Disruption of Endocytosis with the Dynamin Mutant *shibire^{ts1}* Suppresses Seizures in *Drosophila*

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ABSTRACT One challenge in modern medicine is to control epilepsies that do not respond to currently available medications. Since seizures consist of coordinated and high-frequency neural activity, our goal was to disrupt neurotransmission with a synaptic transmission mutant and evaluate its ability to suppress seizures. We found that the mutant *shibire*, encoding dynamin, suppresses seizure-like activity in multiple seizure–sensitive *Drosophila* genotypes, one of which resembles human intractable epilepsy in several aspects. Because of the requirement of dynamin in endocytosis, increased temperature in the *shi^{ts1}* mutant causes impairment of synaptic vesicle recycling and is associated with suppression of the seizure-like activity. Additionally, we identified the giant fiber neuron as critical in the seizure circuit and sufficient to suppress seizures. Overall, our results implicate mutant dynamin as an effective seizure suppressor, suggesting that targeting or limiting the availability of synaptic vesicles could be an effective and general method of controlling epilepsy disorders.

KEYWORDS *Drosophila*; seizure; dynamin; behavior; genetics

ONE of the complexities in understanding and treating epilepsy disorders is that even though understanding of the molecular lesions underlying the conditions has improved, 20–30% patients display resistance to currently available antiepileptic drugs (AEDs) (Schuele and Lüders 2008). Many AEDs aim to decrease excitability by globally restoring ionic balances via binding to Na^+, K^+, or Ca^{2+} channels (Meldrum 1996). However, this global approach may not be sufficient to prevent the action potentials and seizures caused by all epilepsy mutations given that a mutation may affect a single population of neurons differently than the mutation in the context of the interconnected neural circuits. For example, haploinsufficiency of the sodium channel Na(V)1.1 in mice actually caused hyperexcitability and spontaneous seizures as a result of preferential loss of sodium currents in inhibitory interneurons compared to the sodium currents in pyramidal cells (Yu *et al.* 2006). In humans, approximately 70% of the cases of Dravet syndrome (DS) or severe myoclonic epilepsy of infancy (SMEI) are caused by *SCN1A* mutations, a large portion of them resulting in truncations that likely would eliminate the function of the channel, canceling out the effects of any Na^+ channel–targeted drugs (Marini *et al.* 2011).

Seizures are thought to initiate within a localized area and then spread to downstream neurons within the circuit, involving a large number of chemical synapses. For action potentials associated with the seizure to cross a chemical synapse, docked synaptic vesicles must fuse with the plasma membrane and release neurotransmitter into the synaptic cleft via exocytosis. Equally important for synaptic transmission is endocytosis, in which the plasma membrane is recycled to form new synaptic vesicles (Saheki and De Camilli 2012). Given the limited number of vesicles that can fit within the active zone of a synaptic terminal, replenishment of the vesicles locally by endocytosis is essential for sustained or high-frequency synaptic transmission. Because synaptic vesicles are critical for the generation and spread of seizures throughout the nervous system, regardless of the nature of the epilepsy mutation, we hypothesized that synaptic transmission mutations that interact or disrupt synaptic vesicles could be candidates to suppress seizures in *Drosophila* models of seizure disorders and epilepsy (Parker *et al.* 2011a).

Behavioral screens in *Drosophila* have found genes essential for synaptic transmission by isolating temperature-sensitive mutations, which often cause paralysis when shifted to a restrictive temperature because of their important roles in neural

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transmission (Grigliatti *et al.* 1973; Siddiqi and Benzer 1976). One such mutant, *shibire*, encodes dynamin, a GTPase required late in the process of endocytosis and synaptic vesicle recycling that is responsible for fission of the vesicle from the membrane (van der Bliek and Meyerowrtz 1991). The *shibire* mutant shi^{ts1} is a temperature-sensitive missense mutation in the GTPase domain of the protein, and when flies are heated to the restrictive temperature (~29°) and stimulated, nerve terminals become completely depleted of synaptic vesicles, paralyzing the flies (Koenig *et al.* 1983; van der Bliek and Meyerowrtz 1991). Ultrastructural analysis of the depleted nerve terminals shows the presynaptic membrane filled with clathrin-coated pits corresponding to vesicles stuck at the late stage of endocytosis, waiting to be pinched off by dynamin (Koenig and Ikeda 1989). When flies are returned to permissive temperatures, dynamin resumes normal function, and vesicular recycling resumes, refilling the depleted releasable pool of vesicles and allowing the shi^{ts1} mutation to act as a conditional block of endocytosis. In addition to impairments with vesicle endocytosis, ionic currents recorded from the dorsal longitudinal muscle (DLM) neuromuscular synapse in shi^{ts1} flies showed rapid synaptic fatigue within 20 msec to high-frequency stimulation (HFS), suggesting an additional role for dynamin in maintenance of the releasable pool of vesicles because the time to recycle a newly formed vesicle is between 15 and 30 sec (Kawasaki *et al.* 2000).

