants and controls in the number of mitochondria per μm^2 (Figure 4E; p = 0.47, Student's t-test). We therefore propose that it is unlikely that the entire subgroup of *gyr* boutons with profoundly abnormal synaptic vesicle diameter are type Is boutons, although we cannot completely rule out the possibility that the loss of synaptogyrin alters the morphology of boutons such that a minority of type Is boutons are more similar in appearance to type Ib boutons. We conclude that the loss of synaptogyrin causes an overall increase in average synaptic vesicle diameter as well as enhanced variability of synaptic vesicle size, although the penetrance of these phenotypes is variable between individual *gyr* boutons.

gyr mutants have an increased number of endocytic cisternae following intense stimulation

Synaptic vesicles are known to form through several different pathways, including traditional clathrin-mediated endocytosis from the plasma membrane (De Camilli and Takei, 1996) and via endosomal intermediates (Heuser and Reese, 1973; Takei et al., 1996; de Lange et al., 2003). During periods of relatively low activity, clathrin-mediated endocytosis appears to be the predominant form of synaptic vesicle retrieval at central synapses (Granseth et al., 2006). However, intense non-physiological

stimuli or tetanic action potential stimulation can induce bulk endocytosis, a process whereby large plasma membrane invaginations are internalized to form endocytic cisternae from which synaptic vesicles then bud (Miller and Heuser, 1984; Richards et al., 2000; Richards et al., 2003; Evans and Cousin, 2007). The presence of many large, endosomal-like structures in a subset of *gyr* mutant boutons led us to hypothesize that synaptic vesicle recycling via endosomal intermediates might be disrupted in these boutons, resulting in a buildup of endocytic cisternae.

To explore this possibility, we incubated larvae with a high-potassium solution known to induce the formation of endocytic cisternae in response to the massive synaptic vesicle exocytosis caused by continuous membrane depolarization (Marxen et al., 1999; de Lange et al., 2003; Akbergenova and Bykhovskaia, 2009). We then investigated whether *gyr* mutants were impaired either in recovering vesicle membrane via bulk endocytosis or in resolving endocytic cisternae into synaptic vesicles. Under normal resting conditions, *gyr* mutants have a slight but not statistically significant increase in the number of cisternae per μm^2 (here cisternae are defined as structures with a diameter greater than 80 nm; Figure 5 [left]; control = 0.84 ± 0.22; *gyr* = 1.36 ± 0.28; p = 0.21, Student's t-test). Immediately after a five-minute incubation in high K^+ (90 mM) Jan and Jan solution (Jan and Jan, 1976; Akbergenova and Bykhovskaia, 2009), both control and *gyr* animals show a similar increase in the number of cisternae, suggesting that *gyr* mutants are not impaired in this step of bulk endocytosis (Figure 5 [center]; control = 15.0 ± 0.62; *gyr* = 12.7 ± 0.90; p = 0.21, Student's t-test). However, when larvae are subsequently allowed to recover for ten minutes in normal (low K^+) saline prior to fixation, *gyr* animals have a significantly

higher amount (~50% by area) of endocytic cisternae remaining compared to controls (Figure 5 [right]; control = 5.20 ± 0.72; *gyr* = 7.98 ± 0.78, $p = 0.01$, Student's t-test). This observation suggests that the process of resolving synaptic vesicles from endocytic cisternae is delayed in *gyr* mutants.

To further examine synaptic vesicle recycling, we turned to the styryl dye FM1-43, which reversibly binds membranes and dramatically increases in fluorescence intensity upon membrane integration, allowing one to track compartments as they move through the exo-endocytic cycle (Cochilla et al., 1999). Mutations in a variety of endocytic proteins, such as endophilin, eps15, synaptojanin, and dap160, result in decreased loading of FM1-43 dye (Verstreken et al., 2002; Verstreken et al., 2003; Koh et al., 2004; Koh et al., 2007). It should be noted that mutations in these genes also can lead to a dramatic decline in synaptic vesicle number at rest (in the case of endophilin, synaptojanin, and dap160) or result in defects immediately following stimulation with high K^+ (in the case of eps15), neither of which we observed in *gyr* mutants. When we incubated *gyr* and control larvae for five minutes with a high K^+ (60 mM) solution containing 4 μM FM1-43, we found no significant difference in the amount of dye uptake (Figure 6A-B; $p = 0.16$, Student's t-test). This result is unsurprising given that electron micrographs clearly illustrate that *gyr* is capable of taking up large quantities of membrane in the form of endocytic cistenae immediately following a potassium shock (Figure 5). We then examined unloading of FM1-43 dye by incubating preparations briefly (for one minute) in high K^+ saline without dye. *gyr* and control NMJs display a similar decrease in fluorescence levels (~50%) relative to the loading levels, suggesting that an equal fraction of the dye is capable of being released in both

genotypes (Figure 6C; p = 0.29, Student's t-test). Since both loading and unloading were performed with a non-physiological stimulus, it is difficult to draw conclusions about whether *gyr* mutants sequester FM1-43 in endocytic cisternae to a greater extent than controls. The second potassium stimulus used to unload the dye may have caused both synaptic vesicles and cisternae to fuse, which could mask a defect in synaptic vesicle budding from cisternae.

Synaptic vesicle diameter in *gyr* mutants shifts to more normal values following a high K^+ shock

If synaptogyrin is indeed involved in regulating synaptic vesicle budding from endocytic cisternae, one might expect that the dramatic synaptic vesicle recycling induced by high-potassium stimulation might exacerbate the misregulation of synaptic vesicle size seen in *gyr* mutants under non-stimulated conditions. Intriguingly, when we measured the diameter of synaptic vesicles ten minutes after high-potassium stimulation, we found that the distribution of synaptic vesicle diameter was more similar to controls compared with pre-potassium stimulation (Figure 7A-B). The average synaptic vesicle diameter following the potassium shock was not significantly different between *gyr* and controls (Figure 7C; control = 42.95 ± 0.63 nm, n = 19; *gyr* = 43.06 ± 0.48 nm, n = 22; p = 0.89, Student's t-test), and individual *gyr* boutons had much less variation in their mean synaptic vesicle diameter (compare Figure 7C with Figure 4B). So while the number of large cisternae is increased in *gyr* mutants after intense stimulation, synaptic vesicles (less than 60 nm in diameter) that form soon after stimulation are more similar in size to controls (Figure 7D). This shift in distribution is more readily apparent when structures greater than 60 nm in diameter are excluded

and the cumulative frequency plots are normalized (Figure 7E). This allows for a more direct comparison of synaptic vesicle size distribution since the elevated number of endocytic cisternae after the potassium shock dramatically alters the overall cumulative distribution curves. These findings suggest that intense stimulation results in the restoration of normal synaptic vesicle diameter, perhaps by forcing the abnormally large synaptic vesicles to fuse (see Discussion).

***gyr* mutants exhibit increased release probability and facilitation**

The increased synaptic vesicle diameter seen in *gyr* boutons lead us to hypothesize that these synapses may release more neurotransmitter per vesicle on average and might thereby result in an enhanced postsynaptic response. To our surprise, however, both miniature excitatory junctional potential (mEJP) and evoked excitatory junctional potential (EJP) amplitude were not significantly altered in *gyr* mutant larvae under low-frequency stimulation conditions (Figure 8A-B). Although the average mEJP frequency is slightly decreased in the *gyr* mutant compared to controls, the difference is not statistically significant (control = 3.67 ± 0.27 Hz, n = 6; *gyr* = 2.58 ± 0.41 Hz, n = 7; p = 0.06, Student's t-test). The lack of a significant change in mEJP amplitude suggests that either the larger synaptic vesicles and cisternae are resistant to spontaneous fusion or they release approximately the same amount of neuro-transmitter as normal-sized synaptic vesicles. When we measured the diameter of synaptic vesicles located in close proximity to active zones (within 150 nm), we found no significant difference in size between *gyr* mutants and controls, suggesting that larger vesicles are not normally recruited to release sites (control = 41.02 ± 0.56 nm, n = 82 vesicles from 11 active zones; *gyr* = 41.97 ± 0.76 nm, n = 80 vesicles from 12 active

zones; p = 0.31, Student's t-test). This does not, however, indicate that the larger endosomal-like structures are incapable of fusion under other circumstances such as during high-frequency stimulation or after incubation with high-potassium saline.

Although basal evoked release is normal in *gyr* mutants, analysis using two-electrode voltage clamp at higher stimulation frequencies revealed increases in synaptic vesicle release probability. At low (0.2 mM) calcium concentrations, *gyr* larvae display an increase in the paired-pulse ratio at short (50 ms), but not longer (100 ms) interstimulus intervals (Figure 9A). Similarly, when stimulated continuously at 10 or 20 Hz for 500 stimuli, *gyr* animals show an increase in facilitation that becomes more pronounced at 20 Hz (Figure 9B). Interestingly, this enhanced facilitation appears to be transient when examined using a longer stimulation protocol of 1,500 stimuli at 20 Hz. After approximately 500 stimuli, the enhanced facilitation in *gyr* larvae peaks and subsequently declines until facilitation reaches levels similar to that of controls (Figure 9C). Notably, this phenotype can be rescued by presynaptic expression of synaptogyrin cDNA using the pan-neuronal $elav^{c155}$–*GAL4* promoter (Figure 9C).

Interestingly, when we separated the peak current (evoked excitatory junctional current [EJC] amplitude) and the charge transferred (the total amount of current transfer induced by an action potential) during the extended 20 Hz stimulation protocol, we discovered that there was a more pronounced difference in the amount of charge transferred between *gyr* and control larvae than the difference in the peak EJC amplitude (Figure 9D). This suggests that, under high-frequency stimulation conditions, the *gyr* mutant not only has more facilitation as indicated by the EJC amplitude, but also has altered release kinetics such that more current is transferred per stimulus. When

representative traces from *gyr* and control larvae are normalized, there is a clear increase in the width of the *gyr* EJC trace relative to the control (Figure 9E). This finding implies that *gyr* mutants have an increase in the amount of asynchronous synaptic vesicle release that is induced by high-frequency stimulation. Unsurprisingly, the initial enhanced facilitation in *gyr* larvae leads to a greater total amount of charge transferred over the course of the stimulation protocol (Figure 9F); however, after the initial phase of facilitation, *gyr* and control larvae reach the same steady-state level of release. Exactly how these changes in facilitation and release kinetics impact the overall function of the NMJ or the behavior of the animal remains to be determined.

Discussion

Despite decades of research into the function of synaptogyrin and synaptophysin, the exact role of these proteins in the synaptic vesicle cycle remains ambiguous. We have continued our characterization of a *Drosophila synaptogyrin* mutant in an attempt to elucidate synaptogyrin function in a relatively simple genetic background that is free of additional synaptophysin or synaptogyrin paralogs. We examined larval NMJs using both immunofluorescence and electron microscopy and found no significant differences in neuronal architecture or synapse number. This result is unsurprising given that mouse synaptophysin/synaptogyrin double knockouts and *C. elegans* mutants lacking synaptophysin, synaptogyrin, and SCAMP have no significant changes in brain architecture or synaptic wiring, indicating that these proteins are not essential for nervous system development (Janz et al., 1999; Abraham et al., 2006; Abraham et al., 2011). Similarly, synaptogyrin is not required for synaptic transmission *per se*, as all knockouts have normal evoked responses, which demonstrates that the basic release machinery is not significantly altered by the loss of synaptogyrin (Figure 8) and (Janz et al., 1999; Abraham et al., 2006). However, further analysis of the mouse and nematode synaptophysin/synaptogyrin knockouts identified alterations in certain aspects of endocytosis, synaptic plasticity, and/or synaptic vesicle morphology. Our work with the *Drosophila gyr* mutant supports the idea that synaptogyrin and synaptophysin are not essential for neurotransmission, yet they play a potentially important modulatory role in the exo-endocytic cycle and can impact synaptic function.

The relationship between synaptogyrin and synaptic activity

We were intrigued to discover that a subset of *gyr* boutons have quite dramatic changes in vesicle morphology, while others appear indistinguishable from wild-type boutons. Interestingly, work in murine retinal cells lacking both synaptophysin and its paralog synaptoporin indicated that synaptic vesicle density was decreased and that this phenotype became more pronounced during periods of high activity (i.e., during dark adaptation when photoreceptors release more synaptic vesicles). Moreover, during these periods of elevated activity there was an increase in the number of large (>80 nm) vacuolar-like structures as well as an increase in synaptic vesicle diameter in synaptophysin knockouts compared to controls (Spiwoks-Becker et al., 2001). These results suggest that synaptophysin promotes the efficient formation of synaptic vesicles and that enhanced activity can reveal more severe phenotypes.

Our work indicates that synaptogyrin performs a similar role in modulating synaptic vesicle formation in *Drosophila*, and it is possible that the phenotypic variation we see at *gyr* boutons under resting conditions (Figure 3) could be due to dissimilar levels of activity between boutons. Several studies have shown that bouton activity levels are variable at the *Drosophila* NMJ, namely that terminal boutons tend to have higher release probabilities than more proximal boutons (Guerrero et al., 2005; Peled and Isacoff, 2011). Interestingly, there also was a great deal of variability in the severity of the phenotypes in murine retinal photoreceptor cells lacking both synaptophysin and synaptoporin (Spiwoks-Becker et al., 2001), suggesting that synaptophysin and/or synaptogyrin may not be required to the same extent in every nerve terminal. We were unable to retrospectively determine the relative positions of the boutons we examined

by electron microscopy to correlate the severity of phenotype with the location of a bouton along the synaptic arbor. However, it is possible to mark boutons prior to preparation for electron microscopy (e.g., with a cactus needle), so future studies could examine the relationship between synaptic activity and synaptic vesicle morphology.

