Mutations in the Exocyst Component Sec5 Disrupt Neuronal Membrane Traffic, but Neurotransmitter Release Persists

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Summary

The exocyst (Sec6/8) complex is necessary for secretion in yeast and has been postulated to establish polarity by directing vesicle fusion to specific sites along the plasma membrane. The complex may also function in the nervous system, but its precise role is unknown. We have investigated exocyst function in *Drosophila* with mutations in one member of the complex, *sec5*. Null alleles die as growth-arrested larvae, whose neuromuscular junctions fail to expand. In culture, neurite outgrowth fails in *sec5* mutants once maternal Sec5 is exhausted. Using a trafficking assay, we found impairments in the membrane addition of newly synthesized proteins. In contrast, synaptic vesicle fusion was not impaired. Thus, Sec5 differentiates between two forms of vesicle trafficking: trafficking for cell growth and membrane protein insertion depend on *sec5*, whereas transmitter secretion does not. In this regard, *sec5* differs from the homologs of other yeast exocytosis genes that are required for both neuronal trafficking pathways.

Introduction

Within neurons, there are multiple pathways of exocytosis that function to accomplish distinct tasks, such as the polarized insertion of membrane proteins, neurite extension during development and plasticity, the secretion of trophic factors and peptides, and synaptic transmission. These pathways require the transport of vesicles to the cell surface, recognition of appropriate membrane sites, and fusion of membranes. Many of the proteins that underlie neuronal membrane traffic have homologs in yeast and constitute an essential core of related molecules that likely function similarly in all intracellular membrane fusion pathways. Proteins such as those of the SNARE complex and NSF appear to have similar conserved roles whether in the growth of yeast (Finger and

Novick, 1998), membrane addition in mammalian cells (Rothman, 1994), or the fusion of synaptic vesicles (Lin and Scheller, 2000; Sudhof, 2000).

The exocyst complex comprises an additional set of proteins implicated in trafficking to the cell surface in yeast, but one whose function in the nervous system remains unclear. The eight proteins of the exocyst complex, Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84, both in yeast and in mammals, appear to be almost always associated with one another either in the cytosol or bound to membranes (Hsu et al., 1996; TerBush and Novick, 1995). In yeast, mutations in any of the members of the complex block bud growth and cause transport vesicles to accumulate in the cytosol (Novick et al., 1980; Salminen and Novick, 1989). The exocyst complex is involved in the post-Golgi transport of vesicles to the plasma membrane (Finger and Novick, 1997) and has been proposed to function as the vesicle targeting signal because two complex members, Sec8 and Sec3, were found to localize to sites of membrane addition: the tips of small buds during cell division and the bud neck during cytokinesis (Finger and Novick, 1998; TerBush and Novick, 1995). A Sec3-GFP fusion protein localized to the plasma membrane even when other members of the exocyst complex were mutated, and this protein, therefore, may function as a spatial landmark for the site of exocytosis.

Much less is known about the role of the exocyst complex in multicellular organisms. The mammalian complex is expressed in both neuronal and nonneuronal tissue (Hsu et al., 1996). In MDCK cells, the complex was observed at tight junctions, and antibodies to the complex member Sec8, when introduced into permeabilized cells, partially blocked delivery of basal-lateral, but not apical, membrane proteins to the surface (Grindstaff et al., 1998). Similarly, overexpression of Sec10 in MDCK cells increased delivery of a membrane protein to the basal-lateral, but not apical, domain (Lipschutz et al., 2000). Thus, as in yeast, the mammalian complex may mediate fusion at a spatially restricted domain.

The exocyst has a punctate distribution in processes of cultured primary hippocampal neurons and localizes near, but not precisely with, synaptic vesicles (Hsu et al., 1996; Kee et al., 1997). A role for the complex in synapse formation and neurite outgrowth has been suggested by the early presence of Sec6/Sec8 immunoreactivity at sites of synaptogenesis in culture (Hazuka et al., 1999) and the impairment of neurite outgrowth with dominant-negative forms of sec10 and sec8 in PC12 cells (Vega and Hsu, 2001). Though abundant in adult brain, the function of the complex in neurons after development remains uncertain. The exocyst, like SNARE complexes, may be needed for all fusions at the plasma membrane both in developing and mature neurons, or it may be required only for particular forms of traffic.

Thus far, resolving the neuronal function of the exocyst complex has been hampered by the absence of a functional genetic analysis. In the mouse, a *sec8* mutant has been identified, but these mice die shortly after

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gastrulation of the embryo, precluding a more detailed analysis of the role of the complex (Friedrich et al., 1997). In the present study, we have identified and characterized *sec5* mutations in *Drosophila* in order to delineate the role of the protein in neurons and particularly at synapses. We find that this protein is required for many aspects of membrane traffic within neurons, including the elaboration of neurites, but that the release of neurotransmitter at the synapse is independent of this exocyst component. Thus, *sec5* mutations reveal the presence of two mechanistically distinct forms of neuronal membrane traffic and demonstrate that not all proteins required for membrane addition in yeast are essential for synaptic vesicle fusion.

Results

Mapping of *Drosophila sec5* and Identification of Mutations

To understand the role of the exocyst complex in synapse formation, we searched the *Drosophila* genome database for homologs to members of the rat exocyst complex (Hsu et al., 1996). The genome contained only one isoform of each member of the exocyst complex. By in situ hybridization to polytene chromosomes, we identified preexisting chromosomal deficiencies that removed three members of the complex: *sec5*, *sec6*, and *sec10* (Figure 1A). *Df(2L)tim*^{02} and *Df(2L)P22* removed the *sec5* locus, which is located at 23F (Figure 1B). Because Sec5 is a central component of the yeast exocyst complex (Grote et al., 2000; Guo et al., 1999a; Jantti et al., 1999), we concentrated our efforts on *sec5*.

An EMS mutagenesis screen for lethals uncovered by *Df(2L)P22* generated candidate *sec5* mutations. The *sec5* gene resides in an 11 kb Kpn fragment of genomic DNA from 23F that had been previously introduced into flies as a transgene (P[Kpn]) and that also contained the genes *msl-2* and *CG3246* (Figure 1B; Bashaw and Baker, 1995). This transgene was used to identify candidate *sec5* alleles among the lethal mutations isolated from the screen (Figure 1C). In addition to *msl-2*, two complementation groups were rescued with the P[Kpn] transgene. We sequenced the open reading frame of *sec5* in all alleles from both of these complementation groups and found nonsense mutations in *sec5* in alleles *E10* (R31 to STOP) and *E13* (Q361 to STOP) from one complementation group, and we found no mutations in the coding sequence of *sec5* in alleles of the other complementation group.

Allele *E10*, which contains an early stop codon in *sec5*, meets the genetic criterion for a null allele—either in combination with *Df(2L)tim*^{02} or homozygous, it has the same lethal phase. *sec5*^{E10} mutants die within 96 hr of egg laying, as morphologically first instar larvae. These larvae hatch at the same time as their heterozygous siblings, at approximately 24 hr after egg laying (AEL), and their growth between 24 and 48 hr is comparable to that of wild-type larvae. However, *sec5*^{E10} mutants do not grow after 48 hr AEL and remain late first instar larvae, whereas by 96 hr AEL, wild-type larvae progress to the early third instar stage.
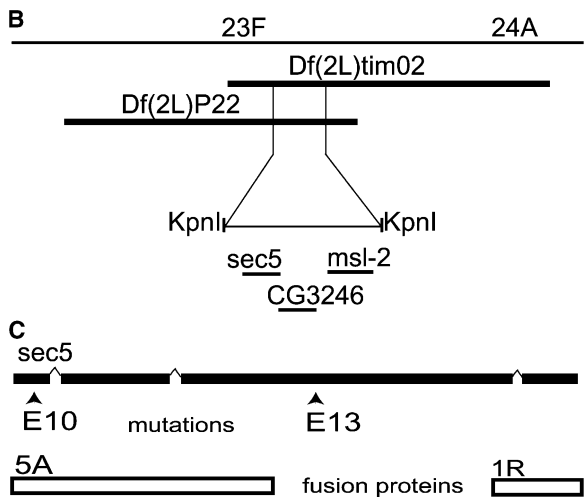
Figure 1. Mutations in *sec5*

(A) The eight members of the *Drosophila* exocyst complex, their chromosomal locations, and deficiencies that remove *sec5*, *sec6*, and *sec10*.

(B) The genomic map of *Drosophila sec5*. EMS mutations in *sec5* were isolated in a screen for lethals uncovered by *Df(2L)P22*. The genomic fragment between the two KpnI sites can rescue mutations in *sec5*.

(C) The four exons of the *sec5* gene. The stop codon in alleles *E10* and *E13* are indicated, as are the GST-fusion proteins used as immunogens.

