Ceramidase Regulates Synaptic Vesicle Exocytosis and Trafficking

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A screen for *Drosophila* synaptic dysfunction mutants identified *slug-a-bed* (*slab*). The *slab* gene encodes ceramidase, a central enzyme in sphingolipid metabolism and regulation. Sphingolipids are major constituents of lipid rafts, membrane domains with roles in vesicle trafficking, and signaling pathways. Null *slab* mutants arrest as fully developed embryos with severely reduced movement. The SLAB protein is widely expressed in different tissues but enriched in neurons at all stages of development. Targeted neuronal expression of *slab* rescues mutant lethality, demonstrating the essential neuronal function of the protein. C₅-ceramide applied to living preparations is rapidly accumulated at neuromuscular junction (NMJ) synapses dependent on the SLAB expression level, indicating that synaptic sphingolipid trafficking and distribution is regulated by SLAB function. Evoked synaptic currents at *slab* mutant NMJs are reduced by 50–70%, whereas postsynaptic glutamate-gated currents are normal, demonstrating a specific presynaptic impairment. Hypertonic saline-evoked synaptic vesicle fusion is similarly impaired by 50–70%, demonstrating a loss of readily releasable vesicles. In addition, FM1-43 dye uptake is reduced in *slab* mutant presynaptic terminals, indicating a smaller cycling vesicle pool. Ultrastructural analyses of mutants reveal a normal vesicle distribution clustered and docked at active zones, but fewer vesicles in reserve regions, and a twofold to threefold increased incidence of vesicles linked together and tethered at the plasma membrane. These results indicate that SLAB ceramidase function controls presynaptic terminal sphingolipid composition to regulate vesicle fusion and trafficking, and thus the strength and reliability of synaptic transmission.

Key words: *Drosophila*; synaptic transmission; synapse; presynaptic; ceramidase; ceramide; sphingolipids; lipid regulation

Introduction

Membrane lipid composition and lipid-based signaling are critical to neuronal function. Synaptic membranes are heterogeneously distributed into specialized domains, ensuring specific lipid and protein interactions required for regulated neurotransmission. Whereas neuronal plasma membrane (PM) and vesicle lipids consist predominantly of phospholipids and cholesterol, sphingolipids, including sphingomyelin, ceramide, and related glycosphingolipids, serve essential structural, modulatory, and signaling roles in surface and internal membranes (Merrill et al., 1997; van Meer and Holthuis, 2000; Hoekstra et al., 2003). Sphingolipids have compact head groups and relatively saturated acyl tails, promoting lipid order and packing, and self-aggregate with cholesterol into lipid raft domains (Brown and London, 2000; Lai, 2003; van Blitterswijk et al., 2003). Lipid rafts are platforms

for protein targeting and sorting, vesicle endocytosis, fusion and trafficking, actin cytoskeleton regulation, and diverse cellular signaling pathways (Brown and London, 2000; Maekawa et al., 2003; Helms and Zurzolo, 2004).

Ceramide is particularly important in membrane domain formation and vesicle trafficking. Ceramide is synthesized in the endoplasmic reticulum (ER) and transported to the PM and generated in the PM by sphingomyelin hydrolysis (van Blitterswijk et al., 2003; Riezman and van Meer, 2004). In the outer PM leaflet, ceramide aggregates into lateral domains in association with sterols and other sphingolipids, aiding raft stabilization (Venkataraman and Futerman, 2000; van Blitterswijk et al., 2003). Ceramidase, a central enzyme in sphingolipid metabolism and signaling, cleaves ceramide to produce the motile second messenger sphingosine. Neutral/alkaline ceramidases (Cdases) share the greatest conservation among disparate organisms. Mammalian neutral Cdases are secreted but localized to the PM by O-glycosylation of a serine–threonine-rich mucin domain absent in invertebrate homologs (Tani et al., 2003). Perturbing Cdase activity disrupts cholesterol and sphingolipid distribution (Pagano et al., 2000a) and raft protein sorting (McMaster, 2001; Watanabe et al., 2002) and modulates endocytic pathways in the *Drosophila* retina (Acharya et al., 2003, 2004).

Lipid rafts have increasingly recognized roles in synaptic do-

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main organization and signaling processes (Martin, 2000; Paratcha and Ibanez, 2002; Tsui-Pierchala et al., 2002). Lipid topology is relevant for synaptic morphological specialization and the extreme membrane structural changes accompanying synaptic vesicle (SV) endocytosis and fusion (van Blitterswijk et al., 2003). Rafts localize and functionally modulate certain neuronal ion channels and neurotransmitter receptors (Bruses et al., 2001; Suzuki et al., 2001; Tsui-Pierchala et al., 2002; Eroglu et al., 2003; Hering et al., 2003; Taverna et al., 2004) and regulate postsynaptic morphology (Hering et al., 2003). In particular, raft lipid and protein interactions potentially regulate neurotransmitter release. Raft and raft-like domains localize essential components of the vesicular exocytic machinery, including syntaxin, SNAP-25 (soluble *N*-ethylmaleimide-sensitive factor attachment protein), and synaptobrevin (Lafont et al., 1999; Chamberlain et al., 2001; Lang et al., 2001; Chamberlain and Gould, 2002). SV recycling may also entail recruitment of endocytic machinery to preassembled domains concentrated in sphingolipids, cholesterol, and SV proteins (Martin, 2000; Mitter et al., 2003). Additionally, multiple proteins regulating SV pool organization and mobilization, including F-actin, synapsin, and synaptophysin, bind or interact directly with SV lipids (Benfenati et al., 1992; Greengard et al., 1993, 1994; Ceccaldi et al., 1995; Thiele et al., 2000; Bloom et al., 2003; Sankaranarayanan et al., 2003).

The *Drosophila* neuromuscular junction (NMJ) is a well studied model for investigating SV trafficking and transmitter release mechanisms (Richmond and Broadie, 2002; Kidokoro, 2003). The homology between *Drosophila* and vertebrate raft composition and function (Rietveld et al., 1999) predicts that genetic and functional analysis in this system will provide insight into the roles of sphingolipids and rafts in synaptic regulation. We identified *slug-a-bed* (*slab*) in a forward screen for novel synaptic dysfunction mutants. The *slab* gene encodes a long-chain Cdase (Yoshimura et al., 2002) essential in the nervous system. Null *slab* mutant embryos characteristically arrest partially hatched from the egg case, thus appearing disinclined to get moving (hence “slug-a-bed”; see *Romeo and Juliet*: scene V). Mutant NMJs have impaired presynaptic transmitter release and a reduced cycling SV pool. Ultrastructurally, *slab* terminals have normally clustered and docked SVs at active zones (AZs), but fewer SVs overall, and increased tethering of vesicles together and to the PM, indicating specific defects in SV fusion and trafficking. These results reveal an essential role for SLAB Cdase in regulating sphingolipid-dependent SV fusion and trafficking processes underlying neurotransmission.

Materials and Methods

Genetics and *Drosophila* stocks. The *slab*¹ mutation was generated in an ethyl methanesulfonate (EMS) screen of an isogenized *rucuca* (*ru*, *h*, *th*, *st*, *cu*, *sr*, *e*, *ca*) third chromosome (Featherstone et al., 2000). For mapping and functional analyses, deficiencies in the 93–100 region were obtained from the Third Chromosome Deficiency kit (Bloomington *Drosophila* Stock Center, Bloomington, IN). Df(3R)20 was a gift from Zhi-Chun Lai (Pennsylvania State University, University Park, PA). Other *slab* alleles were generated by local hop P-element mutagenesis (Grigliatti, 1998), using P{lacW}l(3)j8B9^{j8B9} (Bloomington *Drosophila* Stock Center). The j8B9 flies were crossed to Δ2–3 *CyO/Bc*, and male progeny containing both transposon and transposase were crossed singly to *w*; *Ly/TM6 Tb* virgins (66 crosses). Male progeny lacking the Δ2–3 *CyO* chromosome were mated singly to *w*; *slab*¹/*TM6 Sb*, *Tb* virgins (309 crosses). Seven new independent P-element insertion lines were identified based on failure to complement *slab*¹. An adjacent sequence was cloned by plasmid rescue (O’Kane, 1998) and identified by a BLAST (Basic Local Alignment Search Tool) search of the *Drosophila* genome

database (Adams et al., 2000). The *slab*² mutation contains an 855 bp deletion spanning exons 4 and 5 of *slab* (CG1471). The *slab*³ mutation deletes the *slab* and CG2224 genes, and portions of adjacent genes *aralar* and PH4αEFB (Adams et al., 2000). Other *slab* alleles included larger deletions, in each case with the P element 5′ (downstream) segment retaining its original position in l(3)j8B9 and the 3′ (upstream) segment adjacent to upstream genomic DNA.

To map deletions, homozygous *slab* mutant embryos were selected by the absence of the green fluorescent protein (GFP) balancer *TM3*, P{GAL4-Kr.C}DC2, P{UAS-GFP.S65T}DC10, *Sb*. Single-embryo PCR was performed on four homozygous mutant embryos and two balanced embryos for each allele. The primer pair CCGCAATGAGTGTGATC-TAC and GTTGCATTAAGTGATGACC, which generate an 824 base pair fragment from the coding region of CG1471, was used for screening. These primers produced no band for any single homozygous embryos. Control primers specific for a region of genomic scaffold AE003678 produced a band in each case. To identify the *slab*¹ mutation, homozygous *slab*¹ embryos were selected by the absence of the GFP balancer, and RNA was prepared using TriZol (Invitrogen, San Diego, CA). The cDNA was prepared using the Ominiscript kit (Qiagen, Chatsworth, CA), amplified by PCR using Platinum Pfx (Invitrogen), and the resulting DNA, as well as control cDNA from parental *rucuca* flies, was sequenced. Homozygous and hemizygous *slab*¹, *slab*², and *slab*³ alleles were used for characterization of mutant morphological, functional, and ultrastructural phenotypes and, in all cases, selected at late embryonic stages by the absence of GFP. Controls included wild-type (Oregon R) and *slab*¹ heterozygotes balanced over *TM3 Sb Kr-GFP* (indicated in the text as *slab*¹/*TM3*).

