

A *Drosophila* *SNAP-25* Null Mutant Reveals Context-Dependent Redundancy With *SNAP-24* in Neurotransmission

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ABSTRACT

The synaptic protein SNAP-25 is an important component of the neurotransmitter release machinery, although its precise function is still unknown. Genetic analysis of other synaptic proteins has yielded valuable information on their role in synaptic transmission. In this study, we performed a mutagenesis screen to identify new *SNAP-25* alleles that fail to complement our previously isolated recessive temperature-sensitive allele of *SNAP-25*, *SNAP-25^s*. In a screen of 100,000 flies, 26 F₁ progeny failed to complement *SNAP-25^s* and 21 of these were found to be null alleles of *SNAP-25*. These null alleles die at the pharate adult stage and electroretinogram recordings of these animals reveal that synaptic transmission is blocked. At the third instar larval stage, *SNAP-25* nulls exhibit nearly normal neurotransmitter release at the neuromuscular junction. This is surprising since *SNAP-25^s* larvae exhibit a much stronger synaptic phenotype. Our evidence indicates that a related protein, SNAP-24, can substitute for SNAP-25 at the larval stage in *SNAP-25* nulls. However, if a wild-type or mutant form of SNAP-25 is present, then SNAP-24 does not appear to take part in neurotransmitter release at the larval NMJ. These results suggest that the apparent redundancy between *SNAP-25* and *SNAP-24* is due to inappropriate genetic substitution.

SYNAPTIC transmission requires the fusion of neurotransmitter-filled vesicles with the presynaptic membrane. The protein machinery responsible for this process includes a core set of proteins, the SNARE proteins (reviewed in JAHN and SUDHOF 1999; LIN and SCHELLER 2000), which form an extremely stable complex shown to be sufficient to promote membrane fusion (WEBER *et al.* 1998). This complex is formed by the vesicle protein VAMP or synaptobrevin (TRIMBLE *et al.* 1988) and the presynaptic plasma membrane proteins syntaxin (BENNETT *et al.* 1992) and SNAP-25 (OYLER *et al.* 1989). SNARE proteins also mediate the fusion of transport vesicles at each stage of the secretory pathway in eukaryotic cells (reviewed in WICKNER and HAAS 2000; PELHAM 2001).

A variety of techniques have helped elucidate the characteristics of these proteins. Structural analyses using fluorescence resonance energy transfer (LIN and SCHELLER 1997), electron paramagnetic spectroscopy (POIRIER *et al.* 1998), and X-ray crystallography (SUTTON *et al.* 1998) have shown that the SNARE complex is composed of four parallel amphipathic helices stabilized by hydrophobic interactions. Clostridial toxins, which cleave the SNARE proteins, have shown the requirement for SNAP-25, syntaxin, and synaptobrevin in vesicle fusion (re-

viewed in SCHIAVO *et al.* 2000), and antibody inhibition studies have demonstrated that efficient fusion is dependent on the function of SNAP-25 (LOW *et al.* 1999; XU *et al.* 1999). Genetic perturbation of these proteins has done a great deal to show the physiological effects of mutating or ablating components of the SNARE complex. For example, genetic disruption of syntaxin and synaptobrevin demonstrates that these proteins are essential for evoked synaptic transmission (SCHULZE *et al.* 1995; SWEENEY *et al.* 1995; DEITCHER *et al.* 1998; NONET *et al.* 1998; SAIFEE *et al.* 1998; RAO *et al.* 2001; SCHOCH *et al.* 2001).

In a previous study, we had isolated a recessive temperature-sensitive paralytic mutation in the *Drosophila* *SNAP-25* gene, *SNAP-25^s*, which has two distinct effects on synaptic transmission (RAO *et al.* 2001). At the permissive temperature (22°), evoked release at the third instar larval neuromuscular junction (NMJ) in *SNAP-25^s* homozygotes or hemizygotes is increased twofold, and spontaneous vesicle fusion frequency is increased by a factor of six. At the restrictive temperature (37°), evoked release is reduced by 60% in *SNAP-25^s* larvae. In mice, genetic ablation of *SNAP-25* results in embryonic lethality, a heightened rate of spontaneous vesicle fusion in some synapses, and a block in evoked neurotransmitter release (WASHBOURNE *et al.* 2002). To further investigate the function of the SNAP-25 protein, in this study we performed a screen to isolate new alleles of *SNAP-25* using the existing *SNAP-25^s* allele. Using ethyl methanesulfonate (EMS)- and X-ray-induced mutagenesis we screened >100,000 chromosomes in a large-scale F₁ tem-

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perature-sensitive paralytic screen. We obtained 26 new lines that exhibited temperature-sensitive paralysis in combination with *SNAP-25^{ts}*. Surprisingly, many of these lines are homozygous viable through the larval stage and die only as pharate adults. Western blot analysis revealed that these lines lack any detectable SNAP-25 protein. Nevertheless, electrophysiological recordings from the NMJ of third instar larvae of *SNAP-25* nulls revealed that neurotransmitter release was almost indistinguishable from wild type and was surprisingly much more normal than that of *SNAP-25^{ts}*. *SNAP-25* null mutants do not die until the pharate adult stage, at which point electroretinogram recordings revealed a failure of synaptic transmission.

A substantial body of evidence points to a crucial role for SNAP-25 in vesicle fusion. Our observations therefore suggest that another, related protein may substitute for the function of SNAP-25 at the larval stage in *SNAP-25* null larvae. However, if a redundant SNAP-25 isoform in larvae is capable of supporting normal synaptic function, then we should not expect a recessive point mutation in the *SNAP-25* gene to exhibit any phenotype. Nevertheless, the *SNAP-25^{ts}* point mutant shows a dramatic effect on synaptic transmission. One explanation for this may be found in the phenomenon of inappropriate genetic substitution (MADHANI and FINK 1998). In this situation, two related protein isoforms perform similar functions in distinct biochemical pathways. Normally, an individual isoform does not participate in the other pathway, and so hypomorphic mutations disrupting each protein show a distinct phenotype. However, a null allele that completely abolishes the expression of one protein could allow an isoform to step in and compensate for it. In this way, a null allele of a gene may paradoxically show a phenotype that is much weaker than that of a hypomorphic allele of that gene.

To date, few examples of this phenomenon have been described. Here, we report that SNAP-24 (NIEMEYER and SCHWARZ 2000), a protein closely related to SNAP-25, is present at third instar larval NMJs and within the larval and adult central nervous systems (CNSs). SNAP-24 can, like SNAP-25, form primary (NIEMEYER and SCHWARZ 2000) and higher-order SNARE complexes *in vitro* with syntaxin and neuronal-synaptobrevin (N-syb; DIANTONIO *et al.* 1993). In the *SNAP-25* null background, SNAP-24 levels appear to be sufficient to sustain normal synaptic transmission. However, endogenous SNAP-24 levels appear to be insufficient to rescue the *SNAP25^{ts}* phenotype. Only by the overexpression of SNAP-24 can the phenotype of *SNAP25^{ts}* be suppressed. From these data we conclude that SNAP-24 can substitute for SNAP-25 in neurotransmitter release in a *SNAP-25* null background. However, SNAP-24 does not appear to play a significant role in synaptic transmission if a sufficient level of SNAP-25 is in neurons, even in a mutant form, as in the *SNAP-25^{ts}* allele. We suggest that SNAP-24's ability to functionally replace SNAP-25 at the

larval stage is an example of inappropriate genetic substitution.

MATERIALS AND METHODS

Mutagenesis and rescue constructs: To generate new alleles of *SNAP-25*, we performed an F₁ temperature-sensitive paralytic screen using a previously isolated recessive temperature-sensitive mutant, *SNAP-25^{ts}* (RAO *et al.* 2001). *red ebony* (*red e*) flies were made isogenic as described in WATSON *et al.* (2001). Two rounds of mutagenesis, one using EMS and the other X rays, were performed on a total of >100,000 chromosomes. For EMS, 1000 isogenic *red e* males were fed a sucrose solution containing 25 mM EMS overnight. These male flies were then mated *en masse* to *SNAP-25^{ts}* virgin females to produce ~60,000 F₁ progeny. For X ray, 3000 isogenic *red e* males were exposed to 3000 rad from a cesium source and mated as above.