Maternally Contributed Sec5 Protein Is Depleted in the Null Mutant by 72 hr AEL

Eyes composed exclusively of cells homozygous for a mutation can be generated in an otherwise heterozygous animal by means of mitotic recombination that is induced during development of the eye disc (Stowers and Schwarz, 1999). When this method was applied to either *sec5*^{E10} or *sec5*^{E13}, the eye was completely ablated (data not shown). The presence of the P[Kpn] transgene restored eye development in these flies, demonstrating that the cell lethality was due to the mutations in *sec5* and not to second site mutations on the chromosome. This apparent requirement for *sec5* in cell viability raised the question of why the homozygous null embryos developed and survived for up to 96 hr AEL. This survival can be attributed to maternally deposited *sec5* mRNA and protein. *sec5* transcripts were present at rather constant levels throughout development, including the first
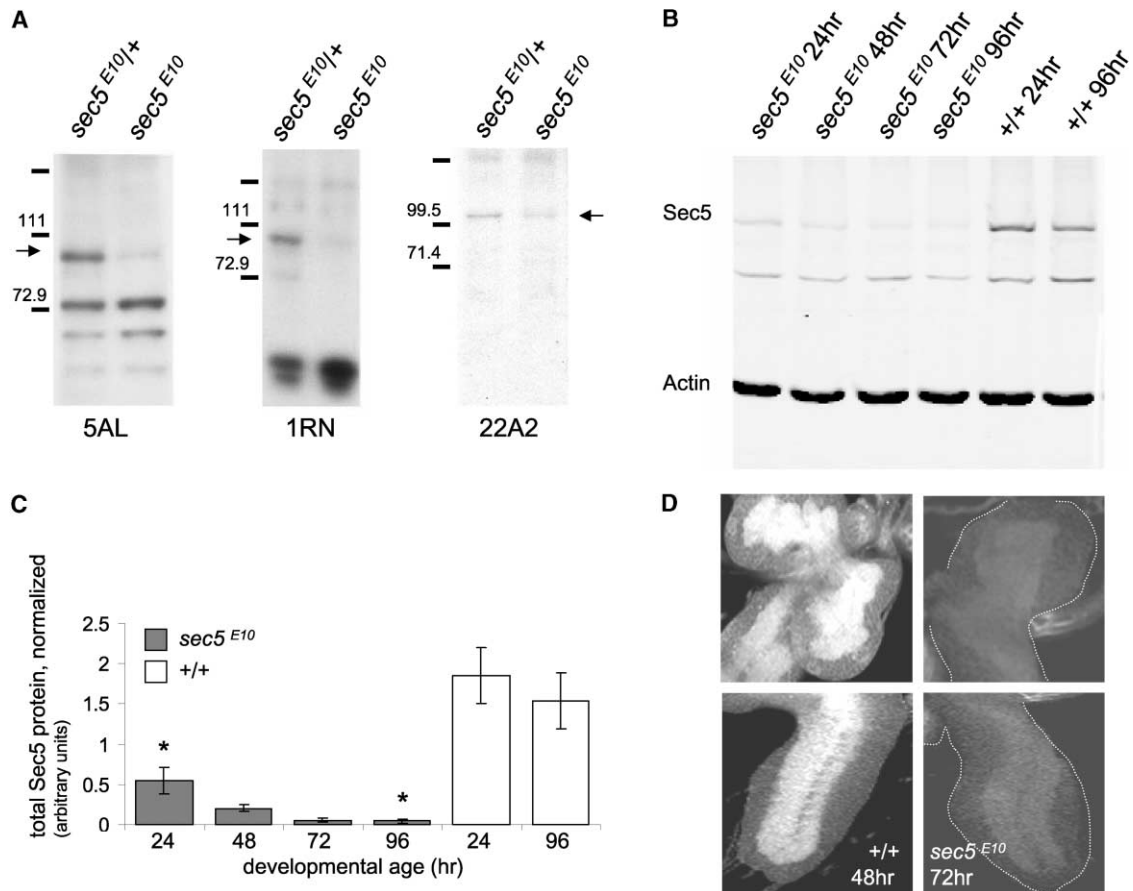
Figure 2. Sec5 Protein Is Substantially Reduced in Null Mutants by 48 hr AEL

(A) Antisera 5AL and 1RN, to two separate regions of Sec5, recognize a band of 100 kDa (arrows) that is decreased in the homozygous null mutant relative to the heterozygote at 72 hr AEL. Monoclonal antibody 22A2 recognizes the same-sized band (arrow) that is decreased in $sec5^{E10}$ homozygotes relative to heterozygotes at 48 hr AEL. 20 μg protein/lane.

(B) Protein was extracted from $sec5^{E10}$ or +/+ larvae at the indicated times, and 15 μg of total protein was loaded per lane. The top of the blot was probed for Sec5 with 5AL and the bottom for Actin as a loading control.

(C) Quantitation of remaining maternally derived Sec5. From immunoblots as in (B), Sec5 levels in $sec5^{E10}$ mutants of 24 to 96 hr AEL were compared to control (n = 3; average ± SEM).

(D) Immunolocalization of Sec5 by monoclonal 22A2 in the ventral nerve cord of +/+ larvae at 48 hr AEL and comparably sized $sec5^{E10}$ larvae of 72 hr AEL under identical conditions and exposures. Immunoreactivity is nearly absent in the $sec5^{E10}$ nerve cord at 72 hr AEL.

2 hr of embryogenesis, before zygotic transcription begins (data not shown). This was confirmed by analysis of Sec5 protein (see below).

Mouse polyclonal 5AL (raised against fusion protein 5A, aa 1–321; see Figure 1C), mouse polyclonal 1RN (raised against fusion protein 1R, aa 634–894), and monoclonal 22A2 (raised against fusion protein 1R) each recognized a band of 100 kDa on immunoblots, consistent with the predicted mass of *Drosophila* Sec5. The intensity of the band was sharply decreased in homozygous null mutant larvae at 48 or 72 hr AEL, relative to heterozygous mutants from the same stock (Figure 2A). Crossreacting bands observed with either unpurified antiserum 5AL or 1RN were unaffected by the mutation, consistent with the identification of the 100 kDa band as the sole product of the sec5 locus.

The amount of maternally derived protein (maternal contribution) remaining in $sec5^{E10}$ null mutants between hatching (24 hr AEL) and 96 hr AEL was quantified with antibody 5AL on immunoblots by means of a fluoro-

phore-coupled secondary antibody, employed in the linear range of detection (Figures 2B and 2C, n = 3). In wild-type, Sec5 protein levels remained constant relative to total protein between 24 and 96 hr AEL. At 24 hr, $sec5^{E10}$ homozygotes had ~29% of Sec5 protein (normalized) that was present in equivalently aged wild-type larvae (p = 0.02). By 48 hr AEL, when the growth of the larva ceased, the protein level in homozygous mutants had dropped to 11% of the 24 hr control. Sec5 protein continued to decline thereafter, and by 72 or 96 hr the remaining Sec5 appeared almost completely gone (3% and 2.5% of the 24 hr control, respectively; p = 0.03). The nearly complete absence of Sec5 in homozygous *E10* alleles was confirmed by immunocytochemistry with monoclonal 22A2 (Figure 2D). While Sec5 immunoreactivity appeared to be present ubiquitously at low levels, within the nervous system, Sec5 was enriched in the neuropil, the synapse-rich region of the ventral nerve cord. In $sec5^{E10}$ mutants at 72 hr AEL, immunoreactivity was barely detectable. Thus, $sec5^{E10}$ larvae, be-
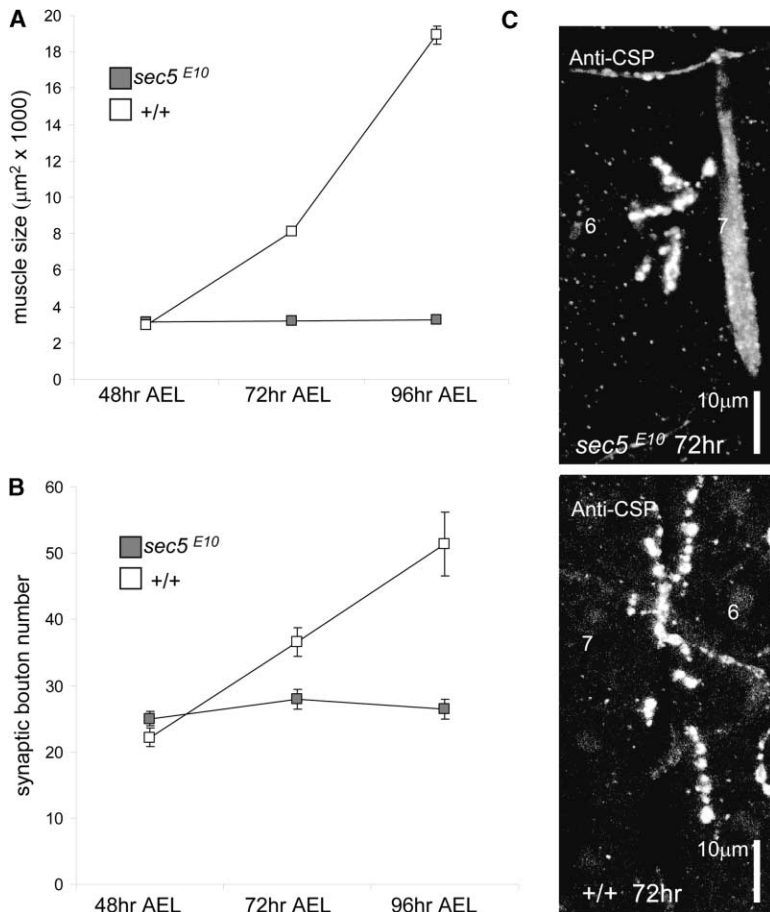
Figure 3. Growth of the Neuromuscular Junction Is Arrested in *sec5^E10*

(A) Whereas in wild-type the average area of muscle 6 increases during early larval development, *sec5^E10* mutant larvae do not substantially grow between 48 and 96 hr AEL. The average (±SEM) 2D area, measured optically, of muscle 6 in abdominal segments A2 or A3 for *sec5^E10* at 48 hr AEL (n = 12), 72 hr AEL (n = 12), and 96 hr AEL (n = 6) and for +/+ at 48 hr AEL (n = 10), 72 hr AEL (n = 9), and 96 hr AEL (n = 6).

(B) Synaptic boutons, labeled with anti-CSP, did not increase in number between 48 and 96 hr AEL in *sec5^E10* mutant larvae. From confocal images, type Ib and Is endings were counted at the junction of muscles 6 and 7 in segments A2 or A3. *sec5^E10* at 48 hr AEL (n = 12), 72 hr AEL (n = 7), and 96 hr AEL (n = 8); +/+ at 48 hr AEL (n = 7), 72 hr AEL (n = 7), and 96 hr AEL (n = 8).

(C) Representative neuromuscular junctions on muscles 6 and 7 are shown for *sec5^E10* and +/+ at 72 hr AEL.

tween 72 and 96 hr, provided a suitable system in which to study the consequences of a loss of Sec5.