Bioinformatics. Entrez-PubMed searches were performed at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/pubmed>). BLASTP searches were performed using search queries for *Drosophila* CG1471, human acid Cdase (Farber’s disease, AAC50907), and the *C. elegans* conjugal proteins 04586 and Q09551. Ceramidase sequences were analyzed using CLUSTALW at the Center for Molecular and Biomolecular Informatics website.

Generation and transformation of transgenic animals. A construct containing the full-length genomic *slab* (CG1471) sequence was generated by PCR of Oregon R genomic DNA with the Expand Long Template system (Roche, Indianapolis, IN). The construct includes 3553 bases upstream of the translation start and the complete genomic coding region. The fragment was cut with *EagI* and *XhoI* and ligated into pUAST (<http://flybase.bio.indiana.edu>). Constructs of UAS-*slab* and UAS-*slab* fused to the coding sequence of enhanced GFP (eGFP) were generated by PCR of the genomic coding region. For the UAS-*slab*-eGFP construct, the stop codon was omitted from the fragment. These fragments were similarly cut and ligated into pUAST-GFP. All three constructs were transformed into DH10B electrocompetent cells (Invitrogen) and isolated in concentrated and purified form for microinjection with a transposase (Δ2–3). Embryos (*w*¹¹¹⁸) were injected for transformation using standard techniques, and progeny were screened for *w*⁺ expression to identify stable insertions of the transgene.

Either the chromosomes carrying the full-length *slab*-eGFP transgene and either the *slab*¹ or *slab*² mutations were made by recombination. The presence of the *slab* mutation was confirmed by PCR and sequencing. UAS-*slab* and UAS-*slab*-eGFP were driven using P{GAL4-da.G32}UH1 (UH1 gal4) (Wodarz et al., 1995) and elav gal4 (Luo et al., 1994). Genotypes of rescued flies were elav gal4; UAS-*slab*, *slab*¹/*slab*²; and UH1 gal4, *slab*²/UAS-*slab*, *slab*¹.

Apoptosis assays. Wild-type and *slab*¹/*slab*³ mutant embryos were selected at early stage 16 [~13 hr after egg laying (AEL) at 25°C]. Embryos were treated for 5 min at room temperature with 5 μg/ml acridine orange (Molecular Probes, Eugene, OR) in PBS, 1:1 with heptane, mounted in halocarbon oil (Abrams et al., 1993), and oriented on their sides. Z-series confocal images were made with a LSM 510 microscope (Zeiss, Oberkochen, Germany), using a 488 nm argon excitation laser. Each series sectioned approximately halfway through the embryo, using identical slice thickness and number for each embryo. Bright spots indicating apoptotic cells were counted and summed in 17 slices for each embryo.

Immunohistology. Immunocytochemistry of mature control and mutant embryos was performed as described previously (Featherstone et al.,

2002). Central neurons, peripheral nerves, and presynaptic NMJ terminals were visualized with Texas Red- or FITC-conjugated anti-horseradish peroxidase (HRP; 1:200; Molecular Probes). Presynaptic vesicle staining was examined with anti-synaptotagmin I (1:200) and Alexa-conjugated secondary antibody (1:500). Glutamate receptor clusters were visualized with anti-GluRIIA antibody (1:20; Iowa Hybridoma Bank, University of Iowa, Iowa City, IA) and Alexa 488-conjugated secondary antibody (1:500; Molecular Probes). Anti-SLAB staining was done in mature embryos and third instar larvae using rabbit polyclonal antibodies against the N-terminal (Ab11.3) or C-terminal (Ab10.3) half of the protein or mouse monoclonal antibodies, at dilutions of 1:200–1:500, and visualized with the appropriate Alexa-conjugated secondary antibody. Confocal images of the CNS, neuromusculature, and additional tissues were acquired and processed with Adobe Photoshop software. NMJ presynaptic terminal areas were measured at muscles 12 and 13 in anti-HRP-stained wild-type and mutant embryonic preparations. Terminal regions were outlined, and area measurements were made using NIH Image (total pixels); areas for one to two NMJs in five animals were averaged for each genotype.

Western analysis. Sixty embryos or 10 fly heads were collected from the appropriately identified genotypes and homogenized in sample buffer containing 2× complete protease inhibitors. Extract from 60 embryos or two fly heads were electrophoresed using 5–20% SDS-PAGE gradient gels and transferred to polyvinylidene difluoride. Ceramidase antibody staining (1:1000 dilution) was performed in PBS–Tween–4% powdered milk. Blots were developed by using alkaline phosphatase-conjugated secondary antibodies with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates.

Fluorescent ceramide staining and analyses. Staged embryos (22–24 hr AEL) or newly hatched larvae, or mature third instar larvae, were dissected in low $[Ca^{2+}]$ recording saline (see below) and transferred to Schneider's *Drosophila* medium (Invitrogen, Gaithersburg, MD). Preparations were incubated for 30–40 min at 18°C with 10 μ M BODIPY FL C_5 -ceramide (C_5 -Cer) complexed to BSA (Molecular Probes) in Schneider's medium, rinsed several times with fresh medium, and then washed for 1–2 hr, with several medium changes, in the dark at room temperature. Transgenic *Fl-slab* animals were processed in parallel with *slab*³/TM3 heterozygotes and/or wild-type larvae in the same chamber in each experiment. Confocal images of BODIPY C_5 -Cer fluorescence were collected using a 40× or 63× water immersion lens, using identical confocal settings for each set of preparations to allow direct comparison of fluorescence among genotypes. Emitted fluorescence was acquired with a 560 nm long-pass filter. Emission spectra of BODIPY-labeled sphingolipids is shifted from green to red wavelengths (~617 nm emission peak) with increasing membrane concentration, allowing regions of accumulated BODIPY-lipid to be distinguished (Pagano et al., 1991, 2000a,b). To confirm synaptic localization of fluorescent sphingolipid, preparations were fixed (4% paraformaldehyde for 30 min at room temperature) after live imaging, stained with rabbit anti-Discs large (DLG; 1:500) and Alexa 488-conjugated secondary antibodies (Beumer et al., 2002), and mounted between two coverslips in Vectashield (Vector Laboratories, Burlingame, CA). The same NMJs were reimaged (40× oil objective) to compare synaptic C_5 -Cer and DLG localization. The synaptic C_5 -Cer fluorescence level was quantified using NIH Image software. Contiguous regions of stained synaptic terminals (NMJ 4) were outlined, and mean brightness (1–256 grayscale) was measured for the outlined region. Several NMJs were averaged for each preparation, and the average value in each experiment was normalized to that of *Fl-slab*.

Electrophysiology. Physiological recordings were made at the NMJ of staged embryos and newly hatched larvae, as described previously (Broadie and Bate, 1993). Unhatched embryos were dechorionated in bleach and removed manually from the vitelline membrane case. Animals were transferred to recording saline, secured at the head and tail to sylgard-coated coverslips using surgical histoacryl glue, dissected open dorsally, and glued flat. Preparations were exposed to collagenase (1.0 mg/ml, type IV; Sigma, St. Louis, MO) in 0.2 mM Ca^{2+} -containing saline for 1–2 min and washed with fresh recording saline. The bath recording saline composition contained (in mM): 135 NaCl, 5 KCl, 1.8 $CaCl_2$, 4 $MgCl_2$, 5 TES, 36 sucrose, and 2 NaOH, pH 7.2.

Standard whole-cell patch-clamp recordings at a holding potential of –60 mV were made from muscle 6 in anterior abdominal segments (A2–A4). Excitatory junctional currents (EJCs) were evoked by peripheral nerve stimulation using a glass suction electrode, or by central stimulation using a 3 M KCl-filled sharp electrode placed in the center of the ventral nerve cord (Yoshihara et al., 2000). Both stimulation techniques produced similar EJC amplitudes, but nerve stimulation was used in the majority of recordings because central stimulation tended to promote segmental muscle contraction. Glutamate (100 mM in dH₂O, pH 9) was iontophoretically applied from sharp microelectrodes positioned in the middle of the muscle 6/7 NMJ (Broadie and Bate, 1993). Brief glutamate applications of 2–10 msec elicited currents with highly reproducible peak amplitudes and <500 msec duration. Hyperosmotic (HO) saline consisted of normal recording saline with 850 mM of added sucrose (Ara-vamudan et al., 1999; Fergestad and Broadie, 2001). HO saline was pressure ejected (2 sec) from an unpolished patch pipette onto the muscle 6/7 NMJ using low (1–2 psi) pressure to reduce movement artifacts and nonsynaptic muscle conductance. The ejected stream was observed visually to encompass the entire NMJ. The number of discrete current peaks in the response and the synaptic charge (measured as area under the current trace relative to baseline, in units of pAS) were quantified for 4 sec beginning with the start of the application.

Postsynaptic currents were filtered (1–2 kHz) and acquired to disk (5 kHz sampling) using hardware and computer interface from Axon Instruments (Foster City, CA) and analyzed with PClamp version 6 or version 8 software. Representative data traces were exported and prepared for display using Igor or standard spreadsheet and graphics software.