While it may be the case that a more active bouton would require synaptogyrin for proper vesicle formation (and would therefore have a more severe phenotype), the finding that a high-potassium shock causes *gyr* synaptic vesicle diameter to more closely resemble controls suggests that the opposite may be true. In other words, it appears as though higher activity leads to more normal synaptic vesicle formation relative to controls, at least for those vesicles less than 60 nm in diameter. However, since *gyr* boutons have an increased number of cisternae following high-potassium stimulation (Figure 5), it is clear that dramatic increases in neuronal activity can cause alterations in endocytic pathways in *gyr* mutants, which subsequently leads to an accumulation of abnormally large structures.

A preliminary model to explain synaptogyrin's role in synaptic vesicle formation

While there are still many unanswered questions about synaptogyrin function, we have developed a preliminary model to account for the *gyr* phenotypes related to abnormal synaptic vesicle diameter and the delay in the recovery of endocytic cisternae. Numerous studies have established the existence of multiple endocytic pathways at the NMJ, including clathrin-mediated and bulk endocytosis, and, according to some, the somewhat controversial kiss-and-run endocytosis (Koenig and Ikeda, 1996; Verstreken et al., 2002; Dickman et al., 2005). We propose that there are at least two pathways responsible for the budding of synaptic vesicles from endocytic cisternae,

one of which is regulated by synaptogyrin and the other that is independent of synaptogyrin function. In a wild-type bouton, both pathways are fully functional and are able to produce vesicles of normal size, resulting in a low level of abnormal synaptic vesicles or structures resembling endocytic cisternae. However, in the absence of synaptogyrin, the synaptogyrin-dependent pathway can no longer function properly and produces vesicles of abnormal size. The synaptogyrin-independent pathway is still functional and capable of generating synaptic vesicles with a normal diameter; nevertheless, there is an overall increase in synaptic vesicle diameter in *gyr* animals. Since these larger synaptic vesicles appear to be excluded from active zones (see Results), it is possible that they are incapable of exocytosis under basal conditions and therefore accumulate over time.

During periods of intense activity that induce bulk endocytosis, a wild-type synapse would have both pathways fully capable of regenerating synaptic vesicles from endocytic cisternae, and these structures quickly would resolve into synaptic vesicles. However, in the *gyr* mutant, the synaptogyrin-dependent pathway is impaired, and the time it takes to resolve cisternae into synaptic vesicles would be increased relative to controls. Furthermore, if the synaptogyrin-independent pathway is dominant in the *gyr* mutant, the synaptic vesicles that form shortly after the high-potassium shock would be more uniform in size because the vast majority would have been generated through the unimpaired synaptogyrin-independent pathway. We anticipate that, if this model were true, as the *gyr* boutons recover from a high-potassium shock, the impairment of the synaptogyrin-dependent pathway would result in the accumulation of abnormally sized vesicles, similar to what we observed under resting conditions.

An alternative possibility to the multiple endocytic pathways described above would be that a single major endocytic pathway is responsible for vesicle biogenesis from cisternae, and the loss of synaptogyrin merely slows the process of synaptic vesicle budding. While this certainly would explain the delay in resolving endocytic cisternae into synaptic vesicles, it is unclear how this model would account for the changes in synaptic vesicle diameter seen following a potassium shock. If synaptogyrin were involved in the budding of all synaptic vesicles from endocytic cisternae, one would expect to see a population of abnormally shaped synaptic vesicles shortly after the high-potassium shock as the cisternae begin to resolve, which we did not observe. However, it is unknown what fraction of vesicles generated after the potassium shock originate directly from the plasma membrane as opposed to cisternae. If the majority of synaptic vesicles observed ten minutes after the potassium shock derive from the plasma membrane and this form of endocytosis is independent of synaptogyrin, then the plasma membrane-derived synaptic vesicles may mask a smaller population of abnormal synaptic vesicles originating from cisternae in the *gyr* mutant. This also raises the need to determine the extent to which synaptogyrin mediates endocytosis from the plasma membrane. In future studies, it will be informative to track the flow of synaptic vesicle and cisternal membranes during various stimulation conditions using a combination of photoconvertible FM dyes and electron microscopy, which may allow us to more precisely determine synaptogyrin's influence on synaptic vesicle recycling.

Synaptogyrin influences synaptic vesicle release probability

The lack of significant changes in miniature or evoked EJP amplitude suggests that the abnormally large synaptic vesicles and endosomal-like cisternae seen in *gyr*

boutons are not capable of fusing under basal conditions. However, the increase in the paired-pulse ratio suggests that synaptic vesicle release probability is enhanced in *gyr* animals. Furthermore, vesicle fusion dynamics are altered during high-frequency stimulation, resulting in enhanced facilitation and a greater amount of total synaptic vesicle release. Some of the enhanced facilitation seen in *gyr* mutants may be due to the recruitment of larger synaptic vesicles during the initial phases of high-frequency stimulation, and the lack of sustained facilitation would be due to the depletion of these larger synaptic vesicles. However, the finding that increased facilitation occurs in the *gyr* mutant by the second action potential (as illustrated by the paired-pulse ratio) suggests that this explanation is insufficient, as the larger synaptic vesicles would have to be trafficking to active zones exceptionally quickly to account for this rapid facilitation.

How might synaptogyrin regulate synaptic vesicle release? One possibility is that synaptogyrin mediates exocytosis through a direct interaction with a component of the vesicle fusion machinery such as synaptobrevin. Although synaptobrevin and synaptogyrin have not been shown to interact directly, synaptobrevin does bind synaptophysin, and synaptophysin has been proposed to regulate synaptobrevin availability in a manner similar to the syntaxin/Munc18 interaction (Hata et al., 1993; Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995). Synaptophysin and/or synaptogyrin may control the ability of synaptobrevin to interact with the other members of the neuronal SNARE complex and may thereby ensure that the SNARE complex only assembles under appropriate circumstances. The loss of

synaptophysin or synaptogyrin could therefore result in inappropriate SNARE complex formation, which could alter synaptic vesicle release properties.

Alternatively, the changes in synaptic vesicle fusion seen in *gyr* mutants may instead be a consequence of the defects in synaptic vesicle formation that we observed by electron microscopy. If vesicle biogenesis is impaired in the absence of synaptogyrin, one of the consequences may be that synaptic vesicles do not receive the appropriate complement of synaptic vesicle proteins, which could lead to alterations in the dynamics of synaptic vesicle exocytosis. A connection between changes in synaptic function and protein sorting via endosomal compartments was recently described in a paper investigating the function of a neuronal Rab35 GTPase activating protein (GAP) named Skywalker (Sky) that is proposed to regulate synaptic vesicle trafficking between endosomal compartments (Uytterhoeven et al., 2011). Sky appears to inhibit the trafficking of synaptic vesicles to sorting endosomes and consequently impacts the degradation of synaptic vesicle proteins. Like *gyr* mutants, *sky* larvae accumulate an excess of cisternal-like structures after intense stimulation and show increased neurotransmitter release relative to controls. Unlike *gyr* mutants, however, *sky* larvae have a basal increase in exocytosis and also display punctate accumulations of FM1-43 after high-potassium stimulation that we did not observe in *gyr* larvae (data not shown). Nevertheless, the *sky* phenotypes demonstrate that changes in synaptic vesicle protein sorting through endosomal compartments can lead to alterations in synaptic vesicle release properties. It remains to be determined whether synaptogyrin might also directly or indirectly regulate the protein composition of synaptic vesicles.

Methods

Fly stocks and transgenics

Flies were cultured using standard media and techniques at room temperature (~22°C) unless otherwise noted. The GAL4/UAS system was utilized to drive neuronal expression of selected transgenes (Brand and Perrimon, 1993). The UAS-myc-gyrin construct used for rescue experiments was subcloned into a modified pValum vector with an N-terminal myc tag (Cho et al., 2010). This construct was injected into the strain *yv;;attP2*, which contains a site for targeted transgene insertion on the third chromosome (Markstein et al., 2008; Ni et al., 2008). The pan-neuronal driver $elav^{c155}$-*GAL4* was used to express the transgene in neurons. Embryo injections were performed at Genetic Services, Inc. (Cambridge, MA).

Immunohistochemistry

Wandering third instar larvae were dissected in calcium-free HL3.1 saline (70 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 4 mM $MgCl_2$, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES, pH 7.2) and fixed for 45 minutes in HL3.1 containing 4% formaldehyde. Following several washes in phosphate-buffered saline (PBS) and PBST (PBS with 0.1% Tween 20), larvae were incubated with primary antibody in PBST overnight at 4°C. After additional washes, larvae were incubated in secondary antibodies in PBST for four hours at room temperature, washed, and mounted in 70% glycerol. The dilutions for primary antibodies were: nc82/bruchpilot, 1:100; Dlg, 1:250. The nc82 antibody developed by Erich Buchner and the Dlg (4F3) antibody developed by Corey Goodman were obtained from the Developmental Studies Hybridoma Bank developed under the

auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Secondary antibodies were used at a dilution of 1:250 and include Alexa Fluor 488-conjugated goat anti-mouse (Invitrogen) and Rhodamine Red-conjugated donkey anti-mouse (Jackson ImmunoResearch). Goat α-HRP antibodies conjugated to DyLight 549 (Jackson ImmunoResearch) were added with secondary antibodies and were used at a concentration of 1:500. Images were acquired using a confocal microscope (Axoplan 2; Carl Zeiss MicroImaging, Inc.) running PASCAL software (Carl Zeiss) with a 40x oil-immersion lens.

Bouton and active zone counting

Age-matched larvae were grown at low density at 25°C and immunohistochemistry was performed as described above. Bouton quantification was performed using antibodies against horseradish peroxidase (HRP), a neuronal membrane marker, and Discs large (Dlg), a postsynaptic scaffolding protein. Type Ib and Is boutons were differentiated by the intensity of Dlg immunofluorescence, since type Ib boutons have significantly higher levels of Dlg (Lahey et al., 1994). Active zone numbers were quantified using monoclonal nc82 antibodies, which recognize the active zone component bruchpilot (Wagh et al., 2006). To control for variability in staining as much as possible, larvae of different genotypes were combined and processed together (tails were cut to differentiate genotypes). All bouton and active zone measurements were done at muscle 6/7 of segment A3. Imaging was performed using an Axoplan 2 confocal microscope with PASCAL software (Carl Zeiss MicroImaging, Inc.). Image analysis was done using ImageJ software (NIH). Bouton number is known to be proportional to

muscle size (Lnenicka and Keshishian, 2000); however, since muscle area did not differ significantly between *gyr* and control larvae, no corrections were necessary (see Results). Bouton and active zone counting were performed blind to genotype.

Electron microscopy

Dissected *Drosophila* third instar larvae were fixed for 1 hour in 4% para-formaldehyde/1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Fixed larvae were then washed in standard HL3 solution for 1 hour and postfixed for 40 minutes in 1% osmium tetroxide. Specimens were then dehydrated in a graded series of ethanol and water mixtures up to 100% ethanol followed by acetone. Samples were pre-infiltrated for 1 hour in a 1:1 mixture of acetone and Epon and then embedded in fresh Epon overnight at 60°C. Thin sections (60-70 nm) were contrasted with 0.2% lead citrate and imaged at 80 kV on an FEW Tecnai G2 Spirit transmission electron microscope equipped with an AMT CCD camera. Imaging was performed at the W.M. Keck Microscopy Facility at the Whitehead Institute, Cambridge, MA.

Quantification of synaptic vesicle diameter and density was performed using randomly sampled images from type Ib boutons. The diameter of synaptic vesicles and cisternae were measured from the outsides of the vesicle membranes along the long axis. A vesicle was measured only if its borders were well defined and it had a clear center (i.e., it was not a dense-core vesicle). Structures larger than 60 nm in diameter were excluded from calculations of mean synaptic vesicle diameter, but all vesicles that could be measured were included in the frequency and cumulative frequency analysis. High-potassium stimulation was performed using a modified Jan and Jan solution (Jan and Jan, 1976; 45 mM NaCl, 90 mM KCl, 36 mM sucrose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM

HEPES, pH 7.3). Cisternae were defined as intracellular single-membrane structures with a diameter greater than or equal to 80 nm. In certain cases the exact size of a cisterna could not be measured; therefore, it was not included in the cumulative frequency plots (Figure 6). However, if a cisterna clearly was greater than 80 nm in diameter, it was included in the analysis of the number of cisternae per unit area (Figure 5). The analysis was done blind to genotype wherever possible. Measurements were performed using ImageJ (NIH) and Adobe Photoshop 7.0 (Adobe Systems, Inc.) software.

Electrophysiology

Intracellular electrophysiology was performed on wandering third instar larvae at room temperature as described (Rieckhof et al., 2003) at muscle fiber 6 of segments A3 and A4 using HL3.1 saline with 0.2 mM $CaCl_2$. Data acquisition and analysis were performed using an Axoclamp 2B amplifier and pClamp 9.0 software (Axon Instruments, Foster City, CA). mEJP amplitude was measured using Mini Analysis Program (Synaptosoft). The resting muscle potential was not significantly different between genotypes in the traces used for mEJP analysis (control = -67.4 ± 2.30 mV, n = 6; *gyr* = -66.9 ± 1.90 mV, n = 7; p = 0.85, Student's t-test).