Loss of Sec5 Prevents Growth of the Neuromuscular Junction

Maternal contribution of Sec5 was essential for appropriate embryonic development—when we attempted to remove it by generating maternal germlines homozygous for either *sec5^E10* or *sec5^E13* (Chou and Perrimon, 1996), no fertilized eggs were produced. However, in *sec5^E10* homozygotes, the maternal contribution was sufficient to allow the nervous system to form. The role of the exocyst complex in neuronal vesicle trafficking could thus be studied in a nervous system that had subsequently run out of Sec5.

The neuromuscular junction (NMJ) of *Drosophila* consists of a string of boutons from a small number of axon branches that form a pattern characteristic for each muscle in each abdominal segment. In wild-type, the motor neuron first contacts the muscle at about 14 hr AEL, and these contacts mature into synapses by 16–17 hr AEL. After hatching at 24 hr AEL, both the muscle and the motor neuron increase in size. In wild-type, the size of muscles increases 10-fold between first and third instar development, with a concurrent increase in synaptic bouton number (Schuster et al., 1996). In *sec5^E10* homozygous embryos, neuromuscular junctions develop with an apparently normal morphology, and the NMJs

of *sec5^E10* mutants are size-matched to wild-type until 48 hr AEL. However, *sec5^E10* mutant larvae do not grow substantially between 48 and 96 hr AEL. Because maternal protein decreases dramatically during larval development in the mutant, we looked for any aberrations in the postembryonic maturation of the NMJ.

The size of muscle 6 was first measured for both mutant and control. While the size of muscle 6 increased 6-fold between 48 and 96 hr AEL in wild-type, there was no growth of muscle 6 in the *sec5^E10* mutant during this time (Figure 3A). The number of synaptic boutons at the NMJ of muscles 6 and 7 was then determined with an antibody to the synaptic vesicle marker Cysteine String Protein (CSP). The bouton number at the wild-type NMJ increased 2.5-fold between 48 and 96 hr AEL, whereas there was no change in the mutant (Figure 3B). By immunocytochemistry, diffuse Sec5 staining was observed in segmental muscles 6 and 7 and along the nerves that innervate these fibers (data not shown). The presence of Sec5 in the muscles, however, obscured the detection of Sec5 in the nerve terminals of the NMJ.

Sec5 Is Required for Neurite Extension

The observed defect in muscle growth and bouton addition may reflect the inability of the muscle and nerve cells to insert new membrane. However, assayed in vivo, it was also possible that the failure of these cells to grow might be secondary to malnutrition of the animals, the
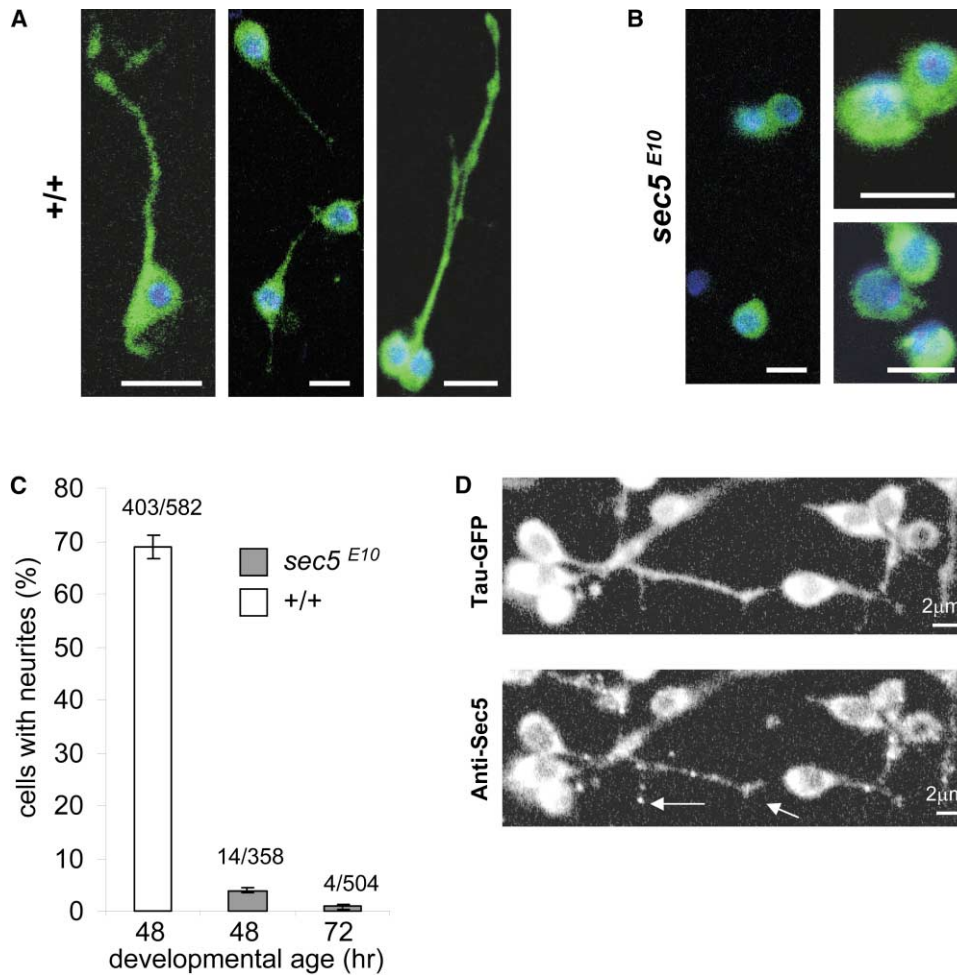
Figure 4. Sec5 Is Required for Neurite Outgrowth

(A and B) Neurons were cultured from the ventral nerve cord of +/+ larvae (48 hr AEL, size-matched to *sec5E10* mutants) or *sec5E10* larvae (48 or 72 hr AEL) and visualized with an *elav*-GAL4 driven UAS-Tau-GFP transgene (green). Cells were cultured for 1 day and fixed, and nuclei were stained with Hoechst 33342 (blue). Only cells with a large nonfragmented nucleus and an identifiable nucleolus were counted. Representative +/+ neurons (A) extend neurites in culture, while neurons cultured from *sec5E10* mutants at 72 hr AEL (B) do not. All scale bars are 2 μm.

(C) Quantification of cells with neurites. +/+ larvae were cultured at 48 hr AEL (582 cells from 4 cultures). *sec5E10* larvae were cultured at 48 hr AEL (358 cells from 3 cultures) or 72 hr AEL (504 cells from 3 cultures).

(D) Dissociated neurons from the ventral nerve cords of third instar larvae were cultured and labeled with mAb 22A2. Sec5 was present in the cell body, puncta along the developing neurite, and the tips of neurites (arrows). Immunolabeling was dependent on the primary antibody (data not shown).

absence of a secreted growth signal, or a similar confounding phenotype. Moreover, because the increase in bouton number is thought to be tightly coupled to muscle size (Schuster et al., 1996), it was not possible to determine in these experiments whether the inability of the synapse to grow was secondary to the failure of the muscle cells to expand. To address more directly the potential requirement of the exocyst in the neuron, neurite extension was studied in vitro.

The ventral nerve cord and some adhering tissues from either control larvae at 48 hr AEL or *sec5E10* mutant larvae at 48 or 72 hr AEL were dissociated and placed in culture for 1 day (see Experimental Procedures). To distinguish neurons in these cultures from other contaminating cell types, both control and mutant cultures were made from *Drosophila* that expressed Tau-GFP under the control of the neuron-specific *elav* promoter. GFP-positive cells were scored for the presence of neurites and the lengths of any processes were measured (Figure 4). The viability of neurons in culture was judged by the integrity of their nuclei, stained with Hoechst 33342 (Dudek et al., 1997). Neurons that were not healthy were excluded from further analysis. The survival of cells in culture was also judged by staining with ethidium homodimer-1, which only enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to DNA. The presence or absence of Sec5 did not alter the overall viability of the 1 day cultures, with comparable numbers of cells from either genotype surviving (data not shown). In control cultures from +/+ larvae at 48 hr AEL, 69% of the neurons had extended neurites (Figure 4C). In contrast, neu-
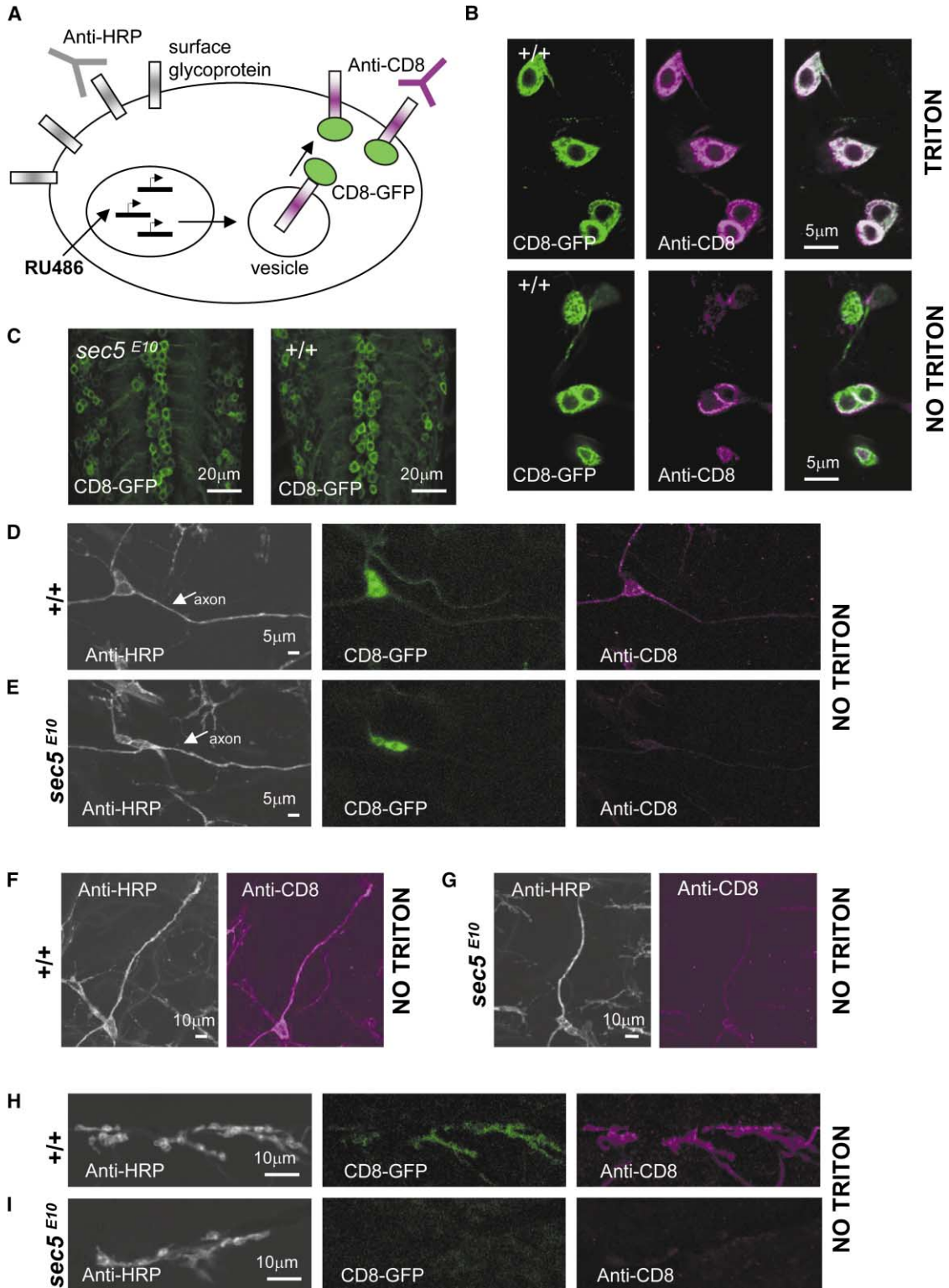
Figure 5. Transport of an Integral Membrane Protein to the Cell Surface Is Impaired in *sec5* Mutants

