A Temperature-Sensitive Allele of Drosophila *sesB* Reveals Acute Functions for the Mitochondrial Adenine Nucleotide Translocase in Synaptic Transmission and Dynamin Regulation

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ABSTRACT

Rapidly reversible, temperature-sensitive (ts) paralytic mutants of Drosophila have been useful in delineating immediate *in vivo* functions of molecules involved in synaptic transmission. Here we report isolation and characterization of *orangi* (*org*), an enhancer of *shibire* (*shi*), a ts paralytic mutant in Drosophila dynamin. *org* is an allele of the *stress sensitive B* (*sesB*) locus that encodes a mitochondrial adenine nucleotide translocase (ANT) and results in a unique ts paralytic behavior that is accompanied by a complete loss of synaptic transmission in the visual system. $sesB^{org}$ reduces the restrictive temperature for all shi^{ts} alleles tested except for shi^{ts1}. This characteristic allele-specific interaction of $sesB^{org}$ with *shi* is shared by *abnormal wing discs* (*awd*), a gene encoding nucleoside diphosphate kinase (NDK). $sesB^{org}$ shows independent synergistic interactions, an observation that is consistent with a shared pathway by which *org* and *awd* influence *shi* function. Genetic and electrophysiological analyses presented here, together with the observation that the $sesB^{org}$ mutation reduces biochemically assayed ANT activity, suggest a model in which a continuous mitochondrial ANT-dependent supply of ATP is required to sustain NDK-dependent activation of presynaptic dynamin during a normal range of synaptic activity.

BEHAVIORAL mutants of Drosophila and, in particular, those that reversibly paralyze on exposure to nonpermissive temperatures have helped to identify molecules essential for neuronal conduction and synaptic transmission. Due to their largely normal development when reared at permissive temperatures, functional analyses of temperature-sensitive (ts) mutants before and after shift to nonpermissive conditions have played a special role in revealing and specifying immediate synaptic functions for the affected proteins (Suzuki 1970; Grigliatti *et al.* 1973; Siddiqi and Benzer 1976; Salkoff and Wyman 1981; Loughney *et al.* 1989; Pallanck *et al.* 1995; Dellinger *et al.* 2000; Littleton *et al.* 2001). Most importantly, ts mutations at the Drosophila *shibire* (*shi*) locus have been decisive in establishing the role of the encoded GTPase, dynamin, in endocytosis at synapses and in other cellular contexts (Suzuki 1970; Kosaka and Ikeda 1983; Poodry 1990; Chen *et al.* 1991; van der Bliek and Meyerowitz 1991). Dynamin is an unusual GTPase that shows a remarkably low affinity for GTP ($K_m \sim 30$ μM) and a high basal rate of GTP hydrolysis (Sever *et al.* 1999, 2000). These biochemical properties of dynamin suggested unusual mechanisms to control GTP loading and GTP hydrolysis, functions traditionally mediated by GDP/GTP exchange factors and GTPase-activating effector proteins. The specific mechanism for GTP loading was suggested by our previous analyses in Drosophila and subsequently supported by biochemical experiments with mammalian dynamin (Krishnan *et al.* 2001; Baillat *et al.* 2002; Palacios *et al.* 2002). Nucleoside diphosphate kinase (NDK) encoded by the Drosophila *abnormal wing discs* (*awd*) locus interacts with dynamin and, by converting GDP to GTP using ATP as a phosphate donor, provides a local supply of GTP required for efficient dynamin activation. Thus, hypomorphic alleles of *awd* substantially enhance the *shibire*ts paralytic phenotype as well as the various physiological and morphological consequences of presynaptic dynamin inhibition (Krishnan *et al.* 2001).

With a view to identifying additional molecules relevant to synaptic vesicle endocytosis, we performed a screen for mutations that, like *awd*, reduce the temperature at which shi^{ts} mutants are paralyzed. One such enhancer that we obtained, *orangi* (*org*), is a hypomorphic mutation in the mitochondrial adenine nucleotide translocase (ANT). *org* reduces ANT activity in Drosophila without obviously reducing the number of presynaptic mitochondria or the amount of ANT encoding mRNA. These observations predict a reduced rate of ATP synthesis in *org*. We first show that synaptic activity is acutely dependent on a continuous local supply of ATP. We additionally present genetic and physiological evidence in support of a model in which lowered rates of ATP synthesis at nerve terminals affect synaptic vesicle

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recycling by inhibiting the rate of NDK-dependent dynamin activation.

MATERIALS AND METHODS

Drosophila stocks: All stocks and crosses were grown at 25° in standard sugar-agar medium with yeast in vials or bottles. Recombinants of *orangi* with the temperature-sensitive paralytic mutants *shi*^{ts1}, *shi*^{ts2}, *shi*^{ts4}, *shi*^{BS1}, *shi*^{CK2}, and *shi*^{SHY} were made by using standard genetics with appropriate balancers and further ascertaining the presence of both the mutants by testing the double recombinant for failure of complementation with the respective single mutants. These are a part of the TATA Institute of Fundamental Research stock collection. Alleles at the *stress sensitive B* (*sesB*) locus and the transgene, which contains the genomic region of *sesB*, *Ant2*, and *P(genomic)*, were obtained from Michael Ashburner (Cambridge, UK). *P(genomic)* is described in Zhang *et al.* (1999); it was made by cloning a 10.3-kb *Eco*RI-*Hin*dIII genomic DNA fragment, which contains the *sesB* and *Ant2* loci in tandem and rescues the lethality and the bang sensitive phenotypes of *sesB* alleles. Deficiencies and other *P*-transposon-tagged lines were obtained from the Bloomington Stock Center (Indiana). Transposon-tagged lines used for recombination mapping were EP0395, EP0345 EP0308, EP0355, EP0317, EP0404, and EP0356. The transposon-tagged lines used in the 9E region for complementation with *orangi* were P1235, P1287, P138, P1286, P108, P1098, P1207, P1645, P2251, P1934, P1427, P405, P1798, P1608, and P107.

Genetics: Mutations that lead to enhancement of the ts paralytic phenotype of *shibire* flies were obtained as follows. All flies carrying the *shi*^{CK2} allele completely paralyze at 35° (Ramaswami *et al.* 1993) in ~2 min. In assaying >1000 flies we did not get any fly moving about at 35° in 3 min. At a temperature of 33° none of ~1000 flies tested paralyzed in 3 min. With this background information, 2- to 4-day-old adult male *shi*^{CK2} flies, all of which paralyzed in 2 min when exposed to 35°, were starved for 8 hr and then transferred for 12 hr into bottles containing filter paper soaked in freshly prepared 1% sucrose and 0.30% ethyl methanesulfonate. The mutagenized males were mated to virgin compound X chromosome (attached X) females and distributed with ~15 males and 25 females in each bottle. Flies were transferred every 3 days to fresh yeasted bottles. Progeny from this cross were tested for paralysis at 33° in the Sushi cooker (Ramaswami *et al.* 1993). Male flies that paralyzed were used to set lines with attached X virgin females. Putative lines were then set up as balancer stocks and their chromosomal location was ascertained by conventional genetic crosses.

P2251 is a homozygous lethal line that failed to complement *orangi*. However, this line had an additional lethality because the lethality could not be rescued by the transgene *P(genomic)*. Meiotic recombination with wild-type flies was carried out to obtain flies that survive in the presence of one copy of the transgene. One such recombinant obtained was *P(3)org*. All the phenotypes of *P(3)org* except rescue by transgene were identical to P2251. Excision lines for *P(3)org* were obtained by crossing the line to a transposase source and selecting males that would survive as the original line is homozygous lethal. A total of 17 perfect excision lines, obtained out of a total of 8000 progeny screened, had no recordable phenotype and complemented the *orangi* ts paralytic defect.