F₁ progeny were tested for temperature sensitivity at 38° as described in WATSON *et al.* (2001). Briefly, ~5000 flies were placed in a prewarmed clear plastic box and the whole box was placed into a 38° incubator for 7 min. The box was tilted and tapped so that any flies that were unconscious on the bottom of the box slid through an opening leading to a reservoir chamber. The box was then inverted to trap the temperature-sensitive flies in the smaller compartment and filled with CO₂, rendering all remaining flies unconscious. The flies in the reservoir were collected and tested for the ability to regain consciousness to ensure that they were not dead and were retested for temperature sensitivity to eliminate any false positives. All flies believed to be mutant were crossed to *TM6B, Tb/Sb* virgin females. From the above cross, stocks balanced with *TM6B, Tb* were generated, and the lines were again crossed to verify that they were temperature sensitive in combination with *SNAP-25^{ts}*. Each line was also tested for lethality with *Df(3L)1-16*, the deficiency that removes *SNAP-25*.

A SNAP-25 rescue line consisted of a *P* insertion containing the SNAP-25 open reading frame driven by the *n-syb* promoter on the X chromosome and *Df(3L)1-16/TM6B, Tb* on the third chromosome (RAO *et al.* 2001). A line expressing SNAP-24 under control of the heat-shock promoter on the second chromosome (generous gift of Barbara Niemeier) was also placed in the background of *Df(3L)1-16/TM6B, Tb* on the third chromosome.

Immunocytochemistry: An antibody specific to the N terminus of SNAP-25, which does not recognize SNAP-24, was used at 1:200 (RAO *et al.* 2001). A peptide was synthesized to a sequence that is identical in SNAP-24 and SNAP-25 (with an additional C-terminal cysteine for coupling purposes) and corresponds to a region in exon 4 of *SNAP-25* (sequence KEAGIRTLVALDDC; Bethyl Laboratories, Montgomery, TX). The exon 4 peptide was coupled to KLH using the Sulfolink kit (Pierce, Rockford, IL) and injected into a guinea pig (Pocono Rabbit Farms, Canadensis, PA). The antiserum, designated Exon 4, was affinity purified to the immobilized peptide and used at a 1:200 dilution.

Dilutions for other antibodies were as follows: CSP, 1:100 (mouse monoclonal, a generous gift of Konrad Zinsmaier); syntaxin, 1:50 (antibody 8C3 from the Developmental Studies Hybridoma Bank); and synaptotagmin, 1:1000 (rabbit polyclonal, a generous gift of Hugo Bellen). Secondary antibodies for tissue were goat anti-rabbit, goat anti-mouse, and donkey anti-guinea pig conjugated to AlexaFluor 594 (red) or 488 (green; Molecular Probes, Eugene, OR); each was used at a 1:500 dilution. Third instar larvae were dissected in HL3 medium (STEWART *et al.* 1994) and fixed in 3.5% paraformaldehyde in PBS overnight at 4°. Larvae were washed five times for 30 min in PBT (PBS + 0.2% Triton X-100), blocked for

1 hr in 5% normal donkey serum or 5% normal goat serum, and incubated in primary antibody overnight at 4°. They were then washed five times for 15 min in PBT + 1% BSA, incubated in secondary antibody for 5 hr, washed five times for 15 min, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Larval neuromuscular junctions were visualized at $\times 400$ on an MRC-600 confocal microscope (Bio-Rad, Richmond, CA), and larval CNSs were visualized at $\times 100$ on an E600FN microscope (Nikon, Melville, NY) fitted with a Spot2 CCD camera (Diagnostic Instruments, Sterling Heights, MI). Experimental and control images were taken with identical camera settings.

For the pharate adult CNS, 4-day-old *SNAP-25* null pupae were screened to select animals whose cuticle had just begun to tan. Pharate adults were removed from the pupal case and the proboscis, antennae, and surrounding cuticle were removed from the head in adult Ringer solution (KAWASAKI *et al.* 1998) and fixed overnight at 4° in nonalcoholic Bouin's. Fixed heads were washed in Tris-buffered saline, cryoprotected in phosphate-buffered saline with 25% sucrose overnight, and then equilibrated in Tissue-Tek medium (Sakura Finetek, Torrance, CA). The heads were then oriented in fresh Tissue-Tek in aluminum molds, frozen in dry ice, cryosectioned at 15 μm onto gelatin-coated slides, and stored at -80° . For antibody staining, the sections were thawed and rehydrated with PBT, blocked for 2 hr in PBT with 5% donkey and 5% goat serum, and incubated in a primary antibody in PBT with either 1% goat or 1% donkey serum, depending on the secondary antibody used. The slides were then washed five times for 15 min in PBT + 1% BSA and incubated in secondary antibody overnight at 4°, washed in PBS, spread with Vectashield, and coverslipped. Tissue was visualized at $\times 400$ as described for the larval CNS.

Electrophysiology: Two-electrode voltage clamp on the third instar larval NMJ was performed as described (STEWART *et al.* 1994). Larvae examined were of the genotypes *red e* (parental control), *Df(3L)1-16/TM6*, *Ubx*, *y*⁺ (deficiency control), *SNAP-25²⁴/Df(3L)1-16* (null), and *SNAP-25²⁴/Df(3L)1-16* containing the *SNAP-25* rescue construct on the X chromosome. Wandering third instar larvae were dissected at room temperature in HL3 saline without calcium and bathed in calcium-containing saline for recording. Traces shown were recorded in 1 mM calcium saline, and each genotype represents between 7 and 10 animals with at least two stable cells recorded from each. Electrode resistances were 15–20 M Ω when filled with 3 M KCl and a holding potential of -80 mV was used. Data were digitized at 10 kHz and low-pass filtered at 2 kHz for excitatory junctional currents (EJCs) or 800 Hz for miniature excitatory junctional currents (mEJCs) and acquired with pClamp8 software (Axon Instruments, Foster City, CA). For evoked release, amplitude in a given cell was an average of 10 events evoked at 0.05 Hz. Data were analyzed for amplitude and, for mEJCs, for frequency using Clampfit software (Axon Instruments, Foster City, CA). Rescue of the *SNAP-25^s* electrophysiological phenotype with the *SNAP-24* protein was accomplished by crossing *hs-SNAP-24; Df(3L)1-16/TM6B, Tb* to a *SNAP-25^s* homozygous line. Evoked potentials at 22° and 37° were recorded as described by RAO *et al.* (2001).

Electroretinograms (ERGs) were performed as described by WATSON *et al.* (2001). Mutant pharate adults were manually removed from the pupal case for the recordings, and only animals that exhibited pumping of the head capsule (indicating appropriate maturity) were used. Control animals of the same age (*Df(3L)1-16/TM6B, Tb*) were chosen: these either were manually removed from the puparium (at which point they typically began to move about) or were allowed to eclose on their own. Mutant pharate adults required no restraints for ERG recording and were placed directly on sections of glass coverslip, while control animals were immobilized with wax.

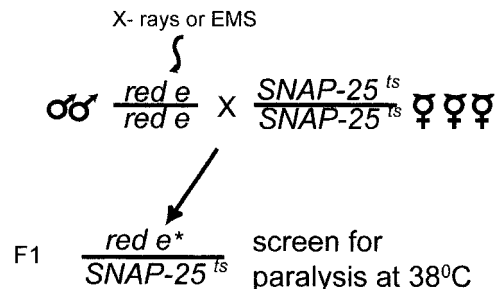


FIGURE 1.—Diagram of the mutagenesis scheme using X rays and EMS. Mutagenized *red e* males were mated *en masse* to virgins homozygous for the *SNAP-25^s* mutation. Male and female F₁ offspring that failed to complement the *SNAP-25^s* phenotype were selected and crossed to a balancer line and stable stocks were established for further analysis.

Western blotting and Coomassie staining: Western blots of *SNAP-25* mutant lines were probed with the *SNAP-25*-specific antibody at a 1:1000 dilution (RAO *et al.* 2001). Relative levels of *SNAP-24* and *SNAP-25* in various tissues were examined by dissecting larvae in calcium-free HL3 saline and then separating and washing each set of organs. Nonneuronal larval tissues were ground in solubilization buffer (50 μl of 10% SDS, 0.2 M Tris base, 20% glycerol) and boiled, and the total protein concentration for each sample was measured using the BCA assay (Pierce). To the remaining sample, dithiothreitol was added to 0.2 M and bromphenol blue was added to 0.1%. A total of 10 μg was loaded in each lane. For larval CNS and pharate adult head samples, animals were raised at 22° and dissected at the appropriate stage. Five larval brains or two pharate adult heads were ground in 25 μl of the above solubilization buffer containing 0.2 M dithiothreitol and boiled. Ten microliters of each of the above samples was loaded per lane of a 15% polyacrylamide gel, run according to standard SDS-PAGE protocols, and blotted on an Immobilon-P PVDF membrane (Millipore, Bedford, MA). Blots were probed with Exon 4 antibody (1:2000 dilution), developed with chemiluminescent reagents (ECL kit, Amersham Pharmacia Biotech, Buckinghamshire, UK), and exposed to BioMAX ML film (Kodak, Rochester, NY). Equal portions of each sample were run, blotted, and probed with an antibody against tubulin (1:2000 dilution, antiserum E7 from the Developmental Studies Hybridoma Bank) to ensure that all samples compared contained equal amounts of total protein.