Using behavioral assays and electrophysiology, we found that temperature-sensitive shi^{ts} mutations are potent seizure suppressors for multiple bang-sensitive mutants, including the difficult-to-suppress $paralytic^{bss1}$ mutant. We used the *GAL4/UAS* system to overexpress shi^{ts1} in different neurons to map out a preliminary seizure circuit and identify the giant fiber (GF) neurons as sufficient to suppress seizures behaviorally and electrically. Our results help to elucidate circuits and neurons involved in the bang-sensitive phenotypes of *Drosophila* and provide evidence that even seizures in strong gain-of-function Na^+ channel mutants can be suppressed by disruption of synaptic vesicle endocytosis, revealing synaptic vesicle disruption or regulation as a potentially important pathway or target for the suppression of seizures and epilepsy.

Materials and Methods

Fly stocks and crosses

Flies were raised on standard agar-molasses-yeast food at room temperature (22°–24°) unless otherwise stated. The bang-sensitive mutant stocks were from the Tanouye laboratory stock collection. The protein encoded by the *easily shocked* gene is ethanolamine kinase. The *eas* allele used in this study is eas^{PC80}, a recessive mutation caused by a 2-bp deletion that introduces a frame shift; the resulting protein lacks a kinase domain and abolishes enzymatic activity (Pavlidis *et al.* 1994). The *slamdance* allele used in this study is $sda^{iso70.8}$, referred to as *sda*, and encodes for an aminopeptidase, and mutants show altered Na^+ currents (Zhang *et al.* 2002; Marley and Baines 2011). The $para^{bss1}$ mutation is a single-base-pair substitution (L1699F) of a highly conserved residue in the third membrane-spanning segment (S3b) of homology domain IV, resulting in a dominant phenotype with ~80% penetrance when heterozygous and 100% penetrance when homozygous (Parker *et al.* 2011b). The *UAS-*shi^{ts1} Kitamoto line, shi^{ts1}, and shi^{ts2} stocks were gifts from Nigel Atkinson. The remaining *UAS-*$shibire^{ts1}$ lines, *pBDGP-GAL4U* control line (Pfeiffer *et al.* 2012), and *R57C10-GAL4* stock were from the laboratory of Gerry Rubin. In this paper, *UAS-*$shi^{ts1\text{-}J100}$ refers to pJFRC100 *20XUAS-TTS-Shibire-ts1-p10* at insertion site VK00005 (Pfeiffer *et al.* 2012). The *a307-GAL4* was obtained from Rod Murphey and was maintained as a stock in our laboratory. The *shibire* deficiency stock used was *Df(1)sd72b/FM7c*. Other *GAL4* lines used were obtained from the Bloomington Stock Center. The shi^{ts1} *eas* and shi^{ts1} $para^{bss1}$ double mutants were made using standard recombination techniques. The shi^{ts}; *sda* stocks were made with the use of a *w*; *TM6,Tb/TM3*, *Sb* balancer line. All double mutants were verified for both mutations by the presence of bang-sensitive behavior at room temperature and paralysis at 30° (restrictive temperature). For *GAL4* experiments reported in Figure 7, master stocks of *w* $para^{bss1}$ *f*; *UAS-*$shi^{ts1\text{-}J100}$ or *eas/FM7a*; *UAS-*$shi^{ts1\text{-}J100}$ were made with a *FM7a*; *TM3*, *Sb/Dr*, *e* balancer stock. Virgins were collected from the master stocks and then crossed to different *GAL4* lines. For experiments reported in Figure 6, a *w* $para^{bss1}$ *f*; *R57C10-GAL4* line was created with a *FM7a*; *TM3*, *Sb/Dr*, *e* balancer stock. Virgins were collected from this line and crossed to different *UAS-*shi^{ts1} transgenes. For Rab experiments, *UAS-Rab* transgenes were obtained from the Bloomington Stock Center (Table 1 shows the stock numbers). Virgin flies from the *w* $para^{bss1}$ *f*; *R57C10-GAL4* line were collected and crossed to different *UAS-Rab* transgenes.