(A) Neuronal expression of murine CD8 fused to GFP was triggered by RU486 activation of *elav*-Geneswitch at 48 or 72 hr AEL to assay transport of the newly synthesized protein to the surface. *sec5^E10* or control larvae were fed RU486 12 hr before dissection and immunostaining for CD8. In the absence of Triton X-100, the antibody can only bind to cell surface CD8. Larvae were also stained with anti-HRP, which labels the surface of all neurons.

(B) +/+ larvae fed RU486 at 48 hr AEL and dissected at 60 hr AEL. mCD8-GFP is abundant in the cell bodies of sensory neurons and, in the presence of detergent, CD8 immunoreactivity (magenta) colocalizes with the GFP signal (green), whereas in the absence of detergent, only the surface pool is labeled.

rites were scarce in *sec5^E10* cultures: only 4% of neurons cultured at 48 hr AEL extended neurites, and in cultures made at 72 hr AEL, less than 1% of healthy neurons possessed neurites. The few neurites that were present in *sec5* mutant cultures were significantly smaller than the neurites observed in control cultures (+/+ 48 hr AEL [n = 34], 27.0 ± 3.2 μm; *sec5^E10* 48 hr [n = 14], 14.5 ± 2.1 μm; *sec5^E10* 72 hr [n = 4], 19.1 ± 1.6 μm). We conclude that Sec5 is required for neurite outgrowth.

In yeast, exocyst proteins have been shown to mark the site of membrane addition (Finger et al., 1998), and so the immunolocalization of Sec5 was undertaken in these cultures. Consistent with the findings of Hazuka et al. (2000) in mammalian cultured neurons, *Drosophila* Sec5 immunoreactivity was present in the cell bodies of neurons, in puncta along developing neurites, and at the tips of neurites (Figure 4D). Membrane addition in growing neurites occurs primarily at growth cones (Craig et al., 1995), and therefore the presence of Sec5 at neurite tips may reflect sites of membrane addition. The widespread distribution of Sec5 in the cell, however, may reflect a broader role in vesicle traffic.

sec5 Mutants Possess Defects in Vesicle Trafficking

To look directly for defects in membrane trafficking, an assay was developed that would allow for a determination of the efficacy with which newly synthesized protein could be added to the neuronal surface in larvae in which little or no Sec5 remained. To this end, the Geneswitch system (Osterwalder et al., 2001) was used to activate a reporter gene at 48 or 72 hr AEL. The neuron-specific *elav* promoter was used to express the Geneswitch product, an inactivated form of Gal4. Upon feeding larvae RU486, the Geneswitch is activated and can bind to an upstream activating sequence, or UAS, which results in transcriptional activation of the transgene. For this assay, a transmembrane protein, murine CD8 fused to GFP at its cytoplasmic end, served as the reporter transgene. An anti-mCD8 antibody was used that recognizes an extracellular epitope. In the absence of Triton X-100, this antibody will recognize exclusively the subset of the CD8 reporter gene that has been expressed on the cell surface, whereas the GFP fluorescence will represent both surface and internal pools of the protein (Figure 5A). Larvae were fed RU486 for 12 hr, which successfully turned on the transgene. When dissected and immunolabeled in the presence of Triton X-100, the GFP signal colocalized with staining for mCD8 throughout the cell. As expected, without detergent, mCD8 immunolabeling was restricted to the cell surface (Figure 5B).

sec5^E10 mutant larvae at 72 hr AEL were fed RU486 and compared with either similarly sized control larvae fed at 48 hr AEL (Figures 5 and 6) or similarly aged controls fed at 72 hr AEL (data not shown). Larvae were dissected 12 hr after introduction to the drug and stained in the absence of detergent. Both control and mutant larvae showed strong expression of the transgene in the central nervous system (Figure 5C), indicating that despite a lack of Sec5, protein synthesis was not impaired. We focused our attention on lateral bipolar dendrite (bd) sensory neurons, because their cell bodies and axons were easily visualized due to their isolation in the periphery and because they lie close to the surface of the dissected larva. In the absence of Triton, the anti-mCD8 antibody was able to access the neuron and label its surface reliably. No difference was observed between mutant and control in the expression of the reporter gene as determined by GFP fluorescence in the somata of these cells (Figures 5D, 5E, and 6A).

To examine the transport of the mCD8 reporter gene to the surface of the bd neurons, antibody staining was performed in the absence of Triton and examined in confocal sections (Figures 5D–5G). Total anti-mCD8 labeling of the surface of the cell body was reduced in the mutant to 13% of control (p = 0.0003) (Figure 6A). Animals were costained with an anti-HRP antibody that labels a surface antigen in all neurons so that the mCD8 signal could be normalized to the surface area of the cell (Figure 5A). The immunostaining for the HRP-like antigen appeared to decrease in the mutant, and this could result from thinner axons or from a defect in the addition of the epitope to the surface. Normalization of CD8 surface staining to this parameter was therefore conservative and may have underestimated the extent to which insertion of the CD8 reporter was impaired in the mutant. The loss of Sec5 reduced the surface mCD8 immunoreactivity to 17% of control (p = 0.0002) when normalized to the HRP signal. Therefore, between 72 and 84 hr AEL in *sec5^E10* mutants, less of the newly synthesized mCD8 is inserted at the membrane, demonstrating a defect in this membrane trafficking pathway. The difference between mutant and control cannot be attributed to the different ages of the animals (72 hr AEL for *sec5^E10* versus 48 hr AEL for +/+) because control larvae fed RU486 at 72 hr AEL also efficiently transported the mCD8 reporter to the plasma membrane (data not shown).

Membrane traffic was also examined in the axons of the bd neurons (Figure 6B). In the axons, the GFP signal was equivalent between mutant and control, demonstrating that Sec5 was not required for axonal transport of the CD8-containing vesicles. Similar to the cell soma, however, the surface mCD8 immunoreactivity was reduced to 13% of control (17% when normalized to HRP; p = 0.0002).

In addition, the synaptic boutons of the NMJ were

(C) *sec5^E10* mutant larvae were fed RU486 at 72 hr AEL and dissected at 84 hr. Mutant and +/+ larvae expressed comparable levels of CD8-GFP as indicated in a confocal section of the ventral nerve cord.
(D) A lateral bd sensory neuron in a +/+ larva that had been fed RU486 at 48 hr AEL and dissected at 60 hr AEL. Anti-HRP immunostaining (grayscale) was used to find the same sensory neuron in each animal.
(E) A lateral bd neuron as in (D) but from a *sec5^E10* mutant fed RU486 at 72 hr AEL and dissected at 84 hr AEL. *sec5^E10* larvae show less cell surface CD8 than the control.
(F and G) Additional contol (F) and *sec5^E10* (G) neurons as in (D) and (E).
(H and I) Comparison of surface and intracellular CD8-GFP at the boutons of the neuromuscular junctions of control (H) and *sec5^E10* (I).
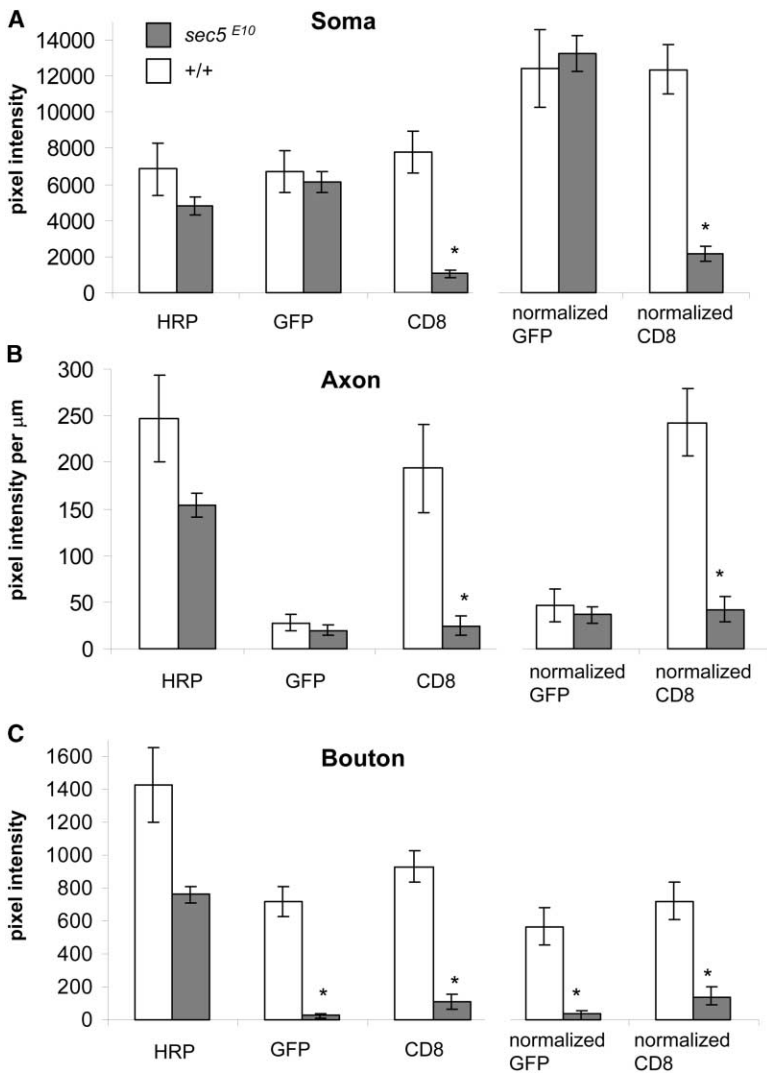
A Soma