The *in vitro* SNARE complex formation assay was performed as described by RAO *et al.* (2001). Recombinant proteins of *Drosophila* neuronal-synaptobrevin (N-syb, minus the transmembrane domain); syntaxin (minus the transmembrane domain); and *SNAP-24*, or *SNAP-25^s* were mixed (10 μM each) in 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% Tween-20, 5 mM dithiothreitol, and 2 mM EDTA and incubated for 20 hr at 4°. After incubation, samples were mixed with an equal volume of sample loading buffer (10% glycerol, 1.34% SDS, 62 mM Tris-Cl (pH 7.5), 0.17 M β -mercaptoethanol) and separated by SDS-PAGE at 4°. Gels were stained with Coomassie blue and scanned and bands were quantitated by ImageQuant 5.0 (Molecular Dynamics, Sunnyvale, CA). Amount of complex formation was calculated from four independent experiments and one-way ANOVA was used for statistical comparisons. Controls lacking individual proteins failed to produce SNARE complexes and higher-order complexes, and boiling the samples likewise abolished all complexes formed (data not shown).

TABLE 1
New mutant lines failing to complement *SNAP-25^{ts}*

Mutant line	Mutagen	Homozygous lethal period	Lethal period over <i>Df(3L)1-16</i>	Rescued by <i>SNAP-25</i> transgene
1-16-0	X ray	Early	Pharate adult	Yes
3	X ray	Early	Early	No
14	X ray	Pharate adult	Pharate adult	Yes
16	X ray	Early	Pharate adult	Yes
22	X ray	Early	Pharate adult	Yes
27	X ray	Early	Pharate adult	Yes
33	X ray	Early	Early	No
34	X ray	Early	Pharate adult	Yes
42	X ray	Early	Pharate adult	Yes
44	X ray	Pharate adult	Pharate adult	Yes
46	X ray	Pharate adult	Pharate adult	Yes
48	X ray	Pharate adult	Pharate adult	Yes
52	X ray	Pharate adult	Pharate adult	Yes
53	X ray	Early	Early	No
70	X ray	Early	Pharate adult	Yes
87	X ray	Pharate adult	Pharate adult	Yes
93	X ray	Early	Early	No
114	X ray	Pharate adult	Pharate adult	Yes
124	X ray	Pharate adult	Pharate adult	Yes
125	X ray	Pharate adult	Pharate adult	Yes
126	X ray	Pharate adult	Pharate adult	Yes
132	X ray	Pharate adult	Pharate adult	Yes
133	X ray	Pharate adult	Pharate adult	Yes
147	X ray	Pharate adult	Pharate adult	Yes
153	X ray	Early	Early	No
164	X ray	Pharate adult	Pharate adult	Yes
695	EMS	Early	Pharate adult	Yes

Fourteen mutant lines are homozygous viable to the late pupal (pharate adult) stage; 13 are homozygous lethal at a stage prior to the third instar larva (early), but are viable to late pupae over the deficiency that removes *SNAP-25*, *Df(3L)1-16*. All 22 lines that survive as hemizygotes to the late pupal stage were rescued by expression of a *SNAP-25* transgene. Five lines are lethal as hemizygotes at a stage prior to third instar larvae and all five were not rescued by expression of *SNAP-25*. Note: line 1-16-0 was recovered from an earlier screen in which lethal X-ray alleles were recovered *in trans* with *Df(3L)1-16*.

RESULTS

Generation and identification of *SNAP-25* null mutants: The objective of our mutagenesis scheme was to produce hypomorph or null alleles of *SNAP-25* that fail to complement our previously characterized recessive temperature-sensitive allele, *SNAP-25^{ts}* (RAO *et al.* 2001). Our screen was designed so that a large number of flies could be screened, but this allowed for the possibility of recovering multiple siblings containing the same mutant chromosome within a batch of progeny (Figure 1). The EMS screen produced only one line that was temperature-sensitive paralytic in combination with the *SNAP-25^{ts}* allele, possibly because the *SNAP-25* gene consists of a relatively short coding sequence of eight exons distributed over 120 kb of genomic DNA (RISINGER *et al.* 1997). This expansive genomic structure, however, potentially made the *SNAP-25* gene a good mutagenesis target for X rays. This prediction was proven correct, as the X-ray screen produced 25 potential *SNAP-25* mutant

lines (Table 1). We also identified an additional X-ray allele (1-16-0, a generous gift of Barry Honda) generated in a previous screen (SCHULZE *et al.* 2001).

Of 27 candidate lines, 13 were homozygous lethal at a stage prior to third instar larvae and 14 were viable until the late pupal stage, at which point the homozygous flies failed to emerge from the pupal case and died. Crossing the 13 lines that died as homozygotes at an earlier stage to *Df(3L)1-16*, a deficiency that removes *SNAP-25* (MARCHANT and HOLM 1988; RAO *et al.* 2001), revealed that eight of these alleles were viable over the deficiency until the late pupal stage. This suggests that the prepupal lethality of some lines was due to unrelated second-site lethal hits and that the actual lethal period of *SNAP-25* mutations is the pharate adult stage.

To identify those lines that contained a mutation only in *SNAP-25*, we sought to rescue the lethality of the potential mutants. We therefore crossed each of the potential mutant lines to a line containing a neurally

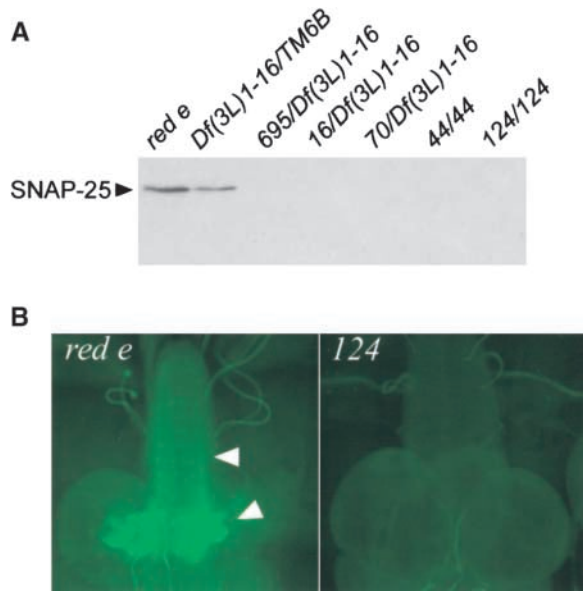


FIGURE 2.—Western blot and immunocytochemical analysis of *SNAP-25* mutant lines using the *SNAP-25*-specific antibody. (A) Western blot of pharate adult head extracts from the parental *red e* strain, the strain heterozygous for *Df(3L)1-16* that removes *SNAP-25*, and five representative mutant lines of the indicated genotypes. Each lane represents extract from 10 heads; the equivalent of 1 head was loaded per lane. A band corresponding to the *SNAP-25* protein appears at ~30 kD in the *red e* lane. The sample heterozygous for *Df(3L)1-16* shows ~50% reduction in band intensity, and all potential *SNAP-25* mutant lines show a complete absence of *SNAP-25* immunoreactivity. (B) Immunocytochemical staining of *red e* and a *SNAP-25* mutant larval CNS from *SNAP-25*¹²⁴. The *SNAP-25*¹²⁴ line shows a complete lack of staining while the *red e* line shows strong staining in synaptic regions of the brain lobes and the ventral ganglion, indicated by arrowheads.

expressed *SNAP-25* transgene and the *Df(3L)1-16* chromosome. We examined the hemizygous progeny containing the transgene for lethality. Of the 27 mutant lines, 22 were rescued with the neurally expressed *SNAP-25* transgene, including all 14 lines that were viable until the late pupal stage as homozygotes. Therefore, these 14 new lines contain mutations solely in *SNAP-25*, albeit some may be duplicates due to the design of our screen. Four of the 5 lines that failed to be rescued by the transgene (lines 3, 33, 53, and 153) were complementation tested to other lethal complementation groups uncovered by *Df(3L)1-16*. All of them failed to complement one or more surrounding complementation groups, indicating that these 4 lines contain deletions that remove *SNAP-25* and additional essential genes in the region.