P(3)org FRT 19A was made by obtaining meiotic recombinants with P (neo FRT19A) and selecting on neomycin. *P(3)org* eye clones were generated by crossing *P(3)org* FRT19A with GMR-Hid FRT19A; Ey Gal4 UAS Flp as described in Stowers and Schwarz (1999). The P(w+ UAS-mito-GFP) stock was obtained from William Saxton (Department of Biology, Indiana University).

Assaying temperature-sensitive paralysis: Temperature-sensitive paralysis was assayed in a double-jacketed glass-walled container (Sushi cooker) in which circulating water was maintained at the desired temperature (Ramaswami *et al.* 1993). Three to five batches of 10 flies each were added and tested for paralysis at each temperature for 3 min to obtain a paralysis profile. Double mutants and corresponding single mutants were tested at the same time. The temperature of 100% paralysis was strictly defined as that at which 100% of the flies paralyze in 3 min. This implies that at 1° below the restrictive temperature several flies remain standing after the 3-min duration.

Cloning: Genomic DNA was prepared from P2251 for a plasmid rescue. The genomic DNA was digested with *Eco*RI, which has a unique restriction site in the *P* element and is suitable for obtaining a DNA sequence to the 3′ of the *P* element. An overnight ligation was performed at 16° using T4 DNA ligase (Roche) and transformation of the ligated product was done in DH5α electro-competent cells grown with ampicillin selection. A plasmid was extracted from the colonies obtained and its identity was confirmed by using suitable restriction digests. A genomic fragment of ~4 kb was obtained. A partial sequence of this fragment was determined to ascertain the position of the transposon insertion (Bangalore Genie, Bangalore, India). The obtained sequence was used for a homology search with the Drosophila BLAST (Berkeley *Drosophila* Genome Project) and found to have 100% identity with *sesB*. To sequence the mutation in the *orangi* allele, multiple rounds of sequencing were done with appropriate primers to the genomic DNA region of the *sesB* locus from homozygous mutant *orangi* flies (DNA sequencing facility, University of Arizona, Tucson).

ATP/ADP translocase assay: Mitochondria were extracted by differential centrifugation. A total of 30 flies were homogenized in 1 ml of 0.32 m sucrose buffered with 10 mm Tris HCl pH 7.6. All the procedures were carried out at 4°. The homogenate was spun at 1500 rpm for 10 min to remove debris and nuclei. The supernate was further spun at 10,000 rpm for 10 min, and the resultant pellet was washed with 0.32 m sucrose in 10 mm Tris HCl pH 7.6 and resuspended in the reaction buffer (110 mm KCl, 20 mm Tris HCl pH 7.4 with 1 mm EDTA). Bradford protein estimation (Bio-Rad, Richmond, CA) was carried out to equalize the protein concentration between mutant and wild-type fly extracts. A total of 1 μg/μl of protein was used for each reaction (Yan and Sohal 1998), 0.9 μCi of ^{3}[H]-ADP (Sigma, St. Louis) was added and incubated for 1 min, and 10 μm atractyloside (Sigma) was added to stop the reaction. The reaction mix was spun at 10,000 rpm and washed three times in reaction buffer. The pellet was lysed in 0.1 m NaOH. The radioactivity was measured in a β-scintillation counter (Betascout, Perkin-Elmer, Norwalk, CT).

Quantitative RT-PCR: Real-time quantitative PCR amplification of a constitutive gene such as *rp49* was used to standardize the concentration of cDNA prepared from mutant and control head RNA. Similar dilutions of cDNA were prepared and used for amplification of an ~500-bp nonoverlapping C-terminal region of *sesB* and *Ant2*. Quantitative RT-PCR for *sesB* and *Ant2* loci was carried out by using the following primers: 5′-ACCAGCTAATTATGTTAG-3′ and 5′-GAAGTT GAGTTTGTTTGTTG-3′ for *sesB* and 5′-TAATGAAGGTGAT CAAGAGAGTG-3′ and 5′-GGGGCTAAAAGTACTTCTTC-3′ for *Ant2*. RNA was extracted from wild-type and *orangi* mutant fly heads (RNAeasy kit, QIAGEN, Chatsworth, CA), quantitated, DNA digested (DNA Free, Ambion, Austin, TX), and reverse transcribed (Multiscript RT, QIAGEN). The concentration of the cDNA thus obtained was equalized after assaying

the concentration by real-time quantitative PCR (Smart Cycler, Cepheid) by using the following primers to *rp49*: 5′-ACGAT ACGGAGCATGTCTCC-3′ and 5′-ATGTCGTTCTCCTTGAA GGC-3′. Further dilutions of the cDNA in the linear range (1:5, 1:50, 1:250, 1:500, and 1:2500) were used to compare the product from *sesB* and *Ant2*.

Electroretinogram recording: Extracellular recordings, called electroretinograms (ERG), of light-evoked visual responses were made from eyes of 2- to 3-day-old flies grown at 25° (Rikhy *et al.* 2002). Flies, lightly immobilized by cooling on ice, were mounted upright on modeling clay with the right eye facing the light. Recording electrodes filled with 3 M KCl (tip resistance of 3–5 MΩ) were placed in contact with the eye and a reference electrode was inserted into the thorax. Light pulses of the duration indicated were delivered from an optical fiber output placed 3–4 cm from the eye of the fixed fly. Signals were amplified using an intracellular preamplifier (IX2-700 dual intracellular preamplifier, Dagan, Minneapolis) and data were acquired directly from an oscilloscope (Tektronix) connected to a computer.

Immunostaining: Wandering third instar larvae were pinned dorsally on a Sylgard dish and cut open to expose the neuromuscular junction. The dissection was carried out in cold Ca-free HL3 saline (70 mM NaCl, 5 mM KCl, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES, pH 7.3). Samples were fixed in 3.5% paraformaldehyde in phosphate-buffered saline (PBS), washed in PBS containing 0.15% Triton X-100, and incubated overnight at 4° with the primary antibody. Fluorophore-coupled secondary antibodies were used to visualize antibody binding on a Bio-Rad Radiance 2000 confocal microscope and images were acquired on Laser Sharp 2000 and processed with Adobe Photoshop 5.5. The pixel density was calculated by using the Metamorph image-processing software (Universal Imaging, West Chester, PA).

RESULTS

Isolation and characterization of *orangi*, an enhancer of *shibire*^{ts}: While a large number of available mutations at the *shi* locus share the property of rapid and reversible paralysis above a sharply defined temperature, they vary widely in the restrictive temperature itself (Kim and Wu 1990; Ramaswami *et al.* 1993). *shi*^{CK2}, isolated as an intragenic suppressor of *shi*^{ts2} (Grigliatti *et al.* 1973; Ramaswami *et al.* 1993), has a restrictive temperature of 35° at which 100% of the mutant flies are paralyzed within 2 min. In a *shi*^{CK2} background, we performed a genetic screen for mutations that caused paralysis at lower temperatures. Following EMS mutagenesis (see materials and methods) we screened a total of 13,760 male progeny. Of 11 putative lines set up, 2 showed consistent enhancement of the ts phenotype and were extragenic, while others were lethal in subsequent generations or intragenic. Of the 2 extragenic enhancer lines, 1 was a recessive enhancer of *shi*^{CK2} paralysis and showed an independent ts paralysis phenotype and so was named *orangi* (*org*). The *org shi*^{CK2} combination paralyzes rapidly at 33°, a temperature at which *shi*^{CK2} animals are functional and active (Figure 1A). *orangi* mapped to the X chromosome to a region proximal to *shi*. When isolated by recombination from *shi*^{CK2}, *org* mutants showed an independent recessive ts paralytic phenotype, albeit at a much higher restrictive temperature than the *org shi*^{CK2} double mutant. Thus, 100% of flies of the *org* genotype paralyze at 37° in 3 min (Figure 1B). The onset and course of *orangi* paralysis differs substantially from that of *shi*^{ts} mutants. While the rate of paralysis at 37° is rapid, the restrictive temperature is not as sharply defined as for *shi*^{ts} alleles: *org* flies paralyze if they are kept at lower temperatures for a longer duration. The recessive ts paralytic phenotype of *org* not only served to distinguish the *org* paralytic phenotype from that of *shi*, but also was of considerable utility for mapping and cloning the affected gene.

