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Stoned B MEDIATES SORTING OF INTEGRAL SYNAPTIC VESICLE PROTEINS

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Abstract—A continuous supply of fusion-competent synaptic vesicles is essential for sustainable neurotransmission. *Drosophila* mutations of the dicistronic *stoned* locus disrupt normal vesicle cycling and cause functional deficits in synaptic transmission. Although both Stoned A and B proteins putatively participate in reconstituting synaptic vesicles, their precise function is still unclear. Here we investigate the effects of progressive depletion of Stoned B protein (STNB) on the release properties of neuromuscular synapses using a novel set of synthetic *stnB* hypomorphic alleles. Decreasing neuronal STNB expression to ≤35% of wild-type level causes a strong reduction in excitatory junctional current amplitude at low stimulation frequencies and a marked slowing in synaptic depression during high-frequency stimulation, suggesting vesicle depletion is attenuated by decreased release probability. Recovery from synaptic depression after prolonged stimulation is also decelerated in mutants, indicating a delayed recovery of fusion-ready vesicles. These phenotypes appear not to be due to a diminished vesicle population, since the docked vesicle pool is ultrastructurally unaffected, and the total number of vesicles is only slightly reduced in these hypomorphs, unlike lethal *stoned* mutants. Therefore, we conclude that STNB not only functions as an essential component of the endocytic complex for vesicle reconstitution, as previously proposed, but also regulates the competence of recycled vesicles to undergo fusion. In support of such role of STNB, synaptic levels of the vesicular glutamate transporter (vGLUT) and synaptotagmin-1 are strongly reduced with diminishing STNB function, while other synaptic proteins are largely unaffected. We conclude that STNB organizes the endocytic sorting of a subset of integral synaptic vesicle proteins thereby regulating the fusion-competence of the recycled vesicle.

Key words: synaptic vesicle cycle, endocytosis, stonin2, cargo sorting, synaptotagmin, vesicular glutamate transporter.

The maintenance of fast synaptic transmission requires the reconstitution of fusion-competent vesicles after phases of activity. Full vesicular fusion events inevitably deliver vesicular proteins to the plasma membrane, a process that occurs even at synapses that otherwise may possess kiss-and-run style release. These "misplaced" vesicle proteins must be retrieved by consecutive sorting and uptake mechanisms. According to the classical model (Heuser and Reese, 1973), clathrin-mediated endocytosis (CME) represents the major mechanism for recovery of vesicle components after full fusion. Studies on receptor internalization during cellular housekeeping processes have identified a large set of proteins generally involved in CME (Schmid, 1997). However, relatively little is known about CME in the context of synaptic vesicle recycling.

Forward genetic screens in *Drosophila* have identified many proteins involved in synaptic vesicle cycling. The *stoned* gene locus was discovered in a screen for temperature-sensitive paralytic mutations 35 years ago (Grigliatti et al., 1973), and its dicistronic gene products were later named Stoned A protein (STNA) and Stoned B protein (STNB) (Andrews et al., 1996). Both Stoned proteins contain motifs common to CME accessory proteins, suggesting an involvement in endocytosis (Stimson et al., 1998). STNB shares partial homology with the μ2-subunit of adaptor protein complex 2. Viable and embryonic lethal *stoned* mutants suffer from compromised synaptic transmission at the neuromuscular junction (NMJ) synapse, and exhibit a delayed vesicular uptake of the styryl dye *N*-(3-triethylammoniumpropyl)-4-(4-dibutylamino) styryl) pyridium bromide (FM1-43), indicating a substantial slowing of vesicle recycling (Stimson et al., 1998; Fergestad et al., 1999; Stimson et al., 2001; Fergestad and Broadie, 2001). Rescue experiments (Estes et al., 2003) attribute all major physiological defects to a specific loss of STNB in *stoned* mutants, with no known function attributed to STNA. However, despite this intriguing evidence that STNB function is essential for synaptic vesicle cycling, its specific role in endocytosis has not been sufficiently defined.