To characterize the nature of the *SNAP-25* mutant lines, we subjected protein extracts from mutant late pupal heads to SDS-PAGE and Western blotting and probed with a *SNAP-25*-specific antibody (RAO *et al.* 2001). Surprisingly, all potential *SNAP-25* mutant lines showed a complete loss of *SNAP-25* immunoreactivity (several representative lines are shown in Figure 2A).

Therefore, in each case, the mutations induced resulted in a null allele, which failed to produce any detectable *SNAP-25* protein. Immunocytochemistry using this antibody on larvae of *SNAP-25* mutant lines likewise showed a complete loss of *SNAP-25* immunoreactivity (Figure 2B). Since these lines showed a complete lack of detectable *SNAP-25* protein, we defined them as protein null alleles. We selected one allele, 124, which was homozygous viable to the pharate adult stage and was rescuable with the *SNAP-25* transgene, for more detailed analysis. We called this *SNAP-25* null allele *SNAP-25*¹²⁴.

Behavior of *SNAP-25* nulls: *SNAP-25* null larvae seem to behave normally; they can crawl, feed, and withdraw upon prodding. Pupal development proceeds normally in these mutants, and pharate adults exhibit a beating heart, tanning of the cuticle, and some weak pumping of the head capsule at the stage in which control animals begin eclosing from the puparium and, when freed, they are capable of slight twitching movements with the forelegs and palps. They are incapable of inflating their wings and may survive for up to 18 hr if kept in a moist enclosed chamber at 22°.

Third instar larval electrophysiology: We investigated the effects of abolishing *SNAP-25* expression on synaptic physiology at the well-characterized third instar larval neuromuscular junction. To our surprise, the size of EJCs was not affected by the lack of *SNAP-25* protein (Figure 3, A and B). This indicates that regulated neuronal exocytosis proceeds normally in these mutants. The calcium dependence of EJCs was likewise unchanged in mutant larvae (data not shown). We then examined the size and frequency of spontaneous mEJCs corresponding to spontaneous fusion of individual synaptic vesicles. While the size and shape of mEJCs was normal in the mutant larvae, the mEJC frequency was ~50% of normal (Figure 3, C and D). Although mild, this phenotype could be rescued by expression of a *SNAP-25* transgene and is likely to be due directly to the lack of *SNAP-25* protein. The grossly normal behavior of these larvae thus reflects the basically normal synaptic physiology of the *SNAP-25* null mutants. A second mutant line, 44 (Table 1), was assayed in the same way, with identical results (data not shown). Therefore, we concluded that all *SNAP-25* null lines are likely to be phenotypically equivalent.

Distribution and relative levels of *SNAP-25* and *SNAP-24* proteins: What could account for the unexpectedly mild effects of abolishing *SNAP-25* at the larval stage? Since *SNAP-25* is thought to be a crucial component of the exocytosis machinery and since these mutants die just prior to eclosion, we reasoned that another related protein, *SNAP-24* (NIEMEYER and SCHWARZ 2000), may be substituting for *SNAP-25* function in the larvae. Although this previous study did not show a specific concentration of *SNAP-24* in synaptic areas, it is possible

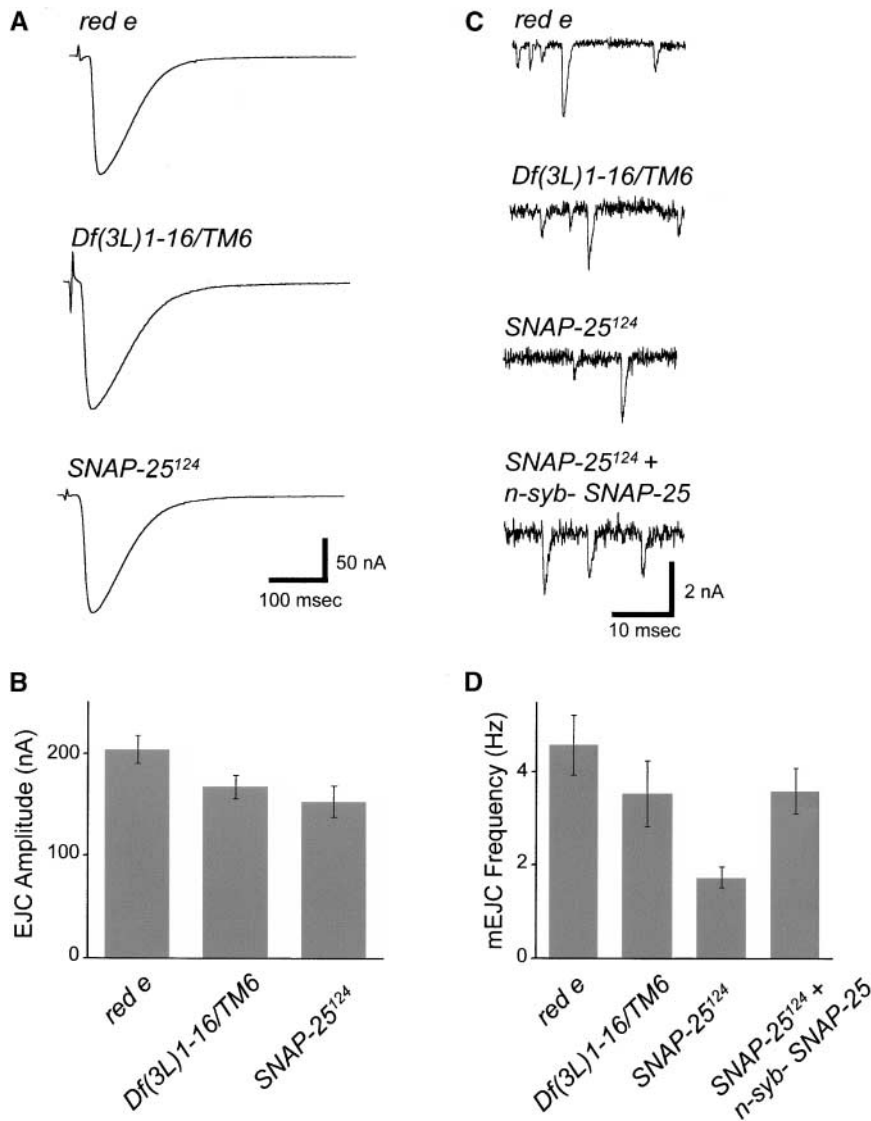


FIGURE 3.—Voltage clamp recording of evoked and spontaneous release at the muscle 6-7 NMJ in third instar control and mutant larvae. (A) Traces of evoked EJCs from the indicated genotypes. (B) No statistical difference was seen in EJC amplitude between mutant and control larvae. (C) Traces of spontaneous mEJCs from the indicated genotypes show that mutant NMJs exhibit 50% reduction in the normal rate of spontaneous vesicle exocytosis, which can be rescued by expression of a *SNAP-25* transgene. (D) Plotting mEJC frequencies shows that, while there is no significant difference between *red e* and *Df(3L)1-16/TM6* animals, *SNAP-25¹²⁴* larvae had mEJC frequencies ~50% of normal. Expression of a *SNAP-25* transgene rescues this mEJC frequency to near-normal levels.

that the antiserum used in that study may not have been sensitive enough to detect SNAP-24 in those regions.

We therefore examined the distributions of SNAP-24 and SNAP-25 in mutant and control animals using an antibody raised against a peptide sequence from exon 4 of SNAP-25 that is identical in the SNAP-25 and SNAP-24 proteins. The Exon 4 antibody recognizes both proteins equally when used on a Western blot containing lanes of equal amounts of SNAP-25 and SNAP-24 (Figure 4A). Western blot analysis of different tissues in larvae (Figure 4, B and C) shows that, while SNAP-25 is specifically neuronal (Figure 4C), SNAP-24 is found in all tissues examined except the larval gut (Figure 4B). In fact, SNAP-24 is found at relatively high levels within the CNS of both control and mutant animals. In the larvae, the level of SNAP-24 in the nervous system is at least as high as that of SNAP-25 (Figure 4C). This ratio shifts during metamorphosis, so that pharate adult heads express more SNAP-25 than SNAP-24 (Figure 4C). We saw no clear evidence that levels of SNAP-24

in mutant animals are upregulated to compensate for the lack of SNAP-25.

We also probed the larval CNS and neuromuscular junctions for immunoreactivity to the Exon 4 antibody (Figure 5). Staining in *SNAP-25* null larvae revealed that SNAP-24 is indeed found within synaptic boutons at the neuromuscular junction and in synaptic regions of the CNS (Figure 5, C and G). The pattern of staining in *SNAP-25* nulls is similar to that of controls, but control larvae show greater staining intensity due to the presence of both SNAP-24 and SNAP-25. Due to the fact that SNAP-24 is also present in larval muscle, detection of SNAP-24 in synaptic boutons required confocal microscopy to separate out the signal in muscle from that in boutons.

