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Synaptophysin Is Required for Synaptobrevin Retrieval during Synaptic Vesicle Endocytosis

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The integral synaptic vesicle (SV) protein synaptophysin forms ~10% of total SV protein content, but has no known function in SV physiology. Synaptobrevin (sybII) is another abundant integral SV protein with an essential role in SV exocytosis. Synaptophysin and sybII form a complex in nerve terminals, suggesting this interaction may have a key role in presynaptic function. To determine how synaptophysin controls sybII traffic in nerve terminals, we used a combination of optical imaging techniques in cultures derived from synaptophysin knock-out mice. We show that synaptophysin is specifically required for the retrieval of the pH-sensitive fluorescent reporter sybII-pHluorin from the plasma membrane during endocytosis. The retrieval of other SV protein cargo reporters still occurred; however, their recapture proceeded with slower kinetics. This slowing of SV retrieval kinetics in the absence of synaptophysin did not impact on global SV turnover. These results identify a specific and selective requirement for synaptophysin in the retrieval of sybII during SV endocytosis and suggest that their interaction may act as an adjustable regulator of SV retrieval efficiency.

Introduction

The localized retrieval and recycling of synaptic vesicles (SVs) after exocytosis is critical for the maintenance of neurotransmission. A key event in this process is the efficient clustering and retrieval of SV proteins from the plasma membrane during endocytosis, which ensures that SVs have the correct molecular composition to participate in the next cycle of neurotransmitter release. The sorting of SV proteins is performed by clathrin adaptor proteins, which recognize specific endocytic cargo motifs (Kelly and Owen, 2011). Not all SV proteins possess such motifs, however, suggesting that other molecules may participate in their retrieval during SV endocytosis.

Synaptobrevin II (sybII) is an integral SV protein that possesses a cytosolic N-terminal tail with an α-helical SNARE (soluble NSF attachment protein receptor) motif (Sutton et al., 1998). This motif allows sybII to interact with the plasma membrane SNARE proteins syntaxin and SNAP-25 to drive membrane fusion, resulting in neurotransmitter release (Sudhof, 2004). The cytosolic sybII tail contains noncanonical cargo recognition motifs (Kelly and Owen, 2011), suggesting it may be potentially recognized by classical adaptor proteins or alternately by a distinct adaptor protein. SybII has an established interaction with the integral SV protein synaptophysin (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995; Hübner et al., 2002). Synaptophysin is an abundant SV protein forming ~10% of total SV protein content (Takamori et al., 2006); however, studies using synaptophysin knock-out (KO) mice have shown no apparent SV recycling phenotype (Eshkind and Leube, 1995; McMahon et al., 1996). Synaptophysin is proposed to be a chaperone for sybII, controlling either its targeting to SVs (Pennuto et al., 2003; Bonanomi et al., 2007) or its entry into the SNARE complex (Calakos and Scheller, 1994; Edelmann et al., 1995; Becher et al., 1999). It has also been implicated in SV endocytosis, with either dominant-negative approaches (Daly et al., 2000) or gene ablation studies (Spiwoks-Becker et al., 2001) highlighting a potential regulatory role in central nerve terminals.

Since synaptophysin is implicated in both SV endocytosis and sybII targeting to SVs, we hypothesized that synaptophysin could be a potential sybII adaptor protein. To test this hypothesis, we monitored the trafficking and retrieval of the fluorescent reporter superecliptic synaptopHluorin (sybII-pHfluorin) in cortical cultures derived from synaptophysin knock-out mice (Eshkind and Leube, 1995). We found that synaptophysin is specifically required for the retrieval of sybII-pHfluorin, while its absence slowed the retrieval of other SV protein cargo. Thus synaptophysin is specifically required for sybII retrieval during SV endocytosis.