Bang-sensitive behavioral testing

Flies were collected with the use of CO_2, and up to 20 were placed in fresh-food vials with foam plugs. The flies were assayed the following day. For testing, the food vial was mechanically stimulated with a VWR vortex mixer at maximum speed for 10 sec. The number of flies experiencing seizure and paralysis owing to the bang-sensitive phenotype was counted immediately after the vortex. Results were pooled. Flies were tested only once and discarded unless otherwise stated. Care was taken to minimize shaking and vibrations of the vials before testing. All flies were 1–5 days posteclosion.

Electrophysiology of the GF system

In vivo electrophysiological assays and recording of seizure-like activity in the DLM were performed as described previously (Kuebler and Tanouye 2000; Lee and Wu 2002). For mounting the flies for electrophysiology, a fly was suctioned onto a hypodermic needle attached to a vacuum line and then immobilized in a channel of dental wax on a glass microscope slide. Uninsulated tungsten electrodes were used for the recordings and stimulation. Seizure threshold tests were

described previously (Kuebler and Tanouye 2000; Lee and Wu 2002). Seizure-like activity was evoked by HFS of 0.4-msec pulses at 200 Hz for 300 msec of varying voltages and monitored by DLM recordings. Flies were given up to 4 HFSs with 10 min of rest between each stimulus. To assess GF neural circuit function, the GF was stimulated with single-pulse stimuli (0.2-msec duration, 0.5 Hz) delivered to the brain. Recordings were obtained with Axoscope software and were analyzed with Axoscope and Stimfit software (Guzman *et al.* 2014). All flies were 1–4 days posteclosion.

Temperature-shift experiments

Flies were collected with the use of CO_2, and up to 10 were placed in fresh-food vials with foam plugs. The following day, vials were submerged upside down in a water bath at the indicated temperature. The plug was fully inserted into the vial so that when held upside down under water, a bubble of air was trapped and prevented water from contacting the plug or flies. Flies were kept in food vials during heating to avoid excessive handling of the flies before testing. If an air incubator was used, vials were placed on their sides at either 18° or 30°. Care was taken to minimize shaking and vibrations during movement of the vials. Flies were exposed only once to a given temperature treatment, tested, and then discarded. Heating flies during the electrophysiology experiment was accomplished by blowing heated air at the fly with a hand-held blow dryer set at a fixed position ~60 cm from the fly. The blow dryer produced heated air at a temperature of ~35°. We used two methods to estimate the temperature of the fly during the recording session: a temperature probe placed near the fly and an infrared (IR) thermometer aimed at the area near the fly. Our goal was to heat the flies consistently across trials by using similar lengths of heat exposure and by determining the temperatures of the flies by thermometers. Flies reached a peak temperature of 28°–31° during the experiments.

Statistics

All error bars shown represent the standard error of the mean (SEM). *N* values are indicated in the text or figure legends. In general, Fisher's exact test was used for comparing two groups, and a chi-square test was used for comparing multiple groups for bang-sensitivity percentages. Additionally, one-way ANOVA or the Student's *t*-test (for two samples) was used.

Data availability

Fly stocks available upon request.

Results

Behavioral suppression of seizure-like activity by disrupting endocytosis

The bang-sensitive (BS) *Drosophila* mutants represent a unique class of behavioral mutants that undergo a seizure-like episode on exposure to mechanical stimulation (Parker *et al.* 2011a) (Figure 1A). Ordinarily, individual mutants of the BS class show normal behavior until subjected to mechanical stimulation. Stimulation causes an immediate initial behavioral seizure (1–2 sec), followed by a period of paralysis lasting about 20–50 sec depending on the BS genotype. After paralysis, a recovery seizure is observed (Figure 1A). These BS behaviors are never observed in wild-type flies, nor are they ever observed in *shibire* (shi^{ts}) mutants.

To test the effects of shi^{ts} mutations, double-mutant combinations with different BS mutations were made using standard crossing techniques, and flies were tested for BS behavioral phenotypes. Three BS mutants were initially tested: *slamdance* (*sda*), *easily shocked* (*eas*), and *bang senseless* ($para^{bss1}$), the latter an allele of the *paralytic* gene. The restrictive temperature required to reach complete paralysis for shi^{ts} was unchanged by the presence of the BS mutation. The presence of shi^{ts} mutations caused changes in response to mechanical stimulation, indicating that they can act as suppressors of BS behavior. The initial indication of suppression is the occurrence of flies that are not paralyzed by mechanical stimulation. These nonparalyzed ("suppressed") flies resemble wild-type flies and appear completely normal. Some flies carrying a suppressor mutation are paralyzed by stimulation, indicating incomplete penetrance of suppression. These flies resemble other BS paralytic flies. Intermediate phenotypes are not apparent: stimulated flies are either paralyzed or not by mechanical stimulation.