To see if elimination of SNAP-25 led to a change in expression or redistribution of other presynaptic proteins, we stained *SNAP-25¹²⁴* larvae with antisera to the synaptic vesicle proteins syntaxin and synaptotagmin. The staining intensities and distributions of these pro-

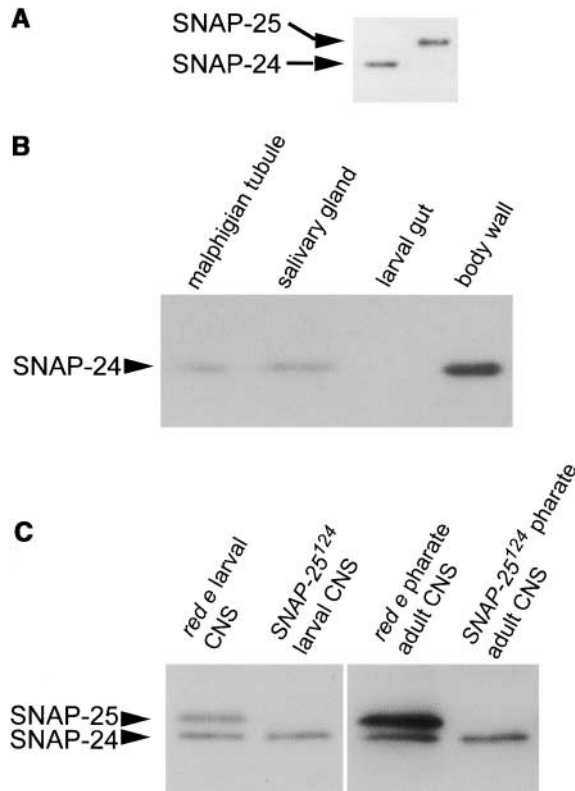


FIGURE 4.—Developmental expression of SNAP-24 and SNAP-25 as revealed by Western blot. (A) The Exon 4 antibody, which was raised against an epitope identical in SNAP-24 and SNAP-25, recognizes SNAP-24 and SNAP-25 equally on Western blots. Equal amounts of recombinant SNAP-24 and SNAP-25 were run side by side, probed with the antibody, and visualized by enhanced chemiluminescence. (B) Ten micrograms of total protein extracted from different nonneuronal tissues of control (*red e*) third instar larvae was probed with Exon 4 antiserum. SNAP-24 expression is seen in all tissues except gut and is higher in the body wall muscle than in other nonneuronal larval tissues. SNAP-25 expression is not seen in any nonneuronal tissues. (C) Protein extracts from CNS derived from *red e* and *SNAP-25¹²⁴* larvae and pharate adult heads were probed with Exon 4 antiserum to determine the relative levels of SNAP-25 and SNAP-24 at these stages. In the larvae, SNAP-24 and SNAP-25 levels are approximately equal in the *red e* CNS, but only SNAP-24 expression is seen in the CNS of *SNAP-25¹²⁴* animals. In *red e* pharate adult heads, the amount of SNAP-25 is now notably greater than that of SNAP-24. We did not detect any upregulation of SNAP-24 in *SNAP-25¹²⁴* larvae or pharate adult animals. All larval and pharate adult CNS samples were also probed against tubulin to ensure that mutant and control lanes contained the same amount of total protein (data not shown). Larval samples are not directly comparable to pharate adult samples as they consisted of different amounts of tissue. The lanes pictured are representative of at least six independent samples.

teins did not differ between *SNAP-25* null larvae and controls (data not shown).

SNARE complex formation by SNAP-25 and SNAP-24: If SNAP-24 can substitute for the function of SNAP-25, how well does SNAP-24 interact with other SNARE proteins? An earlier report showed that SNAP-24 can

form the characteristic 73-kD SNARE complex with syntaxin and neuronal-synaptobrevin (NIEMEYER and SCHWARZ 2000). We compared the ability of SNAP-24, SNAP-25, and the temperature-sensitive form of SNAP-25, SNAP-25^{ts}, to form SNARE complexes using an *in vitro* assay (RAO *et al.* 2001). Our results show that, while SNAP-24 forms less 73-kD complex than SNAP-25 does, it forms more complex than SNAP-25^{ts} does (Figure 6, A and B). Interestingly, SNAP-24 is much better than either SNAP-25 or SNAP-25^{ts} at forming a higher-order SNARE complex, indicating that the biochemical functions of SNAP-24 and SNAP-25 may have differences more subtle than those apparent in our electrophysiological assays. In addition, SNARE complexes containing SNAP-24 migrate faster on SDS-PAGE gels than do those containing SNAP-25. The apparent size difference of SNARE complexes containing SNAP-24 cannot be accounted for by the small difference in molecular weight between SNAP-25 and SNAP-24 (131 D). Thus, the size differences likely represent structural differences between complexes formed by these two proteins.

SNAP-24 rescues the phenotype of *SNAP-25^{ts}*: In larvae, the endogenous level of SNAP-24 is sufficient to compensate for a lack of SNAP-25 protein in the *SNAP-25* null. Why then does the recessive *SNAP-25^{ts}* allele of SNAP-25 confer such a marked phenotype at the larval NMJ, given the presence of an apparently redundant homolog? We tested whether increased levels of SNAP-24 can rescue the phenotype of *SNAP-25^{ts}*. *SNAP-24* driven by the heat-shock promoter was overexpressed in *SNAP-25^{ts}/Df(3L)1-16* larvae, and evoked potentials and spontaneous vesicle fusion frequency were measured (Figure 7). Overexpression of SNAP-24 completely rescued the increased evoked potentials of *SNAP-25^{ts}* at 22° to control levels (Figure 7A). Increasing levels of SNAP-24 partially, though not completely, reduced the increased miniature excitatory junctional potential (mEJP) frequency of *SNAP-25^{ts}* larvae at 22° (Figure 7B). At 37°, overexpression of SNAP-24 partially rescues the reduction in EJP amplitude of *SNAP-25^{ts}* larvae. At the adult stage, overexpression of *SNAP-24* driven by the heat-shock promoter also partially rescues the temperature-sensitive paralysis of *SNAP-25^{ts}* flies, so that their temperature of paralysis shifts from 35° to 38° (data not shown). Therefore, while SNAP-25 normally excludes SNAP-24 from participating in neurotransmitter release, overexpression of SNAP-24 can drive more SNAP-24 to participate in this process, thereby overcoming the *SNAP-25^{ts}* phenotype.

Physiology of pharate adults: *SNAP-25¹²⁴* flies die as pharate adults: therefore, the critical phase for SNAP-25 activity seems to be the late pupal stage. To investigate what physiological properties are disrupted by a lack of SNAP-25 protein, we assayed synaptic transmission in mutant animals by examining activity in the laminar neuropil using ERG recordings (HOTTA and BENZER 1969). ERGs from mutant animals show a complete

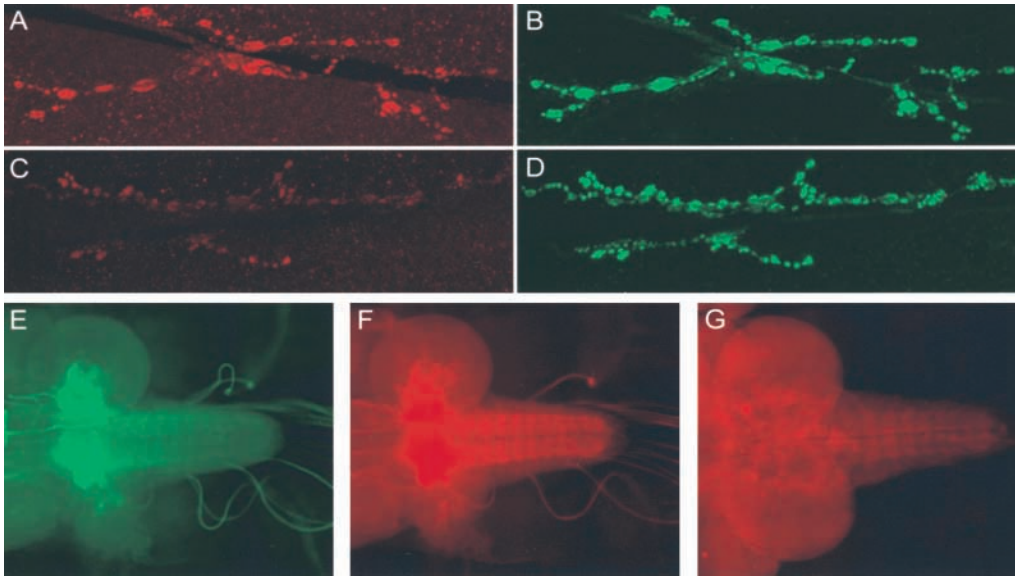


FIGURE 5.—Immunocytochemistry shows the synaptic distribution of SNAP-24 and SNAP-25 in larvae. (A) *red e* larval NMJ boutons are stained with the Exon 4 antibody. Staining represents immunoreactivity of both SNAP-24 and SNAP-25 proteins. (B) The same nerve terminal as in A is stained against the synaptic bouton marker CSP. (C) *SNAP-25¹²⁴* larval NMJ boutons stained with the Exon 4 antibody. Staining represents immunoreactivity of only the SNAP-24 protein. (D) The same nerve terminal as in C, stained against the synaptic bouton marker CSP. (E) *red e* larval CNS probed with the