***Orangi* is allelic to the Drosophila *stress sensitive B* locus encoding adenine nucleotide translocase:** Recombination mapping of the *org* paralytic phenotype with respect to visible markers on the X chromosome established that it lies between *cut* (cytological map position: 7B) and *garnet* (cytological map position: 12B) on the X chromosome. Deficiency stocks in the region between *cut* and *garnet* (7B–12B on the polytene chromosome) were obtained to map *orangi* more accurately. Stock 903 contains a deficiency [Df(1)v-L3: missing 9F10–10A8] and a duplication of a genomic region [Dp(1;2)v^{+}63iv: covers 9E1–10A11]. The deficiency uncovered the recessive ts paralysis and the duplication complemented the *org* paralytic defect. Further, the deficiency that is a part of 954 [Df(1)v-L15: missing 9B1–10A2] failed to complement *org*. This established *org* in the 9F10–10A2 region of the polytene chromosome (Figure 2A).

Transposon insertion lines throughout the X chromosome were used for recombination mapping. We found that it is closely linked to a w^{+} marked transposon insertion in line P1284 (recombination frequency is 0.3 cM) at the 9E position on the polytene chromosome (Figure 2B). We performed complementation with existing transposon-tagged lines in the database in the 9 region (see materials and methods). One homozygous lethal P line, 2251, failed to complement *org*. Perfect excisions of *P(3)org* (a derivative of P2251; see materials and methods) obtained using a transposase source did not show any phenotype, confirming that the above phenotype was due to the transposon. We used P2251 flies to isolate and sequence the DNA flanking the transposon by plasmid rescue. The DNA sequence obtained was found to be 100% identical to the coding region of *sesB*. The position at which the open reading frame is disrupted by the transposon is indicated by an inverted triangle in Figure 2C. This encodes the mitochondrial ATP transporter, ANT, a component of the mitochondrial inner membrane that exchanges lumenal ATP for cytosolic ADP. The position of the *P*-element insertion suggests that the translation of the *sesB* gene would be completely abolished in P2251 because the transposon disrupts the third exon.

Two genes arranged in tandem in the Drosophila genome, and each encode ATP/ADP translocase (*sesB*,

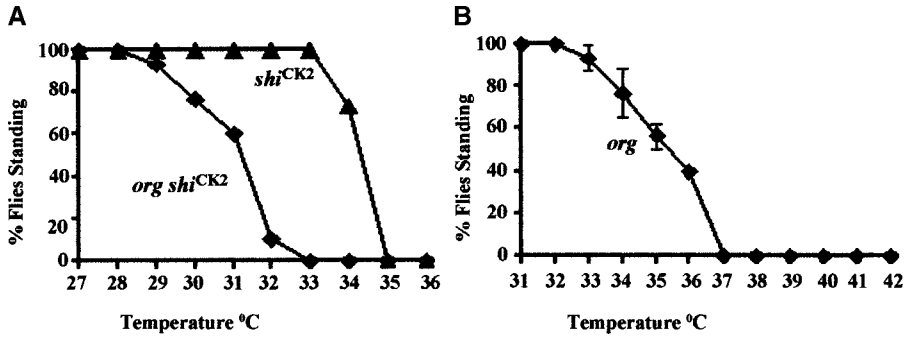

FIGURE 1.—Temperature-sensitive paralytic phenotypes of *orangi*. (A) *org shi*^{CK2} paralyzes at a lower temperature than *shi*^{CK2} does. (B) *org* exhibits independent ts paralysis. In 3 min 100% of flies homozygous for *orangi* paralyze at 37°.

which is the same as CG16944 and *Ant2*, which is the same as CG1683). They share 80% amino acid homology. We sequenced the genomic DNA region for *sesB* and *Ant2* in *orangi* and found a point mutation at the 266th amino acid position, resulting in a glutamate-to-lysine change in the *sesB* gene. This change is in a KQEG motif, which is conserved in all sequenced ANT-encoding eukaryotic genes (ClustalW analysis). Electrostatic interactions between the K and E (a charge pair) in the KQEG peptide are predicted to be important for normal ANT activity (NELSON *et al.* 1998). In *org*, the amino acid change from glutamate (negatively charged) to lysine (positively charged) disrupts the charge pair (Figure 2C). The amino acid residues mutated in the three existing, non-temperature-sensitive *sesB* alleles are shown in Figure 2C. Note that the residues are distinct from *org*.

To investigate a cell-autonomous requirement for *sesB*, we used a recently described strategy to create and analyze *P(3)org* (a recombinant line from P2251; see MATERIALS AND METHODS for details on its isolation from P2251) heterozygous animals whose eyes were composed of exclusively homozygous *P(3)org/P(3)org* mutant cells (STOWERS and SCHWARZ 1999). *P(3)org FRT19A* animals were crossed to *GMR Hid FRT19A; Ey Gal4 UAS Flp* mates. *Eyeless* (*Ey*) expressing cells carrying GMR-Hid are killed by Hid-induced apoptosis. *P(3)org/P(3)org* eyes showed severe defects in eye geometry and morphology compared to control eyes similarly generated by *Flp*-mediated recombination (Figure 2C). Thus, *P(3)org* disrupts a gene important for normal differentiation and/or function of photoreceptor neurons (Figure 2D). It also argues that *sesB* encodes the major mitochondrial ATP/ADP translocase in photoreceptor cells. Examination of multiple *sesB* enhancer trap lines confirms that the gene is highly and widely expressed in the nervous system (BOURBON *et al.* 2002).

Further evidence to suggest that *org* is allelic to *sesB* is provided by genetic complementation and transgene rescue analyses (Figure 2E). A transgene containing a 10.3-kb genomic fragment that encodes the genomic DNA region of *sesB* and *Ant2*, *P(genomic)*, rescues the *P(3)org* lethality and *orangi/P(3)org* paralysis and lethality, as well as the *orangi* ts paralytic phenotypes and the *org* enhancement of *shibire* paralysis (Figure 2E). On the basis of multiple criteria, the *Ant2* locus has been suggested to have limited function, if any. First, as with *org*, all *sesB* mutations that cause detectable phenotypes such as stress sensitivity or reduced viability affect *sesB* coding sequences (Figure 2C). More significantly, an inversion that clearly disrupts the *Ant2* locus while leaving *sesB* sequences intact causes no detectable phenotype (ZHANG *et al.* 1999). Despite these varied lines of data to support our working hypothesis that *org* affects *sesB*, but not ANT2, we performed additional detailed complementation analyses with known alleles of *sesB*.