STNB clearly appears to regulate the trafficking of synaptotagmin (SYT) -1, as the synaptic localization of the protein is disrupted in *stoned* mutants (Fergestad et al.,

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Abbreviations: AP50, μ2 subunit of adaptor protein complex 2; AZ, active zone; CME, clathrin-mediated endocytosis; ECP, exo/endo cycling vesicle pool; EJC, excitatory junctional current; FM1-43, *N*-(3-triethylammoniumpropyl)-4-(4-dibutylamino) styryl) pyridium bromide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HFS, high-frequency stimulation; HRP, horseradish peroxidase; ISI, interstimulus interval; MHD, μ2 homology domain; NMJ, neuromuscular junction; OE, over-expressing; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PPF/D, paired-pulse facilitation/depression; RP, reserve pool; RRP, readily releasable pool; STNA, Stoned A protein; STNB, Stoned B protein; *stn-l*, Stoned B hypomorphic mutants with low expression; *stn-m*, Stoned B hypomorphic mutants with moderate expression; *stn-vl*, Stoned B hypomorphic mutants with very low expression; SYB, synaptobrevin; SYN, synapsin; SYT, synaptotagmin; TEVC, two-electrode voltage-clamp; UAS, upstream activating sequences; vGLUT, vesicular glutamate transporter; VNC, ventral nerve cord; *wt*, wild type.

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1999). Consistently, SYT-1 interacts directly with STNB *in vitro* (Phillips et al., 2000). Interestingly, Stonin2, the closest STNB vertebrate ortholog, is also proposed to act as a sorting factor for SYT-1, since Stonin2 overexpression stimulates the uptake of a SYT-GFP protein from the plasma membrane (Diril et al., 2006). Since SYT-1 is an essential modulator of Ca^{2+}-dependent neurosecretion (Tucker and Chapman, 2002), its mislocalization presumably contributes to the physiological impairments in *stoned* mutants. SYT-1 is also suggested to be directly involved in the endocytic pathway of the synaptic vesicle cycle (Poskanzer et al., 2003; Nicholson-Tomishima and Ryan, 2004). Therefore, the mislocalization of SYT-1 in *stoned* mutants might directly participate in perturbing synaptic vesicle recycling.

In this study, we have engineered a set of novel *stnB* hypomorphic mutants to further investigate the function of STNB. We demonstrate that these *stnB* alleles have compromised basal synaptic transmission and altered synaptic depression during high frequency stimulation. However, these defects are not due to a diminished vesicle pool, but rather appear to arise from the compromised functional competence of synaptic vesicles in the mutants. In support of this idea, we demonstrate a selective depletion of the integral synaptic vesicle proteins SYT-1 and the vesicular glutamate transporter (vGLUT) in mutant presynaptic boutons, while other synaptic proteins are properly maintained. These results suggest that STNB is part of an endocytic sorting complex specific for a particular subset of integral membrane proteins during the reconstitution of synaptic vesicles.

EXPERIMENTAL PROCEDURES

Transgenic construct generation

A polymerase chain reaction (PCR) fragment containing the STNB sequence was produced using the full-length cDNA clone RH38069 (BDGP collection; GenBank BT011172) as template. The PCR fragment was first inserted into the cloning vector pGEM-t (Promega, Madison, WI, USA) and subsequently cloned into the pUASt vector (Brand and Perrimon, 1993) to yield the final construct (restriction sites: *Eco*RI and *Bgl*II). A truncated STNBΔMHD variant (amino acids 1–903) was generated using the alternative reverse primer 5′-GGA TCC TTA TGT CAA CGC TCG CTC TCG GAG AGC-3′. In addition, targeted mutations were introduced using Stratagene's QuickChange Kit employing the following primers: 5′-CC TCC GGA CAG GCC AAA GGC GAG CAT CAT CAC CG-3′ (Y1125G) and 5′ GCC ATT GTG TGG GCT TGT CCC CGT TTG CCC AAA G-3′ (R1135A). The double mutation Y1125G, R1135A was obtained by two successive rounds of mutagenesis. The chimeric STNB–μ2 subunit of adaptor protein complex 2 (AP50) construct was generated by fusion of three PCR fragments representing the N-terminal portion of STNB (amino acids 1–903), the homologous part of AP50 (amino acids 166–432; derived from cDNA clone SD05403), and the very C-terminal tail of STNB (amino acids 1219–1263). The strategy followed the widely used "splicing by overlap extension" method (Horton, 1995), and employed the overlapping primers: 5′-GAG CGA GCG TTG ACA TAC CGG CGC AAC GAG CTT TTC C-3′ and 5′-C ATC GGA CGC AGT GGC TTG GAG ACC ACG CAC GGC GAG-3′. 6xHis,myc-tagged STNB variants were produced by N-terminally attaching the sequence HHHHHHEQKLISEEDLNGGPR (c-myc epitope underlined).