FM1-43 dye imaging. Hatching-stage (22–24 hr AEL) control and mutant embryos were dissected in Ca^{2+} -free recording saline. One control and one mutant embryo were always prepared on the same coverslip to ensure identical processing and imaging conditions (Fergestad and Broadie, 2001). Confocal settings underwent little or no variation between experiments. Embryos were exposed to 10 μ M FM1-43 (Molecular Probes) in 90 mM K^+ saline containing 1.8 mM Ca^{2+} (Fergestad and Broadie, 2001) for 2.5 min to load synaptic terminals, then washed in Ca^{2+} -free recording saline (2 mM K^+) for a minimum of 5 min to remove background fluorescence. Confocal fluorescence images were acquired with a 63× water lens. In each animal, one to two loaded muscle 12 and 13 NMJs were imaged (segments A2–A4) and then re-imaged after a second high K^+ treatment to destain terminals. Successive images for each NMJ were exported into Adobe Photoshop and aligned precisely as layers and quantified as a multi-image stack in NIH Image. The density slice function, adjusted to twofold to threefold over background, was used to outline the loaded terminal boutons (>1 μ m), and the identical region was superimposed onto the aligned destained terminals. Mean synaptic loading intensity was measured for each loaded and destained NMJ 12/13 pair. Background fluorescence from a nonsynaptic region of muscle surface was subtracted from loaded and destained synaptic levels, and values from multiple NMJs were averaged for each animal. Normalized, background-subtracted pixel intensities are reported.

Electron microscopy and ultrastructural analysis. Mutant (*slab*¹/*slab*³) and control (wild-type or *slab*/TM3) embryos from timed egg lays were collected, fixed, sectioned, and visualized in parallel using standard transmission electron microscopy techniques, as reported previously (Broadie et al., 1995; Featherstone et al., 2001). Briefly, embryos were dechorionated, removed from the vitelline membrane, and the anterior and posterior ends were excised in 5% glutaraldehyde in 0.05 M PBS. Embryos were then transferred to 2.5% glutaraldehyde in 0.05 M PBS for 1 hr, washed three times in PBS, transferred to 1% OsO₄ in distilled water (dH₂O) for 1 hr, and washed three times in dH₂O. Preparations were then stained *en bloc* in 1% aqueous uranyl acetate for 1 hr, washed three times in dH₂O, dehydrated in an ethanol series (30–100%), passed through propylene oxide, transferred to a 1:1 araldite:propylene oxide mixture, and removed and embedded in Epon 812 epoxy resin. Ultrathin serial sections (50–60 nm) were obtained on a UCT Ultracut microtome (Leica, Nussloch, Germany), transferred to formvar-coated grids, and examined on a Phillips CM12 transmission electron microscope. Synaptic boutons were serially sectioned, and profiles for each identified bouton were quantified in the section where an electron-dense AZ and T-bar

structure were most prominent. Only sections containing a single AZ were quantified. SVs in the “clustered” pool were defined as those within 250 nm of an AZ. Docked vesicles were defined as those within 0.5 vesicle diameter (<20 nm) of the electron-dense PM at the AZ. Measurements and quantification were made using Image J. Docked, clustered, and total vesicles were scored for each profile, as well as vesicles linked together or tethered to the PM outside the clustered zone, by electron-dense material. Linked and tethered vesicles were also examined in one to two serial sections adjacent to the principle AZ-containing section. Mean quantified parameters were statistically compared using the Mann–Whitney test, and presentation images were processed in Adobe Photoshop.

Results

Identification and characterization of the *slug-a-bed* Cdase gene

An EMS mutagenesis screen of the third chromosome (comprising ~40% of the *Drosophila* genome) was performed to identify novel molecules essential for neurotransmission. Mutant lines resulting in late embryonic or early postembryonic lethality were sequentially screened for normal gross anatomy, impaired movement, correctly patterned neuromusculature, and finally for synaptic dysfunction using whole-cell patch-clamp recordings at the embryonic NMJ (Broadie and Bate, 1993). The EMS-induced *slab*¹ mutation was isolated on an isogenized *rucuca* (*ru*, *h*, *th*, *st*, *cu*, *sr*, *e*, *ca*) chromosome and mapped by recombination with wild type (Oregon R) to a region distal to the *e* locus. Genomic deficiency mapping showed that *slab*¹ fails to complement Df(3R)*tll-g* (99F1–2;100B5) but complements Df(3R)20 (100A1–2;100B1–2), placing the *slab* locus in 99F1–2;100A1–2 or 100B1–2;100B5 (Fig. 1A).

All available lethal P-element insertions in the region complement *slab*¹. To create additional mutant alleles and to facilitate cloning the gene, P{lacW}1(3)j8B9^{j8B9} (j8B9) was mobilized in a P-element local hop mutagenesis. New insertions were complementation tested for lethality against *slab*¹ to isolate seven new *slab* mutant alleles. All insertion alleles retain a 5′ (distal) P-element sequence adjacent to prolyl-4-hydroxylase-α EFB (PH4αEFB), the original insertion site of j8B9 (Fig. 1B). Six insertion alleles have a 3′ (proximal) P-element sequence adjacent to genes proximal to j8B9 (Fig. 1B and data not shown). PCR on single homozygous embryos with CG1471-specific primers verified that the CG1471 sequence was deleted in three of these lines. The *slab*² deletion allele has an intragenic deletion of 855 bases removing parts of exons 4 and 5; *slab*² retains its original insertion site both 5′ and 3′ of the j8B9 P-element and has a new insertion in CG1471 (Fig. 1B). A second larger deletion allele, *slab*³, removes CG1471 and CG2224 and portions of the adjacent genes *aralar* and PH4αEFB (Fig. 1B). CG1471 maps to 99F5–99F6

Figure 1. Genomic mapping and identification of the *slug-a-bed* ceramidase gene and mutants. *A*, Genomic map showing the right tip of chromosome 3R. Genomic regions deleted by complementing and noncomplementing deficiency (Df) lines are represented below. *B*, Molecular organization of the *slab* locus, schematizing genomic scaffold AE003774. The boxes represent known and predicted genes in the region, with *slab* indicated by the shaded box. The deleted sequence in the *slab*³ mutation (line and triangles) is indicated above; the right triangle is the original point of insertion of j8B9. The expanded region below shows the intron/exon structure of *slab* to scale. The site of the *slab*¹ point mutation (arrow) and the region deleted in the *slab*² mutation are indicated. *C*, CLUSTALW alignment of five related Cdases, with overall homology to D.mel SLAB Cdase indicated by the percentages at right: human mitochondrial acid (H.sap), mouse alkaline/neutral (M.mus), *Arabidopsis* neutral (A.thal), and *Dictyostelium* predicted gene “random slug cDNA25 protein” (D.disc) Cdases. The *slab*¹ point mutation occurs in a highly conserved valine (arrow). *D*, Phylogenetic trees (midpoint rooted) showing relationship of Cdases. al, Alkaline; n, neutral; a, acid; conj, conjectural (predicted from a genome sequence annotation). In addition to Cdases listed in *C*, related proteins include rat neutral (R.ratt), *Dermatophilus congolensis* alkaline (D.cong), and *Pseudomonas* alkaline (P.aer) Cdases. D.mel *bwa* is the gene *brainwashing*, and H.sap acid Cdase is implicated in Farber’s disease.

(The FlyBase Consortium, 2003), consistent with the deficiency mapping data. The *slab*¹ (EMS) mutation results in a single point mutation of valine 264 to methionine in exon 4 of CG1471 (Fig. 1B,C).

BLAST searches against sequenced genomes revealed CG1471 to be a recently identified *Drosophila* Cdase (Renault et al., 2002; Yoshimura et al., 2002), with demonstrated functional Cdase activity when expressed in *Drosophila* S2 cultured cells (Yoshimura et al., 2002). Comparison among different organisms reveals three major groups of Cdases; the best characterized and most highly conserved are “long” Cdases (670–761 amino acids),

Figure 2. Expression of *slab* in embryonic, larval, and adult neuronal and non-neuronal tissues. *A–D*, Larval and embryonic preparations stained with antibodies against SLAB Cdase and HRP, showing SLAB is enriched in central neurons. *A*, Left, Wild-type (wt) larval ventral ganglion and central brain lobes, showing prominent SLAB staining (red) within neuronal cell bodies and large central neuroblasts but weaker staining in central and peripheral neuronal processes. Insets at $2\times$ greater magnification show cytoplasmic staining in the indicated regions. Middle, Wild-type embryonic ventral ganglion, showing similar cellular localization of SLAB (red) within neuronal cell bodies; the inset shows several cells at $3\times$ greater magnification. Embryonic central brain cells (data not shown) also displayed strong SLAB expression similar to that in larvae. Right, Neuronal UAS-*slab* overexpression in the embryonic ventral ganglion (elav GAL4; UAS-*slab*). Confocal gain was substantially reduced to illustrate the cellular staining pattern of SLAB (red). The inset image at left shows the fluorescence of the boxed region captured at the wild-type (center) confocal gain settings for comparison. HRP in all panels is shown in green. Scale bars: left, $40\ \mu\text{m}$; middle and right, $20\ \mu\text{m}$. *B*, Anti-SLAB (green) and anti-HRP (red) double-staining at larval NMJs. Genotypes shown are wild type (left), elav GAL4 UAS *slab* neuronal overexpression line (middle), and transgenic Fl-*slab* line with two additional copies of full-length genomic *slab* (right). SLAB appears to be expressed in muscle cells but not detectably enriched at the majority of synapses; the insets show SLAB staining alone at $2\times$ greater magnification for a region of the NMJ indicated by arrows. SLAB antibody staining is detectably enriched at a subset of NMJs in Fl-*slab* larvae (bottom). Scale bars: left and middle, $20\ \mu\text{m}$; right, $10\ \mu\text{m}$. *C*, Embryonic NMJs in wild-type preparations stained for SLAB (green) and HRP (red). The insets show SLAB staining alone for the indicated NMJ regions. Scale bar, $5\ \mu\text{m}$. *D*, SLAB cellular expression in other tissues, including epidermis (green; top left), twist cells (green; bottom left), and salivary gland (red; right). *E*, Western analysis of SLAB expression in embryos and adult flies. Extracts from 60 embryos (left; lanes 1–3) or two adult heads (right; lanes 4–6) of the respective genotypes were probed with SLAB antibody. The predicted size of SLAB protein is $78.2\ \text{kDa}$ and $\sim 76\ \text{kDa}$ with and without signal sequence, respectively. A band of expected size (bottom arrowhead) is present in wild-type adult heads and embryos. The SLAB band is markedly increased in intensity when UAS *slab*-GFP expression is driven by elav GAL4 (lane 2; SLAB-GFP protein size is increased to $\sim 95\ \text{kDa}$) and absent in homozygous *slab*³/*slab*³ mutants. In adults, the SLAB level relative to wild type is predictably increased by transgenic overexpression (Fl-*slab*) and decreased in *slab*³/*TM3* heterozygotes.