The *sesB* mutants have been graded in an allelic series on the basis of the severity of the phenotypes exhibited (HOMYK and SHEPPARD 1977; HOMYK *et al.* 1980; JANCA *et al.* 1986; ZHANG et al. 1999). Three different strengths of phenotype were obtained with *org in trans*-allelic combination with known alleles of *sesB*. The combination of *org* with *sesB*^{9Ed1}, *sesB*^{9Ed3}, and *sesB*^{9Ed6} and null alleles such as *Df(954)* and *P(3)org* are semilethal when maintained at 25°. The escapers have a slow development, are flightless, show a temperature-sensitive paralysis, and are stress sensitive. The phenotype of 100% paralysis at 37°, however, is unique to *orangi*. However, *orangi* alone does not show any lethality during development. The weaker alleles of *sesB* did not show a remarkable phenotype with *orangi* (Table 1). Further, *sesB*^{1}, which is the previously characterized stress-sensitive allele, did not show paralysis similar to that of *org* (ZHANG *et al.* 1999). These additional observations make an overwhelming case for *org* being allelic to *sesB*. However, *org* (now termed *sesB*^{org}) shows a unique conditional phenotype that enables detailed and more meaningful analysis of neural functions that depend on immediate activity of the *sesB*-encoded mitochondrial ATP/ADP translocase.

Synaptic transmission is disrupted at restricted temperature in *sesB*^{org}: Synaptic transmission in the visual system of *sesB*^{org} mutants is lost at the same temperature at which behavioral ts paralysis occurs. Light-evoked visual response from the Drosophila eye can be measured in the form of a simple extracellular recording, the ERG. A typical ERG consists of a compound potential with different components. A slow receptor potential is due to the response of the photoreceptor cells to the light stimulus by depolarization. The fast transient spikes, which occur when light is switched on or off

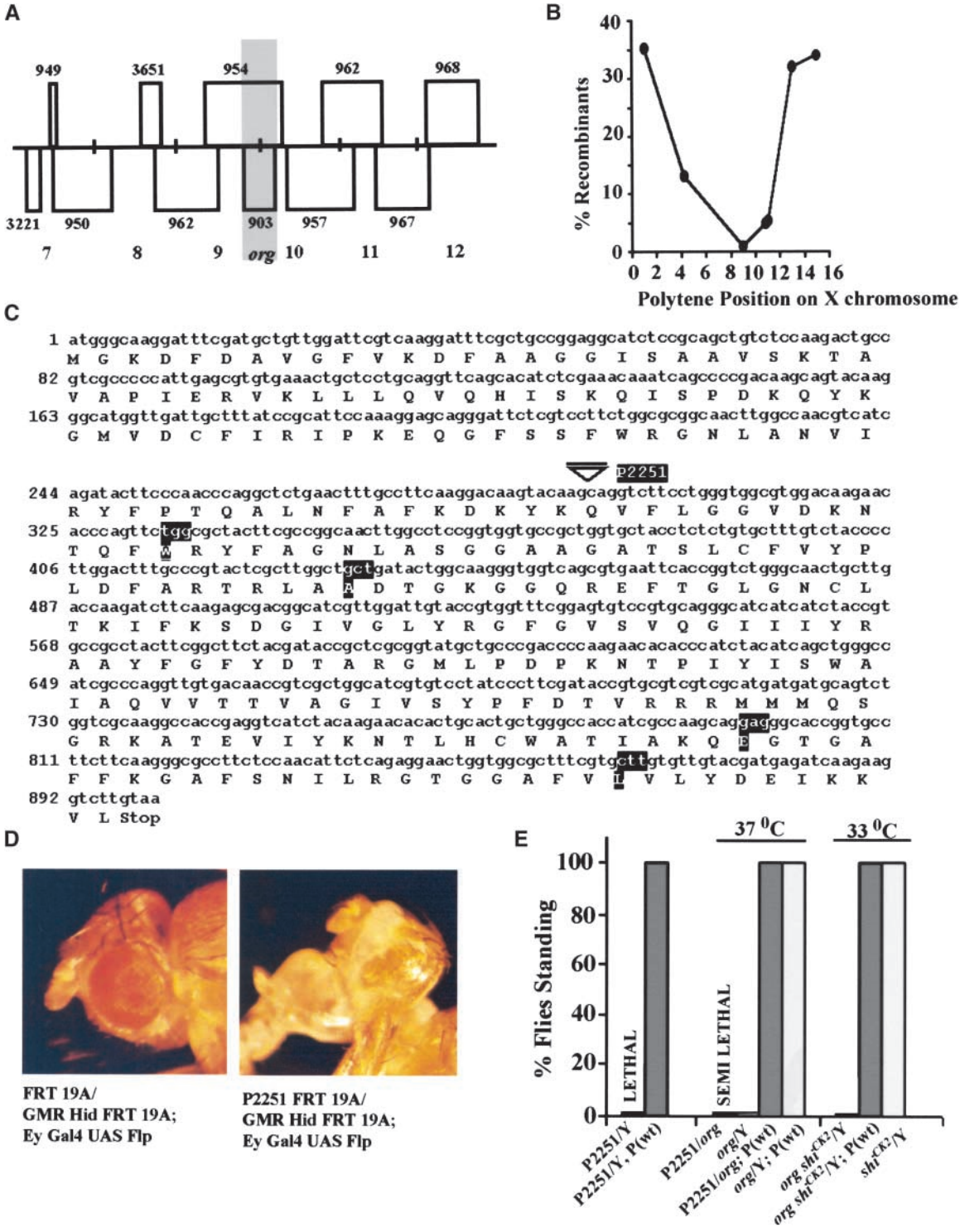

FIGURE 2.—*orangi* is an allele of *sesB* encoding adenine nucleotide translocase. (A) Deficiency mapping of *org* using overlapping deficiency stocks: each rectangle indicates the approximate region of overlap; 954 and 903 fail to complement *org*. The shaded bar represents the overlapping region of the polytene chromosome (9F10–10A2) between the two deficiency stocks to which *org* maps. (B) Recombination mapping of *org*: each point indicates the recombination frequency between a *P*-transposon-tagged line and the *org* paralytic phenotype on the X chromosome. P1284 at 9E has the lowest recombination distance of 0.3 cM from *org*. (C) *org* is a point mutation in *sesB*: the cDNA sequence and corresponding amino acids encoded for *sesB* are shown. An inverted triangle shows the position at which P2251 disrupts the *sesB* cDNA. The codons and the amino acid residues highlighted are those where mutations exist in different alleles of *sesB*: $sesB^{9Ed6}$ tgg to tga, $sesB^{9Ed1}$ gct to act, *org* gag to aag, and *sesB1* ctt to ttt. (D) *P(3)org* eye clones fail to develop: *P(3)org* eye clones generated by crossing a *P(3)org FRT19A* with *GMR-Hid FRT19A; Ey Gal4 UAS Flp* fail to develop. (Left) Eye clones from a control where *FRT19A* was crossed to *GMR-Hid FRT19A; Ey Gal4 UAS Flp*. The control clones have normal eye morphology whereas the *P(3)org* clones do not show eye formation. (E) The transgene referred to as *P(wt)*, which is the same as *P(genomic)*, rescues the lethality of *P(3)org*. It reverses both the semilethal and the paralytic defect of *P(3)org/org* and *org/org*. With the presence of one copy of the transgene, the paralysis of *org* shi^{CK2} at 33° is reversed.

TABLE 1

Complementation analysis of *org* with alleles at the *sesB* locus

Genotype	Survival (%)	Phenotype
Class 1		
org/Df 954	2	Lethal when grown at 25°, escapers paralyze at 35°, development is slow, and flightless
org/P(3) org	2	
org/sesB^{9Ed1}	2	
org/sesB^{9Ed3}	1.6	
org/sesB^{9Ed6}	1.2	
Class 2		
org/org	100	100% paralysis at 37°
Class 3		
org/sesB^{9Ed2}	56	Paralysis at 40°–41°
org/sesB^{9Ed4}	100	
org/sesB^{9Ed5}	36	
org/sesB^{1}	90	
org/Y;P(genomic)	100	The *org* paralysis defect is rescued

The number of flies emerged in each case has been normalized as percentage with respect to expected numbers. Note that P2251 had an unlinked lethality, which was removed by recombination with a wild-type chromosome. The line obtained after recombination is referred to as *P(3)org*. This line fails to complement *org* and the lethality in this line is rescued by the transgene *P(genomic)*.