SNAP-25-specific antibody shows a staining pattern characteristic of synaptic neuropils within the brain lobes and ventral ganglia. (F) The same CNS stained with the Exon 4 antibody shows a pattern similar to that in E. (G) *SNAP-25¹²⁴* larval CNS probed with the Exon 4 antibody shows a synaptic staining pattern, although the staining intensity is greatly reduced compared to *red e* as staining here represents only SNAP-24 immunoreactivity.

absence of on- and off-transients (Figure 8), indicating a severe reduction or abolition of synaptic transmission within the laminar neuropil. Mutant animals rescued by expressing a *SNAP-25* transgene or a *SNAP-24* transgene also show a rescue of synaptic activity, indicating that synaptic failure in mutant animals is due to the absence of SNAP-25 protein. Significantly, transgenic expression of *SNAP-24* was also able to completely rescue the pupal lethality of *SNAP-25¹²⁴* flies. This suggests that, in adult flies, SNAP-24 is not distributed in the right locations and/or is not present at high-enough levels to support normal synaptic transmission, but that, in principle, SNAP-24 is able to functionally substitute for SNAP-25, even in adult flies.

Data from our Western blot assays have shown that in both mutant and wild-type flies the adult CNS contains a reasonable amount of SNAP-24. How is it that in larvae, the presence of SNAP-24 in neurons is able to compensate for a lack of SNAP-25, while in the adult, normal SNAP-24 expression cannot do likewise? To answer this question, mutant and control brains were stained against SNAP-25 and against the conserved SNAP-24/SNAP-25 peptide sequence using the Exon 4 antiserum (Figure 9). Immunocytochemistry on parate adult heads shows that SNAP-24 is present at relatively low levels in optic lobes of *SNAP-25¹²⁴* animals and is not specifically concentrated in synaptic areas (as defined by the synaptic marker CSP; Figure 9D). Control animals reveal that

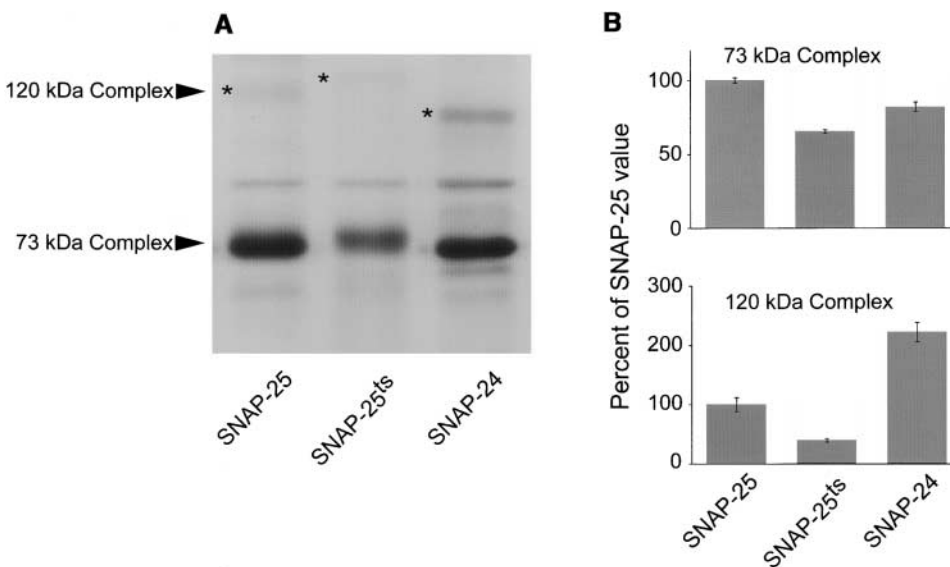


FIGURE 6.—SNARE complex formation with SNAP-25, SNAP-25^{ts}, or SNAP-24. (A) Representative Coomassie gel showing the characteristic 73-kD and higher-order SNARE complexes of ~120 kD, indicated by asterisks. Complexes were formed by incubating syntaxin and N-syb with SNAP-25, SNAP-25^{ts}, or SNAP-24. The band directly above the 73-kD complex is a bacterial protein contaminant. (B) Plotting the amounts of SNARE complex formed shows that SNAP-24 forms less 73-kD complex than SNAP-25 does, but still more than SNAP-25^{ts}. Conversely, SNAP-24 is more efficient at forming the higher-order SNARE complex than is SNAP-25 and much more efficient than SNAP-25^{ts}.

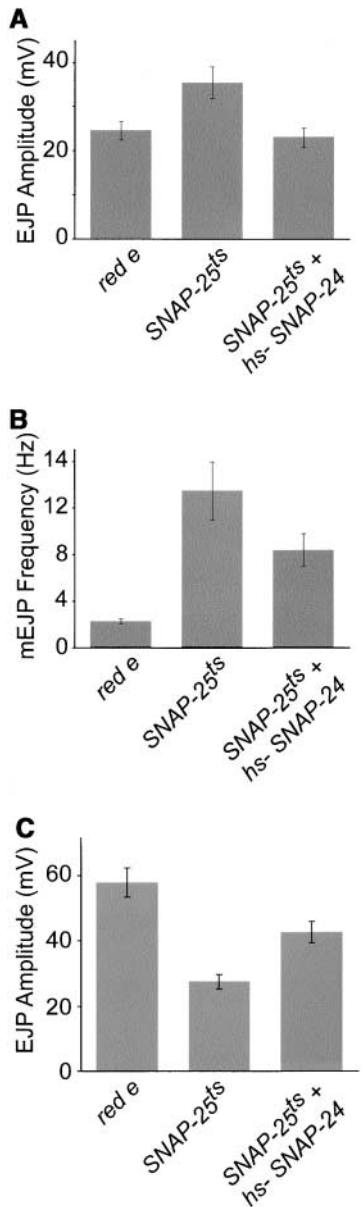


FIGURE 7.—SNAP-24 overexpression suppresses the *SNAP-25^{ts}* phenotypes at permissive and restrictive temperatures. (A) Evoked potentials are increased from a mean of 24.7 mV in control ($n = 8$) to 35.5 mV ($n = 7$) in *SNAP-25^{ts}* animals at 22° (at Ca^{2+} concentrations of 0.5 mM): SNAP-24 overexpression reduces this to 23.1 mV ($n = 8$). (B) mEJP frequency is increased from 2.3 Hz in control ($n = 6$) to 13.5 Hz in *SNAP-25^{ts}* animals at 22° ($n = 7$): SNAP-24 overexpression reduces this to 8.4 Hz ($n = 7$). While this is still not at control levels, it is significantly below the *SNAP-25^{ts}* frequency. (C) At 37°, evoked potentials in *SNAP-25^{ts}* animals are decreased by >50% to 27.5 mV ($n = 6$) from a control value of 57.7 mV ($n = 5$, at Ca^{2+} concentrations of 2 mM). SNAP-24 overexpression increases this to 46.7 mV ($n = 6$), which is now 80% of control levels. All comparisons are statistically significant (one-way ANOVA, $P < 0.01$ or better).