which share 23–39% amino acid homology and function predominantly in alkaline/neutral environments (Fig. 1*D*). The *slab* gene encodes a long Cdase that functions predominantly as an alkaline/neutral form *in vitro* but reportedly also has significant

activity at acidic pH (Yoshimura et al., 2002). The valine altered by the *slab*¹ mutation is a highly conserved residue in human, mouse, *Drosophila*, and plant Cdases (Fig. 1*C*). Other Cdase classes consist of “short” acidic and alkaline forms (264–395 amino acids) (Fig. 1*D*), which lack homology or common motifs between members of other short or long classes. In *Drosophila*, the only additional Cdase is a short alkaline form encoded by the *brainwashing* gene (Boquet et al., 2000).

To confirm that lethality is attributable to mutations in *slab*, we rescued lethality with a *slab* construct consisting of a full-length *slab* genomic coding sequence including 3553 bases of endogenous upstream regulatory sequence (Fl-*slab*). A single copy of the Fl-*slab* transgene in *slab*¹/*slab*² and *slab*¹/*slab*³ mutants is sufficient to confer adult viability at percentages near expected for full rescue. In addition, the genomic *slab* coding sequence was cloned into a pUAS-T vector, and this construct transformed into *Drosophila*. A single copy of UAS-*slab* expressed under the control of either a ubiquitous (UAS-GAL4) (Wodarz et al., 1995) or a nervous system-specific GAL4 (elav-GAL4) (Luo et al., 1994) driver is sufficient to fully rescue *slab*¹/*slab*² flies to adulthood. In contrast, *slab* expression targeted to muscle using a myosin heavy chain GAL4 fails to rescue *slab*¹/*slab*² embryonic lethality. These studies demonstrate that mutation of the Cdase encoded by *slab* is the sole cause of mutant lethality and demonstrate an essential requirement for *slab* Cdase within the nervous system.

Expression of SLAB Cdase

Slab Cdase expressed-sequence tags are present in libraries from *Drosophila* embryo, larva, pupa, and adult heads as well as from Schneider cells (Rubin et al., 2000), indicating that SLAB is expressed at all stages of the *Drosophila* life cycle. We assessed SLAB protein expression in embryos, larvae, and adult flies using antibodies including two polyclonal antibodies raised against either the N terminus (Ab11.3) or C terminus half (Ab10.3) of the protein and three mouse monoclonal antibodies raised against the entire protein. The predicted size of the SLAB protein including signal sequence is $78.2\ \text{kDa}$. In Western blots of adult whole fly or head extracts, all antibodies appear highly specific and recognize a prominent band of $\sim 80\ \text{kDa}$ (Fig. 2*E*). Ab10.3 also recognizes a second band of $\sim 130\ \text{kDa}$ that may represent a modified/glycosylated adult form of Cdase (data not shown). Immunoreactive band intensity is detectably increased over control in Fl-*slab* flies with two extra

copies of *slab* and decreased in *slab*³/*TM3* heterozygotes with a single copy (Fig. 2*E*). Anti-SLAB signal is greatly increased when UAS *slab* expression is driven by a ubiquitous or neuronal-specific GAL4 driver (data not shown). Western blots of wild-type embryonic extracts (30–60 embryos) revealed the expected ~80 kDa SLAB band. The SLAB signal is strongly increased by neuronally driven expression of SLAB-GFP and cannot be detected in *slab*³/*slab*³ mutants, indicating that this allele represents a protein null, as predicted (Fig. 2*E*).

The SLAB protein is strongly enriched in neurons, including central neurons in the brain and ventral nerve cord of embryos and larvae (Fig. 2*A*). The protein is clearly concentrated within the cytoplasm of neuronal cell bodies and weakly expressed or absent from neuronal processes within the central neuropil and in peripheral axons (Fig. 2*A*). Neuronally driven *slab* expression greatly increases the staining intensity in central neurons, but the cytoplasmic localization remains similar to that of endogenous SLAB in wild-type neurons (Fig. 2*A*, middle and right). SLAB protein is also evident at a lower level in muscle cells (Fig. 2*B,C*). In Fl-*slab* preparations, a few NMJs exhibit clearly enriched synaptic SLAB localization (Fig. 2*B*, right). However, SLAB staining is not detectably enriched at most NMJs, even when neuronally overexpressed (Fig. 2*B*, left and middle). We also examined a SLAB-GFP protein driven by neuronal elav-GAL4 (data not shown). Consistent with antibody staining results, neuronally expressed SLAB-GFP is present at high levels within neuronal cell bodies and some central axons. SLAB-GFP also appears cytosolic, and its cellular localization is indistinguishable from the intracellular pattern of anti-SLAB staining. The SLAB protein is widely expressed and also present in other tissues *in situ*, including epidermis, salivary gland, and adult muscle precursor (twist) cells (Broadie and Bate, 1991) (Fig. 2*D*). In these cells, as in neurons, the SLAB protein appears cytoplasmic with no clear subcellular localization.

Null *slab* mutants show no detectable increase in cell death

Ceramide derivatives are known signaling molecules in programmed cell death and induce apoptosis in some cell lines (Merrill et al., 1997; Dbaibo and Hannun, 1998; Spiegel et al., 1998; Acharya et al., 2003). During embryogenesis, apoptosis is a normal process for programmed removal of unneeded cells and sculpting of tissues (Abrams et al., 1993). In flies, increased embryonic expression of the death-inducing signal Reaper triggers ceramide generation and activation of caspases, effectors of cell death (Pronk et al., 1996; Bose et al., 1998). A block of Sph-1-phosphate hydrolysis in *sply* mutants results in increased embryonic apoptosis (Herr et al., 2003). The Reaper and ceramide pathway is modulated in part by stress-activated protein kinase/Jun kinase, a target of ceramide signaling (Dbaibo and Hannun, 1998). To address the possibility that ceramide-mediated cell death contributes to the mutant phenotype, we assayed apoptosis in *slab* null mutant (*slab*³/*slab*³) and wild-type embryos.

Apoptotic profiles were quantitatively examined in mid-stage embryos (stage 16; ~13 hr AEL) by comparison of numbers of acridine orange-stained cells in confocal sections. There was no evidence for increased cell death in *slab* mutant embryos; 532 ± 50 and 492 ± 126 acridine orange-stained cells were counted in wild-type and *slab* preparations, respectively (mean ± SD; *n* = 5 for both genotypes). The absence of detectably increased cell death in *slab* Cdase mutants is likely attributable to cellular checkpoints preventing unwanted cell death. For example, *Drosophila* caspase activity is inhibited by binding of DIAP1 (inhibitor of apoptosis 1); this inhibition is relieved by several proteins

including Reaper, which thus regulates apoptosis both upstream and downstream of ceramide generation (Holley et al., 2002). Additionally, Bcl-2 can protect mammalian cells from ceramide-induced apoptosis (Dbaibo and Hannun, 1998). The *Drosophila* homologs *buffy* and *death executioner* Bcl-2 likely perform similar protective functions in the fly. We conclude that *slab* mutant lethality is unlikely to be a result of decreased neuronal viability or cell maintenance.

SLAB Cdase regulates synaptic sphingolipid level and distribution

Drosophila raft lipid content has been investigated in extracted embryonic membranes (Rietveld et al., 1999); however, no known specific probes or markers exist for endogenous *Drosophila* sphingolipids or raft domains. Fluorescent sphingolipid analogs have been widely used to study sphingolipid trafficking and metabolism in mammalian *in vitro* systems (Putz and Schwarzmann, 1995; Pagano and Chen, 1998; Pagano et al., 2000b). Ceramide analogs with a fluorophore attached to a shortened fatty acid chain are rapidly incorporated into the PM, internalized and concentrated in sphingolipid-rich domains within the Golgi, and subsequently metabolized to higher fluorescent sphingolipids and glycosphingolipids and trafficked via vesicles back to the surface membrane (Lipsky and Pagano, 1985; Pagano and Sleight, 1985; Pagano et al., 1989, 1991, 2000b; Putz and Schwarzmann, 1995).

We assessed the distribution and cellular trafficking of BODIPY Fl C₅-ceramide (C₅-Cer) (Pagano and Chen, 1998; Pagano et al., 2000b) at living NMJ synaptic membranes and asked whether localization of fluorescent ceramide over a 1–2 hr time course is dependent on the SLAB expression level. Dissected third instar larvae and mature embryos (22–24 AEL) were incubated in C₅-Cer (5–10 μM) in Schneider's medium for 30–45 min, washed at room temperature for 1–2 hr to remove excess surface labeling, and imaged on the confocal microscope. Significant fluorescence is present in many cells, consistent with expected surface incorporation and internal trafficking of the lipid. In muscle cells, for example, C₅-Cer is diffusely incorporated throughout the PM as well as accumulated within internal organelle membranes, including the ER membrane surrounding nuclei (Fig. 3 and data not shown). Little or no fluorescence is found in the CNS and ventral ganglion, presumably because of the glial sheath barrier.