Materials and Methods

Materials. SybII-pHfluorin, vGLUT1-pHfluorin, and synaptotagmin-pHfluorin constructs were provided by Prof. G. Miesenböck (Oxford University, Oxford, UK), Prof. R. Edwards (University of California, San Francisco, CA) and Prof. V. Haucke (Free University of Berlin, Berlin, Germany) respectively. Rabbit anti-sybII antibody was from Abcam. Synaptophysin-mCerulean was generated by replacing EGFP from synaptophysin-EGFP (gift from Jane Sullivan, University of Washington, Seattle, WA) with mCerulean (gift from David Piston, Vanderbilt University, Nashville, TN) using the enzymes AgeI and BsrGI. Neurobasal media, B-27 supplement, penicillin/streptomycin, Minimal Essential
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Medium (MEM), Lipofectamine 2000, AlexaFluor 568 antibody, and FM2-10 were from Invitrogen. All other reagents were from Sigma-Aldrich.

Cortical neuronal cultures. Synaptophysin knock-out mice were maintained as heterozygous breeding pairs, and genotyped as described previously (Schmitt et al., 2009). Dissociated primary cortical neuronal cultures were prepared from E17.5 KO and wild-type embryos of either sex by trituration of isolated cortices to obtain a single-cell suspension, which was plated at a density of 15–17 × 10^3 cells/coverslip on poly-lysine- and laminin-coated 25 mm coverslips. Cultures were maintained in Neurobasal media supplemented with B-27, 0.5 mM l-glutamine, and 1% w/v penicillin/streptomycin. After 72 h, cultures were further supplemented with 1 μM cytosine β-arabinofuranoside to inhibit glial proliferation. Cells were transfected after 7 d in culture with Lipofectamine 2000 according to the manufacturer’s instructions, with the following alterations: cells were preincubated in 2 ml of MEM at 5% CO2 for 30 min at 37°C, and then incubated for 2 h with 2 μl of Lipofectamine and 1 μg of DNA construct/well. Cells were subsequently washed with MEM before replacement of conditioned Neurobasal media. Cells were imaged after 14–21 d in culture.

Fluorescent imaging protocols for pHluorin reporters. Cortical cultures were mounted in a Warner imaging chamber with embedded parallel platinum wires (RC-21BFRS) and placed on the stage of a Zeiss Axio Observer D1 epifluorescence microscope. Neurons transfected with synaptophysin-mCerulean were visualized with a 4× objective objective at 430 nm excitation, whereas neurons transfected with pHluorin reporters were visualized at 500 nm (all >525 nm emission). In all experiments, cultures were stimulated with a train of 200 action potentials delivered at 10 Hz (100 mA, 1 ms pulse width). Cultures were subjected to continuous perfusion with imaging buffer (in mM: 136 NaCl, 2.5 KCl, 2 CaCl2, 1.3 MgCl2, 10 glucose, 10 HEPES, pH 7.4). Fluorescent images were captured at 4 s intervals using a Hamamatsu Orca-ER digital camera and processed offline using ImageJ 1.43 software (NIH). Regions of interest of identical size were placed over nerve terminals that displayed an increase in stimulation and the total fluorescence intensity was monitored over time. All statistical analyses were performed using Microsoft Excel and GraphPad Prism software. The pHfluorin fluorescence change was calculated as ΔF/F0, in all cases.

Estimation of surface sybII-pHluorin was performed by perfusing acidic imaging buffer (substituting 20 mM MES for HEPES, pH 5.5) over cultures for 30 s (to quench surface sybII-pHluorin) followed by a 1 min perfusion with standard imaging buffer, pH 7.4. Cultures were then subjected to alkaline imaging buffer (50 mM NH4Cl substituted for 50 mM NaCl) for 30 s to reveal total sybII-pHluorin. The surface fraction of sybII-pHluorin as a percentage of total was estimated using the following equation: ([neutral fluorescence – acidic fluorescence]/[alkali fluorescence – acidic fluorescence]) × 100.