At room temperature (23°), shi^{ts1} *eas* and shi^{ts1} $para^{bss1}$ double-mutant flies showed no changes in bang sensitivity compared to control. However, a minor decrease in bang sensitivity was seen in the shi^{ts}; *sda* double mutant (Figure 1B). Bang sensitivity in *sda* has been described previously as easier to suppress than *eas* or $para^{bss1}$ (Zhang *et al.* 2002; Song *et al.* 2007). Because *SCN1A* sodium channel mutations in human patients are heterozygous, we also tested heterozygous $para^{bss1}/+$ flies, which show semidominant bang sensitivity. There were no significant difference in bang sensitivity between $para^{bss1}/+$ flies and $para^{bss1}/shi^{ts1}$ double heterozygotes when tested at room temperature, with both genotypes showing BS paralysis in ~80% of flies. Increasing the dosage of mutant shi^{ts1} did not significantly alter bang sensitivity (Figure 1C). Therefore, at room temperature, in which no significant alteration of the synaptic vesicle pools has been observed in previous studies of shi^{ts}, BS behaviors are largely unaffected.

To trigger a stronger block of endocytosis, we preincubated flies at an elevated temperature immediately before bang tests. Because incubation of shi^{ts1} flies at temperatures ≥29° results in a complete paralysis of the fly and eliminates the BS phenotype, as well as all other behaviors, we limited preincubation to temperatures of 26° or 27°. At 27°, shi^{ts1} flies are still able to walk and maintain posture and apparently normal behavior. Flies display some hyperactivity and uncoordinated walking compared to controls, behaviors reported previously for shi^{ts1} mutants at this temperature (Koenig and Ikeda 1980). After 3 min of preincubation at an elevated temperature, BS behavior in *sda*, *eas*, and $para^{bss1}$ flies was partially suppressed by the shi^{ts1} mutation compared