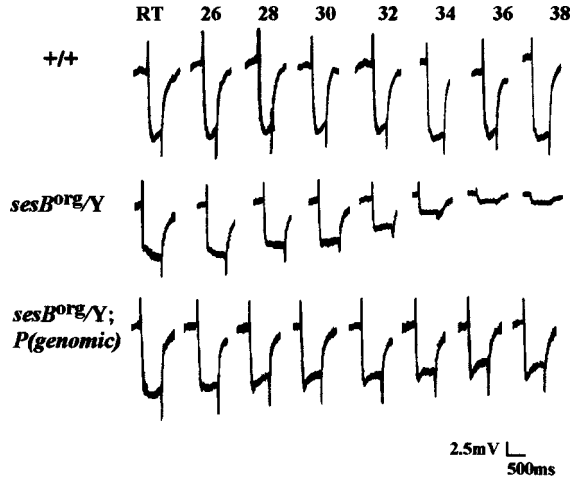

FIGURE 3.—Complete block of neurotransmission in *sesB*^{org} at restrictive temperature. The wild-type ERG shows no significant difference with increase in temperature (trace 1, top). All the components of the electroretinogram are lost at the restrictive temperature in *sesB*^{org} flies (trace 2, middle). The combination of *sesB*^{org} with one copy of the transgene *P(genomic)* rescues the loss of synaptic transmission similar to that of wild type (trace 3, bottom). The *n* values are cited in the text and the ERG traces represent typical responses obtained for the mutants analyzed.

and which flank the receptor potential, are due to the response of the second-order neurons in the optic lobe (HEISENBERG 1971). In wild-type flies, there is no significant difference in the ERG at different temperatures (Figure 3, trace 1; $n = 5$). In *sesB*^{org}, as the temperature is raised, the amplitude of all ERG components decreases; on-and-off transients as well as most of the total light response is abolished at restrictive temperatures (Figure 3, trace 2; $n = 10$). Upon shifting back to permissive temperature, the characteristic ERG response is restored. One copy of the genomic transgene completely rescues the ERG defect in *sesB*^{org} mutants (Figure 3, trace 3; $n = 5$); thus, these ts defects in the light response also map to *sesB*. Taken together, the ERG phenotype of *sesB*^{org} suggests that it affects multiple components of the ERG, including but not limited to, synaptic transmission. To isolate and study *sesB* function in synaptic transmission, we further analyzed the effect of *sesB*^{org} on *shi* mutants.

Allele-specific interactions between *sesB*^{org} and *shibire*: We initially isolated the *sesB*^{org} mutation as an enhancer of *shi*^{CK2}. The double recombinant *sesB*^{org} *shi*^{CK2} paralyzes at 33°, which is 2° lower than *shi*^{CK2} paralyzes. This enhancement of *shi* by *sesB*^{org} does not occur in the presence of the *sesB* transgene *P(genomic)*, indicating that this phenotype depends on the *sesB*^{org} mutation in *sesB*. To address the mechanism of *sesB*^{org} *shi* interactions, we used meiotic recombination to generate *sesB*^{org} *shi* double mutants with several different ts alleles of *shi*, including *shi*^{ts1}, *shi*^{ts2}, *shi*^{ts4}, *shi*^{BS1}, *shi*^{CK2}, and *shi*^{SHY}, and analyzed their paralytic behavior (Table 2). As observed for *shi*^{CK2}, *sesB*^{org} unequivocally reduces the restrictive temperature for paralysis of *shi*^{ts2}, *shi*^{ts4}, *shi*^{BS1}, *shi*^{CK2}, and *shi*^{SHY} by 2° (Table 2). None of these genetic interactions are observed in the presence of one copy of the genomic *sesB* transgene. Remarkably, the *sesB*^{org} *shi*^{ts1} combination paralyzes at the same temperature as *shi*^{ts1} alone (Table 2). This pattern of allele specificity is strikingly similar to that shown by hypomorphic mutations in *awd* (KRISHNAN *et al.* 2001; CHEN *et al.* 2002). We further examined

TABLE 2

Interaction between *sesB*^{org} and *sesB*^{1} and different *shi*^{ts} alleles

Genotype	Temperature	Genotype	Temperature
sesB^{org} *shi*^{ts2}	26°	*shi*^{ts2}	28°
sesB^{org} *shi*^{ts4}	27°	*shi*^{ts4}	29°
sesB^{org} *shi*^{BS1}	30°	*shi*^{BS1}	32°
sesB^{org} *shi*^{SHY}	33°	*shi*^{SHY}	35°
sesB^{org} *shi*^{CK2}	33°	*shi*^{CK2}	35°
sesB^{org} *shi*^{CK2}; *P(genomic)*	35°	*shi*^{CK2}	35°
sesB^{org} *shi*^{ts1}	27°	*shi*^{ts1}	27°
sesB^{1} *shi*^{ts2}	28°	*shi*^{ts2}	28°
sesB^{1} *shi*^{BS1}	32°	*shi*^{BS1}	32°

TABLE 3

Interaction among *sesB*^{org}, *awd*^{Msm95}, and *shi*^{ts}

Genotype	Paralysis temperature
shi^{CK2}/Y	35°
sesB^{org} *shi*^{CK2}/Y	33°
shi^{CK2}/Y; *awd*^{Msm95}/*awd*^{Msm95}	29°
sesB^{org} *shi*^{CK2}/Y; *awd*^{Msm95}/*awd*^{Msm95}	27°
sesB^{org}/Y; *awd*^{Msm95}/*awd*^{Msm95}	34°
+/Y; *awd*^{Msm95}/*awd*^{Msm95}	40°
sesB^{org}/Y	37°

the effect of *sesB*^{1}, a stress-sensitive allele of *sesB* (Zhang *et al.* 1999), on paralysis of *shi*^{ts2} and *shi*^{BS1}. No significant effect on *shi* paralysis was observed, implying that *sesB*^{1} is a weaker allele than *sesB*^{org}.

Of all *shi*^{ts} mutants previously tested, *shi*^{ts1} has previously been shown to be unique due to the complete absence of interactions with *awd* alleles that cause severely reduced NDK activity (Krishnan *et al.* 2001; Chen *et al.* 2002). The similarity in patterns of allele-specific interactions of *sesB*^{org} with *shi* and *awd* with *shi* suggested a common mechanism in which *sesB*-encoded ANT activity might contribute to NDK function. Specifically, a constant supply of ANT-derived ATP might be required for NDK to generate GTP stores essential for presynaptic dynamin activation. Such a hypothesis predicts independent genetic interactions between *sesB*^{org} and *awd*. We tested this and other genetic predictions of such a model.