Fluorescent imaging protocols for FM2-10 experiments. FM2-10 uptake was evoked with a train of 900 action potentials (10 Hz). FM2-10 (100 μM) was present either during and after stimulation (3 min) or only after stimulation was terminated in knock-out neurons, in contrast to wild-type or knock-out cultures transfected with exogenous synaptophysin-mCerulean (rescued, rescued, rescued). A parallel decrease in the CV of endogenous sybII also occurred in knockout neurons (K–O/C: CV 47.6 ± 3.4; Rescue 74.8 ± 3.1, both n = 4; p = 0.001 Student’s t test) showing that the absence of synaptophysin affects both exogenous and endogenous sybII (Fig. 1D).

To confirm the mislocalization of sybII-pHluorin to the plasma membrane in knock-out neurons, we determined its surface expression by sequentially applying acidic (to reveal surface expression) and alkaline (to reveal total expression) buffers to wild-type and knock-out cultures (Fig. 1E,F) (Sankaranarayanan and Ryan, 2001). These experiments confirmed that almost 50% of sybII-pHluorin was present on the cell surface in knock-out cultures, in contrast to wild-type [sybII-pHluorin surface expression (% of total): wild-type 20.8 ± 2.7%, n = 3; knock-out 43.7 ± 4.0, n = 8; p = 0.009 Student’s t test].

Increased plasma membrane sybII-pHluorin could result from its defective targeting to SVs, increased delivery to the plasma membrane, or inefficient retrieval from the plasma membrane. To delineate between these possibilities, we stimulated either wild-type or knock-out cultures with a train of 200 action potentials (10 Hz) to trigger SV recycling. A robust stimulation-dependent increase in sybII-pHluorin fluorescence occurred in synaptophysin knock-out cultures, indicating that the reporter is efficiently delivered to the cell surface by SV exocytosis (Fig. 2A). However sybII-pHluorin fluorescence remained elevated once stimulation was terminated in knock-out neurons, in contrast to wild-type neurons, which decayed back to baseline with first-order kinetics (Fig. 2A). The rate of sybII-pHluorin fluorescence decay after stimulation contains contributions from both SV exocytosis and acidification (Sankaranarayanan and Ryan, 2000),
either surface fraction (neutral pH 100% in alkaline buffer (green bars) and to 0% in acidic buffer (yellow bars). Arrows indicate WT (blue circles, tentative traces displaying the proportion of sybII-pHluorin expressed on the cell surface in either wild-type (WT, blue circles) and synaptophysin KO (red circles) cultures were transfected with either vGLUT-pHluorin (A) or syt-pHluorin (B). Cultures were stimulated with a train of 200 action potentials (10 Hz, indicated by bar). Averaged traces for either WT and KO (A) or KO and Rescue (B) are displayed as ±SEM, n = 10 for WT, n = 8 for KO, n = 9 for rescue, **p < 0.001 two-way ANOVA for KO against both WT and rescue.

Figure 2. Synaptophysin is required for sybII-pHluorin retrieval. SybII-pHluorin transfected wild-type (WT, blue circles), synaptophysin KO (red circles), or KO neurons expressing synaptophysin-mCerulean (Rescue, purple circles) were stimulated with a train of 200 action potentials (10 Hz, indicated by bar). Averaged traces for either WT and KO (A) or KO and Rescue (B) cultures. Scale bar represents 15 μm. E, F, Representative traces displaying the proportion of sybII-pHluorin expressed on the cell surface in either WT (blue circles, E) or KO (red circles, F) neurons. SybII-pHluorin fluorescence is normalized to 100% in alkaline buffer (green bars) and to 0% in acidic buffer (yellow bars). Arrows indicate either surface fraction (neutral pH — acidic pH, SF) or total sybII-pHluorin (alkaline pH — acidic pH, Tot).

Since SV acidification is unaltered in synaptophysin knock-out cultures (Kwon and Chapman, 2011), this indicates that the sustained elevation in sybII-pHluorin fluorescence after stimulation is due to its defective retrieval from the plasma membrane.