SNAP-25 is normally found within the optic lobe neuropil and is also more generally distributed within the CNS (Figure 9, B and E). This suggests that *SNAP-25* null flies experience synaptic failure and subsequently

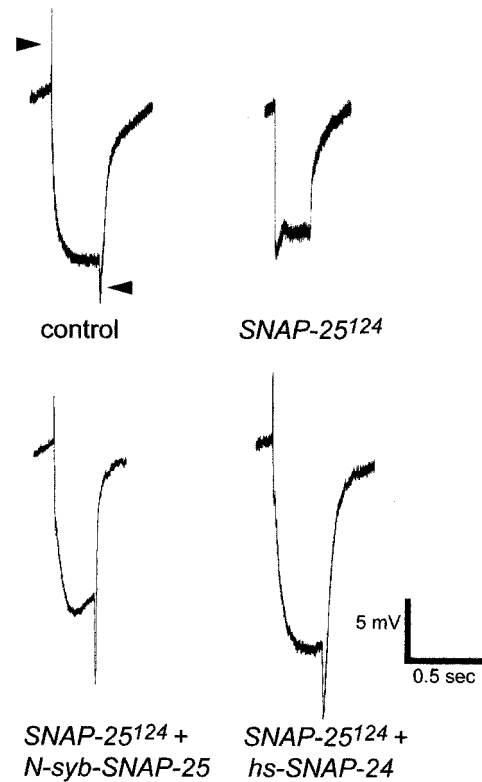


FIGURE 8.—Electretinogram recordings of control, *SNAP-25¹²⁴*, and *SNAP-25¹²⁴* flies rescued by expression of *SNAP-25* or *SNAP-24* transgenes. The traces show a severe reduction or complete loss of synaptic transmission in the lamina of *SNAP-25¹²⁴* pharate adults, as evidenced by the lack of on- and off-transients (indicated by arrowheads in control trace). Synaptic transmission is restored by transgenic expression of *SNAP-25* driven by the *N-syb* promoter (bottom left trace) or overexpression of *SNAP-24* driven by the heat-shock promoter (bottom right trace). ERG traces of control (*Df(3L)1-16/TM6B,Tb*) and rescued animals may appear more robust than those of the mutant because these animals had emerged from the pupal case on their own by the time recordings were performed.

die due to lack of SNAP-25. Critical synapses within the adult nervous system do not have a high-enough concentration of SNAP-24 to allow continued activity in the absence of SNAP-25.

Where then is SNAP-24 found in the adult CNS? Staining revealed that SNAP-24 is distributed at low levels throughout the brain, but is specifically concentrated in the mushroom bodies (Figure 10). The synaptic area of the mushroom body calyx contains some SNAP-25 in control animals, but SNAP-25 expression is noticeably lower in the calyx (Figure 10A), throughout the peduncle (Figure 10D), and within the α - and β/γ -lobes (Figure 10G) than in the surrounding neuropil. SNAP-24 immunoreactivity is highest in those regions: this is especially evident in *SNAP-25* null animals, where all immunoreactivity is due to SNAP-24 protein (Figure 10, C, F, and I). SNAP-24 expression is also higher in the fan-shaped body of the central brain than in the surrounding neuropil in both wild-type and *SNAP-25* null

animals (data not shown). Thus in the adult CNS, SNAP-25 and SNAP-24 show a segregation of expression between different neuropil regions.

DISCUSSION

As a step toward understanding the function of SNAP-25 in vesicle fusion and synaptic transmission, we have generated *SNAP-25* null alleles in *Drosophila*. We showed that SNAP-25 is required for viability at the pharate adult stage. Surprisingly, the synaptic physiology of the *SNAP-25* null larvae is normal. Western blot and immunocytochemical analysis of another SNAP iso-

form, SNAP-24, revealed its presence at the larval NMJ and CNS, as well as at the adult CNS. Our findings suggest that at the larval neuromuscular junction, endogenous levels of SNAP-24 are able to support synaptic transmission in the context of a *SNAP-25* null. However, in the context of *SNAP-25^{ts}*, endogenous SNAP-24 levels are not sufficient to restore normal synaptic transmission. SNAP-24 is also unable to restore normal synaptic transmission in the optic lobes of *SNAP-25* null animals, where endogenous SNAP-24 levels are low. Thus, the apparent redundancy of SNAP-25 and SNAP-24 is highly dependent on the genetic and temporal context. It is still possible that it is not SNAP-24, but another protein that substitutes for the absence of SNAP-25. While a definitive answer must await isolation of SNAP-24 mutant lines, rescue of *SNAP-25* null and *SNAP-25^{ts}* phenotypes by a SNAP-24 transgene strongly suggests that SNAP-24 can functionally substitute for SNAP-25.

SNAP-25¹²⁴ larvae, which lack any detectable SNAP-25 protein, show a surprisingly mild synaptic phenotype. If, as our data suggest, larvae contain enough SNAP-24 to support exocytosis in the absence of SNAP-25, how then can a recessive *SNAP-25* missense mutation, *SNAP-25^{ts}*, have such a significant effect in third instar larvae? One possibility is that the temperature-sensitive form of SNAP-25, *SNAP-25^{ts}*, acts as a poison subunit. Poison subunits are thought to function by impairing the normal activity of a protein complex (STEARNS and BOTSTEIN 1988) and may be phenotypically dominant or recessive. In *SNAP-25^{ts}* heterozygotes, the amount of wild-type SNAP-25 is sufficient to outcompete the *SNAP-25^{ts}* poison subunit: hence, *SNAP-25^{ts}* has a recessive phenotype. However, in *SNAP-25^{ts}* homozygotes, our evidence suggests that endogenous SNAP-24 levels are insufficient to outcompete *SNAP-25^{ts}*, resulting in an altered synaptic physiology. In the null allele of *SNAP-25*, no poison subunit is present and SNAP-24 is therefore able to bind SNAP-25's partners and support normal synaptic transmission. A similar phenomenon was ob-

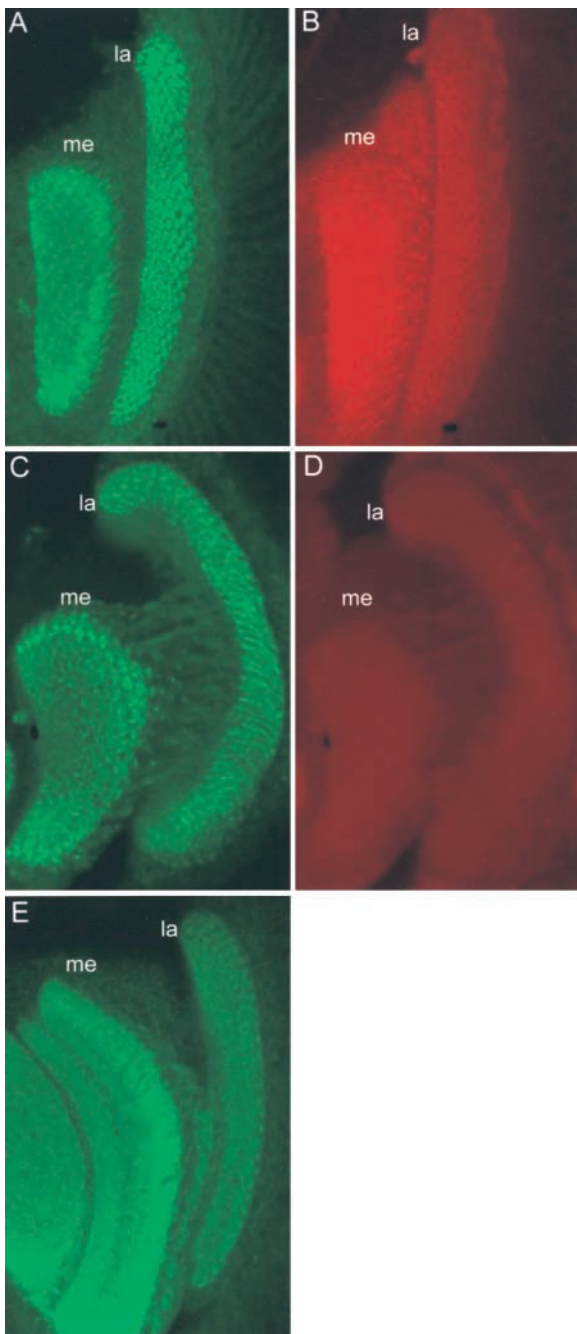


FIGURE 9.—*SNAP-25* and *SNAP-24* expression in optic neuropils of *SNAP-25¹²⁴* and white-eyed control pharate adults. (A) CSP staining of control animals shows synaptic regions of the optic neuropil. (B) The same section as in A stained with the Exon 4 antibody shows strong immunoreactivity in synaptic regions, representing the combined signal of SNAP-25 and SNAP-24. (C) CSP staining of *SNAP-25¹²⁴* animals shows synaptic regions of the optic neuropil, indicating that neuropil organization is generally intact in *SNAP-25* nulls. Small variations in cartridge shape and clarity may reflect slight differences in plane of sectioning between preps. (D) The same section as in C stained with the Exon 4 antibody, which shows very weak staining, reflecting a low level of SNAP-24 expression. (E) Control animal optic neuropil stained with the SNAP-25-specific antibody shows a pattern similar to that seen in B, indicating that the vast majority of Exon 4 antibody staining in controls is due to the SNAP-25 protein. la, lamina; me, medulla.