B Axon

C Bouton

Figure 6. Cell Surface Expression of a CD8-GFP Reporter Gene Is Impaired by Loss of Sec5

From quantitative fluorescent microscopy of the transport assay in Figure 5, surface-expressed CD8 immunofluorescence, total GFP fluorescence, and cell surface area (measured by HRP-immunoreactivity) were expressed as fluorescent units after background subtraction. To control for differences in cell size, GFP and surface CD8 were also normalized to the anti-HRP signal.

(A) In the sensory neuron soma (n = 10 +/+ and $sec5^{E10}$ larvae), comparable levels of expression of the transgene are indicated by the GFP fluorescence, but significantly less CD8 has been trafficked to the cell surface.

(B) In the axons of the bd neurons, (n = 10 +/+ and $sec5^{E10}$ larvae) surface CD8 is also severely reduced in the $sec5^{E10}$ mutant.

(C) At neuromuscular nerve terminals (n = 7 +/+, n = 6 $sec5^{E10}$), cell surface expression of the reporter is also severely reduced and this is accompanied by a decline in total GFP retained in the terminals (p = 0.004 after normalization).

examined to analyze integral membrane protein insertion at the same nerve endings that had failed to increase with age (Figure 3) and at which electrophysiological studies were conducted (see below). In these terminals, there was also a dramatic reduction in surface expression of mCD8 (11% of control, 19% normalized to HRP; p = 0.002) (Figures 5H, 5I, and 6C). Thus, in these terminals, transport vesicles carrying the newly synthesized protein do not appear to fuse with the plasma membrane. Interestingly, a decrease in GFP signal relative to control boutons was also observed, although the axons entering the muscle had abundant GFP. It is likely, therefore, that transport vesicles that fail to fuse with the membrane are not retained in the terminal but may return to the axon for retrograde transport.

The axonal transport of post-Golgi vesicles was examined further with a second reporter gene, a Synaptotagmin (Syt)-GFP fusion (Zhang et al., 2002). RU486 was again fed to control larvae at 48 hr AEL and mutant larvae at 72 hr AEL, and both were dissected 12 hr later. As with the mCD8 transgene, the axons of both mutant and control contained abundant Syt-GFP, at equivalent levels, demonstrating that there was no defect in synthesis of the transgene or transport of vesicles along the

axon (Figures 7A and 7C). As with the CD8 transgene, Syt-GFP did not accumulate in the boutons of the $sec5^{E10}$ larvae (Figure 7B), though it accumulated as expected in +/+ boutons. To locate the boutons, dissected larvae were stained with anti-CSP. Several boutons from each animal were selected for quantifying the amount of GFP at the synapse (Figure 7C). The Syt-GFP signal was reduced in the bouton to 5% of the +/+ control (p = 0.001), although CSP-immunolabeling did not significantly differ. Thus, in the absence of Sec5, vesicles containing newly synthesized Syt-GFP are present along the length of the axon but are rarely seen in the synaptic boutons of the NMJ. However, synaptic vesicles, visualized with antibodies either to Synaptotagmin (data not shown) or to CSP (Figures 3C, 7B, and 7C), continue to be concentrated at the synapse. We conclude that the Syt-GFP-labeled vesicles fail to fuse with the membrane and are not retained in the mutant terminals.

Synaptic Transmission Persists Despite Run Down of Sec5 Maternal Protein

The ability of synapses to form in the homozygous mutant allowed us the opportunity to inquire whether the
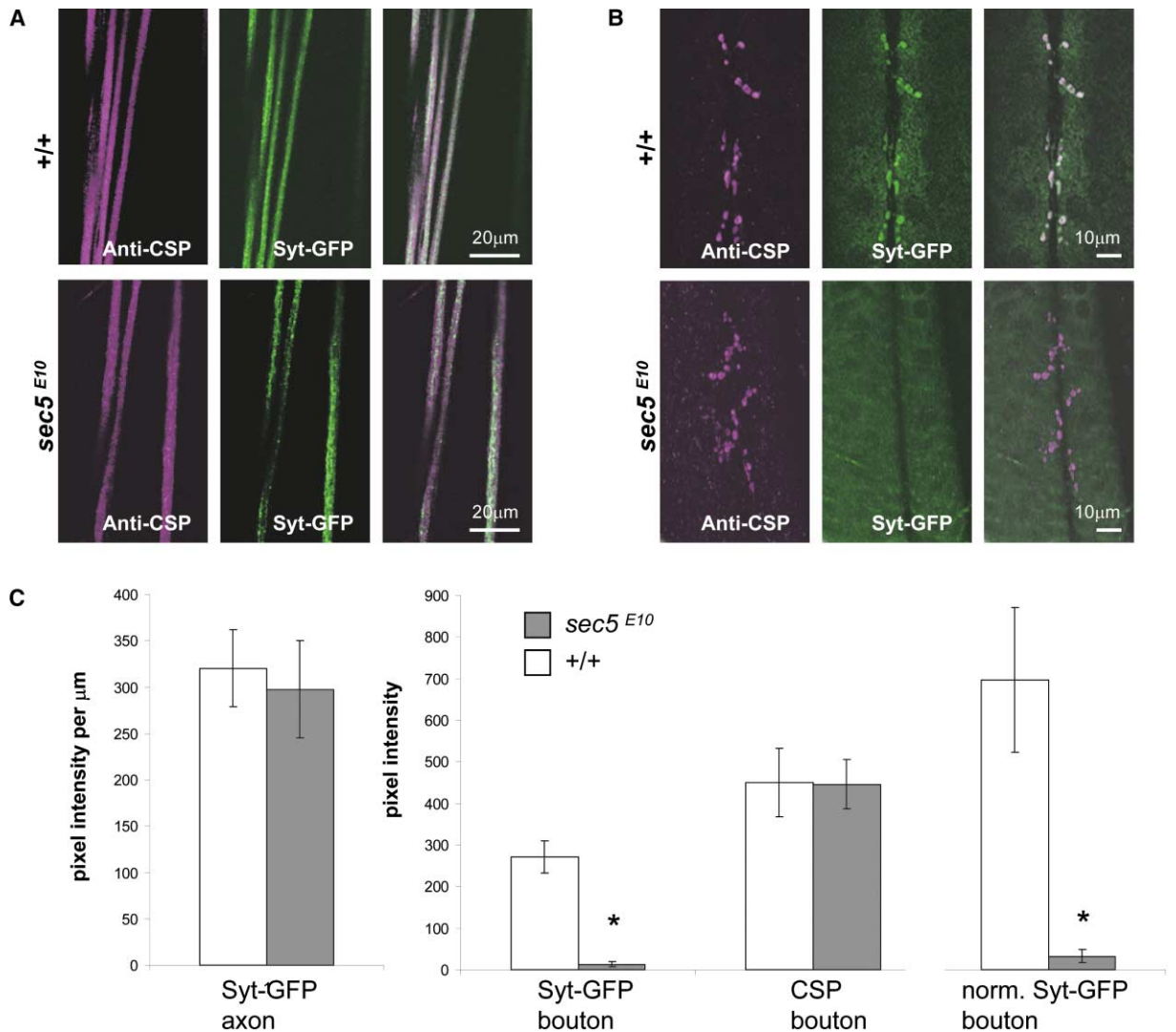
Figure 7. Vesicles Containing Newly Synthesized Synaptotagmin-GFP Do Not Localize to the NMJ in sec5^{E10}

RU486 feeding at 72 hr AEL induced expression of the reporter protein in sec5^{E10} larvae that were dissected and fixed at 84 hr AEL. +/+ control larvae of comparable size were fed RU486 at 48 hr AEL and dissected at 60 hr AEL.

(A) Images of segmental nerves of sec5^{E10} and control larvae indicated that the Syt-GFP transgene is robustly expressed and transported into the axons.

(B) The NMJ was imaged for Syt-GFP and for the synaptic vesicle marker CSP. Newly synthesized Syt-GFP did not concentrate in the synapses of mutant NMJs, though they remained immunoreactive for CSP.