Voltage clamp electrophysiology was performed as described (Acharya et al., 1998) using two-microelectrode voltage clamp (OC725, Warner Instruments, Hamden, CT) at -80 mV holding potential using a programmable stimulator (Master-8, A.M.P.I., Jerusalem, Israel). Data acquisition and analysis were performed using pClamp 9.0 software (Axon Instruments, Foster City, CA).

FM1-43

Wandering third instar larvae were dissected in HL3.1 solution with 1 mM $CaCl_2$ and the nerves were cut to prevent spontaneous muscle contractions. Larvae were stimulated for five minutes with high-potassium (60 mM) HL3.1 containing 4 μM FM1-43 (Invitrogen). Preparations were briefly rinsed in standard HL3.1 and momentarily incubated in HL3.1 containing 100 μM Advasep-7 (Biotium), which has been shown to reduce background staining (Kay et al., 1999). Larvae were briefly washed again in HL3.1 and then imaged to quantify the level of FM1-43 uptake. After ten minutes, boutons were unloaded with a one-minute incubation with high-potassium (60 mM) HL3.1, briefly washed in normal HL3.1, and then imaged again. Images were acquired using an UltraVIEW VoX confocal imaging system (PerkinElmer) equipped with an ImagEM camera (C9100-13, Hamamatsu) and a Yokogawa CSU-X1 spinning disk head. Images were acquired with a 63x water-immersion lens, and analysis was performed using Volocity 3D Image Analysis Software (PerkinElmer).

Graphing and statistics

Statistical analysis was performed using GraphPad Prism for Mac OS X version 5.0a. Error measurements are standard error of the mean (SEM). Statistical significance was determined using Student's t-tests, one-way ANOVA, or Pearson's correlation coefficient (*, $p < 0.05$; **, $p < 0.01$).

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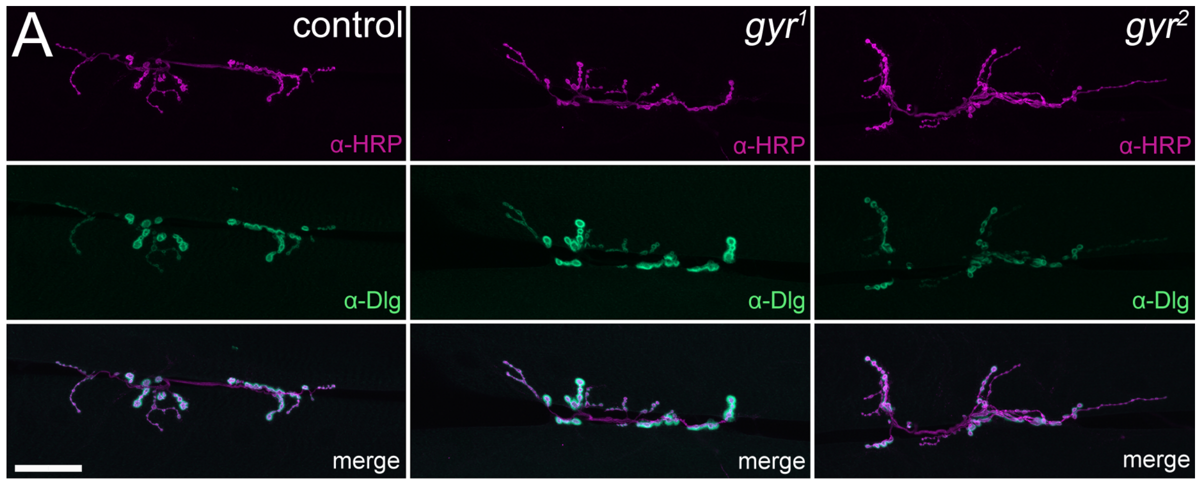
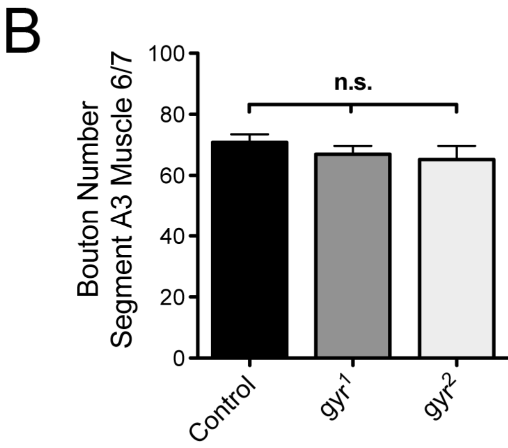
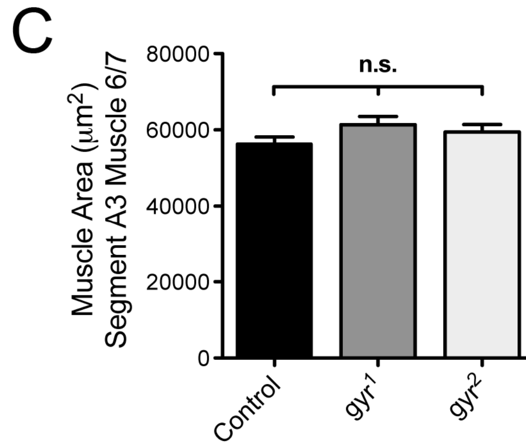
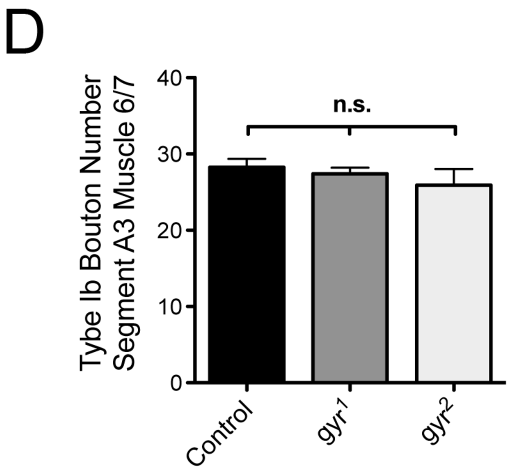

A
control
gyr1
gyr2
α-HRP
α-Dlg
merge


B
Bouton Number
Segment A3 Muscle 6/7
n.s.
100
80
60
40
20
0
Control
gyr1
gyr2


C
Muscle Area (μm2)
Segment A3 Muscle 6/7
n.s.
80000
60000
40000
20000
0
Control
gyr1
gyr2


D
Tybe Ib Bouton Number
Segment A3 Muscle 6/7
n.s.
40
30
20
10
0
Control
gyr1
gyr2

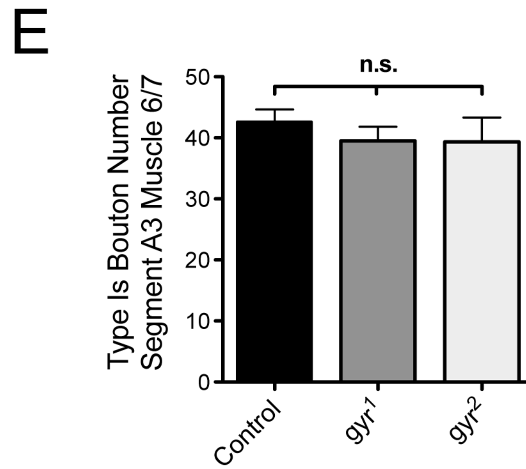
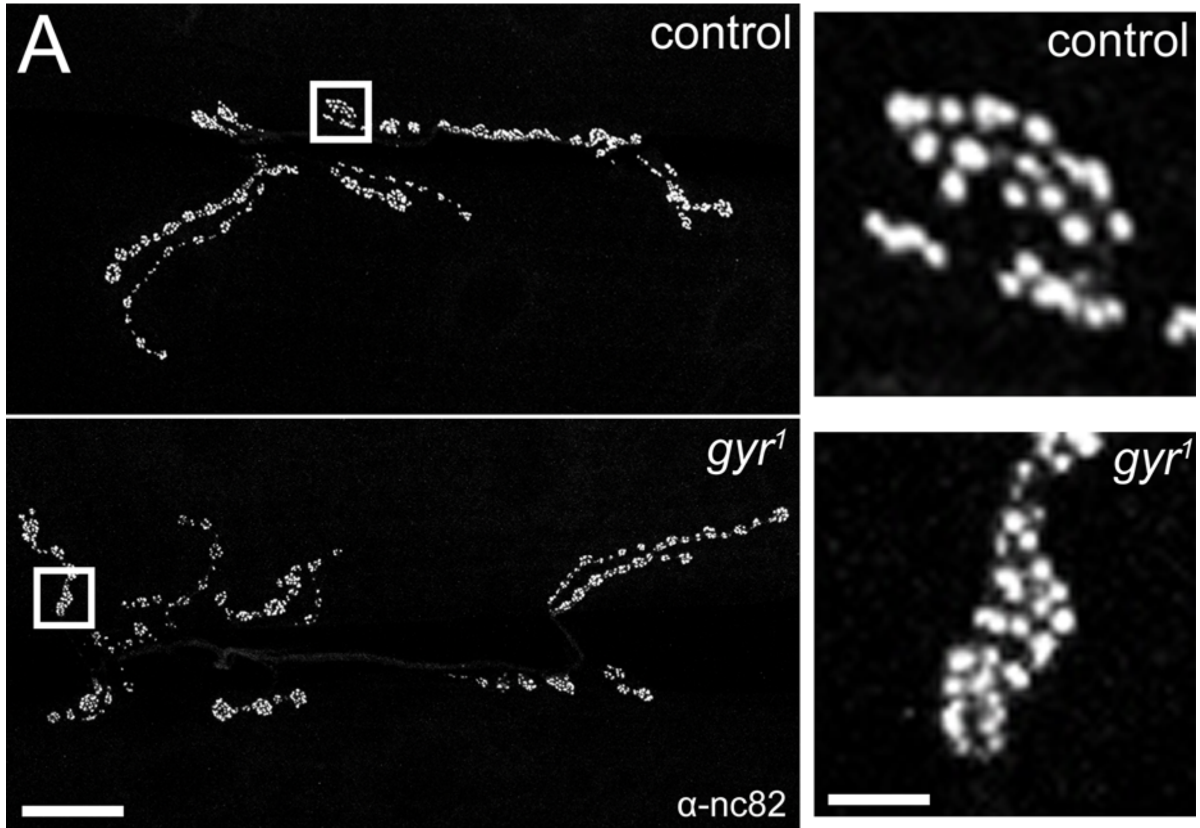
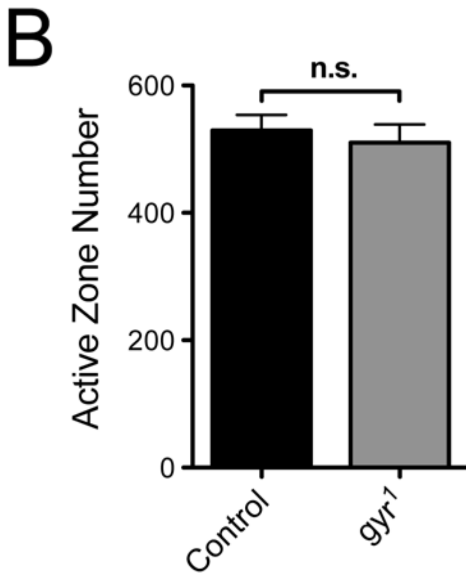

E
Type Is Bouton Number
Segment A3 Muscle 6/7
n.s.
50
40
30
20
10
0
Control
gyr1
gyr2

Figure 1. The number of synaptic varicosities is unchanged in *gyr* mutants. **A)** Synaptic bouton number and morphology at muscle 6/7 in body segment A3 appear normal in gyr^1 and gyr^2 third instar larvae relative to controls (gyr^{PE}), as determined by immunostaining against HRP (magenta) and Dlg (green). Dlg thickness is increased at type Ib boutons. **B)** Quantification of the total bouton number at muscle 6/7 revealed no significant difference between control, gyr^1, and gyr^2 (p = 0.48, one-way ANOVA). Average total bouton number ± SEM: control = 70.8 ± 2.62, n = 23; gyr^1 = 66.9 ± 2.80, n = 22; gyr^2 = 65.2 ± 4.45, n = 21. **C)** The average muscle area at segment A3 muscle 6/7 is not significantly different between controls and *gyr* mutants (p = 0.20, one-way ANOVA). Average muscle area (in μm^2) ± SEM: control = 56,301 ± 1,832, n = 22; gyr^1 = 61,337 ± 2,172, n = 23; gyr^2 = 59,433 ± 1,976, n = 20. **D)** The number of type Ib boutons (as defined by thick Dlg immunofluorescence) is not significantly different between genotypes tested (p = 0.50, one-way ANOVA). Average type Ib bouton number ± SEM: control = 28.3 ± 1.10, n = 23; gyr^1 = 27.4 ± 0.80, n = 22; gyr^2 = 25.9 ± 2.13, n = 21. **E)** Type Is bouton number also is unchanged in *gyr* mutants relative to controls (p = 0.41, one-way ANOVA). Average type Is bouton number ± SEM: control = 42.6 ± 2.09, n = 23; gyr^1 = 39.5 ± 2.33, n = 22; gyr^2 = 39.3 ± 4.01, n = 21. Error bars are ± SEM. Scale bar = 40 μm.