***sesB*^{org} effect on *shi*^{ts} mutants is synergistic to NDK mutants (*awd*^{Msm95}):** We used NDK mutants (*awd*^{Msm95}) described previously to identify any interaction with *sesB*^{org}. Interestingly, double recombinants of the *sesB*^{org}*; awd*^{Msm95}/*awd*^{Msm95} paralyze at a lower temperature than *orangi* alone. While *sesB*^{org} mutants are paralyzed in 2 min at 37° and *awd*^{Msm95}/*awd*^{Msm95} flies at 40°, double-mutant *sesB*^{org}*; awd*^{Msm95}/*awd*^{Msm95} flies are paralyzed at 34°. This is indicative of a shared biochemical pathway essential for normal synaptic transmission in which both ANT and NDK participate. That this pathway also involves dynamin is suggested by the independent genetic interactions of *sesB* and *awd* with *shi*. Consistent with this model, while *shi*^{CK2}*; awd*^{Msm95}/*awd*^{Msm95} paralyzes at 29° (compared to 35° for *shi*^{CK2}), triple mutant *sesB*^{org} *shi*^{CK2}*; awd*^{Msm95}/*awd*^{Msm95} paralyzes at 27°, 2° lower than the *shi*^{CK2}*; awd*^{Msm95}/*awd*^{Msm95} double mutant paralyzes (Table 3). These data, combined with the similar allele specificity of *sesB*^{org} and *awd* interactions with *shibire*, strongly argue that the effect of *sesB*^{org} on *shi* occurs via an NDK-dependent pathway. To obtain additional evidence to address this issue, we performed neurophysiological experiments to analyze such genetic interactions at the level of the electroretinogram.

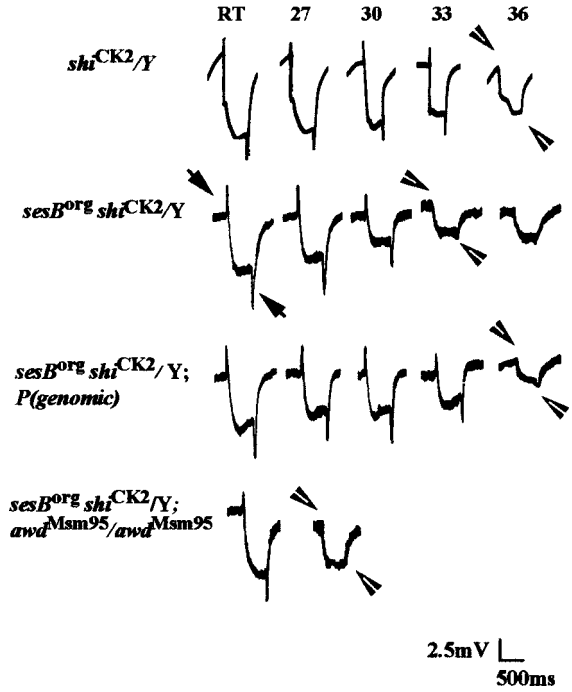

Figure 4.—*sesB*^{org} decreases the temperature of synaptic failure in *shi*^{ts} flies. The fast spikes in an electroretinogram shown by arrows are a result of synaptic transmission from first-order photoreceptor neurons to second-order lamina neurons. They are absent in *shi*^{CK2} at 36° (shown by open arrowheads in trace 1). The loss of these transients in the *sesB*^{org} *shi*^{CK2} combination occurs at a temperature lower than that of *shi*^{CK2} alone (33° in trace 2). The transgene rescues synaptic failure to the background *shi*^{CK2} level (trace 3). There is a loss of the on-and-off transients at the same temperature as paralysis in *sesB*^{org} *shi*^{CK2}*/Y; awd*^{Msm95}/*awd*^{Msm95} (trace 4). The *n* values are cited in the text and the ERG traces represent typical responses obtained for the mutants analyzed.

***sesB*^{org} *shibire* double mutant enhances the *shibire* synaptic failure:** ERG recordings show that *shi*^{ts} mutants suffer selective loss of synaptic on-and-off transients at (and above) their restrictive temperatures; receptor potentials, although slightly reduced, remain robust when synaptic components are lost (Kelly and Suzuki 1974). In *shi*^{CK2}, the on-and-off transients (indicated by arrows in Figure 4, trace 1) are lost at 36° ($n = 5$). In double-mutant *sesB*^{org} *shi*^{CK2} flies, on-and-off transients are lost at 33° ($n = 6$), a temperature at which the *shi*^{CK2} ERG appears normal (Figure 4, trace 2). The effect of *sesB*^{org} on the *shi*^{CK2} ERG is abrogated by the presence of one copy of the *sesB*$^{+}$ transgene *P(genomic)* (Figure 4, trace 3; $n = 4$); this confirms that a mutation in *sesB* underlies *sesB*^{org} enhancement of the *shi* ERG phenotype. The electroretinogram recordings were also performed for the *sesB*^{org} *shi*^{ts4} combination with respect to *shi*^{ts4} alone and the results obtained were similar to those shown for *sesB*^{org} *shi*^{CK2} *vs. shi*^{CK2} alone (data not shown).

Analysis of ERGs in multiple mutant combinations of *sesB*^{org}, *awd*, and *shi* indicate that genetic interactions observed in behavioral paralysis are reflected at the level

of synaptic transmission in the visual system. Thus, triple-mutant *sesB*^{org} *shi*^{CK2}*; awd*^{Msm95}*/awd*^{Msm95} (but not double-mutant *shi*^{CK2}*; awd*^{Msm95}*/awd*^{Msm95}) flies lack on-and-off transients at 27°, the same temperature at which behavioral paralysis occurs (Figure 4, trace 4; $n = 3$). The physiological data thus confirm and extend conclusions from the behavioral analyses. Because the *sesB*^{org} and *shi* effects on the ERG are clearly distinguished by the relative specificity of *shi* for the synaptic components of the ERG, these analyses clearly show that *sesB*^{org} mutations affecting the ATP/ADP translocase aggravate the consequences of dynamin inhibition in *shi*. Synaptic failure characteristic of *shi* mutants is enhanced by *sesB*^{org} and/or *awd*.

To interpret the effect of the *sesB*^{org} mutations in terms of its effects on the activity of the ANT protein, we carried out a series of experiments to assess the effects of the *sesB*^{org} mutation on mitochondrial localization and concentration of ANT, as well as on ANT expression and activity.

***sesB*^{org} effects are due to a decrease in translocation of ATP into the cytoplasm:** The *sesB*^{org} mutation that alters the *sesB*-encoded ANT protein could affect mitochondrial function in multiple ways. In principle, a severely misfolded translocase could reduce the number of mitochondria by, for instance, stimulating autophagic removal of aberrant organelles. Alternatively, by reducing the availability of a structural protein or otherwise perturbing mitochondrial membrane components, *sesB*^{org} could affect organelle biogenesis or axonal transport. To test these possibilities as well as the more economical hypothesis that *sesB*^{org} simply reduces ANT activity, we performed a set of experiments presented in Figure 5.

We assayed mitochondrial number at presynaptic terminals using a transgene-encoded, mitochondrion-targeted green fluorescent protein (mito-GFP) developed by W. Saxton (personal communication). When expressed in Drosophila neurons, mito-GFP labels all neuronal mitochondria, including a large concentration in motor terminals on larval longitudinal muscles. The brightness and density of mito-GFP labeling within presynaptic boutons provides a rough estimate for mitochondrial concentration in the presynaptic axoplasm (Figure 5A). A quantitative analysis of the GFP pixel intensity normalized to the bouton area reveals no significant difference between heterozygous *sesB*^{org}/+ and hemizygous *sesB*^{org}/Y animals (Figure 5B). This indicates no substantial defect in biogenesis, stability, or axonal transport of mitochondria in *sesB*^{org} mutants.