C₅-Cer is concentrated to NMJ synapses, most prominently at large (>3 μm diameter) "type Ib" synaptic boutons, which are surrounded by multi-layered postsynaptic subsynaptic reticular (SSR) membranes (Fig. 3*A,B,D*). As confirmation of synaptic localization, we compared C₅-Cer distribution with that of anti-DLG, a synaptic marker associated primarily with the SSR of type I boutons. After labeling and imaging of C₅-Cer at living NMJs, preparations were fixed and stained with anti-DLG, and the identical DLG-stained NMJs were then re-imaged, confirming that C₅-Cer fluorescence is consistently enriched at synaptic boutons (Fig. 3*A*). In addition, C₅-Cer is strongly accumulated at many small type II boutons that do not have associated postsynaptic SSR membranes (Fig. 3*B*). C₅-Cer is also clearly concentrated at embryonic synaptic boutons before SSR development (Fig. 3*D*). These results provide evidence that ceramide, the substrate for SLAB, is characteristically enriched and localized in embryonic synapses at the time of essential requirement (see below) as well as in mature larval stages. To address whether altered SLAB expression modifies the synaptic sphingolipid environment *in vivo*, we compared and quantified C₅-Cer levels at type I larval boutons between transgenic Fl-*slab* larvae and *slab*³/*TM3* heterozygotes (Fig. 3*D,E*). Mean synaptic fluorescence incorporated at Fl-*slab*

Figure 3. Fluorescent C₅-ceramide is trafficked to NMJ membranes, and synaptic accumulation is dependent on the level of SLAB expression. *A*, Left, The fluorescent ceramide analog BODIPY FL C₅-ceramide (C₅-Cer) is incorporated into neuronal and muscle membranes and strongly localized to living larval NMJ boutons within 1–2 hr after a 30 min incubation. Shown is the NMJ at muscle 4 in a living larval preparation. Right, The identical NMJ imaged after fixation and staining with an antibody against DLG, which localizes to type Ib synaptic boutons. Scale bar, 10 μ m. *B*, Wild-type NMJ after incubation in C₅-Cer, showing fluorescence concentrated at both large type Ib boutons (I; arrowheads) and small type II boutons (II; arrows). Labeling of the presynaptic axonal membrane and type II boutons lacking postsynaptic SSR indicates that sphingolipid-enriched environments are present presynaptically as well as postsynaptically. Scale bar, 20 μ m. *C*, C₅-Cer accumulated at embryonic synapses (NMJ 12/13, Fl-slab embryo). Synaptic boutons (arrowhead) and presynaptic nerve (n) are indicated. Scale bar, 2 μ m. *D*, Comparison of NMJ C₅-Cer fluorescence in Fl-slab larva with two additional copies of genomic *slab* (left), with that in *slab*³/TM3 heterozygote with a single copy of *slab*. Both images shown are Z-series of 5 μ m total thickness for comparison of synaptic accumulation and are representative of quantified fluorescence differences in eight paired experiments. Scale bar, 10 μ m. *E*, Quantified mean NMJ synaptic C₅-Cer fluorescence (muscle 4) in *slab*³/TM3 larvae (filled bar), normalized to that in Fl-slab larvae in eight paired experiments. *Significant reduction in mean fluorescence relative to Fl-slab ($p < 0.02$).

NMJs is significantly increased compared with *slab*³/TM3 NMJs ($p < 0.02$) (Fig. 3*E*). Because C₅-Cer was acutely applied and imaged (within ~ 2 hr), this result indicates that the localized endogenous synaptic sphingolipid environment is regulated by SLAB function and altered by the level of SLAB activity.

Essential requirement for SLAB Cdase in neuronal function

The *slab* mutant lethal phenotype is highly penetrant in all allelic combinations of *slab* mutants (*slab*¹, *slab*², *slab*³ homozygotes and transheterozygote combinations) (Fig. 1*B*), with $\sim 99\%$ of mutant animals dying either during or very shortly after hatching. Mutant embryos appear anatomically and morphologically

Figure 4. Null *slab* mutant NMJs have normal presynaptic terminal morphology and postsynaptic glutamate receptor localization. *A*, NMJ terminals on identified muscles (7, 6, 13, 12) within a single ventral hemisegment in mature (20–22 hr AEL) wild-type (wt; left) and *slab*¹/*slab*³ null mutant (right) embryos, visualized with anti-HRP antibody staining. These NMJ terminals are the subject of synaptic electrophysiology, SV dye imaging, and ultrastructural studies (see Figs. 5–8). Scale bar, 5 μ m. *B*, Localization and distribution of anti-synaptobrevin I, a SV protein, showing normal morphology and differentiation of NMJ synaptic boutons in *slab* null mutants. *C*, NMJs of wt and *slab* null mutants double-stained against HRP (α HRP; red) and the GluRIIA postsynaptic glutamate receptor (α GluR; green). The left pair of panels shows NMJ 6/7; the right pair shows NMJ 12/13. GluR puncta are closely opposed to presynaptic boutons. GluR puncta size and appearance are indistinguishable between control and *slab* NMJs, and *slab* GluR functional responses show no postsynaptic impairment (see Fig. 5*C*). Scale bar, 2.5 μ m.

normal, including a normally segmented epidermis, tracheal system, gut, nervous system, and musculature (data not shown and Fig. 4). Mutants move spontaneously and in response to touch before hatching, although both spontaneous and evoked movement are less vigorous than in control embryos, initiate hatching (21–22 hr AEL at 25°C), and protrude their heads from the egg case. The majority of mutant animals exhibit only weak head movements for a few minutes and fail to move further. A minority briefly generate peristaltic body contractions sufficient to emerge partly or completely from the egg case; a few succeed in hatching completely but rarely move more than a few body lengths. When moved to an aqueous environment before or immediately after hatching, most mutant animals maintain spontaneous and touch-evoked movements for 1–2 hr. However, persistence of coordinated whole-body peristaltic locomotion behavior typical of normal animals is never observed. Heterozy-

gous *slab*¹/*TM3* balanced siblings display wild-type movement and behavior as well as physiological transmission properties (see below).

Examination of the nervous system and somatic muscles with the light microscope reveals no obvious anatomical defects in *slab* mutants. NMJ morphology and differentiation was examined at a confocal level with presynaptic and postsynaptic antibody markers to investigate possible defects in synaptic morphogenesis or molecular differentiation. In mature *slab* mutant embryos (22–24 hr AEL), NMJs are correctly and stereotypically formed, with presynaptic terminal elaboration, branching, and synaptic bouton formation indistinguishable from control synapses (Fig. 4*A,B*; see also Fig. 7). We measured areas of NMJ presynaptic terminals in anti-HRP-stained embryos and confirmed that NMJ arbor size is not significantly altered in *slab* mutants ($p > 0.5$; *slab*¹/*slab*¹ vs wild type; muscles 12 and 13 NMJs; $n = 5$ animals). Mutant NMJs also exhibited normal levels and distribution of antibody staining against SV proteins such as Syt1 (Fig. 4*B*). Likewise, staining against postsynaptic glutamate receptors (GluRs; GluRIIA subunit) (Petersen et al., 1997; Saitoe et al., 1997) reveals GluR punctae closely opposed to presynaptic boutons at both *slab* and control NMJs (Fig. 4*C*). GluR cluster appearance and number do not differ detectably between control and mutant NMJs; furthermore, direct assays of GluR receptor function show no impairment in *slab* mutants (see below), suggesting that the postsynaptic receptor field is normally formed and aggregated. These studies indicate that the severe movement impairment of *slab* mutants is likely associated with impaired neuromuscular function.

Impaired neurotransmission at *slab* mutant NMJs is attributable to a presynaptic defect

Electrophysiological assays were performed in *slab* mutants at mature embryonic stages (20–21 hr AEL) and in larvae immediately after hatching (22–23 hr AEL). Direct electrical stimulation of the muscle or application of glutamate to the postsynaptic muscle membrane results in visible muscle contraction in *slab* mutants similar to that observed for control (wild-type or *slab*¹/*TM3* heterozygotes) animals, indicating that mutants retain muscle function. In control animals, electrical stimulation of the ventral ganglion or peripheral motor nerves evokes strong segmental muscle contraction and usually patterned waves of peristaltic muscular activity. In contrast, such stimulation evokes only weak, local muscle contraction in *slab* mutants, consistent with behavioral movement defects indicating a specific defect in the nervous system or NMJ synaptic transmission.