Transgenic stock generation

Chimeric animals carrying insertions of upstream activating sequences (UAS) constructs were generated by embryo injection using standard methods (Spradling and Rubin, 1982). Injected animals were mated to the w^{1118} line, and progeny expressing the red eye-color marker w^{+} due to successful p-element insertions were collected. All stocks were repeatedly back-crossed to w^{1118}, while selecting for red-eyed progeny to retain the insertion. After at least three rounds of crossing, test-crosses were performed to map the chromosomal location of each p-element insertion. Homozygous and balanced stocks were generated using standard genetic techniques. The w^{1118} stock was used as the genetic background control in all experiments. All experiments conformed to international and local guidelines on the ethical use of animals, and thus use of animals and their suffering was minimized as much as possible.

Western blot analysis

Heads of CO_2-anasthesized adults were manually removed with a razor blade and immediately frozen in liquid nitrogen. The tissue was then homogenized in sample buffer containing a protease inhibitor cocktail "2x complete" (Roche) as well as leupeptin (10 μmol/ml) and pepstatin A (10 μmol/ml). Protein extracts were subsequently electrophoresed using 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gradient gels (Ready Gel, Bio-Rad) and transferred to a polyvinylidene difluoride membrane (Millipore). Stainings with antibodies directed against STNA/B (1:2000; Andrews et al., 1996; kind gift of Dr. L. Kelly), SYT (1:2000; kind gift by Dr. H. Bellen) and DAP160 (1:5000; Roos and Kelly, 1998) were performed in phosphate-buffered saline (PBS) (0.02 M phosphate buffer and 0.1 M NaCl, pH 7) containing Tween (Sigma) and 4% powdered milk. Immunopositive protein bands were visualized by using alkaline phosphatase-conjugated secondary antibodies with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates (Bio-Rad). For analysis, blots were scanned, and band intensities were quantified using ImageJ software (NIH). Intensities for the different protein bands were normalized to the value of w^{1118} controls on the same blot.

Electrophysiology

Two-electrode voltage-clamp (TEVC) recordings of the third instar larval NMJ were performed as previously described (Rohrbough et al., 1999). In brief, wandering third instar larvae were dissected in calcium-free standard saline (in mM: 128 NaCl, 2 KCl, 4 $MgCl_2$, 70 sucrose, 5 Hepes, pH 7.1). The dissected animal was transferred into fresh saline containing the appropriate extracellular calcium concentration and washed several times. Extracellular calcium concentration in recording bath saline was 1.8 mM, unless otherwise noted. Recordings were done at 18 °C in a temperature-controlled room. All recordings were performed in muscle 6 in anterior abdominal segment 3, using TEVC techniques (Axoclamp 2B amplifier; Axon Instruments). The holding potential was set to −60 mV in all recordings. Intracellular electrodes were filled with 3 M KCl and had an average resistance of 10 MΩ. Excitatory junctional currents (EJCs) were evoked by application of brief stimuli (0.4–1 ms; Grass Instruments S88 stimulator) to the cut motor nerve, using a glass suction electrode. Data acquisition and analysis was performed using pClamp software (version 8.0; Axon Instruments).

Electron microscopy

Mutant and wild-type (*wt*) control wandering third instar larvae were dissected, fixed, sectioned and visualized in parallel using standard transmission electron microscopy techniques, as re-