To confirm that the defect in sybII-pHluorin retrieval was due to the absence of synaptophysin, we expressed exogenous synaptophysin-mCerulean in knock-out cultures. This resulted in a complete rescue of sybII-pHluorin retrieval kinetics (Fig. 2B). Thus synaptophysin is required for the retrieval of sybII-pHluorin from the nerve terminal plasma membrane.

Deletion of synaptophysin slows retrieval of other SV protein cargo, but global SV turnover is unaltered

We next determined whether synaptophysin was required for the retrieval of other SV protein cargo, or whether the requirement is specific to sybII. To achieve this, we examined the retrieval of two independent pHluorin-tagged reporters of SV protein cargo, the vesicular glutamate transporter (vGLUT-pHluorin) and synaptotagmin (syt-pHluorin) (Diril et al., 2006; Voglmaier et al., 2006), in wild-type and knock-out neurons. When expressed in wild-type neurons, both reporters were retrieved with first-order kinetics after a train of 200 action potentials (Fig. 3 A, B). vGLUT-pHluorin and syt-pHluorin were also retrieved in synaptophysin knock-out cultures, albeit with slower kinetics when compared against wild-type (Fig. 3 A, B). Thus the general retrieval of SV protein cargo is slowed in the absence of synaptophysin.

We next determined whether the slowing of SV protein cargo retrieval in synaptophysin knock-out cultures translated into a global defect in SV turnover. To achieve this, we examined the loading and unloading of FM2-10, a fluorescent dye that labels SVs during endocytosis (Clayton et al., 2009). A second stimulus of FM2-10-loaded neurons reports both the extent of SV turnover (total unload) and rate of SV exocytosis (unloading kinetics).

There was no significant difference in the extent of SV turnover (endocytosis followed by exocytosis) between knock-out and rescued neurons for either the total SV recycling pool (% of knock-out: knock-out 100 ± 7.2, rescue 103.7 ± 9.6, n = 3, p = 0.64 Student’s t test; Fig. 4C) or the pool of SVs that were retrieved after stimulation (% of knock-out: knock-out 100 ± 4.9, rescue 102.5 ± 3.5, n = 4, p = 0.69 Student’s t test; Fig. 4D). Knock-out and rescued neurons also had identical FM2-10 unloading kinetics, indicating no role for synaptophysin in SV exo-
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Global SV turnover is normal in synaptophysin knock-out neurons. A. Synaptophysin KO cultures were transfected with synaptophysin-mCerulean (Rescue) and loaded with FM2-10 (100 μM) using a train of 900 action potentials (10 Hz). FM2-10 was applied either during and after stimulation to load the total SV recycling pool or only after stimulation (to load the poststimulus recycling SV pool). B. Representative images display synaptophysin-mCerulean transfected neuron (left panel), FM2-10 loading (middle panel) and merged image (right panel). Loading in KO neurons indicated by white arrowheads and in rescued neurons by green arrowheads. Scale bar represents 20 μm. C, D. Representative traces display dye unloading in KO (red circles) and rescued (purple circles) neurons evoked by 900 action potentials (10 Hz, indicated by bar) in the same field of view for either the total recycling SV pool (C) or the poststimulus SV recycling pool (D).

Figure 4.

cytosis (KO—knock-out 28.8 ± 3.1 s, rescue 31.1 ± 3.4 s, n = 3 independent experiments, p = 0.77 Student’s t test). Thus while SV protein cargo retrieval is slowed in synaptophysin knock-out neurons, the number of SVs that are turned over is unchanged.

Discussion

We have shown that synaptophysin is specifically required for the retrieval of synbII, but not other SV protein cargo from the nerve terminal plasma membrane during endocytosis. This is the first direct functional evidence of a molecular role for synaptophysin in SV physiology.