Whole-cell patch-clamp recordings were made from voltage-clamped (−60 mV) embryonic muscle (m6) (Broadie and Bate, 1993). Spontaneous EJC events are recorded at nearly all *slab* mutant NMJs, indicating the presence of functional glutamatergic transmission. However, EJCs evoked by direct electrical stimulation of the motor nerve are substantially impaired at *slab* NMJs. At control NMJs, nerve-evoked EJCs are robust and reproducible, with mean amplitudes of ~1000 pA (963 ± 81 pA, wild type, $n = 4$; 987 ± 59 pA, *slab*¹/*TM3*, $n = 7$) at basal stimulation frequencies of 0.2–0.5 Hz (Fig. 5*A*). In contrast, *slab* mutant NMJs display significantly reduced, low-fidelity evoked transmission. Evoked EJC amplitudes display considerable variation and are reduced, on average, by 50–70% overall in different *slab* alleles [*slab*¹/*slab*¹ (327 ± 92 pA; $n = 7$; $p < 0.01$) vs *slab*¹/*TM3*; *slab*¹/*slab*² (490 ± 59 pA; $n = 6$; $p < 0.01$) vs *slab*¹/*TM3*] (Fig. 5*A*). The incidence of transmission failures and near-failures at *slab* NMJs is progressively increased at higher (5–10 Hz) stimu-

Figure 5. Impaired neurotransmission at *slab* mutant NMJs is attributable to a presynaptic defect. *A*, Representative nerve-evoked (arrow) EJCs from control (*slab*¹/*TM3*; top traces) and homozygous *slab*¹ mutant NMJs (bottom traces; examples from 2 animals are shown). In controls, EJCs are robust (~1 nA) and reproducible. In *slab* mutants, EJC amplitude and fidelity are reduced and include transmission failures and near-failures. The mean EJC amplitudes are plotted at right for controls (wild-type, *slab*¹/*TM3*), *slab*¹/*slab*¹ mutants (* $p < 0.01$ vs *slab*¹/*TM3*) and *slab*¹/*slab*² mutants (* $p < 0.01$ vs *slab*¹/*TM3*). The two *slab* alleles are not significantly different ($p > 0.13$). *B*, Higher-frequency (10 Hz) evoked EJC trains illustrate well maintained transmission under elevated demand at control NMJs. In contrast, *slab* mutant NMJs show severely reduced amplitudes and erratic transmission, including failures (marked with symbols). *C*, Postsynaptic current responses elicited by direct iontophoretic application of glutamate. Representative traces (left) show glutamate-gated currents at control and *slab* NMJs. No difference in mean glutamate current amplitudes (plotted at right) is observed between controls (wild-type, *slab*¹/*TM3*) and *slab* mutant (*slab*¹/*slab*¹, *slab*¹/*slab*²) alleles.

lation frequencies, suggesting a reduced efficacy of presynaptic transmitter release in response to evoked nerve activity (Fig. 5*B*). The decreased mutant EJC amplitudes are not attributable to a failure in evoked action potential induction or propagation, be-

cause this direct nerve stimulation technique is readily able to support release by electronic presynaptic depolarization (Broadie and Bate, 1993; Auld et al., 1995). Thus, although a level of endogenously generated transmission and weakened evoked transmission persists at *slab* mutant synapses, normal coupling of transmission to presynaptic depolarization is greatly reduced, with transmission fidelity increasingly compromised at higher levels of activity.

As noted above, direct visualization of postsynaptic GluR fields at *slab* mutant NMJs indicates that receptors appear localized and clustered similarly to wild type. We directly tested GluR function by recording postsynaptic current responses evoked by brief (2–10 msec) iontophoretic application of glutamate directly to the muscle 6/7 synapse (Fig. 5C). Glutamate application produced visible muscle contraction in all recorded *slab* preparations, and robust glutamate current responses were recorded at all *slab* mutant NMJs, with current amplitudes indistinguishable from controls [e.g., 2516 ± 381 pA (*slab*¹/*slab*¹) vs 2292 ± 235 pA (*slab*¹/*TM3*); $p > 0.3$] (Fig. 5C). Strong postsynaptic function was present regardless of the level of presynaptic function suggested by endogenous transmission present at individual NMJs. These results indicate that the postsynaptic GluR field is normally differentiated and functional and that *slab* mutants are impaired specifically in presynaptic function.

Reduction in fusion of readily releasable SVs at *slab* synapses

The preceding analyses demonstrate a defect in the coupling of presynaptic depolarization to neurotransmitter release in *slab* mutant synapses. Several potential mechanisms, including a reduced presynaptic Ca^{2+} signal, a reduction in the readily releasable pool (RRP) of SVs, or an impairment in the SV fusion mechanism, could explain the release defect. To differentiate between these possibilities, we next assayed fusion evoked by HO saline, which specifically triggers the release of the RRP independently of Ca^{2+} influx (Rosenmund and Stevens, 1996). Whole-cell postsynaptic currents were recorded in response to brief (2 sec) focal applications of HO saline to the m6/7 NMJ (Aravamudan et al., 1999; Fergestad et al., 2001).

At control NMJs, HO saline evokes a well defined, sustained episode of vesicular fusion events (Fig. 6A). HO-evoked current responses were quantified by measuring both total synaptic charge as well as the number of discrete fusion events (number of current peaks) (Fig. 6B) over the 4 sec period after the onset of the application. For both parameters, responses at wild-type and *slab*/*TM3* control NMJs are indistinguishable (*slab*/*TM3*: 76.4 ± 7 pA·s mean current area; 83 ± 6 current peaks; $n = 12$). In contrast, HO responses at *slab*¹/*slab*¹ mutant NMJs are reduced by 70% (22.8 ± 5.5 pA·s; 22 ± 6 current peaks; $n = 10$). Likewise, *slab*¹/*slab*² and *slab*¹/*slab*³ allelic combinations were also significantly reduced in both response parameters (Fig. 6A,B). A straightforward interpretation of these results is that *slab* synapses have a reduced pool of readily releasable SVs. If the defect arises from a loss of docked SVs at the presynaptic membrane, this should be apparent in ultrastructural analysis (see below). Alternatively, the loss of the RRP in *slab* mutants may indicate a direct impairment of the SV fusion mechanism itself.

FM1-43 dye assays indicate a smaller cycling pool of SVs at *slab* mutant synapses

A loss of readily releasable SVs at *slab* mutant synapses may be a result of impairments in vesicle biogenesis, endocytosis, or trafficking of SV pools. Assays of FM1-43 dye uptake and release in living terminals provide an optical assay of activity-dependent SV

Figure 6. Presynaptic vesicle fusion evoked by HO saline is reduced at *slab* mutant synapses. *A*, Representative responses to 2 sec focal applications of HO saline (1175 mosM; stippled bar) to NMJs in control (*slab*¹/*TM3*) and *slab* (*slab*¹/*slab*¹, *slab*¹/*slab*², *slab*¹/*slab*³) mutants. Control responses typically consist of a dense burst of SV fusion events, representing release of the docked SV population. Mutant responses ranged from slight reduction in fusion compared with controls to nearly complete absence of response; overall mean reduction is significant for each *slab* allelic combination. *B*, Quantification of synaptic charge (left) and number of fusion events (synaptic current peaks; right) evoked by HO saline in controls (wild-type, *slab*¹/*TM3*) and *slab* mutants. Asterisks indicate statistical significance ($p < 0.05$) compared with *slab*¹/*TM3* control.

pool size and trafficking dynamics to functionally test these possibilities (Fergestad and Broadie, 2001). NMJ synapses in *slab* mutant and control preparations were loaded with FM1-43 during a 2.5 min depolarization with high K^+ (90 mM)-containing saline and identified NMJ terminals imaged on the confocal microscope. Terminals were then destained with a second high K^+

depolarization of varying duration (30 sec to 2.5 min) and re-imaged. These conditions reliably and consistently load and unload the “endo/exo” cycling SV pool at embryonic NMJs (Fergestad and Broadie, 2001), similar to studies at larval NMJs (Kuromi and Kidokoro, 1998, 2000).

In loaded terminals, dye fluorescence is typically localized to the periphery of larger ($\geq 2 \mu\text{m}$ diameter) embryonic boutons (Fig. 7*A,B*), showing that SVs are concentrated in cortical regions underlying the presynaptic PM, as in mature larval boutons. Dye is also incorporated throughout the interior of some boutons, in particular smaller boutons (Fig. 7*A,B*), although usually at less intensity, corresponding to a lower SV density in interior regions. The pattern of dye uptake suggests that most or all of the SV population in embryonic NMJ terminals may participate in active endo/exo cycling, differing from larval NMJs, which contain a large reserve pool that is not cycled under these conditions (Kuromi and Kidokoro, 1999, 2000).

A 2.5 min loading protocol appears to nearly maximally load the cycling SV pool in both controls and *slab* mutants. The spatial pattern and distribution of loaded dye within normal and *slab* mutant boutons appears qualitatively similar (Fig. 7*A,B*). However, *slab* terminals display significantly reduced quantified mean loaded fluorescence compared with control values ($69 \pm 4\%$ of control; $p < 0.0001$) (Fig. 7*C*), indicating a reduction in the size of the actively cycling mutant SV pool. A 1–2.5 min 90 mM K^+ depolarization effectively destains $\sim 80\%$ of loaded fluorescence in both control and mutant terminals ($84 \pm 3\%$ control vs $77 \pm 5\%$ *slab*; $p > 0.4$), showing that *slab* synapses are capable of cycling and releasing the smaller SV pool in a similar period of time (Fig. 7*C*). No significant difference in the unloaded fractions between control and *slab* NMJs was measured for 30 sec, 1 min, or 2.5 min depolarizing stimuli, suggesting a cycling pool turnover time of < 30 sec when challenged with 90 mM K^+ . To target possible differences in the rate of SV pool release, we further assayed destaining using more moderate depolarizing conditions. As shown in Figure 7*D*, fractional release with 30 mM K^+ for either 1 or 2 min is comparable between *slab* and wild-type NMJs. Therefore, during sustained high K^+ -mediated depolarization, *slab* mutant synapses are capable of trafficking and releasing SVs similarly to control synapses. However, the actively cycling *slab* SV pool is reduced in size, consistent with a reduced ability to maintain neurotransmitter release during repetitive stimulation.