(C) Quantitation of Syt-GFP in the axon (n = 3 +/+, n = 6 sec5^{E10} larvae) and Syt-GFP and anti-CSP in the boutons (n = 6 +/+, n = 7 sec5^{E10}). The Syt-GFP intensity at the bouton is reduced in the mutant (p = 0.01 after normalization to anti-CSP to account for any differences in bouton size).

fusion of synaptic vesicles at the terminal was dependent on Sec5. Therefore, we examined the strength of synaptic transmission at the NMJ when the maternal contribution was no longer adequate to support other forms of membrane traffic. We recorded from wild-type first instar larvae at 48 hr AEL, and sec5^{E10} mutants at 48, 72, and 96 hr AEL. We compared mutants from these three ages to 48 hr control larvae, because these mutants were comparable in muscle size and bouton number to the 48 hr control (Figure 3). If synaptic vesicle fusion was dependent on Sec5, as was the incorporation of mCD8-containing transport vesicles into the terminal membrane, release per bouton would be expected to decline over this period and to be negligible by 96 hr AEL.

Surprisingly, synaptic transmission persisted during this period despite the decline in Sec5 protein (Figure 8A). At 96 hr AEL, although the size of the NMJ had not changed (Figure 3) and Sec5 protein represented 3% of control (Figure 2), the evoked response was actually increased 2.5-fold over its amplitude at 48 hr.

The amplitude and frequency of spontaneous synaptic events (mEJCs or minis) was examined. First instar larval muscles contain two populations of minis: fast, large events (Type 1) corresponding to synapses onto the muscle cell from which the recording is made and slow, small events (Type II) that are passively propagated from nearby electrically coupled muscle cells (Ueda and Kidokoro, 1996). Only Type I events, discrimi-
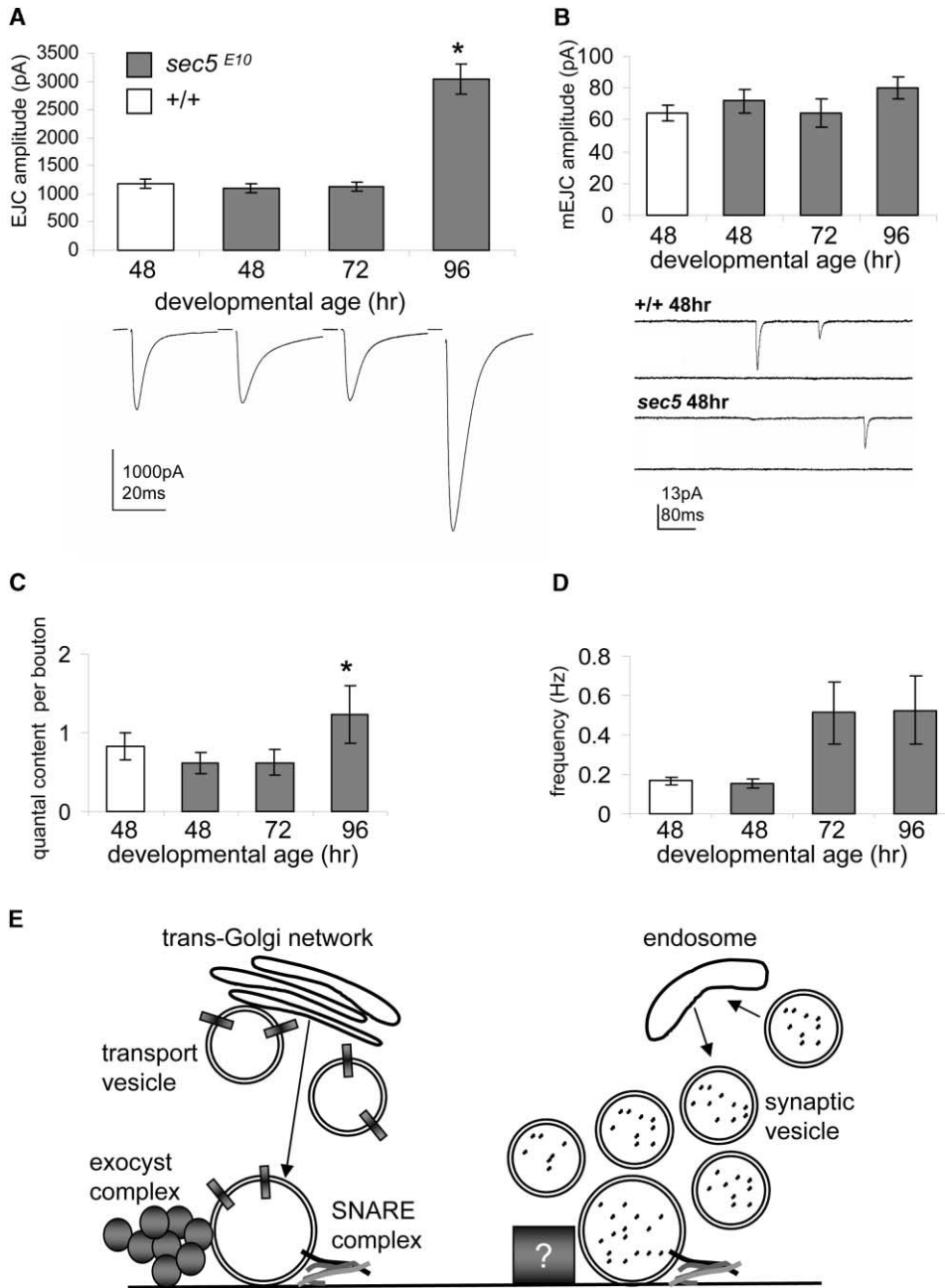
Figure 8. Synaptic Transmission Persists in *sec5* Null Mutants

(A) Average evoked (EJC) amplitudes (±SEM) for +/+ at 48 hr AEL (n = 4) and *sec5^{E10}* at 48 hr (n = 7), 72 hr (n = 8), and 96 hr (n = 5). The amplitude of the evoked current does not decrease between 48 and 72 hr in the mutant, and significantly increases by 96 hr (p = 0.006). Representative averaged responses are shown below each bar. Stimulus artifacts have been deleted.

(B) Average type I miniature EJC amplitudes (±SEM) and representative traces for +/+ at 48 hr (n = 8) and *sec5^{E10}* at 48 hr (n = 7), 72 hr (n = 8), and 96 hr (n = 5).

(C and D) Quantal content per bouton of evoked responses and average frequency of type 1 spontaneous miniature EJCs for each genotype and developmental age. Quantal content per bouton increases slightly by 96 hr AEL when compared to *sec5^{E10}* at 48 hr (p = 0.01). A small increase in mini frequency is also observed but is not statistically significant (p = 0.06).

(E) Mechanistically distinct pathways of vesicle fusion in neurons. Transport vesicles derived from the TGN fuse with the plasma membrane in an exocyst-dependent manner. This pathway adds the CD8 reporter to the cell surface and likely mediates muscle growth, neurite outgrowth, and the addition of new synapses. In contrast, synaptic vesicles can fuse with the membrane independent of Sec5 and the exocyst complex. While the fusion of both types of vesicles requires SNARE proteins, the exocyst complex distinguishes between these pathways.

nated on the basis of their rate of rise, were analyzed. The amplitude of minis was unchanged in the mutant (Figure 8B), indicating that the number of postsynaptic receptors and the amount of transmitter per vesicle was not significantly altered in the mutant between 48 and 96 hr AEL.

From the size of the nerve-evoked responses and the size of the individual minis, the quantal content, the number of vesicles released per stimulus (see Experimental Procedures), could be calculated. Consistent with the robust evoked responses in the mutants, the quantal content of the homozygous null mutants was also observed to increase even as the maternally derived protein declined (+/+ 48 hr, 18 ± 3; $sec5^{E10}$ 48 hr, 15 ± 3; $sec5^{E10}$ 72 hr, 17 ± 4; $sec5^{E10}$ 96 hr, 33 ± 8). To take into account the difference in size between the neuromuscular junctions of different stages and genotypes, we normalized quantal content to bouton number (see Figure 3). This parameter increases slightly with development in wild-type animals. In +/+ embryos at 48 hr, we observed 0.83 ± 0.18 quanta per bouton. In third instar larvae, in similar ionic conditions, approximately 1.3 quanta per bouton are released (Schuster et al., 1996; Stewart et al., 2000). In sec5 mutants, the quantal content per bouton similarly increased (0.61 ± 0.13 at 48 hr; 1.24 ± 0.37 at 96 hr; Figure 5C). Therefore, despite the decline in Sec5 protein, sec5 mutants between 48 and 96 hr AEL secrete equivalent amounts of neurotransmitter per bouton to wild-type animals throughout larval development. Finally, we found that the frequency of minis is somewhat increased in the mutants between 48 and 96 hr AEL, although not significantly (Figure 8D). The persistence of minis indicates that synaptic vesicle fusions that are not driven by action potentials can also persist at rates appropriate to the anatomical size of the synapse. In conclusion, these data demonstrate that sec5 is not required for the exocytosis of synaptic vesicles.

Discussion

Within neurons, multiple pathways are known to transport proteins and transmitters to the cell surface. In some cases, the exocytotic vesicles derive from the Golgi apparatus and consist of newly synthesized materials, while in others the vesicles derive from the plasma membrane and cycle locally. Another distinction that has been drawn contrasts the constitutive and the regulated pathways (Burgess and Kelly, 1987) to distinguish the ongoing transport of protein and lipid to the cell surface from the ability to secrete hormones and transmitters in response to specific stimuli. Subsequently, further distinctions in trafficking pathways have been invoked to explain different forms of regulation and fusion at different domains of the cell. Two important pathways were examined in the current study: (1) a basic pathway of exocytosis that brings new proteins to the cell surface and permits the cell to grow, and (2) synaptic transmission, a specialized form of exocytosis, regulated by Ca^{2+} entry, in which vesicles already present at synapses fuse with the membrane and recycle locally.