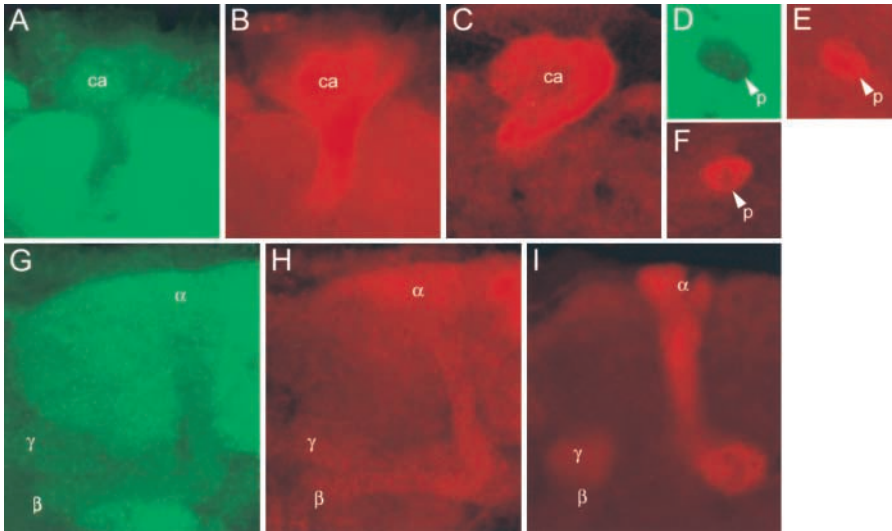


FIGURE 10.—Segregation of *SNAP-25* and *SNAP-24* expression in the mushroom bodies of pharate adults revealed by immunocytochemistry. Positions of the calyx (ca), peduncle (p), and the α -, β -, and γ -lobes are indicated. (A) Control calyx stained with the SNAP-25-specific antibody. Note that the neuropil surrounding the calyx shows strong SNAP-25 immunoreactivity, but the calyx itself is weakly stained. (B) The same section as in A, stained with the Exon 4 antibody. Note that here the calyx is strongly stained, while surrounding neuropil is relatively dim, indicating that SNAP-24 levels are higher in the calyx. (C) Exon 4 antibody staining of a *SNAP-25¹²⁴* animal shows strong SNAP-24 immunoreactivity in the calyx. (D) Control peduncle cross section stained with the SNAP-25-specific antibody, again showing

less immunoreactivity than the surrounding neuropil. (E) The same section as in D, stained with the Exon 4 antibody, now showing brighter immunoreactivity due to levels of SNAP-24 higher within the peduncle than in the surrounding neuropil. (F) Exon 4 antibody staining of a peduncle cross section in a *SNAP-25¹²⁴* animal shows strong SNAP-24 immunoreactivity, indicating that the staining in F is almost entirely due to SNAP-24 immunoreactivity. (G) Control mushroom body lobes stained with the SNAP-25-specific antibody. (H) The same section as in G, stained with the Exon 4 antibody. (I) Exon 4 antibody staining of a *SNAP-25¹²⁴* animal shows strong SNAP-24 immunoreactivity in the mushroom body lobes, while SNAP-25 staining is relatively weaker here than in the surrounding neuropil.

served in the nonallelic noncomplementation of genes important for synaptic vesicle trafficking in *Caenorhabditis elegans* (YOOK *et al.* 2001). Here, *trans*-heterozygotes of recessive point mutations in *unc-13* and *unc-64* show a synaptic phenotype that is more severe than that of *trans*-heterozygotes of null alleles.

Our evidence suggests that SNAP-25 and SNAP-24 serve similar but distinct functions within neurons. Both the limited contexts in which SNAP-24 can substitute for SNAP-25 and its relatively low levels in the larval NMJ suggest that this protein normally plays a minor role in neurotransmitter release at this synapse. When SNAP-24 sustains synaptic transmission in *SNAP-25* nulls, we believe this is a synaptic example of inappropriate genetic substitution. This type of effect has been described for the mitogen-activated protein kinase signaling pathway, where the kinases KSS1 and FUS3 normally act in two different pathways to transduce distinct signals in yeast (MADHANI *et al.* 1997). In this case, missense mutations in either protein exhibit a phenotype, whereas null mutations show no effects due to inappropriate cross-talk between the signal transduction pathways. The authors argue that, in this case, missense mutants may therefore have a stronger phenotype and may be ultimately more informative as to the function of the gene than complete null mutants. This may well be the case for *SNAP-25⁶*, which has a larval phenotype stronger than that of the null allele *SNAP-25¹²⁴*.

While SNAP-25 is critical for adult *Drosophila*, our data suggest that SNAP-24 is sufficient for the animals during the larval stage. This division of function between developmental stages is not unprecedented for proteins

involved in synaptic transmission and exocytosis. In mammals, SNAP-25 is alternatively spliced into two forms, SNAP-25a and SNAP-25b, which differ in the composition of the palmitoylated cysteine-rich domain thought to be responsible for association of the protein with membranes (BARK and WILSON 1994). SNAP-25a is the dominant form throughout embryonic development, while levels of SNAP-25b rise and exceed SNAP-25a postnatally (BOSCHERT *et al.* 1996). There is evidence that the developmentally significant SNAP-25a form is expressed in adult neurons that retain morphological plasticity or undergo regrowth (BOSCHERT *et al.* 1996; JACOBSSON *et al.* 1996). In *Drosophila*, SNAP-24 also differs from SNAP-25 in the cysteine-rich domain, where it contains three instead of four cysteine residues that could potentially affect its membrane association dynamics. SNAP-24 is found at high levels relative to SNAP-25 during the larval stage; during metamorphosis into adulthood SNAP-25 expression rises significantly relative to SNAP-24. Therefore, the roles of SNAP-24 and SNAP-25 in *Drosophila* may be in some ways analogous to the roles of SNAP-25a and SNAP-25b in mammals.

Another parallel may be drawn between SNAP-24 and mammalian SNAP-23, a protein that is expressed widely throughout the body and is not restricted to the nervous system (RAVICHANDRAN *et al.* 1996; STEEGMAIER *et al.* 1998). While SNAP-23 is not highly expressed in the nervous system (WONG *et al.* 1997), it has been demonstrated that SNAP-23 can functionally substitute for SNAP-25 in at least some exocytosis processes (SADOUL *et al.* 1997), and it is found in regions of the hippocam-

pus and cortex where SNAP-25 is absent (CHEN *et al.* 1999). In flies, SNAP-24 is concentrated in mushroom body neuropil in adult brains, a region where SNAP-25 levels are very low. This segregation of expression between specific neuropils may point to more subtle differences in functional requirements for vesicle fusion. Thus, the role of SNAP-24 in the adult CNS may have parallels with the role of SNAP-23 in the mammalian brain.

In *Drosophila* other synaptic proteins have developmentally specific isoforms that roughly parallel that of SNAP-25 and SNAP-24. One example is *N*-ethylmaleimide-sensitive factor (NSF), a chaperone that uses ATP to dissociate SNARE complexes and is thought to be important for recycling SNARE proteins after a round of vesicle exocytosis (BOULIANNE and TRIMBLE 1995; PALLANCK *et al.* 1995). NSF has two isoforms in *Drosophila*, dNSF1 and dNSF2. *dNSF1* and *dNSF2* are expressed in the larval and adult CNS, with *dNSF-2* also being expressed more widely in nonneuronal tissues (BOULIANNE and TRIMBLE 1995). Like *SNAP-25* nulls, *dNSF1* loss-of-function mutants die at the pharate adult stage and are rescued by neuronal expression of a wild-type transgene (GOLBY *et al.* 2001). However, unlike the *SNAP-25^{ts}* allele, temperature-sensitive paralytic alleles of *dNSF1* have no synaptic phenotype at the third instar larval NMJ, presumably due to the presence of dNSF2 in the larval nervous system (MOHTASHAMI *et al.* 2001). Future studies may resolve how different isoforms of NSF may interact with different members of the *SNAP-25* gene family.

In conclusion, we have shown that *SNAP-25* is an essential gene required for synaptic transmission in adult *Drosophila*. In the larvae, it appears that SNAP-24 can substitute for SNAP-25, depending upon the genetic background, but is not likely to play a major role in neurotransmitter release in normal larval NMJs. This genetic redundancy observed between SNAP-25 and SNAP-24 may represent a new instance of inappropriate genetic substitution. The different distribution of these two proteins in the adult CNS and the observed differences in their respective SNARE complexes point to distinct roles for these proteins *in vivo*.

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