Figure 7. FM1-43 imaging indicates a reduced cycling SV pool size in *slab* mutant synapses. FM1-43 dye ($10 \mu\text{M}$) was loaded into NMJ boutons with 2.5 min depolarizing stimulation with saline containing 90 mM K^+ . After imaging of loaded fluorescence at identified NMJ 12/13 terminals, dye was unloaded with 1–2.5 min stimulation with 90 or 30 mM K^+ saline to maximally or partially destain the terminal. *A*, Representative images of loaded and unloaded (1 min, 90 mM K^+) wild-type control (left) and *slab*¹ (right) NMJs. The insets show individual large boutons at twofold increased magnification. *B*, Representative images of loaded and partially destained NMJs (1 min, 30 mM K^+) in wild-type and *slab*¹ NMJs. *C*, Quantified mean fluorescence after loaded (solid bars) and remaining after subsequent destaining with 90 mM K^+ (stippled bars; 1 and 2.5 min unloading experiments are pooled) in wild-type control (Con) and *slab*¹ mutants (left). Values are normalized to mean control loaded fluorescence (100%). Mean dye loading of *slab* NMJs is significantly reduced ($*p < 0.0001$) compared with control. The right plot shows the percentage of loaded fluorescence released by destaining stimulation (% Released) for the same experiments. Control and *slab* mutant 90 mM K^+ percentage unloading shows no significant difference. *D*, Percentage release of loaded fluorescence by 1 and 2 min stimulation with 30 mM K^+ saline; 90 mM K^+ unloading is shown for comparison (rightmost pair of bars). Unloading for *slab* mutants is not significantly different from that in controls for each destaining condition, indicating cycling of the mutant SV pool under these conditions is not detectably altered.

Figure 8. *slab* mutant terminals have normal clustered and docked RRP but a reduction in nonclustered SVs and an accumulation of linked and tethered RP SVs. Electron micrographs of synaptic ultrastructure at the NMJ of fully developed (20–22 hr AEL) wild-type control (left; *A–D*) and *slab*¹/*slab*³ (*slab*; right; *A–D*) mutant embryos. *A*, Representative profiles of wild-type (left) and *slab* (right) boutons, each displaying an electron-dense presynaptic T-bar and electron-dense PM marking the AZ (large arrowheads). SVs are clustered near the AZ and distributed throughout the bouton interior and periphery. Similar profiles were used for all quantitative analyses. cs, Cisternae; m, mitochondria. Scale bar, 100 nm. *B*, Detailed images of wild-type (1, 2) and *slab* (3, 4) AZ regions, showing the population of SVs clustered near the AZ and docked SVs (arrowheads) at the PM. *C*, Detailed images illustrating SVs tethered by filamentous structures (arrows) to the non-AZ PM. Linked SVs, defined as those either in direct contact or connected by electron-dense structures, are present among the SV visible in *slab* panels (3, 4, arrowheads). *D*, Linked SVs in wild-type and *slab* terminals; arrowheads indicate linking structures or point of contact between linked SVs. Linked/tethered SVs in *slab* boutons often appear as arrays or rows aligned near the PM as in panels 3 and 4. Significantly increased numbers and percentages of linked and tethered SVs are present in *slab* mutant boutons (see Table 1). Scale bars, *A–D* (in right panels), 100 nm.

Electron microscopy reveals aberrant SV trafficking in *slab* mutant synapses

Ultrastructural analyses of embryonic NMJ terminals were undertaken to assess the hypothesis that loss of SLAB Cdase function alters SV pool size or distribution. Ultrastructural features examined included PM appearance and integrity; bouton cross-sectional area; AZ regions, defined by electron-dense PM and synaptic cleft; electron-dense “T-bar” bodies at AZ fusion sites; SV morphology and diameter; number of SVs clustered and docked at the AZ; and number/distributions of SVs within the bouton interior (Fig. 8, Table 1). As in previous studies (Ara-
vandan et al., 1999; Featherstone et al., 2001), clustered SVs were defined as those within 250 nm of an AZ, and “docked” SVs as those within <0.5 SV diameter (<20 nm) of the AZ membrane in the section containing the most prominent T-bar structure.

In all fundamental respects, synaptic ultrastructural differentiation at the EM level appears unaltered at *slab* mutant presyn-

aptic terminals (Fig. 8*A*). Membrane integrity is preserved, and terminal organelles, including SVs, maintain normal morphology and size. Mutant terminals exhibit electron-dense AZ membranes and T-bar densities, and SVs clustered at the AZ. The clustered population at the AZ contains the same number of SVs in control and mutant synaptic profiles (13 ± 1 SV/AZ for both wild type and *slab*) (Table 1). Surprisingly, we found that *slab* AZs also exhibited normal or greater numbers of docked SVs (2.5 ± 0.2) compared with wild-type AZs (2.1 ± 0.2) (Table 1). This result indicates that the *slab* release impairment is not attributable to deficiencies in localizing SVs to release sites or in docking of RRP SVs to the membrane, but rather a downstream defect in SV priming/fusion. One possible explanation for this phenotype would be a disrupted assembly or maintenance of the mutant AZ. We therefore quantified the length of mutant AZ membranes and cross-sectional areas of presynaptic T-bar structures, which are known to serve a fusogenic function. There were no significant differences between wild-type and *slab* mutants in either of these features. Thus, *slab* mutants must be specifically defective in SV priming/fusion immediately upstream of neurotransmitter release.

In addition, *slab* mutant terminals exhibit several significant differences in the size and distribution of the internal non-clustered SV pool, suggesting defects in vesicle trafficking. First, although overall SV density throughout the bouton is not significantly reduced, *slab* boutons have, on average, ~30% fewer SVs within the nonclustered population removed from the AZ ($p < 0.05$ vs wild type) (Table 1). This reduction in SV number closely matches the reduction in mutant FM1-43 uptake, supporting the conclusion that *slab* synapses have a smaller functional cy-

cling pool. Second, *slab* boutons contain increased numbers of SVs linked together in multi-vesicle clusters or chains (Fig. 8*C,D*; Table 1). These vesicles appear either in direct contact or to be connected by electron-dense globular or filamentous structures and are located both within the bouton interior as well as near the PM. Similar SV–SV linkages are present in normal terminals but are increased twofold to threefold in *slab* boutons. Third, *slab* mutant boutons exhibit increased numbers of SVs tethered by filamentous structures to the PM. Tethered vesicles resemble docked vesicles but are not localized to AZs and are typically located at a greater distance (~1 vesicle diameter) from the membrane. To confirm that tethered SVs were not associated with nearby AZs in adjacent sections, we scored and counted tethered SVs in serial sections in a subset of boutons (two to four sections/bouton in 32 boutons). This analysis indicated that tethered SVs tended to be localized to PM regions removed from an AZ. The incidence of both linked and tethered SVs is increased by twofold

Table 1. Summary of *slab* mutant synaptic ultrastructural features

Ultrastructural features	Wild type	<i>slab</i>	Significance
Number of synaptic profiles scored	39	24	
Mean terminal cross-sectional area (μm^2)	1.50 ± 0.14	1.27 ± 0.16	NS
Mean SV density (total number of SVs per profile/ μm^{-2})	46.0 ± 3.6	46.2 ± 6.3	NS
Number of clustered SVs (<250 nm from AZ)	12.7 ± 0.5	12.5 ± 0.6	NS
Number of docked SVs at AZ	2.1 ± 0.2	2.5 ± 0.2	NS
Number of nonclustered SVs (>250 μm from AZ)	43.0 ± 3.9	30.0 ± 3.2	$p < 0.05^*$
Number of linked SVs per profile	3.5 ± 0.6	7.5 ± 1.0	$p < 0.002^{**}$
Percentage of synaptic profiles with linked SVs	57% (17/30)	86% (19/22)	
Percentage of linked SVs per profile	$6.0 \pm 1.0\%$	$17.7 \pm 2.4\%$	$p < 0.0005^{***}$
Number SVs per section tethered to non-AZ membrane	2.8 ± 0.4 (43)	6.6 ± 0.7 (41)	$p < 0.005^{**}$

Wild-type and *slab* null mutant (*slab*¹/*slab*³) embryos were fixed and identically processed in parallel. Single sections exhibiting a clear AZ profile were quantified for each identified synapse. When multiple serial sections of the same synapse were obtained, the profile containing the most prominent AZ was selected for analysis. Analysis of tethered SVs included serially sectioned boutons (1–4 sections), with the mean number of tethered SVs per section scored for each bouton. The parentheses show the number of profiles examined were different from that in the first row. Values are mean $\pm$ SEM. Asterisks indicate statistical significance determined by Mann-Whitney nonparametric test. NS, Not significant.

to threefold in *slab* mutant boutons compared with normal terminals ($p < 0.005$ vs wild type) (Table 1). Linked and tethered SVs together represent $\sim 50\%$ of the smaller nonclustered internal pool in *slab* bouton profiles, compared with $\sim 16\%$ for wild type. These latter two phenotypes, which are both characterized by aberrantly increased SV sequestration, may reflect a related trafficking defect leading to an abnormal accumulation of a normal SV trafficking state.

Discussion

Loss of Cdase impairs SV priming/fusion

The *slug-a-bed* gene was identified in a forward screen for synaptic dysfunction mutants and encodes a long-chain Cdase that regulates the ceramide level. At *slab* mutant synapses, postsynaptic receptor function is unaltered, whereas nerve-evoked transmission and HO saline-evoked SV fusion is decreased by 50–70%. These results indicate a specific presynaptic impairment consistent with a loss of the RRP. However, *slab* synapses exhibit normal numbers of clustered and docked SVs at the AZ, ruling out defects in forming a localized releasable pool. We therefore conclude that the primary transmission impairment is a reduced ability of SVs to complete priming/fusion steps after docking.