A
control
control
gyr¹
gyr¹
α-nc82


B
n.s.
Active Zone Number
600
400
200
0
Control
gyr¹

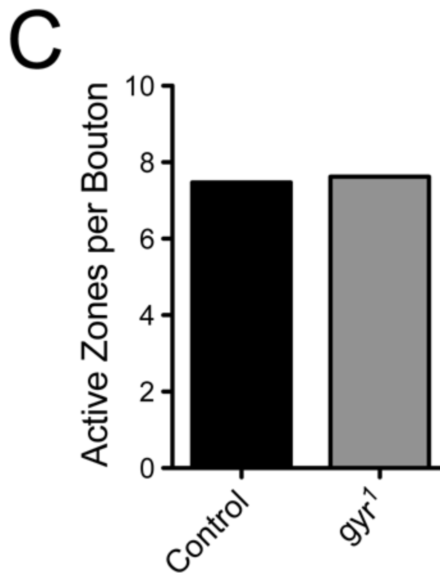
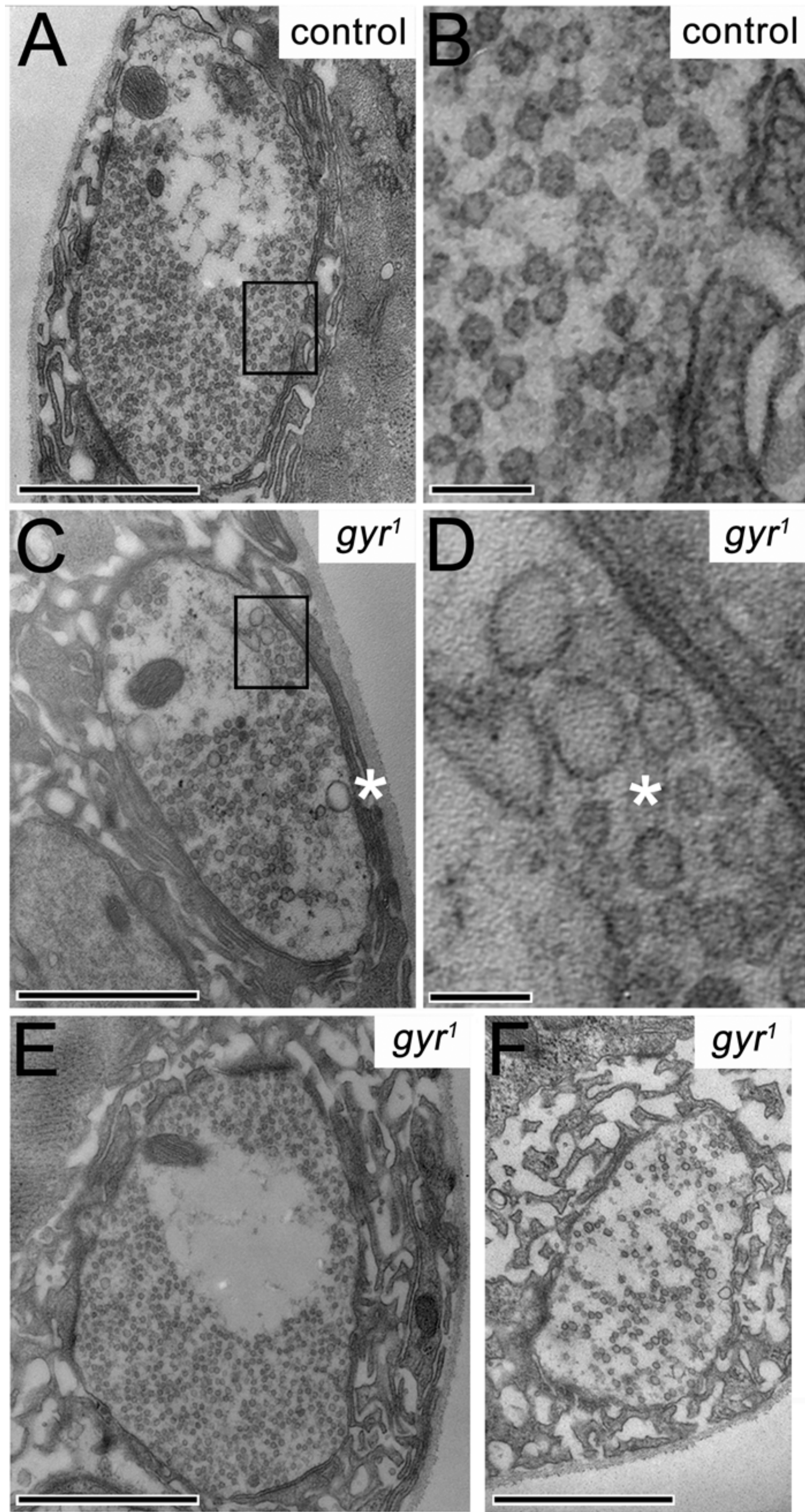
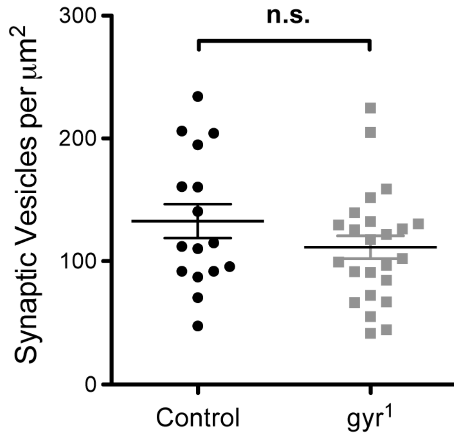

C
Active Zones per Bouton
10
8
6
4
2
0
Control
gyr¹

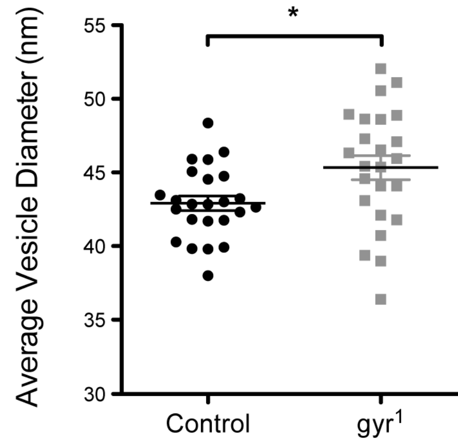
Figure 2. Quantification of active zone number at *gyr* NMJs. **A)** Representative images of control and *gyr* NMJs stained with the monoclonal antibody nc82 to label active zones. Boxed areas are shown at higher magnification in the panels on the right. **B)** Active zone number is not significantly different in *gyr* mutants compared to controls ($p = 0.61$, Student's t-test). Average active zone number ± SEM: control = 529 ± 24.4, $n = 14$; *gyr* = 510 ± 28.7, $n = 14$. **C)** The number of active zones per bouton is similar in *gyr* mutants (7.62 active zones/bouton) relative to controls (7.47 active zones/bouton). Scale bars in (A): left = 20 µm; right = 2.5 µm.

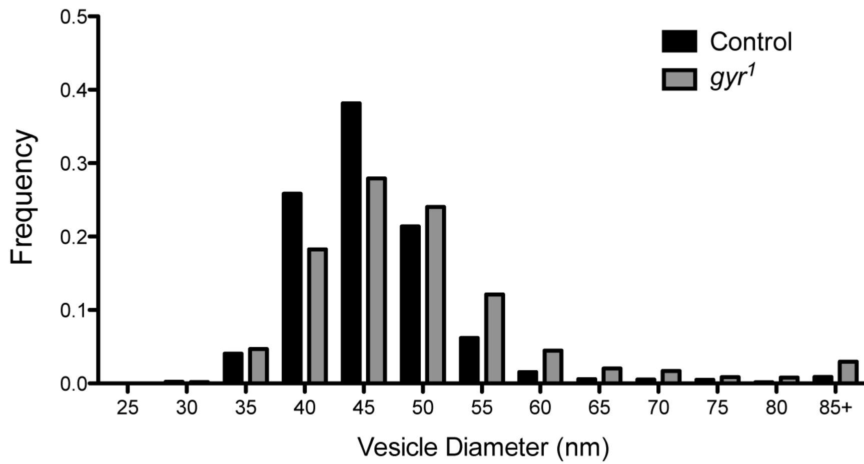


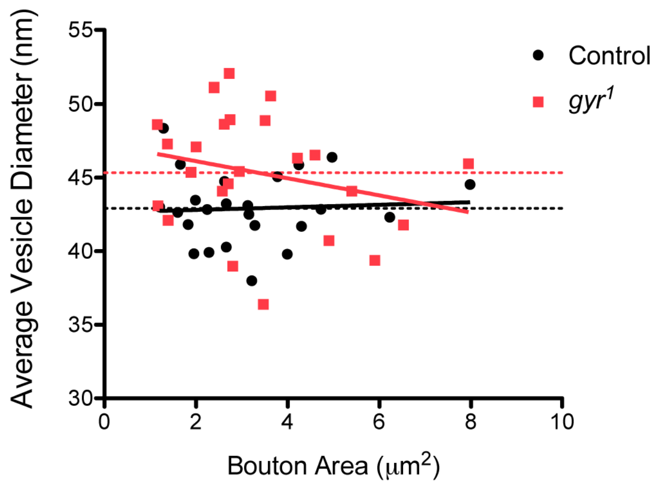
A
control
B
control
C
gyr1
D
gyr1
E
gyr1
F
gyr1

Figure 3. Ultrastructural analysis reveals alterations in synaptic vesicle diameter in *gyr* mutants. **A)** A representative image of a synaptic bouton from a control larva. **B)** The boxed region in (A) shown at higher magnification to illustrate the homogeneity of synaptic vesicle diameter in control animals. **C)** Example of a *gyr* bouton with abnormal synaptic vesicle diameter and large cisternal-like structures (indicated by an asterisk). **D)** The boxed region in (C) is shown at higher magnification to exemplify the variability in synaptic vesicle diameter (see asterisk). **E)** A representative *gyr* bouton with similar synaptic vesicle diameter and density relative to controls. **F)** Example of a *gyr* bouton with decreased synaptic vesicle density. Overall, there is not a statistically significant difference in synaptic vesicle density between *gyr* mutants and controls (see Figure 4; $p = 0.19$, Student's t-test). Scale bars: A, C, E, and F = 1 μm; B and D = 100 nm.


A
Synaptic Vesicles per μm2
n.s.
300
200
100
0
Control
gyr1


B
Average Vesicle Diameter (nm)
*
55
50
45
40
35
30
Control
gyr1


C
Frequency
Control
gyr1
0.5
0.4
0.3
0.2
0.1
0.0
25 30 35 40 45 50 55 60 65 70 75 80 85+
Vesicle Diameter (nm)


D
Average Vesicle Diameter (nm)
Control
gyr1
55
50
45
40
35
30
0 2 4 6 8 10
Bouton Area (μm2)

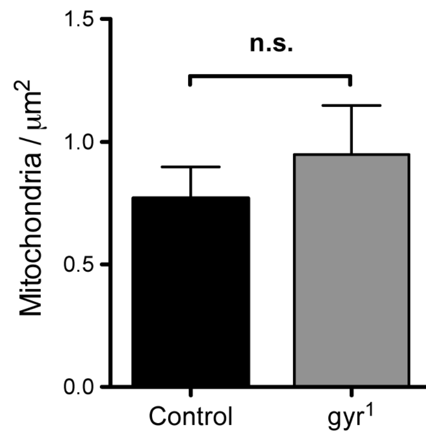
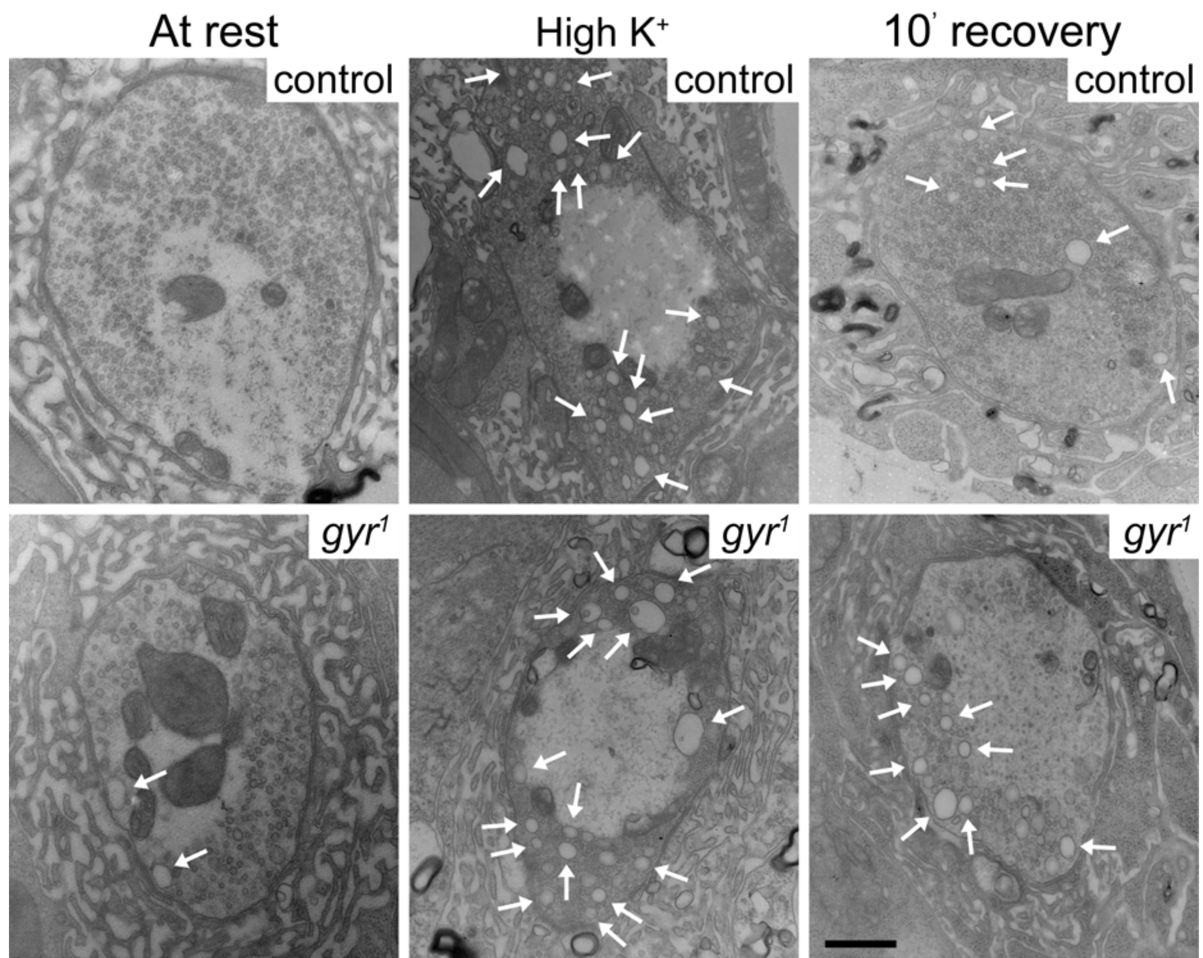