A careful quantitative RT-PCR analysis of *sesB* and *Ant2* mRNA in *sesB*^{org} mutants did not reveal any perceptible difference in the levels of these mRNAs in total RNA extracted from mutant and control heads (Figure 5C; materials and methods). However, a significantly reduced ATP/ADP translocase activity was evident in extracts of mitochondrial membrane prepared from *sesB*^{org} flies. To assay the activity of the ATP/ADP transfer across the mitochondrial membrane, we prepared a crude mitochondrial extract from mutant and wild-type adult flies. The transfer of ^{3}[H]-ADP into mitochondria was assessed. We found that there is an ~60% decreased uptake of the label in the mutant as compared to wild type ($P < 0.01$; Figure 5D). Because adenine nucleotide translocase transfers ATP into the cytoplasm in exchange for ADP, this observation suggests that the point mutation at the ANT locus primarily causes a low rate of ATP pumping into the cytoplasm.

DISCUSSION

Observations presented here indicate that continued mitochondrial ATP/ADP translocase activity is essential for neurotransmission. This is an important, rather than trivial, insight because it is based on analysis of the immediate consequence of ATP/ADP translocase inhibition *in vivo*. Our data indicate that steady-state levels of ATP at the presynaptic terminal may be rapidly depleted by relatively normal levels of synaptic activity, unless rapidly replenished by a local mitochondrial supply of ATP. While it has been speculated that the high concentration of mitochondria at presynaptic terminals is required to provide a local, rapid supply of ATP, there has been little direct analysis of this issue. Our results provide, to our knowledge, the first direct evidence that ATP is turned over at nerve terminals at a high rate with respect to the available cytoplasmic reservoir. The observed consequences of reduced ATP on synaptic transmission and synaptic vesicle recycling offer new hypotheses and insight into biochemical pathways that underlie vesicle traffic at the nerve terminal. Below we discuss the significance of our data for (a) synaptic transmission, (b) synaptic vesicle recycling, and (c) mitochondrial function at nerve terminals.

The *orangi* effect on synaptic transmission: Although mutations in the *sesB* locus have been previously described (Zhang *et al.* 1999), our identification and analysis of a novel conditional *sesB* allele provide new insight into metabolic and biochemical pathways that underlie neural excitability and synaptic transmission. While the importance of ATP in almost all biological functions, including creating ionic gradients across membranes, vesicle fusion, and membrane recycling, is well appreciated, ATP dynamics and its spatial regulation are almost entirely unexplored in neurons. This is of special significance at nerve terminals, whose relatively remote placement with respect to the somatic cellular machinery often necessitates unique regulatory mechanisms.

The high concentration of mitochondria apparent in electron microscopic images of nerve terminals has suggested that ATP replenishment is especially important at synapses. The extent of this requirement is addressed by our analysis of *sesB*^{org}, a mutation that, by affecting the mitochondrial ATP/ADP translocase, causes abnormal ATP replenishment. While no defects are apparent at permissive temperatures, indicating nor-

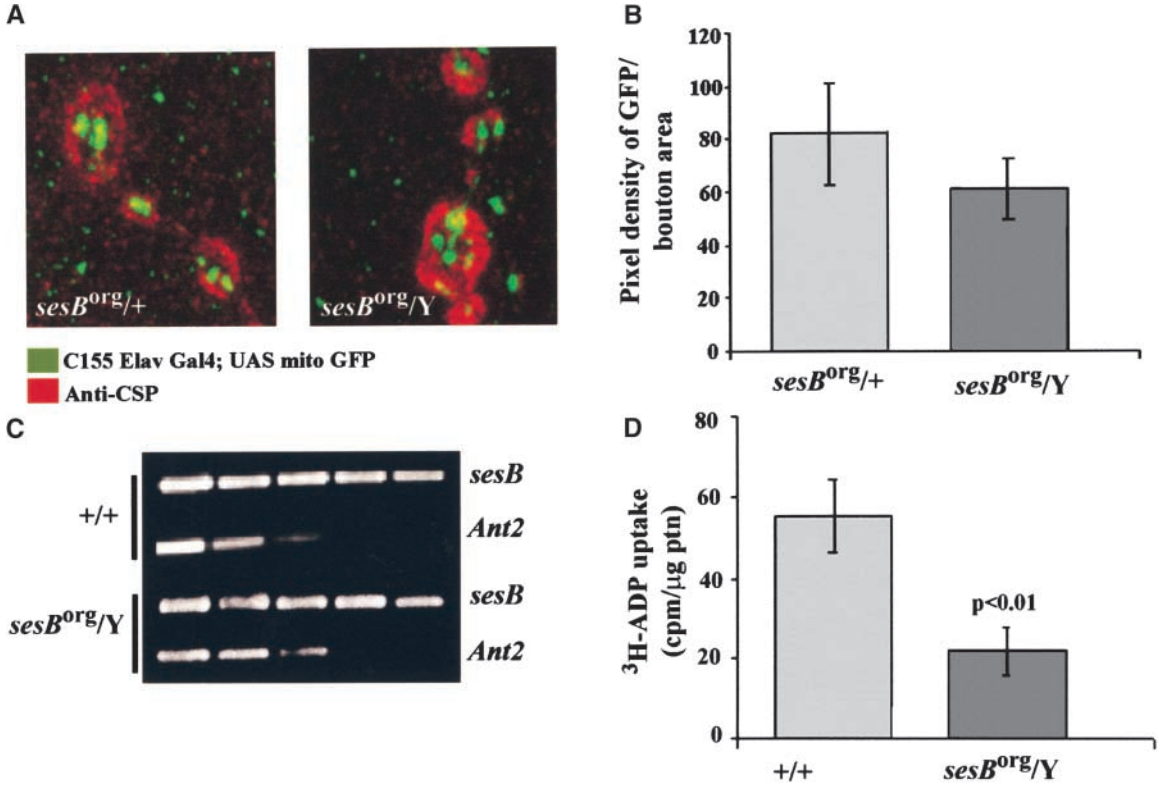

FIGURE 5.—Analysis of *sesB*^{org} function. (A) Mitochondria are visualized using *C155 Elav Gal4; UAS mito-GFP* in heterozygous *sesB*^{org}/+ and *sesB*^{org}/Y combination at the segment A2 muscle 6 and 7 of the larval neuromuscular junction. (B) Quantitation of pixel density normalized to the area of the boutons on muscle 6 and 7 of the larval neuromuscular junction (type 1b) in *sesB*^{org} and *sesB*^{org}/+ shows no significant difference ($P > 0.1$). The values are quantitated using the image-processing software of Metamorph. (C) Quantitative RT-PCR using specific nonoverlapping cDNA probes designed to C terminus of *sesB* and *Ant2* shows that there is no change in the transcript levels of *sesB*^{org} as compared to wild type. The different lanes indicate PCR from cDNA diluted at 1:5, 1:50, 1:250, 1:500, and 1:2500. (D) The *sesB*^{org} and wild-type mitochondrial extract are assessed for the uptake of ^{3}[H]-ADP. This shows a significant decrease ($P < 0.01$) in uptake of the label by mitochondria containing mutant ATP/ADP translocase.

mal development of the organism, shifts to nonpermissive temperatures cause immediate and striking phenotypes. First, behavioral paralysis that is complete within 2 min at restrictive temperature argues that at lowered rates of replenishment, cytoplasmic ATP drops very rapidly to levels that are at least locally too low to sustain normal neural transmission *in vivo* (Table 1). ERG recordings from adult Drosophila show multiple neural excitability processes that are rapidly affected by such acute inhibition of the ATD/ADP translocase. Two distinct components of the electroretinogram, the receptor potential generated by the concerted depolarization of photoreceptor neurons and the on-and-off transients deriving from synaptic transmission in the visual system, are rapidly lost at nonpermissive temperatures. This suggests that more than one aspect of neuronal activity is dependent on a rapid replenishment of ATP mediated by the *sesB*-encoded adenine nucleotide translocase. Specific insight into one of these neuronal processes and pathways is offered by analysis of *sesB*^{org} interactions with *shi* mutations that cause defects in synaptic vesicle recycling.