We observed a mislocalization of both synbII-pHluorin and endogenous synbII to the plasma membrane in synaptophysin knock-out neurons. In agreement, overexpression of synbII in either neurons or a heterologous expression system resulted in a similar phenotype, a defect that was corrected by transfection of endogenous synbII to the plasma membrane in synaptophysin knock-out neurons (Deák et al., 2004), strongly suggesting that the interaction between synbII and synaptophysin occurs via their transmembrane domains, with formation of the complex determined by both the local lipid microenvironment and neuronal activity. Future experiments will focus on these parameters to determine how synbII retrieval can be manipulated during SV endocytosis.

We found that the absence of synaptophysin had no global effect on SV turnover, in agreement with previous studies (Eshkind and Leube, 1995; McMahon et al., 1996; Janz et al., 1999). Recent work has shown that SV endocytosis is slowed in synaptophysin knock-out neurons (Kwon and Chapman, 2011), which is consistent with our observation of a decrease in retrieval kinetics of both vGLUT-pHluorin and synt-pHluorin. The impact of this slowing in terms of SV endocytosis is small, however, with a reduction in endocytic capacity of only ~10% for a defined time period (Kwon and Chapman, 2011). This explains why we did not observe a significant difference in the extent of global SV turnover between synaptophysin knock-out and rescued neurons. Thus, while the absence of synaptophysin has minor effects on SV endocytosis kinetics, the major consequence of its deletion is the arrest of synbII retrieval from the plasma membrane. Interestingly, a similar slowing of SV endocytosis is reported in synbII knock-out neurons (Deák et al., 2004), strongly suggesting that
evidenced by the lack of retrieval after its delivery to the plasma membrane by exocytosis.

The synaptophysin–synbII interaction occurs in resting nerve terminals and is decreased during neuronal activity (Prekeris and Terrian, 1997; Pennuto et al., 2002; Reisinger et al., 2004; but see Khvotchev and Südhof, 2004), suggesting that manipulation of intracellular free calcium levels may also influence synbII retrieval. SynbII interacts with synaptophysin via its transmembrane region (Yelamanchili et al., 2005), indicating the local lipid microenvironment may also be critical in determining the strength of their interaction. In agreement, depletion or enhancement of the cholesterol membrane content resulted in decreased or increased complex formation respectively (Mitter et al., 2003). Some factors do not influence the stability of the synbII–synaptophysin complex, however. For example, protein kinase or phosphatase antagonists do not alter complex levels in vivo, suggesting that the phosphorylation of the cytoplasmic tails of either protein does not influence their interaction (Khvotchev and Südhof, 2004). Indeed the presence of the cytosolic synaptophysin C terminus is not required for its binding to synb II (Bonanomi et al., 2007; Felkl and Leube, 2008). Therefore it seems likely that the interaction between synbII and synaptophysin occurs via their transmembrane domains, with formation of the complex determined by both the local lipid microenvironment and neuronal activity. Future experiments will focus on these parameters to determine how synbII retrieval can be manipulated during SV endocytosis.

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the endocytic phenotype observed in synaptophysin knock-out neurons is due to defective sybII retrieval.

Other isoforms of syb such as sybIII and sybIV possess acidic dileucine cargo recognition motifs that allow their recognition by the endosomal adaptor proteins AP-3 and AP-1, respectively (Darsow et al., 1998; Peden et al., 2001). This motif is not conserved in sybII, suggesting that it may not be recognized by the plasma membrane adaptor protein AP-2. In contrast, the synaptophysin C terminus contains a large number of potential tyrosine-based cargo motifs, which are classic AP-2 interaction sites. Therefore synaptophysin may act as a molecular bridge between classical adaptor proteins and sybII to allow efficient retrieval of the latter (Felkl and Leube, 2008). Experiments are currently in progress to test this hypothesis and to determine how both the structure and molecular properties of synaptophysin control sybII retrieval.

Note added in proof. Genetic deletion of synaptogyrin in C. elegans also results in mislocalization of sybII from nerve terminals (Abraham et al., 2011).

References