The mechanistic basis for these distinct pathways remains obscure. Presently, all membrane trafficking steps are thought to possess a similar underlying mechanism for membrane fusion, and yet clear distinctions in these trafficking steps must also occur. In many cases, synaptic transmission is thought to consist of the basic exocytotic pathway with additional regulatory processes superimposed. We have, in this study, found a distinction between these two trafficking pathways in which the basic pathway depends on a protein, Sec5, that does not appear to be required for synaptic transmission.

We have isolated mutations in sec5, a component of the Drosophila exocyst complex, and demonstrated that the basic exocytotic pathway is impaired in those mutants, and yet vesicle fusion at the synapse persists. Despite a small amount of maternal protein remaining in the mutant at the end of its life span, we observed dramatic phenotypes with respect to membrane addition. Muscles lacking Sec5 did not increase their surface area and motor neurons did not increase their arborizations on the muscle. When cultured, neurons lacking Sec5 survived but proved incapable of extending neurites. This defect was not due to a general malaise of the mutant neurons: neurons at this time point were capable of synthesizing new proteins, conducting action potentials, and releasing neurotransmitter, and they were equally viable in culture. Instead, the likely mechanism for this deficit in cell growth was due to a specific defect in membrane traffic, as revealed in the trafficking assay with which we could follow the cell surface insertion of a newly made transmembrane protein in first instar larvae. Whereas endogenous proteins had inserted into the cell surface while the maternal contribution persisted, induced CD8-GFP was synthesized only after the maternal contribution was no longer functionally adequate. CD8-GFP failed to accumulate in the plasma membrane. These dramatic phenotypes in membrane addition are consistent with data in yeast, where exocyst mutants fail to add new membrane during cell division and accumulate vesicles in the presumptive daughter bud (Novick et al., 1980, 1981).

What step in membrane trafficking requires Sec5? Members of the exocyst complex have been shown to be recruited to budding vesicles in the trans-Golgi network (Liljedahl et al., 2001), and antibodies to Sec6 and Sec8 are reported to interfere with the ability of these proteins to exit the Golgi (Yeaman et al., 2001). In the sec5 null mutants, however, we observed normal levels of synthesis of the CD8 and Synaptotagmin-GFP constructs. They were both distributed throughout the cytoplasm and transported down the axon with equal efficiency to wild-type. The requirement for Sec5 would therefore appear to lie at a later step in membrane traffic, most likely at docking to and fusion with the plasma membrane. This finding is consistent with the phenotype of exocyst mutants in yeast, where vesicles are appropriately transported into the bud but fail to fuse with the membrane (Novick et al., 1980, 1981).

In marked contrast to the arrest of growth and the impairment of the pathway for membrane protein addition, synaptic transmission continued to be robust. Each action potential that arrived at the neuromuscular junction of a 96 hr AEL $sec5^{E10}$ homozygous larva caused an average of 33 ± 8 vesicles to fuse. Though Sec5 protein declined between 24 and 96 hr in these null mutants, the quantal content increased and, if anything, attained a level slightly higher than would be expected for a junction with the observed number of boutons. This persistence of vesicle fusion cannot be attributed to a residuum of maternally derived Sec5, as there were severe defects in the other assays conducted at these

times. At the same time point that the larvae are capable of repeatedly secreting numerous synaptic vesicles, the lack of Sec5 so effectively prevented net membrane addition that almost no neurites could be extended and that little CD8 was successfully expressed on the cell surface. Thus, the synaptic response in larva lacking Sec5 would appear to derive from the combination of a defect in neuronal growth and an unaltered capacity for synaptic vesicle fusion. The growth defect prevented the creation of the normal number of boutons and release sites for a 96 hr animal; however, those boutons that had formed remained fully functional.

What we report here for mutations in *sec5* is likely to represent the function of the entire exocyst complex. From biochemical studies in yeast and mammalian cells, the component proteins of the exocyst are found primarily in a complex (Guo et al., 1999b; Hsu et al., 1996; TerBush and Novick, 1995). This model is consistent with the similarities of the mutant phenotypes for each component of the complex in yeast. Moreover, Sec5p binds in the center of the complex (Guo et al., 1999a), and its absence causes Sec10p and Exo84p to be dissociated from Sec8p (Guo et al., 1999b). We hypothesize that loss of this individual component will be equivalent to loss of most or all exocyst function.

Heretofore, synaptic transmission could be viewed as an elaboration of the basic exocytosis pathway, in which regulatory mechanisms were superimposed on a scaffold equivalent to the exocytotic machinery of yeast. Thus far, of all of the genes studied at the nerve terminal that are orthologs of genes isolated in yeast for defects in the final steps of exocytosis (Novick et al., 1980), all have essential roles in synaptic vesicle fusion (Lin and Scheller, 2000; Rothman, 1994; Sudhof, 2000). These genes include the proteins of the SNARE complex, syntaxin (yeast Sso1p), SNAP-25 (yeast Sec9p), and VAMP (yeast Snc1p), as well as nsec1 (yeast Sec1p), rab (yeast Sec4p), NSF (yeast Sec17p), and α-SNAP(yeast Sec18p). The same screen in yeast identified six of the eight members of the exocyst complex, but, as described above, the exocyst appears to be an exception to this established pattern: it is essential for exocytosis in yeast but dispensable for synaptic vesicle fusion.

Why is vesicle fusion at the synapse distinct from the vesicle fusion required for neurite outgrowth or the addition of CD8 in the membrane? Our data indicate that the synapse has evolved away from a need for the exocyst in fusion. In yeast, the exocyst complex is known to be required before the assembly of the SNARE complex and the fusion of secretory vesicles with the plasma membrane (Grote et al., 2000), which would place the exocyst complex upstream of the final stage of vesicle fusion. At the synapse, the complicated cytoskeletal network at the active zone may take the place of the exocyst complex in tethering vesicles at the release site and priming them for fusion. Such active zone proteins as Munc-13 and RIM, likely regulators of SNARE complex formation, which do not have homologs in yeast, may fulfill at the active zone the role assigned to the exocyst elsewhere in the cell. A second possibility is that the fundamental distinction between exocyst-dependent and -independent pathways in neurons depends on the source of the vesicle; the local cycling of vesicles not derived primarily from the TGN, but directly

from the plasma membrane or through an endosome, may be independent of the exocyst complex (Figure 8E). In this case, exocyst-independent fusions of endosome-derived vesicles may occur at many places in the cell and not just at synapses. However, TGN-derived vesicles, exemplified in our study by the CD8-GFP containing vesicles, would require the exocyst. It remains to be determined whether peptide-containing dense core vesicles, which fuse in a regulated manner like synaptic vesicles, but which, unlike synaptic vesicles, bud directly off of the TGN, can fuse with the membrane in the absence of Sec5. A recent study (Moskalenko et al., 2002) may point in this direction; inhibition of Ral function, which binds to the N terminus of Sec5, blocked the stimulated release of human growth hormone, contained in dense core vesicles, from PC12 cells.

After the run down of maternal contribution in *sec5* mutants, newly synthesized vesicles labeled with Synaptotagmin-GFP did not concentrate at the synapse. Though the boutons contained a large population of preexisting synaptic vesicles, as evidenced by immunostaining with the vesicle marker CSP and by the electrophysiology of the terminals, the boutons did not accumulate Synaptotagmin-GFP. We attribute this to the nature of the biosynthetic pathway for synaptic vesicles. The current model is that transport vesicles containing synaptic vesicle proteins fuse with the plasma membrane via the constitutive pathway of exocytosis from the TGN. Thereafter, they are internalized and sorted in endosomes to become mature synaptic vesicles (Calakos and Scheller, 1996; Hannah et al., 1999). This model is supported by studies following the movement of radioactively labeled synaptophysin in neuroendocrine cells (Regnier-Vigouroux and Huttner, 1993) and studies following Synaptotagmin with a luminal antibody in cultured neurons (Matteoli et al., 1992). It seems likely that in *sec5* mutants, transport vesicles have not fused with the membrane and are not matured into synaptic vesicles. Therefore, the Synaptotagmin-GFP, though transported down and visible in the axon, does not enter the pool of mature synaptic vesicles that are clustered in the bouton. The transport vesicles that fail to fuse with the plasma membrane likely remain in the axon or are sent retrogradely back to the soma.

Neurite outgrowth failed in the absence of Sec5 (Figure 4). This role for the exocyst complex was predicted from its localization to the tips of neurites in cultured hippocampal neurons (Hazuka et al., 1999) and from studies with a dominant-negative sec10 construct in cultured PC12 cells (Vega and Hsu, 2001). Neurite outgrowth requires a substantial increase in the surface area of the neuron and, not surprisingly, requires membrane addition. This membrane addition has been shown primarily to occur at the growth cone rather than in the soma or along the length of the neurite (Bray, 1970; Craig et al., 1995). Thus, as in yeast, the localization of the exocyst complex, both in *Drosophila* (Figure 4) and hippocampal cultures (Hazuka et al., 1999), is consistent with a role in targeting vesicles to the site of membrane addition.

The surface expression of CD8 in neuronal cell bodies and along the axon also was impaired by loss of Sec5. These defects in traffic to the cell surface have parallels in studies of the MDCK epithelial cell line. In MDCK cells,

the exocyst complex localizes to tight junctions between cells, which are thought to be the sites of membrane addition (Grindstaff et al., 1998). Antibodies to Sec8 block the delivery of proteins to the basolateral membrane, with little effect on apical membrane addition. With regard to the trafficking and sorting of proteins, parallels have been drawn between the neuronal axon and the apical domain of epithelial cells (Dotti and Simons, 1990; Jareb and Banker, 1998). In the case of Sec5, however, that equation would not appear to apply in that removing Sec5 affected surface expression of CD8 along the axon and in nerve terminals as well as in the soma. Alternatively, there may have been an exocyst dependence of apical transport in MDCK cells in the Grindstaff et al. (1998) study that was resistant to the blockade by the antibody protocol. The examination of polarized transport in the epithelia of the *sec5* mutants may clarify the question of the exocyst complex and apical transport.