E
Mitochondria / μm2
n.s.
1.5
1.0
0.5
0.0
Control
gyr1

Figure 4. Quantification of ultrastructural morphology. **A)** Synaptic vesicle density is not significantly different in *gyr* mutants compared to controls ($p = 0.19$, Student's t-test). Each point on the graph represents the synaptic vesicle density of a single bouton. Average synaptic vesicle density per bouton area (μm^2) ± SEM: control = 132.6 ± 13.8, n = 16; *gyr* = 111.4 ± 9.3, n = 24. **B)** The average synaptic vesicle diameter (vesicles < 60 nm in diameter) is increased in *gyr* mutants relative to controls ($p = 0.02$, Student's t-test). Each data point represents the mean synaptic vesicle diameter of a single bouton. At least 30 synaptic vesicles were measured in each bouton to obtain the mean synaptic vesicle diameter. In total, 2,518 synaptic vesicles from control boutons were measured, and 1,535 synaptic vesicles from *gyr* mutants were measured. Average synaptic vesicle diameter (in nm) ± SEM: control = 42.91 ± 0.49, n = 24; *gyr* = 45.33 ± 0.82, n = 24. **C)** Synaptic vesicle diameter is shifted to higher values relative to the control in *gyr* mutant larvae. Since varying numbers of synaptic vesicles were measured in each bouton, the frequency distribution of each bouton was calculated and normalized to obtain the overall frequency distribution. The measurements are from the same data set as in (B). **D)** Examination of the relationship between bouton area and mean synaptic vesicle diameter. Each bouton used to determine the average synaptic vesicle diameter is plotted with the bouton area along the abscissa and the mean synaptic vesicle diameter along the ordinate. The horizontal dotted lines represent the overall average for control (black) and *gyr* (red). The solid lines indicate the linear regression best-fit values. The slopes are not significantly different from zero for either control ($p = 0.79$, Pearson's correlation) or *gyr* ($p = 0.23$). **E)** The average number of mitochondria per bouton area is not significantly different between *gyr* and control larvae ($p = 0.47$, Student's t-test). Average number of mitochondria per μm^2 ± SEM: control = 0.77 ± 0.13, n = 24; *gyr* = 0.95 ± 0.29, n = 28.


At rest
High K+
10' recovery
control
control
control
gyr1
gyr1
gyr1

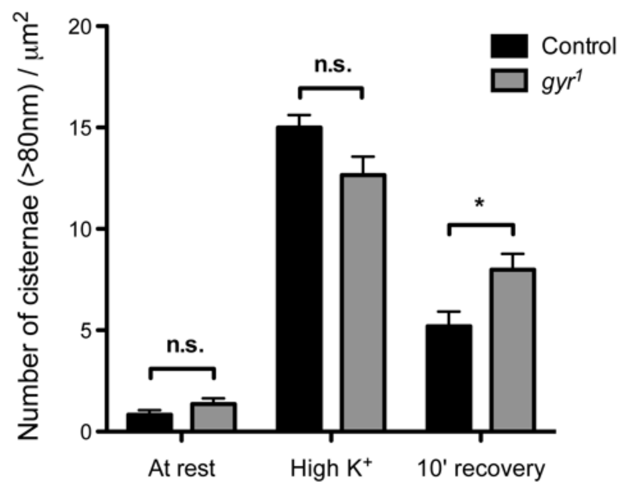
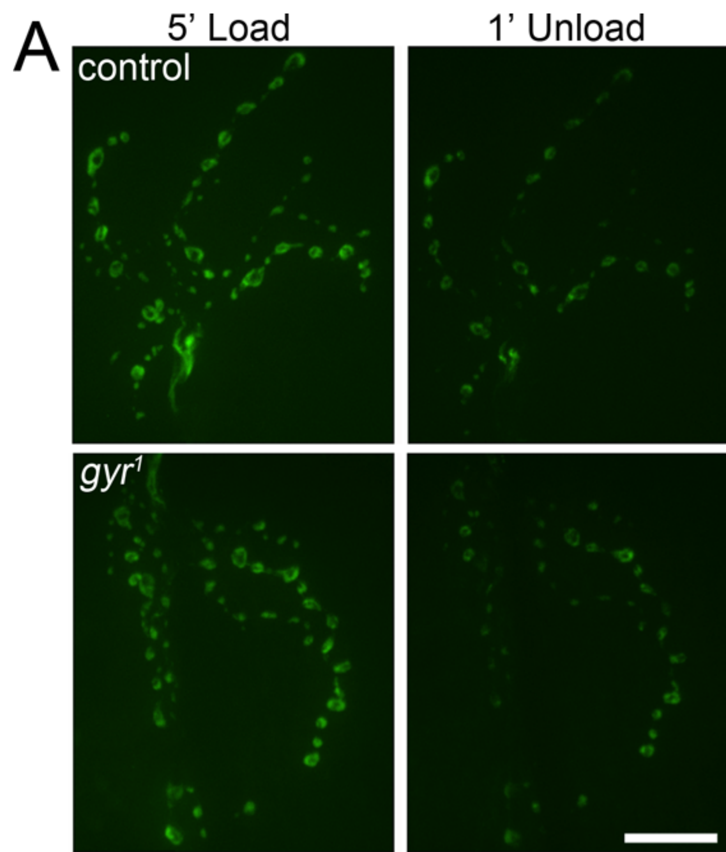
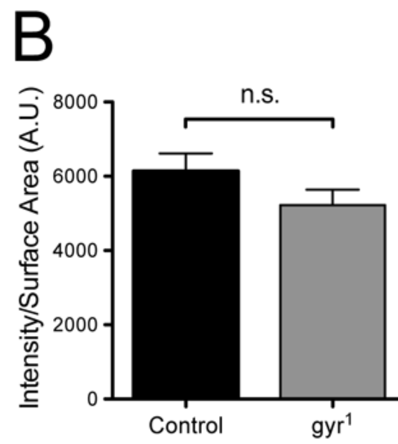

Number of cisternae (>80nm) / μm2
Control
gyr1
n.s.
n.s.
*
At rest
High K+
10' recovery
0
5
10
15
20

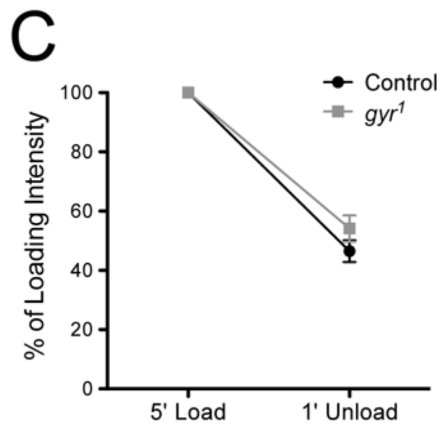
Figure 5. *gyr* mutants are delayed in resolving endocytic cisternae following a high-potassium shock. Left panels: At rest, *gyr* mutants have a slight, but not statistically significant, increase in the number of cisternae (endosomal-like structures larger than 80 nm in diameter) per μm^2 compared to controls ($p = 0.21$, Student's t-test). Middle panels: Immediately following a five-minute incubation with high K^+ (90 mM) Jan and Jan solution, control and *gyr* boutons have a similar dramatic increase in the number of endocytic cisternae due to bulk endocytosis ($p = 0.21$, Student's t-test). Right panels: Ten minutes after high K^+ stimulation, *gyr* boutons have ~50% more endocytic cisternae than controls, suggesting that they cannot resolve endocytic cisternae into synaptic vesicles as quickly as controls ($p = 0.01$, Student's t-test). Average number of cisternae per μm^2 ± SEM: At rest, control = 0.84 ± 0.22, n = 16; *gyr* = 1.36 ± 0.28, n = 29; High K^+, control = 15.0 ± 0.62, n = 8; *gyr* = 12.7 ± 0.90, n = 5; 10' recovery, control = 5.20 ± 0.72, n = 24; *gyr* = 7.98 ± 0.78, n = 23. Scale bar = 500 nm.



A
5' Load
1' Unload
control
gyr1



B
n.s.
Intensity/Surface Area (A.U.)
8000
6000
4000
2000
0
Control
gyr1



C
Control
gyr1
% of Loading Intensity
100
80
60
40
20
0
5' Load
1' Unload

Figure 6. *gyr* mutants are not defective in loading or unloading FM1-43. **A)** Representative images of control (top) and *gyr* (bottom) NMJs from FM1-43 uptake experiments. Both control and *gyr* boutons display prominent fluorescence in synaptic boutons following incubation with a high K^+ (60 mM) HL3 solution containing FM1-43 (4 μM, left panels). After extensive washing to remove excess dye, terminals were unloaded with a one-minute stimulation with high K^+ solution in the absence of dye (right panels). **B)** Quantification of the FM1-43 uptake experiment reveals no significant difference in mean fluorescence intensity between *gyr* and control boutons ($p = 0.16$, Student's t-test). Mean fluorescence intensity (arbitrary units) ± SEM: control = 6,143 ± 473, n = 9; *gyr* = 5,222 ± 417, n = 9. **C)** Control and *gyr* NMJs unload similar levels of FM1-43 following a one-minute incubation with high K^+ solution. Mean fluorescence intensity as a percentage of loading fluorescence ± SEM: control = 48.04 ± 4.16, n = 9; *gyr* = 55.64 ± 5.58, n = 9. Scale bar = 14 μm.

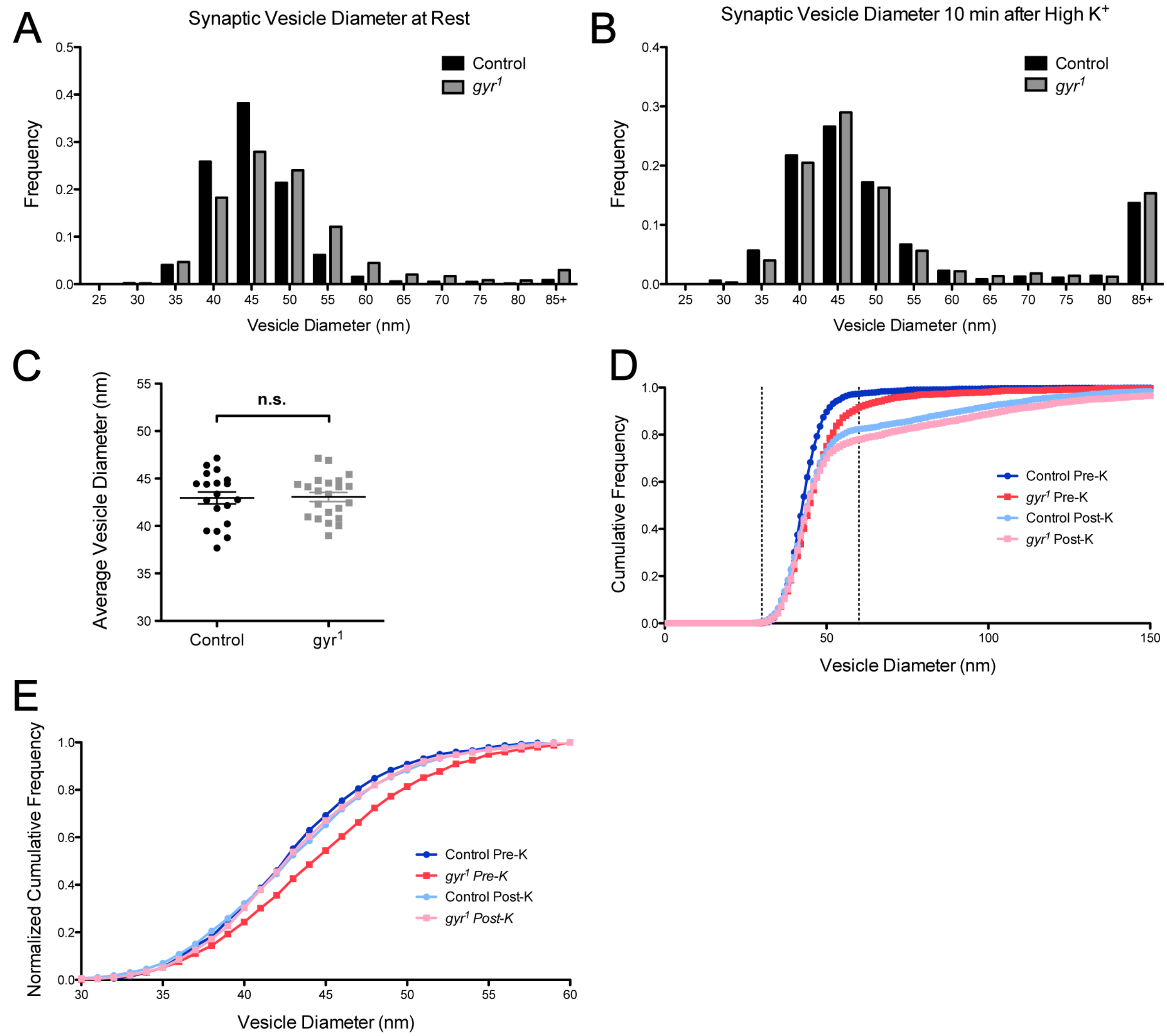

A
Synaptic Vesicle Diameter at Rest
Frequency
Control
gyr1
Vesicle Diameter (nm)
B
Synaptic Vesicle Diameter 10 min after High K+
Frequency
Control
gyr1
Vesicle Diameter (nm)
C
Average Vesicle Diameter (nm)
n.s.
Control
gyr1
D
Cumulative Frequency
Control Pre-K
gyr1 Pre-K
Control Post-K
gyr1 Post-K
Vesicle Diameter (nm)
E
Normalized Cumulative Frequency
Control Pre-K
gyr1 Pre-K
Control Post-K
gyr1 Post-K
Vesicle Diameter (nm)