The *orangi* effect on dynamin-dependent synaptic vesicle recycling: When synaptic vesicle recycling is slowed down, synaptic transmission becomes especially sensitive to its further inhibition. In *shi*^{ts} mutants held just below their restrictive temperatures, such a sensitized situation may be created *in vivo*. Under these conditions, *sesB*^{org} is revealed as a strong enhancer of *shi*. Both paralysis and synaptic failure of *shi*^{ts} mutants occurs at a lower temperature in a *sesB*^{org} mutant background. Because *shi*-induced behavioral paralysis and synaptic failure are easily distinguished from superficially comparable effects of the *sesB*^{org} mutation, these data indicate that a reduced rate of ATP replenishment rapidly reduces the efficiency of dynamin-dependent synaptic vesicle recycling.

The mechanism of this effect is indicated by three lines of convergent evidence (Figure 4; Tables 2 and 3). First, *sesB*^{org}, like *awd* a locus that encodes a NDK required to activate dynamin, is an enhancer of *shi* paralysis. Second, *sesB*^{org}'s interactions with *shi* follow a very unusual pattern of allele specificity that is accurately mirrored by previously described interactions between *awd* and *shi*. Third, *sesB*^{org} strongly enhances a weak ts

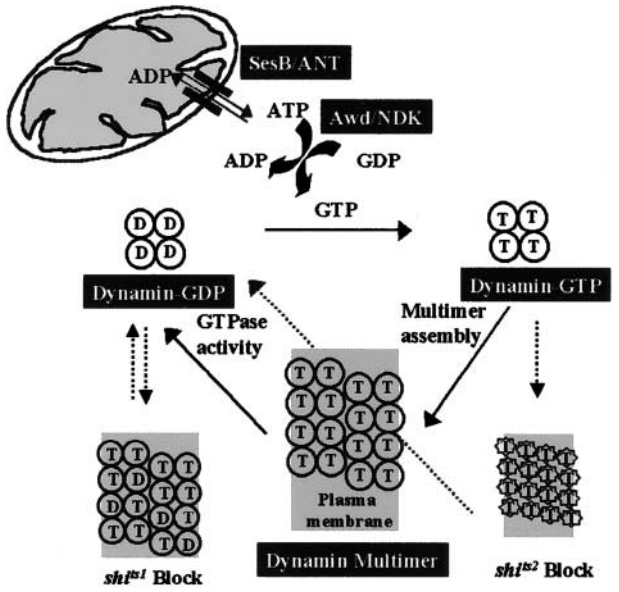

FIGURE 6.—Schematic showing the interaction between *sesB*^{org} and *shibire*. A model assaying the effect of ATP depletion exclusively on Dynamin-mediated synaptic vesicle recycling (adapted from CHEN *et al.* 2002 and KRISHNAN *et al.* 2001). SesB/ANT in the mitochondrial membrane functions to transport ATP into the cytoplasm in exchange for ADP. This regulates the local concentration of ATP. Awd/NDK uses ATP as a phosphate donor for converting GTP to GDP. NDK is required in the vicinity for mediating a conversion of the tetrameric Dynamin-GDP state to the active Dynamin-GTP state. The GTP bound state can load onto the plasma membrane and oligomerize. GTP hydrolysis correlates with the scission of the vesicle as well as the release of Dynamin-GDP tetramers. *shi*^{ts1} may not require GTP bound tetramers to load the plasma membrane for endocytosis. *shi*^{ts2}, however, is enhanced by both *awd*^{Msm95} and *sesB*^{org} and therefore is compromised in a GTP stimulated activity.

paralytic phenotype of *awd* and also further enhances ts paralysis of *shi; awd* double mutants. The most compelling explanation for these data is that rapid ATP replenishment is required for optimal activity of presynaptic NDK, an enzyme that provides GTP essential for dynamin function. The special property of *shi*^{ts1}, its insensitivity to both *awd* and *sesB*^{org} mutations, is easily explained by hypothesizing that dynamin:ts1 is unique in being affected in an enzymatic step that occurs after GTP binding. Such a mutant is predicted to be relatively insensitive to slight reductions in the rate of GTP loading. This hypothesis is consistent with recent studies demonstrating unique biochemical properties for the ts1 mutant dynamin (CHEN *et al.* 2002). A model in which we integrate the various genetic and neurophysiological observations of *sesB*^{org}, *shi*, and *awd* into a single biochemical pathway that underlies dynamin-dependent synaptic vesicle recycling is shown in Figure 6. The only likely source of ATP at the synapse is the mitochondrion and SesB/adenine nucleotide translocase; this enzyme is essential for transporting ATP across the mitochondrial membrane into the cytoplasm. ATP is preferentially used as a phosphate donor by NDK to make GTP. NDK acts to convert inactive dynamin-GDP to active dynamin-GTP. Activated dynamin-GTP associates with the plasma membrane and after oligomerizing proceeds through an orchestrated series of molecular events that underlie synaptic vesicle recycling. GTP hydrolysis that occurs at some step during these events results in the formation of inactive dynamin that requires further NDK-provided GTP for activation. It must be clarified, however, that this model deals exclusively with dynamin-dependent endocytosis and its ATP requirement. It is likely, as mentioned before, that ATP depletion could affect several steps in synaptic release, including neurotransmitter packaging, vesicle fusion, and vesicle cycling. However, the current study emphasizes the genetic interactions among *sesB*, *shi*, and *awd* mutations, suggesting a specific functional relationship in synaptic vesicle recycling. While the model described above fits our initial genetic and phenotypic characterization, it must at this stage be regarded as tentative although useful for guiding future lines of investigation.

Taken together, our observations provide support for the above model in which GTP loading onto dynamin, facilitated by an ATP-dependent enzyme NDK, requires a very dynamic ATP pool that is replenished by sustained activity of the ATP/ADP translocase on mitochondrial membrane. Experiments carried out in dissociated retinal bipolar neurons suggest that ATP is required for compensatory endocytosis, and our observations are consistent with these results (HEIDELBERGER 1998, 2001).

Implications on mitochondrial functions at the synapse: Mitochondria have been functionally implicated in various contexts at the synapse. The behavior and physiological characterization of *orangi* suggest a need for critical levels of ATP for synaptic activity. These effects are perhaps due to the small volume of the synaptic end and localized generation of ATP in the bouton. It is noteworthy that a mitochondrial mutant *milton* identified in Drosophila shows a complete lack of synaptic transmission due to a deranged trafficking of mitochondria to the nerve terminals (STOWERS *et al.* 2002). More recent analyses have revealed that photoreceptor terminals of *milton* mutants show aberrant synaptic vesicles, characteristic of defective synaptic vesicle recycling. Remarkably, these vesicles appear normal if photoreceptor activity (and hence vesicle fusion) is blocked in a *norp*^{A} mutant background (GÓRSKA-ANDRZEJAK *et al.* 2003).

The localized requirement of mitochondrial function is highlighted in large mammalian synapses found in the auditory system, called Calyx of Held. There are specialized structures called the mitochondria-associated adherens complexes in these synapses. Electron microscopic studies suggest that these structures typically contain an attachment of mitochondria with a presynaptic membrane via filaments and are found closely associated with vesicle release sights. These arrange-

ments are thought to be important for various mitochondrial functions at the nerve terminal, such as calcium buffering and supplying energy for synaptic vesicle cycling (Rowland *et al.* 2000). In the present study we demonstrate that if the local needs are not met, then there is synaptic failure due to effects on the synaptic vesicle cycle.

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