Intriguingly, the motor neurons of *sec5* mutants failed to form new synaptic boutons after the maternal contribution of Sec5 was gone. This observation, and the apparent requirement for the exocyst complex in membrane addition but not synaptic transmission, suggests that sec5 and the exocyst complex may play a very particular role in synaptic plasticity. Anatomical plasticity, the formation of a distinct synaptic contact between a neuron and its targets, may require the exocyst protein to be targeted to the site from which the new branch must sprout. By recruiting new membrane and synaptic components to this area, the exocyst may permit the growth of a new synapse that will subsequently function independently of the exocyst. The identification of mutations in *sec5* and other components of the complex should permit tests of this hypothesis.

Experimental Procedures

***Drosophila* Stocks and Clones**
Sources of stocks: *yw; Df (2L)tim⁰²/CyO y+* (M. Young, Rockefeller); *yw; Df(2L)tim⁰²/CyO; P[Kpn]/TM2* (G. Bashaw and B. Baker, Stanford); *w; Sp/CyO; elav-Geneswitch* (T. Osterwalder and H. Keshishian, Yale); *yw; Pᵢₙ/CyO; UAS-mCD8-GFP* (Bloomington Stock Center); *UAS-Syt-GFP* (III) (Y. Zhang, Utah); *sec5ᴱ¹⁰* and *sec5ᴱ¹³* were recombined onto FRT40 chromosomes to yield: (1) *yw; FRT40 sec5ᴱ¹⁰/CyO y+*, (2) *yw; FRT40 sec5ᴱ¹⁰/CyO, Kr-Gal4, UAS-GFP*, (3) *yw; FRT40 sec5ᴱ¹³/CyO y+*, and (4) *yw; FRT40 sec5ᴱ¹³/CyO, Kr-Gal4, UAS-GFP*. Controls were *yw* unless otherwise indicated. Other stocks used were: for neuronal cultures, *yw; sec5ᴱ¹⁰, UAS-Tau-GFP/CyO y+; elav-Gal4* or *yw; UAS-Tau-GFP/CyO; elav-Gal4*; for the trafficking assay, (1) *w; FRT40 sec5ᴱ¹⁰/CyO, Kr-Gal4, UAS-GFP; UAS-CD8-GFP*, (2) *w; FRT40 sec5ᴱ¹⁰/CyO, Kr-Gal4, UAS-GFP; elav-Geneswitch*, and (3) *w; FRT40 sec5ᴱ¹⁰/Cyo, Kr-Gal4, UAS-GFP; UAS-Syt-GFP*.

Larvae were isolated by collecting eggs on grape caps with yeast paste for 2 hr and then raising at room temperature. At 24 hr AEL, homozygous mutant larvae were chosen by an absence of the GFP or y+ marker and transferred to fresh yeast paste until 48, 72, or 96 hr AEL. Control larvae were raised similarly. Genomic clones EC5 and EC8 and *sec5* cDNA were gifts from G. Bashaw and B. Baker.

Generation of Antibodies and Quantitative Immunoblotting
Three portions of the *sec5* cDNA were amplified by PCR and cloned into pGEX-KG. Full-length *sec5* was cloned into pMAL. GST and Maltose fusion proteins were insoluble in bacteria. After French-pressing, the insoluble pellet was resuspended in 8 M urea, 50 mM Tris (pH 8.0), 1% Triton X-100, 1 mM DTT, 300 mM NaCl, and prote-

ase inhibitors, homogenized, and then dialyzed against decreasing concentrations of Urea to 0 M overnight. The refolded, soluble fusion proteins were purified over glutathione or amylose beads, concentrated in a centricon chamber (Millipore), and GST fusions were injected into rabbits and mice. Bleeds from the mice were saved and used as polyclonal antibodies (5AL and 1RN), before their spleens were used to generate monoclonal antibodies. Monoclonals were screened by ELISA, against the pMAL-Sec5 fusion protein, and retested on immunoblots of larval proteins from control and *sec5* mutants. Three monoclonal lines were injected into mice to generate ascites fluid. 5AL and 1RN were used at 1:300 on immunoblots, anti-Actin (ICN Biomedicals) at 1:1000, and supernatant from monoclonal line 22A2 at 1:2.

To quantitate Sec5 levels, larvae were homogenized in 2% SDS, 5% βME, 6 M urea, and 125 mM Tris (pH 6.8). Immunoblots were probed with 5AL and anti-Actin and Alexa Fluor 680 goat anti-mouse (Molecular Probes) at 1:5000 and scanned (Odyssey, Li-Cor). Bands were quantitated by infrared detection in the linear range. Pixel intensities were averaged for Sec5 bands from three separate experiments and normalized to an unrelated band on the same blot.

Immunohistochemistry
Larvae, attached to Sylgard with Nexaband glue (Veterinary Products Laboratories), were dissected in HL-3 media (see below) with pulled-glass dissecting needles and subsequently fixed with 3.7% formaldehyde in 100 mM potassium phosphate (pH 6.8), 450 mM KCl, 150 mM NaCl, 20 mM MgCl₂. Cultured cells were fixed in 3.7% formaldehyde in PBS with 120 mM sucrose. Monoclonal anti-Sec5 (22A2) ascites was used at 1:200, anti-CSP (gift of K. Zinsmaier, U. Penn) at 1:25, rat monoclonal anti-CD8α (CALTAG Laboratories) at 1:100, and Cy5-conjugated goat anti-HRP (Jackson Immunoresearch) at 1:200. Secondary antibodies (Jackson Immunoresearch) used were FITC-conjugated goat anti-mouse (1:100) and Cy3-conjugated goat anti-mouse (1:400).

Electrophysiology
Recordings were made from muscle 6 in either segment A2 or A3 from first instar larvae dissected as described above and treated with 0.5 mg/ml collagenase Type IV (SIGMA) for 1 min. Patch clamp recordings in whole-cell configuration were performed in HL-3 solution (70 mM NaCl, 5 mM KCl, 11 mM MgCl₂, 1.8 mM CaCl₂, 10 mM NaHCO₃, 5 mM Trehalose, 115 mM Sucrose, and 5 mM HEPES [pH 7.2]), and 1 μm TTX was added for mini EJC recordings. Intracellular solution contained 120 mM KCl, 4 mM MgATP, 5 mM HEPES, 5 mM EGTA, 36 mM Sucrose, 0.25 mM CalCl₂, 20 mM KOH (pH 7), and patch electrodes were pulled from capillary glass (ID 1.00 mm, OD 1.50 mm) with resistances of 5–15 MΩ. An Axopatch 1C amplifier (Axon Instruments) was used to hold muscles cells at −60 mV (leak current <100 pA). Stimulating electrodes were placed in the ventral nerve cord just anterior to the appropriate segmental nerve. Amplifier gain and frequency were telegraphed to the Digidata 1322A, and data were acquired using pCLAMP v8.2 (Axon Instruments). Analysis was carried out in pCLAMP or MINI ANALYSIS (Synaptosoft).

Statistics and Quantal Content Calculation
All p values reported in this study are two-tailed values and derived from a Student's t test, assuming unequal variances. To calculate quantal content, the average evoked amplitude (A), for each age and genotype, was divided by the average mini amplitude (B). Standard error of the mean on A/B was calculated as $1/B$(standard error on A) + A/B^2(standard error on B). Standard error of the mean for quantal content per bouton was calculated similarly. We determined if quantal content values were significantly different by finding values for t' and df according to the following formulas: t' = (quantal content$_1$ − quantal content$_2$)/SQRT(variance$_1^2$/n$_1$ + variance$_2^2$/n$_2$) and df = (variance$_1^2$/n$_1$ + variance$_2^2$/n$_2$)^2/((variance$_1^2$/n$_1$)^2/n$_1$ − 1 + (variance$_2^2$/n$_2$)^2/n$_2$ − 1). From t' and df we were able to calculate a two-tailed p value.

Neurite Outgrowth Assay
Culturing of first instar neurons was adapted from Wu et al. (1983). Brain and ventral nerve cord complexes were rinsed in 70% EtOH,

ddH20, and dissecting media (27% Leibovitz, 73% saline, 50 units/ml pennicilin, 50 μg/ml streptomycin). With Sigmacoted (SIGMA) glass pipettes, complexes were transferred to saline (no added Mg^{2+} or Ca^{2+}) with collagenase (0.5 mg/ml) and incubated for 45 min, washed, and then triturated in culture media (dissecting media with 10% HI-FBS) to disperse into single cells. 200 μl of cell suspension were plated in 8-well chamber slides (Lab-Tek) that had been coated with poly-L-lysine (SIGMA). Cultures were maintained at 25°C for 24 hr. Fixed cells were stained with Hoechst 33342 (1:4000) and imaged on a Zeiss LSM 510 microscope. Cells expressing Tau-GFP under the *elav* promoter were scored as neurons, and only those with a healthy nucleus were counted in our assay. Cells were scored for the presence or absence of neurites through the eye piece, and the lengths of any neurites were measured using the LSM 510 software.

Trafficking Assay

Appropriately staged larvae were fed 0.5 mM RU486 (mifepristone, SIGMA) dissolved in wet yeast paste, mixed with instant fly food and sucrose. Confocal data were acquired as image stacks of multitracked, separate channels with a Zeiss LSM 510 microscope. Identical gain, offset, pinhole, and laser settings were used for mutant and control. For quantification of total pixel intensity in cell bodies of sensory neurons, a region of interest was drawn around the cell body located in Cy5 channel, and the sum of pixel intensities for that ROI calculated for each of three channels, Cy5 (HRP), Cy3 (CD8), and GFP. An equal area of background was subtracted to obtain the final values. For sensory axons and segmental nerves, several ROIs were drawn along portions of the axons and nerves, and total pixel intensity values for each channel, with background subtraction, were summed. For quantification of pixel intensity at synaptic boutons of the NMJ, individual boutons were selected at random using either CSP(Cy3) or HRP(Cy5) immunofluorescence, and total pixel intensity values for each channel, with background substraction, were averaged.

Acknowledgments

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