Figure 7. Ten minutes after incubation with a high-potassium solution, *gyr* synaptic vesicle size is more homogeneous. **A)** Same as Figure 4C. At rest, *gyr* synaptic vesicles are larger and more variable in size. **B)** Following a five-minute potassium shock and ten-minute rest period, the distribution of synaptic vesicle diameter in *gyr* mutants is much more similar to controls. **C)** Scatter plots of the average synaptic vesicle diameter (excluding vesicles >60 nm) of each bouton included in the analysis. Only boutons in which more than 30 synaptic vesicles could be measured were used in the analysis. Mean synaptic vesicle diameter following the potassium shock is not significantly different in *gyr* mutants relative to controls (p = 0.89, Student's t-test). Average synaptic vesicle diameter (in nm) ± SEM: control = 42.95 ± 0.63, n = 19; *gyr* = 43.06 ± 0.48, n = 22. **D)** A cumulative frequency diagram illustrates the differences in vesicle diameter before (Pre-K) and after (Post-K) potassium shock. The vertical lines mark the region between 30 and 60 nm, which is displayed in part (E) to emphasize the region of the graph that includes synaptic vesicles. All synaptic vesicles and cisternae (up to 150 nm) that could be measured are represented in this graph, although some data points lie beyond 150 nm (especially in the Post-K condition), and therefore the cumulative frequency does not reach 1.0. **E)** The same data presented in (D) including only the values between 30 nm and 60 nm following normalization. Due to the increased number of cisternae in both control and *gyr* boutons following the potassium shock, only ~80% of the measured structures lie in the region from 30 to 60 nm, as compared to controls at rest where ~97% of structures are in this range. Therefore, we normalized the data within this region to more accurately compare the distribution of vesicles 30 to 60 nm in diameter. To normalize the data, we recalculated the cumulative frequency distributions of each bouton using only the structures less than 60 nm in diameter. These cumulative frequency distributions were then averaged together to give the overall distribution. Prior to the potassium shock, the *gyr* distribution is shifted to the right compared to the control. Following the potassium shock, *gyr* and control synaptic vesicles have much more similar distributions.

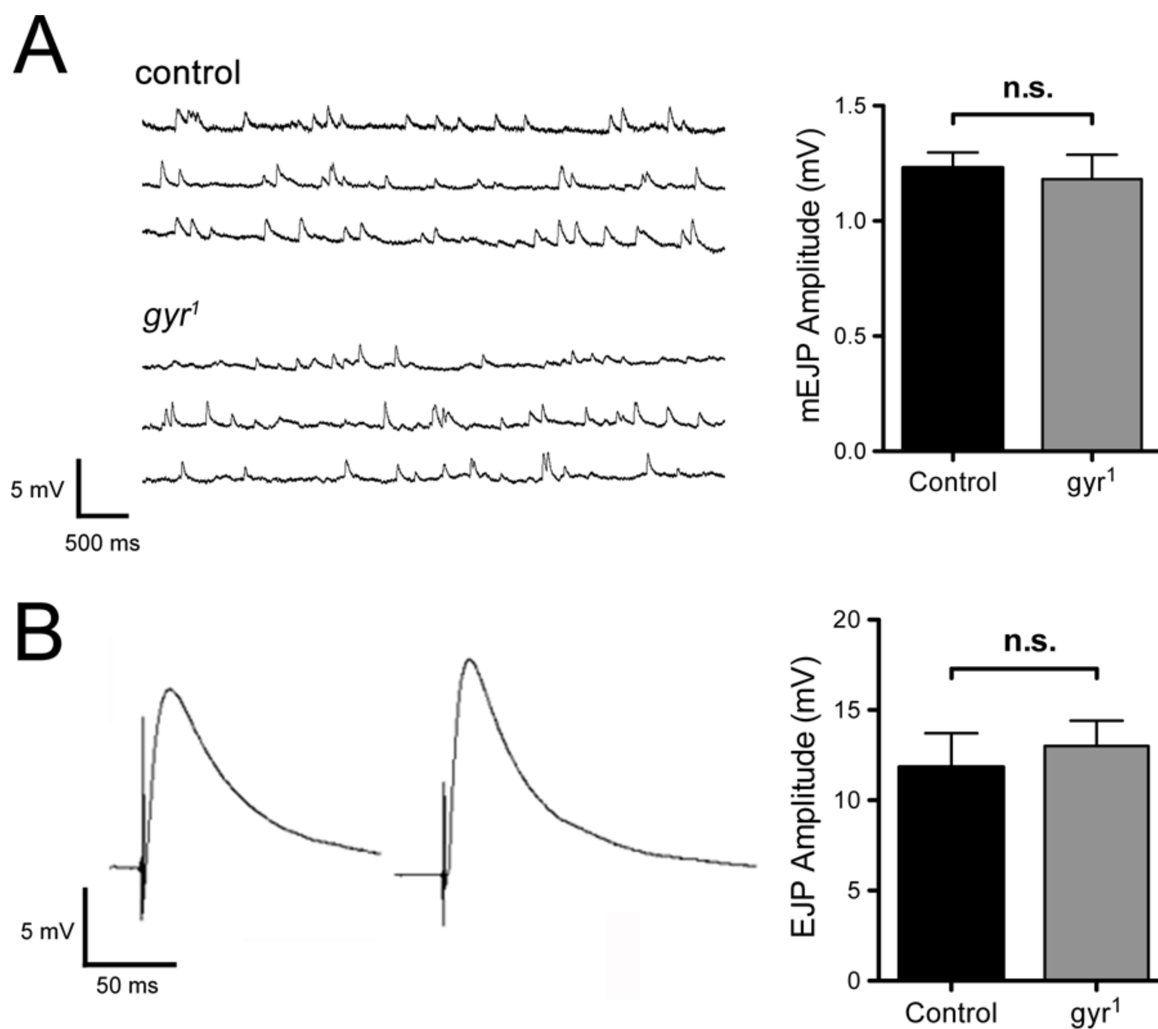
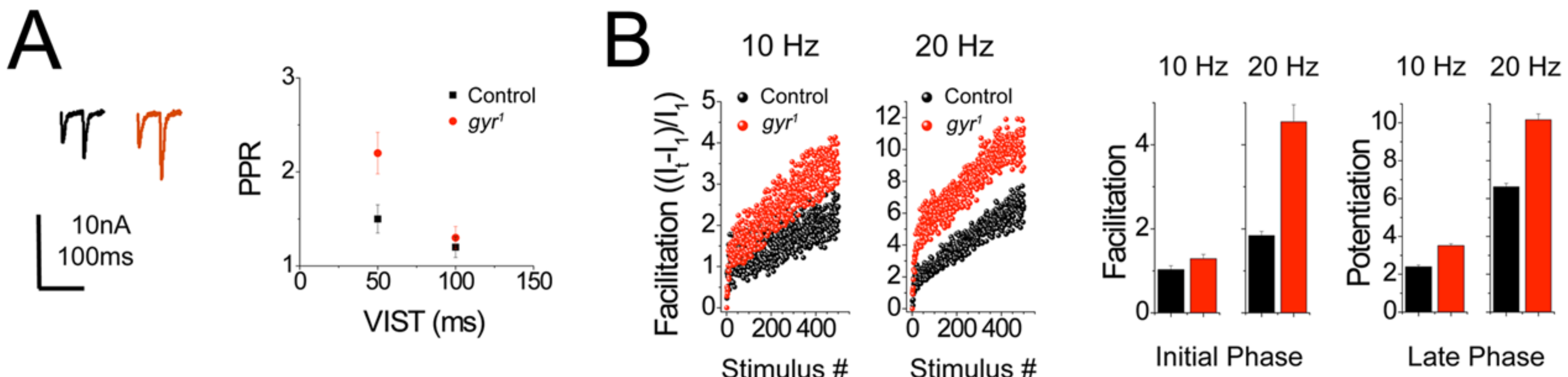
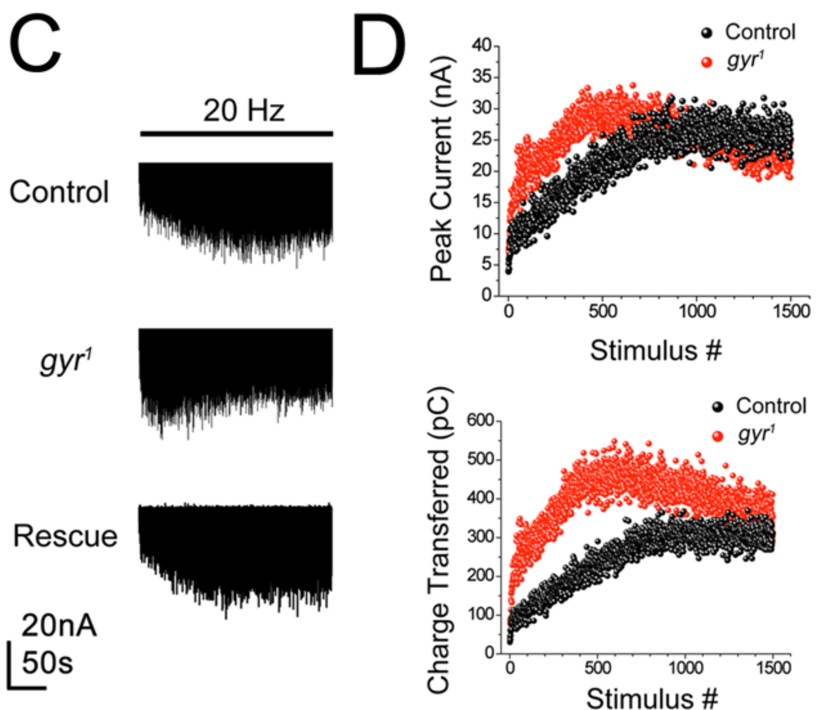


Figure 8. mEJP and EJP amplitude is normal in *gyr* mutants. **A)** Although synaptic vesicle diameter is increased in *gyr* larvae, mEJP amplitude is not significantly different between *gyr* and control (p = 0.71, Student's t-test). Several representative traces from control and *gyr* larvae are depicted. Average mEJP amplitude (in mV) ± SEM: control = 1.23 ± 0.07, n = 6; *gyr* = 1.18 ± 0.11, n = 7. **B)** Evoked release also is not significantly different between *gyr* and control larvae (p = 0.63, Student's t-test). Average EJP amplitude (in mV) ± SEM: control = 11.8 ± 1.87, n = 9; *gyr* = 13.0 ± 1.40, n = 8.