Neurotransmitter release is most directly mediated by the RRP, a small cycling SV pool that includes a subpopulation of docked, fusion-competent vesicles at the AZ (Schikorski and Stevens, 1997, 2001; Sudhof, 2000; Mozhayeva et al., 2002; Richards et al., 2003; Rizzoli and Betz, 2004). Provided that the essential vesicle fusion machinery is functional, release efficacy is predictably correlated to the size of this docked pool (Dobrunz and Stevens, 1997; Reist et al., 1998; Pozzo-Miller et al., 1999; Delgado et al., 2000; Voets et al., 2001; Mozhayeva et al., 2002), which may be functionally assayed by hypertonic saline-evoked fusion (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). This correlation is genetically supported by *C. elegans* mutations in the Sec-1-related protein UNC-18, which causes a parallel loss of docked SVs and hypertonic saline-evoked fusion (Weimer et al., 2003). Similarly, mutants in the mammalian UNC-18 homolog reduce docking and exocytosis of dense core secretory vesicles (Voets et al., 2001). Disruptions to downstream priming and fusion steps, including mutations in the SNARE (SNAP receptor) core complex proteins syntaxin-1, *N*-synaptobrevin, and SNAP-25 as well as in UNC-13 (uncoordinated-13) and RIM (Rab3-interacting molecule), which regulate SV priming, also severely reduce or eliminate Ca^{2+} - and hypertonic saline-evoked release (Broadie et al., 1995; Schulze et al., 1995; Aravamudan et al., 1999; Kidokoro, 2003). In contrast, however, these priming/fusion mutants typically exhibit increased SV docking and a

larger overall vesicle pool because of selective block of the exocytosis step(s) (Broadie et al., 1995; Aravamudan et al., 1999; Richmond and Broadie, 2002).

The *slab* mutant phenotypes place the functional transmission requirement downstream of docking at the level of vesicle priming/fusion, with the insignificant increase ($\sim 20\%$) in docked SVs consistent with a pronounced but incomplete inhibition of fusion. SNARE complex proteins have been shown to localize to sphingolipid- and sterol-enriched membrane raft environments in secretory cells (Chamberlain et al., 2001; Lang et al., 2001; Chamberlain and Gould, 2002). Other exocytic proteins, such as nSEC-1 and α SNAP, are reported to localize to distinct sterol-rich raft-like domains (Salaun et al., 2004). An accumulation and spatial misregulation of ceramide, the predominant sphingolipid in flies (Rietveld et al., 1999), is expected to disrupt the topology, lipid environment, and protein content of membrane exocytic domains. Ceramide has established roles regulating membrane domain fluidity and curvature, cholesterol aggregation, vesicle formation, and fusion (Li et al., 1999; Venkataraman and Futerman, 2000; van Blitterswijk et al., 2003). Increased membrane ceramide levels may therefore interfere with SV fusion competency by inhibiting lipid restructuring required for exocytosis, by inhibiting functional interactions between release machinery proteins, or by a combination of both lipid- and protein-mediated processes.

Loss of Cdase increases SV pool sequestration

Our results indicate that sphingolipids are also involved in regulating SV number and distribution. In *slab* mutant terminals, the endo/exo cycling pool labeled by FM1-43 is reduced by $\sim 30\%$. Although overall mutant SV density is not changed, the number of SVs not localized to AZs is reduced by a similar percentage. Most interestingly, *slab* terminals exhibit a striking increase in the percentage of SVs linked together or tethered to the PM. Filamentous tethers often clearly appear to connect SVs and to connect SVs to the PM, singly and in multi-vesicle arrays. SVs sometimes appear both linked and tethered (Fig. 8C,D), suggesting that these forms of linkage are similar structurally and serve to sequester SVs from the AZ. Although previously overlooked by us and others (Prokop, 1999), normal synapses exhibit a small number of linked/tethered SVs, indicating they represent normal intermediate trafficking steps that are abnormally accumulated in the absence of SLAB.

C. elegans and *Drosophila endophilin* (*endo*) and *synaptojanin* (*synj*) mutants also exhibit prominent arrays or “strings” of linked SVs and SVs tethered to the PM by cytoskeletal filaments (Harris et al., 2000; Schuske et al., 2003; Verstreken et al., 2003).

Both *endo* and *synj* SV trafficking phenotypes are associated with severe primary defects in endocytosis, resulting in a substantial depletion (50–70%) of SVs from the entire terminal, including AZ regions, and an accumulation of intermediate endocytic structures at or near the PM (Harris et al., 2000; Guichet et al., 2002; Schuske et al., 2003; Verstreken et al., 2003). The qualitative similarities between the *slab*, *endo*, and *synj* phenotypes reinforce the conclusion that presynaptic lipid environments are important for regulating SV pool size, trafficking, and availability for fusion. However, the *slab* ultrastructural phenotype differs from these endocytic mutants in two significant respects. First, the reduction in vesicles is far less pronounced and restricted to non-AZ domains, whereas the clustered and docked populations appear unaffected. Second, the *slab* phenotype is more specific to an accumulation of later SV trafficking states.

How might SLAB Cdase and ceramide regulate SV trafficking? Ceramide resides both in plasma and vesicle membranes and is concentrated in raft domains, interacts with raft-associated proteins, and modulates general membrane endocytosis and trafficking (Brown and London, 2000; van Blitterswijk et al., 2003; Acharya et al., 2004) depending on its production and topological location in the membrane. Asymmetrical generation of ceramide, as in rafts, promotes negative membrane curvature, vesicle budding, and vesicle aggregation (van Blitterswijk et al., 2003). Conversely, certain ceramide analogs inhibit membrane internalization and trafficking (Chen et al., 1995; Li et al., 1999). Ceramide may therefore have similar roles in synaptic PMs and SVs and is likely trafficked between the inner PM leaflet and the SV surface by endocytosis. SV recycling is thought to occur in specialized, spatially defined regions in which lipid and protein constituents are preassembled before vesicle budding (Martin, 2000). This process is potentially modulated by changes in PM ceramide levels and raft distribution. The *slab* ultrastructural and FM1-43 loading phenotypes do not indicate a severe endocytosis defect at the NMJ. However, given the pronounced SV fusion impairment in the absence of SLAB, a reduced level or spatial specificity of endocytosis may be sufficient to maintain a pool of SVs trafficked to release sites. A recent analysis of RRP organization at the frog NMJ suggests that, contrary to general assumption, the RRP may be widely dispersed throughout the overall vesicle population and not necessarily recruited from regions nearest the AZ (Rizzoli and Betz, 2004). If this is the case in *Drosophila* NMJ terminals, the increased SV sequestration observed in *slab* boutons could more directly underlie reduced RRP availability and weakened transmission, particularly during maintained activity. Raft domains have functions consistent with a role in SV tethering, including serving as sites for actin nucleation and polymerization and regulating actin cytoskeleton stability (Rozelle et al., 2000; Bruses et al., 2001). In the absence of SLAB, altered raft environments may inhibit F-actin-mediated vesicle trafficking or interactions between SV and the cytoskeletal scaffold. For example, disruption of membrane sphingolipid composition may interfere with SV binding to the tethering protein synapsin, which mediates the activity-dependent sequestration and mobilization of SVs (Chi et al., 2001, 2003).

Cdase, ceramides, and lipid rafts in *Drosophila*

Drosophila detergent-insoluble embryonic membranes are enriched in sphingolipids, sterols, and proteins (Rietveld et al., 1999), supporting the existence of functional raft domains in flies analogous to those in vertebrates. *Drosophila* sphingolipids consist predominantly of saturated long-chain ceramides, glycosphingolipids, and phosphoethanolamine ceramide (PECer), a sphingolipid analog present in insects. Ceramide may therefore be produced in the membrane by PECer hydrolysis (Rietveld et al., 1999; Renault et al., 2002). Sterols constitute ~18% of *Drosophila* membranes and over 30% of raft lipid fractions, relative to phospholipids (Rietveld et al., 1999). Cholesterol, an essential vertebrate membrane component, contributes only a fraction of sterols but may, nevertheless, have an important role in membrane and raft structure and in the regulation of proteins such as signaling protein Hedgehog, known to undergo cholesterol modification. Notably, Hedgehog and numerous glycosylphosphatidylinositol (GPI)-linked proteins are localized to *Drosophila* rafts (Rietveld et al., 1999), indicating that GPI linkage is a conserved mechanism for targeting proteins to rafts by (Tsui-Pierchala et al., 2002).

SLAB Cdase contains a secretory signal sequence and is secreted in an *N*-glycosylated form when expressed in *Drosophila* S2 cells (Yoshimura et al., 2002). SLAB overexpression in the *Drosophila* eye suppresses retinal degeneration by reducing ceramide levels and facilitating membrane recycling (Acharya et al., 2003, 2004), consistent with our findings. We have used protein localization and mutant loss-of-function studies to examine expression and functional requirement of the endogenous Cdase for the first time. SLAB is expressed in numerous tissues and cells and prominently enriched in central neurons. Mutant lethality is rescued by neuronally targeted gene expression, showing that SLAB neuronal expression is essential. C₅-ceramide, an analog known to be trafficked and metabolized in cells (Pagano et al., 2000b), is rapidly accumulated at synaptic boutons, and its synaptic concentration is dependent on the *slab* expression level. These results suggest that endogenous sphingolipids are enriched and dynamically trafficked at *Drosophila* synapses and regulated by SLAB activity. SLAB may regulate synaptic sphingolipid environments by several plausible pathways. In neurons, SLAB is clearly cytoplasmically localized, consistent with either an intracellular or secreted function. SLAB may be present at low levels and function in the synaptic terminal; alternatively, it may be primarily secreted and act on the neuronal PM. If the essential protein function is secreted, however, our rescue results support the conclusion that neuronal rather than general secretion is required. Finally, SLAB may regulate sphingolipid production and content in membrane trafficked to and incorporated at the synapse.

Our results provide further evidence for the involvement of sphingolipid raft domains in specialized SV trafficking and exocytosis. A role for ceramide-rich membrane domains in SV priming or fusion processes represents the most direct potential involvement in neurotransmitter release. Likewise, sphingolipid-dependent interactions between SV and tethering proteins potentially regulate recycling and trafficking steps. Future studies will investigate mechanistic links between SLAB, lipid raft domains, and the established SV fusion and trafficking machinery.

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