A
10nA
100ms
PPR
Control
gyr1
VIST (ms)
B
10 Hz
20 Hz
Facilitation ((It-I1)/I1)
Stimulus #
Facilitation
Initial Phase
Potentiation
Late Phase


C
Control
gyr1
Rescue
20 Hz
20nA
50s
D
Peak Current (nA)
Charge Transferred (pC)
Stimulus #
Control
gyr1

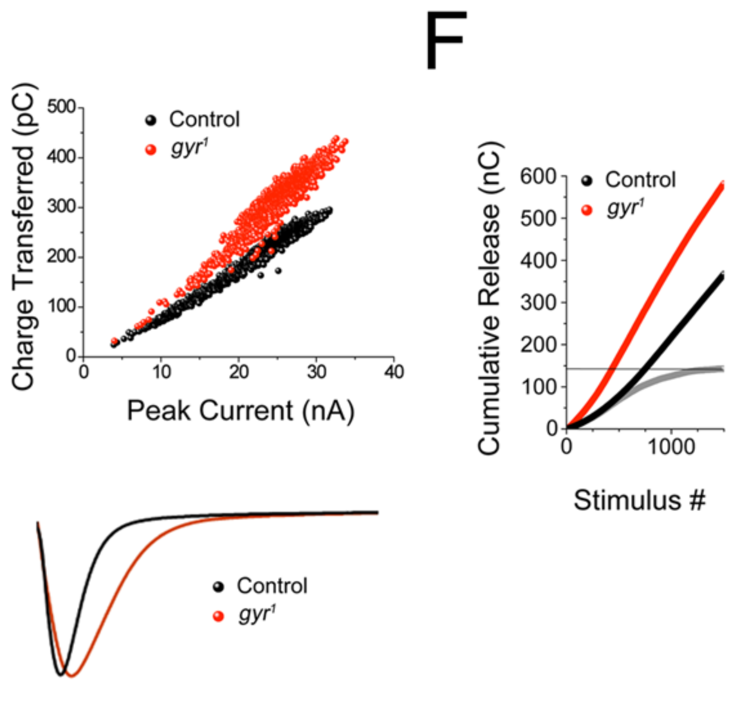

E
Charge Transferred (pC)
Peak Current (nA)
Control
gyr1
F
Cumulative Release (nC)
Stimulus #
Control
gyr1

Figure 9. *gyr* mutants display increased facilitation and have more asynchronous release at higher stimulation frequencies. **A)** At a calcium concentration of 0.2 mM, *gyr* mutants have an increase in the paired-pulse ratio (PPR) compared to controls at a variable interstimulus time (VIST) of 50 ms but not 100 ms. The PPR is defined as the amplitude of the second peak divided by the first peak. **B)** At 10 Hz and 20 Hz stimulation frequencies, the *gyr* mutant displays an increase in facilitation that is further enhanced at 20 Hz. The bar graphs indicate the average facilitation at the beginning of stimulation (Initial Phase) and at the end of 500 stimuli (Late Phase). While *gyr* (red) has higher levels of facilitation compared to controls (black) at both 10 and 20 Hz, this difference is more pronounced at 20 Hz. **C)** When stimulated at high frequency for an extended period of time (1,500 stimuli at 20 Hz), *gyr* larvae display an increase in facilitation that persists for approximately 500 stimuli and then declines to levels similar to controls (see [D]). Presynaptic expression of synaptogyrin rescues the phenotype ($elav^{c155}$*-GAL4; gyr*^{1}*; UAS-myc-gyrin*/+). **D)** For each stimulus from the traces in (C), we plotted the peak current (EJC amplitude) and the amount of charge transferred (total current) separately. While both plots depict an increase in *gyr* relative to controls during the early phase of stimulation, the difference in the amount of charge transferred between *gyr* and control is greater than the difference in peak amplitude. **E)** The same data from (C) and (D) are plotted to compare the amount of charge transferred by a stimulus relative to its peak amplitude. In control and *gyr* larvae, the amount of current transferred increases as the EJC amplitude increases. However, compared to controls, stimuli from *gyr* animals transfer a greater amount of charge at similar amplitudes. This suggests that synaptic vesicle release is more asynchronous in *gyr* mutants. When traces from the 20 Hz stimulation protocol are normalized (lower panel), the *gyr* peak is broader than the control peak, indicating that there is a component of release that is delayed in the mutant larvae. **F)** Due to the increased amount of charge transferred by EJCs in *gyr* larvae, the cumulative release is also enhanced. After 1,500 stimuli, *gyr* larvae release a total of ~600 nC of charge, while controls release ~375 nC, which is about 40% less total charge over the course of stimulation. However, as stimulation proceeds, a steady state is reached in which *gyr* and controls transfer charge at a similar rate. This is indicated by the gray line, which represents the difference in release between *gyr* and control. This line plateaus around 150 nC, indicating the point after which *gyr* and control larvae begin to transfer charge at a more similar rate.

Chapter 4

Conclusions and Perspectives

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Summary

Although synaptogyrin and synaptophysin were identified as integral synaptic vesicle proteins decades ago, their exact role in the synaptic vesicle cycle remains obscure. We have attempted to elucidate the function of synaptogyrin by generating a null mutant in *Drosophila*, which has only a single gyrin isoform and no physin homolog, thereby allowing us to avoid issues of redundancy. *Drosophila* synaptogyrin is abundantly expressed in neurons and enriched in synaptic vesicles, which suggests that it is likely a true synaptogyrin homolog. Initial characterization of a *gyr* mutant revealed no measurable changes in viability, gross synaptic morphology, basal neuro-transmission, or male courtship behavior. However, in-depth analysis revealed defects in the regulation of synaptic vesicle diameter and in the recovery of synaptic vesicles from endocytic cisternae, as well as alterations in synaptic facilitation. These results suggest that synaptogyrin, while not required for neurotransmission, does modulate synaptic vesicle exo-endocytosis, especially during high-intensity stimulation.

The loss of synaptogyrin and synaptophysin results in relatively mild phenotypes

While we were able to find several alterations in synaptic vesicle recycling and neurotransmission in *gyr* mutants, it is intriguing that the loss of a highly conserved synaptic vesicle protein does not result in more dramatic phenotypes. Mouse and nematode synaptogyrin/synaptophysin double knockouts also have relatively mild phenotypes, suggesting that this observation is not unique to *Drosophila* (Janz et al., 1999; Abraham et al., 2006; Abraham et al., 2011). Synaptogyrin and synaptophysin are not essential for synaptic vesicle exocytosis, endocytosis, or basic behaviors. However, this does not necessarily mean that these proteins do not impact

neurotransmission in a manner that is evolutionarily relevant. Indeed, several lines of evidence suggest that synaptophysin and synaptogyrin promote the efficient recycling of synaptic vesicles. For example, an increase in clathrin-coated vesicles was seen in the *C. elegans* triple knockout (Abraham et al., 2006), the mouse synaptophysin knockout (Spiwoks-Becker et al., 2001), as well as in the squid giant axon when the synaptophysin-dynamin interaction was inhibited (Daly et al., 2000). These observations raise the possibility that synaptophysin and/or synaptogyrin are involved in a clathrin-independent version of endocytosis and that clathrin-mediated endocytosis is upregulated in their absence to counteract for the loss of the synaptogyrin/ synaptophysin-mediated endocytic pathway. Under relatively mild stimulation conditions, it appears as though clathrin-mediated endocytosis is able to compensate for the loss of synaptophysin and/or synaptogyrin. However, under more intense stimulation, the clathrin-mediated endocytic pathway alone is insufficient and alterations in synaptic function begin to emerge. While this hypothesis is intriguing, the rapid inactivation of clathrin in *Drosophila* through the use of the FlAsH-FALI technique results in a complete block in synaptic vesicle reformation, suggesting that all synaptic vesicles are formed through clathrin-dependent mechanisms at the *Drosophila* larval NMJ (Heerssen et al., 2008).

Studies in mice have established that synaptophysin and synaptogyrin impact synaptic plasticity as well as certain aspects of learning and memory (Janz et al., 1999; Schmitt et al., 2009). While we were unable to identify learning and memory deficits in the *Drosophila gyr* mutant, it certainly is possible that the loss of synaptogyrin does have important evolutionarily relevant behavioral consequences that we were simply

incapable of isolating in our initial examination. For example, synaptogyrin may be required during periods of intense motor activity such as flight and predator avoidance, or it may impact visual acuity, olfaction, temperature tolerance, or social interactions. Behavioral experiments in a controlled laboratory setting cannot fully replicate the unique stresses of life in the natural environment, and it is therefore difficult to determine exactly how synaptogyrin may impact the fitness of an organism in the wild. Interestingly, several recent studies have associated mutations in the *synaptogyrin* locus with schizophrenia susceptibility in humans (Verma et al., 2004; Verma et al., 2005; Cheng and Chen, 2007; Iatropoulos et al., 2009). It may be the case that the very same alterations in synaptic plasticity and synaptic vesicle recycling observed in model organism knockouts also may have the ability to impact human thought and behavior.

Phylogenetic analysis of synaptophysin and synaptogyrin

When we searched for the evolutionary emergence of gyrins, physins, and other MARVEL proteins, we were surprised to find synaptogyrin and synaptophysin homologs in organisms without nervous systems. The ancestral functions of physins and gyrins are unknown, although one likely possibility is that their MARVEL transmembrane regions were used in some aspect of membrane organization. For example, other MARVEL proteins such as MAL and MAL2 mediate trafficking of proteins and lipids to the apical membrane (Puertollano and Alonso, 1999; de Marco et al., 2002), while occludin localizes to tight junctions (Furuse et al., 1993). Many MARVEL domain-containing proteins are associated with lipid rafts, which suggests that the MARVEL domain itself has the ability to form membrane microdomains enriched in lipids and proteins that mediate processes such as cellular adhesion, vesicle biogenesis,

intracellular trafficking, or intercellular signaling (Thiele et al., 2000; de Marco et al., 2001; Sanchez-Pulido et al., 2002; Raleigh et al., 2010). The ancestral gyrins and physins may have performed similar roles in early metazoans or in their unicellular ancestors, and more recently in evolution they were co-opted by the nervous system to perform specific functions related to synaptic vesicle exo-endocytosis. The presence of two distinct physin and gyrin families in vertebrates with multiple paralogs suggests that several rounds of gene duplication occurred after the split between the ancestral gyrin and the ancestral physin (Hubner et al., 2002). It is currently unknown whether the ancestral physins and gyrins were more similar to the ubiquitous pantophysin and cellugyrin or if they more closely resembled neuronal-specific synaptogyrin and synaptophysin.

The absence of a *Drosophila* synaptophysin homolog and an *N. vectensis* synaptogyrin homolog raises the possibility that the functions of physins and gyrins are interchangeable. Indeed, the observation that synaptic plasticity defects are enhanced in the mouse double knockout strongly supports the idea that synaptogyrin and synaptophysin are to some extent functionally redundant (Janz et al., 1999). However, it seems unlikely that both proteins would be so highly conserved across evolution if they did not serve important and unique functions in the organisms that have both a synaptogyrin and a synaptophysin homolog. Due to the absence of other physin and gyrin isoforms and the relative ease of transgene expression in *Drosophila*, the *gyr* mutant provides an opportunity to examine the relative contributions of gyrins and physins to different aspects of the exo-endocytic cycle. In the future, we would like to perform rescue experiments with the mammalian synaptogyrin and synaptophysin

isoforms to determine if any (or all) of the phenotypes we identified in *gyr* mutants can be rescued by mammalian synaptophysin or synaptogyrin. These experiments could provide insight into whether *Drosophila* synaptogyrin performs a role more similar to mammalian synaptogyrin or synaptophysin as well as information about the level of functional conservation between mammalian synaptophysin and synaptogyrin.

Structure-function analysis of synaptogyrin and synaptophysin

Relatively little is known about how synaptophysin and synaptogyrin function is regulated at the synapse. Mammalian synaptogyrin and synaptophysin contain several tyrosine residues in their C-termini that are phosphorylated by the tyrosine kinases $pp60^{c\text{-}src}$ and c-fyn (Sudhof et al., 1987; Barnekow et al., 1990; Linstedt et al., 1992; Janz and Sudhof, 1998; Janz et al., 1999), and synaptophysin appears to be a calcium-dependent substrate of CaM kinase II (Rubenstein et al., 1993). Interestingly, synaptophysin tyrosine phosphorylation increases in parallel with changes in synaptic plasticity, as indicated by higher levels of synaptophysin phosphorylation following LTP induction in hippocampal slices (Mullany and Lynch, 1998). However, exactly which residues of mammalian synaptogyrin and synaptophysin undergo phosphorylation and the functional consequences of protein modification are currently unknown. Part of the difficulty in isolating the targets of phosphorylation in mammalian synaptophysin has been due to the lack of trypsin cleavage sites in the protein's C-terminus, which hinders the identification of phosphorylated residues via mass spectroscopy (Evans and Cousin, 2005).

We have not yet determined whether *Drosophila* synaptogyrin is a target of phosphorylation, although this protein does contain several cytoplasmic tyrosine and

serine residues. However, since the N- and C-termini are among the least conserved regions of physins and gyrins, it is difficult to identify evolutionarily conserved phosphorylation sites (Hubner et al., 2002). Nevertheless, it would be informative to perform *in vitro* kinase assays on *Drosophila* synaptogyrin to determine if the protein can undergo phosphorylation. If so, identification of phosphorylated residues followed by generation of phosphomimetic and phospho-incompetent versions of *Drosophila* synaptogyrin for use in rescue experiments may provide critical insight into the protein's function and regulation. If mammalian synaptophysin and/or synaptogyrin are capable of rescuing some (or all) of the *gyr* phenotypes, additional rescue experiments could be performed with phosphomimetic and phospho-incompetent versions of these proteins as well. However, since mammalian synaptophysin contains nine tyrosine and four serine residues on its C-terminus alone, this may be an ambitious undertaking (Evans and Cousin, 2005).

Additional information about synaptogyrin function in *Drosophila* could come from deletion and mutagenesis studies to determine the importance of various protein domains in synaptogyrin's role at the synapse. Since the C-termini of mammalian synaptophysin and synaptogyrin are known to interact with dynamin in a calcium-dependent manner (Daly and Ziff, 2002), it would be informative to determine if the loss of this region impacts synaptic vesicle recycling in *Drosophila*. However, the C-terminus of synaptogyrin also has been identified as a critical region for protein targeting to the synaptic terminal in *C. elegans* (Zhao and Nonet, 2001). Therefore, it would be difficult to examine the influence of the *Drosophila* synaptogyrin C-terminus

on endocytosis through deletion studies if the loss of this region results in protein mislocalization or degradation.

In-depth structure-function analysis of the synaptogyrin paralog cellugyrin demonstrated that vesicle biogenesis in PC12 cells was greatly influenced by two short hydrophobic regions, one located in the lumen between the first two transmembrane domains and the other found in the cytoplasm directly after the fourth transmembrane domain (Belfort et al., 2005). Furthermore, the length of the short cytoplasmic loop (between the second and third transmembrane helices) also significantly impacted cellugyrin function. Taken together, these observations led to a hypothesis proposing that cellugyrin forms a conical structure with the luminal ends of the transmembrane helices situated more closely together than the cytoplasmic ends, thereby directly imparting curvature on membranes (Belfort et al., 2005). Given that the lengths of the regions between the transmembrane helices are conserved among the gyrin paralogs, it is possible that synaptogyrin's transmembrane domains may also directly promote membrane curvature and thereby impact vesicle formation (Hubner et al., 2002). Thus it would be worthwhile to perform similar structure-function analysis of *Drosophila* synaptogyrin to determine if the aforementioned regions of cellugyrin are also essential for synaptogyrin function.

The role of synaptogyrin and synaptophysin in endocytosis

Synaptic vesicle biogenesis, whether from an endosomal compartment or the plasma membrane, requires the recruitment of a wide variety of essential synaptic vesicle proteins (e.g., synaptotagmin and synaptobrevin) and the preferential exclusion of certain proteins such as the t-SNARE syntaxin. Maintaining the correct complement

of synaptic vesicle proteins could theoretically be achieved through a "kiss-and-run" endocytic mechanism, which would allow a transiently fused vesicle to directly reform following fusion pore closure rather than fully collapsing into the plasma membrane. While rapid endocytosis has been observed at certain synapses such as the calyx of Held (Wu et al., 2005), the contribution of kiss-and-run endocytosis at the *Drosophila* NMJ is highly controversial (Verstreken et al., 2002; Dickman et al., 2005; He and Wu, 2007). An alternative way to promote proper synaptic vesicle sorting would be through the creation of microdomains on the plasma membrane or endosomes that are enriched in the lipids and proteins found on synaptic vesicles. Synaptophysin, through its interactions with cholesterol and synaptobrevin, in addition to its ability to multimerize, is an intriguing candidate to seed this sort of microdomain to facilitate vesicle biogenesis (Rehm et al., 1986; Thomas et al., 1988; Calakos and Scheller, 1994; Washbourne et al., 1995; Thiele et al., 2000). Whether synaptogyrin could perform a similar function is unclear because an interaction between synaptogyrin and cholesterol was not tested (Thiele et al., 2000), and mammalian synaptogyrin and synaptobrevin do not appear to interact directly (Edelmann et al., 1995). While we have not yet investigated the lipid-binding capabilities of *Drosophila* synaptogyrin, preliminary results of a GST pull-down experiment using *Drosophila* synaptobrevin identified a potential interaction with synaptogyrin (data not shown). However, this result will need to be verified to ensure that this protein interaction is specific and not merely a consequence of the two proteins colocalizing to synaptic vesicles.

Synaptophysin also has been suggested to promote endocytosis via a calcium-dependent interaction with dynamin, and it may recruit dynamin to sites of synaptic

vesicle exocytosis (Daly and Ziff, 2002). Like synaptophysin, mammalian synaptogyrin appears to bind dynamin in a calcium-dependent manner, and therefore may contribute to the recruitment of endocytic machinery; however, the synaptogyrin-dynamin association is not as strong as the synaptophysin-dynamin interaction (Daly and Ziff, 2002). We have conducted preliminary experiments to investigate whether *Drosophila* synaptogyrin is capable of binding dynamin in a calcium-dependent manner. We performed a GST pull-down experiment using the C-terminus of synaptogyrin – the region of synaptophysin and synaptogyrin that binds dynamin in mammals (Daly and Ziff, 2002). Preliminary results indicate that *Drosophila* synaptogyrin and dynamin do not significantly interact in either the presence or absence of calcium (data not shown), suggesting that this interaction is not conserved in *Drosophila*. Therefore, it remains unclear whether *Drosophila* synaptogyrin can promote exo-endocytosis through the same protein interactions observed with mammalian synaptophysin or synaptogyrin.

Further analysis of synaptogyrin's role in vesicle biogenesis and endocytosis

We were intrigued to discover that the loss of synaptogyrin results in an increase in endocytic cisternae ten minutes after intense stimulation with high potassium. While we put forward a model in which synaptogyrin promotes the budding of synaptic vesicles from endosomal-like compartments (see Chapter 3, Discussion), we currently cannot rule out an alternative explanation, namely that synaptogyrin normally inhibits homotypic fusion of synaptic vesicles or that it prevents the fusion of synaptic vesicles with endosomal compartments after periods of extreme neuronal activity. If this were the case, the loss of synaptogyrin would enhance the frequency of

this type of fusion, which could account for the increase in cisternae we observed ten minutes after potassium stimulation.

Indeed, the small GTPase Rab5, which regulates endocytic trafficking to early endosomes, has been identified as an inhibitor of homotypic fusion, indicating that there are mechanisms in place at the presynaptic terminal to prevent synaptic vesicles from fusing with one another (Bucci et al., 1992; Shimizu et al., 2003). Mutations in Rab5 lead to an increase in homotypic fusion and consequently result in significant misregulation of synaptic vesicle size, and this effect is exacerbated when synaptic activity is low (Shimizu et al., 2003). Furthermore, there also is evidence suggesting that synaptic vesicles can fuse with endocytic cisternae at *Drosophila* larval NMJs under intense stimulation conditions. When FM1-43 dye was loaded for 1.5 minutes and subsequently photocoverted to create an electron-dense product visible by electron microscopy, a subset of cisternae had irregular membrane labeling, as if loaded and unloaded vesicles had fused together (Akbergenova and Bykhovskaia, 2009). Furthermore, labeled synaptic vesicles were occasionally observed in extremely close proximity to unlabeled cisternae, potentially indicating fusion between these structures. Thus, the possibility that synaptogyrin may inhibit homotypic fusion is worthy of further investigation.

To distinguish between the two possibilities that synaptogyrin either promotes synaptic vesicle biogenesis from endocytic cisternae or inhibits synaptic vesicle fusion with endosomal-like compartments, we could repeat the previously described FM1-43 experiment in *gyr* mutants. If synaptogyrin merely regulates synaptic vesicle budding from cisternae, we would expect that the extent of irregular membrane labeling would

not be enhanced in the *gyr* mutant background. However, if synaptogyrin inhibits homotypic fusion, there should be an increase in the degree of uneven cisternal labeling in *gyr* boutons. Examination of the genetic interaction between Rab5 and synaptogyrin may provide further insights into whether these proteins function in a similar pathway.

Furthermore, if synaptogyrin promotes vesicle biogenesis from endocytic cisternae, we would predict that *gyr* mutants would retain a greater amount of FM1-43 dye in cisternae following potassium-induced uptake via bulk endocytosis. Our initial FM1-43 experiments did not reveal a significant difference in dye loading or unloading in *gyr* mutants relative to controls. However, our unloading experiments were performed with non-physiological high-potassium saline, which may induce exocytosis of cisternae in addition to synaptic vesicles. This may have prevented us from identifying a delay in the movement of FM1-43 from cisternae to synaptic vesicles. If we were to unload the FM1-43 dye with more physiologically relevant stimuli (i.e., with nerve stimulation), we would expect to see greater retention of FM1-43 in *gyr* boutons, as more of the dye would be trapped in cisternae compared to controls. Other stimulation protocols, including those that selectively label the recycling synaptic vesicle pool or the reserve pool, may provide additional insight into how the loss of synaptogyrin affects synaptic vesicle recycling.

Examination of genetic interactions with *Drosophila* synaptogyrin

Previous studies in *Drosophila* have examined many of the critical proteins involved in regulating clathrin-mediated endocytosis at the larval NMJ. Comparing the endocytic phenotypes caused by mutations in these genes with those observed in our *gyr* mutant may provide further insight into synaptogyrin function. Intriguingly,

several of these endocytic mutants share phenotypes in common with *gyr* animals. For example, the loss of synaptojanin, a polyphosphoinositide phosphatase that participates in the uncoating of the clathrin coat (Cremona et al., 1999), or AP-180/lap (lap is the *Drosophila* homolog), a clathrin adaptor that promotes clathrin assembly (Ahle and Ungewickell, 1986), leads to a decrease in synaptic vesicle density and a dramatic increase in synaptic vesicle size, which we also observed in a subset of *gyr* boutons (Zhang et al., 1998a; Verstreken et al., 2003; Dickman et al., 2005). The loss of endophilin, a protein that binds dynamin and recruits synaptojanin to sites of endocytosis, results in many of the same phenotypes as those seen in the *synaptojanin* mutant, suggesting it may also play a role in removing clathrin coats (Ringstad et al., 1997; Verstreken et al., 2002; Verstreken et al., 2003; Dickman et al., 2005). However, disruption of synaptojanin, AP-180/lap, or endophilin function also resulted in reduced uptake of FM1-43, indicating a general defect in endocytosis that we did not observe in *gyr* larvae (Verstreken et al., 2002; Verstreken et al., 2003; Dickman et al., 2005). Under basal conditions, *endophilin* and *lap* mutants have an increase in mEJP amplitude, while *synaptojanin* mutants display an increase in quantal amplitude following a tetanus (Zhang et al., 1998a; Verstreken et al., 2002; Dickman et al., 2005). Although we did not examine mEJP amplitude after intense stimulation, *gyr* larvae did not have a significant increase in quantal size under basal conditions. It should be noted that, while *gyr* larvae have a subset of boutons with dramatically enhanced synaptic vesicle diameter, many boutons are comparable to controls in terms of synaptic vesicle size and density, which may explain why we did not observe a measurable increase in mEJP amplitude, especially if the more abnormal *gyr* boutons have a lower level of synaptic activity.

Furthermore, the synaptic vesicles near active zones in *gyr* larvae were normal in size, while the other endocytic mutants often had large synaptic vesicles in close proximity to release sites (Zhang et al., 1998a; Verstreken et al., 2002; Dickman et al., 2005).

Overall, the endocytic phenotypes observed in *synaptojanin*, *endophilin*, and *lap* mutants are much greater in severity than those in *gyr* animals. While *gyr* larvae also have an increase in synaptic vesicle size, they do not have defects in FM1-43 uptake, a dramatic decrease in synaptic vesicle density, or alterations in quantal size. Therefore, it is difficult to determine where synaptogyrin might act in the clathrin-mediated endocytic pathway based on phenotypes alone. Synaptogyrin likely does not play an essential role in clathrin coat formation, as the inactivation of clathrin via FlAsH-FALI results in the complete loss of vesicles (Heerssen et al., 2008). Similarly, a severe loss-of-function mutation in the *α-adaptin* gene, a component of the AP-2 complex, results in embryonic lethality and the absence of vesicles and collared pits at synapses (Gonzalez-Gaitan and Jackle, 1997). Synaptogyrin is also not necessary for synaptic vesicle fission, since the inactivation of dynamin using the *shibire* temperature-sensitive mutation also completely blocks vesicle formation and induces the formation of membrane invaginations and collared pits (Koenig and Ikeda, 1983, 1989). Instead, synaptogyrin likely plays a modulatory or regulatory role in synaptic vesicle formation. Alternatively, it may only promote synaptic vesicle endocytosis under certain conditions or function in a subset of endocytic pathways. Interestingly, a recent study investigating a *C. elegans synaptogyrin* mutant indentified synthetic phenotypes with *synaptojanin* and *endophilin* mutants (Abraham et al., 2011). The observation that the loss of synaptogyrin enhances the phenotypes of known regulators of clathrin-mediated endocytosis promotes the

previously discussed hypothesis that synaptogyrin may participate in an alternative clathrin-independent recycling pathway. However, synaptogyrin may instead promote efficient clathrin-mediated endocytosis through a mechanism that parallels the function of synaptojanin and/or endophilin. We would like to repeat these genetic interaction experiments in *Drosophila* by combining the *gyr* mutation with mutations in genes such as *endophilin*, *lap*, and *synaptojanin* to gain further insight into how synaptogyrin function impacts synaptic vesicle endocytosis.

We also would like to determine the extent to which *Drosophila* synaptogyrin promotes synaptic vesicle endocytosis from the plasma membrane as opposed to endosomes or cisternae. To accomplish this, we could inhibit bulk endocytosis in our *gyr* mutant using a temperature-sensitive allele of *rolling blackout* (rbo^{ts}), a *Drosophila* EFR3 integral membrane lipase (Huang et al., 2006; Vijayakrishnan and Broadie, 2006; Vijayakrishnan et al., 2009). At the restrictive temperature, rbo^{ts} mutants fail to form endocytic cisternae in response to incubation with high-potassium saline but are still capable of generating synaptic vesicles, presumably by endocytosis from the plasma membrane (Vijayakrishnan et al., 2009). If *Drosophila* synaptogyrin solely participates in synaptic vesicle budding from cisternae, the *gyr* mutation should not enhance the rbo^{ts} phenotype. However, if *gyr* also mediates synaptic vesicle endocytosis from the plasma membrane, a *gyr*/rbo^{ts} double mutant would be expected to have endocytic deficits beyond those seen in the rbo^{ts} mutant alone. Further analysis of the genetic interactions of *gyr* in combination with additional mutations known to affect endocytosis and exocytosis may provide added insight into the role of synaptogyrin in the synaptic vesicle cycle.

Conclusions

While we have provided additional evidence implicating synaptogyrin in the regulation of synaptic vesicle exo-endocytosis, many questions still remain. Although we identified alterations in synaptic vesicle recycling dynamics and synaptic plasticity in *gyr* larvae, we still do not know exactly how synaptogyrin impacts synaptic vesicle fusion and biogenesis. Further studies examining the protein-binding and genetic interactions of synaptogyrin, along with comprehensive structure-function analysis may help illuminate the mechanisms behind synaptogyrin function at the synapse. Finally, we would like to determine how (or if) the loss of synaptogyrin impacts *Drosophila* on a behavioral level and whether the observed changes in synaptic function lead to deficits in learning and memory.

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