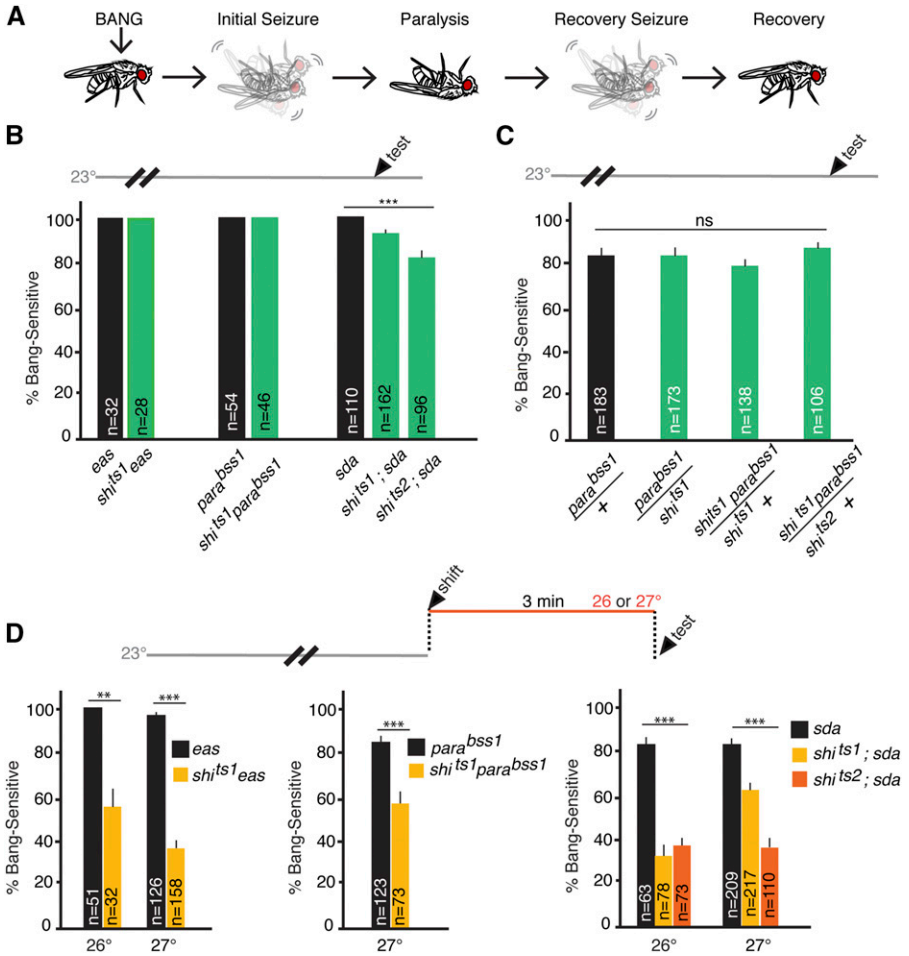

Figure 1 The mutation *shibire^{ts1}* suppresses bang-sensitive behavior in different bang-sensitive mutants. (A) An overview of the response to mechanical stimulation in the bang-sensitive mutants. The bang-sensitive mutant class undergoes a seizure-like episode following mechanical stimulation (a "bang"). After the initial seizure, there is a paralysis period of ~20–50 sec depending on genotype, followed by a recovery seizure of varying length, also dependent on genotype. Wild-type flies do not experience this phenomenon. (B) The bang sensitivities seen in *sda* mutant flies combined with either *shi^{ts1}* or *shi^{ts2}* mutations differ from those in wild-type flies. At room temperature, control flies were 99% bang sensitive, while *shi^{ts1}*; *sda* and *shi^{ts2}*; *sda* double mutants showed bang sensitivities of 90% and 81%, respectively ($P < 0.0001$, chi-square test). There was no change in bang sensitivity at room temperature with *shi^{ts1} eas* or *shi^{ts1} para^{bss1}* double-mutant combinations, which are harder mutations to suppress than *sda*. (C) At 23°, no differences in bang sensitivity were seen in *para^{bss1}*/+ mutants with increasing dosages of mutant *shi* ($P = 0.26$, chi-square test). (D) To further inactivate *shi* and test responses to more severe disruptions of endocytosis, flies were raised at room temperature and then shifted to an elevated temperature for 3 min in a water bath; then the flies were tested at room temperature after removal from the water bath. While there was no effect at room temperature, double-mutant *eas shi^{ts1}* flies were 56 and 36% bang sensitive after a 3-min incubation at 26 and 27°, respectively, while controls were still highly bang sensitive ($P = 0.0007$ at 26° and $P < 0.0001$ at 27°, Fisher's exact test). The bang sensitivity seen in *para^{bss1}* is suppressed by *shi^{ts1}*. At 27°, bang sensitivity was 58% for the *shi^{ts1} para^{bss1}* double mutant, while that of the *para^{bss1}* control was 83% ($P < 0.0001$, Fisher's exact test). At higher temperatures, the trend continues with decreased levels of bang sensitivity. At 27°, *shi*; *sda* flies often displayed hyperactive behavior ($P < 0.0001$ at 26° and 27°, chi-square test).

to controls (Figure 1D). Generally, suppression of BS behavior was good for *eas* and *sda* flies at 26° and better at 27°. One surprising observation was for one genetic combination *shi^{ts1}*; *sda* (but not for *shi^{ts2}*; *sda*): BS suppression appeared to be significantly better at 26° compared to 27°. We are examining the *shi^{ts1}*; *sda* genetic combinations further to determine whether there are any other peculiarities with this genotype. The elevated-temperature preincubation protocol did not lead to complete synaptic vesicle depletion because the flies were upright and able to maintain posture before testing, and flies were moving in the bottom of the vial after heat treatment. This suggests that even partial disruption of synaptic vesicle endocytosis prior to initiation of the seizure was sufficient to suppress the BS behavior in mutant flies.

The effects seen at "near restrictive" temperatures are consistent with previous observations that the *shi^{ts1}* mutant is minimally defective at 18° (the "permissive temperature"), with endocytosis becoming increasingly impaired with elevated temperatures and reaching a critical state that manifests as paralysis at temperatures above 29° (Kilman *et al.* 2009). Suppression of BS behavior was seen with both *shi^{ts1}* and *shi^{ts2}* alleles when tested in the *sda* background, suggesting that the mutant *shi* allele is responsible, rather than an unlinked mutation. Additionally, all three BS genotypes were suppressed to roughly the same degree, suggesting that *shi^{ts1}* acts as general seizure suppressor; it is not particularly specific to any one BS molecular pathway.

Seizure suppression at elevated temperatures by shi^{ts1} is transient and disappears quickly

Because the process of synaptic vesicle endocytosis is thought to occur in ~30 sec (Pyle *et al.* 2000) via classical clathrin-mediated endocytosis, clathrin-coated pits corresponding to trapped vesicular intermediates are found in heated *shi^{ts1}* flies; these intermediates disappear rapidly when endocytosis is allowed to proceed (Koenig and Ikeda 1989). To determine the timescale of BS suppression and whether flies regain BS sensitivity by the time that endocytosis potentially would have time to proceed and regenerate synaptic vesicles, flies were incubated at 27° for 3 min to disrupt vesicle endocytosis and then downshifted to 23° to resume endocytosis for various time periods before bang testing (Figure 2). Our results

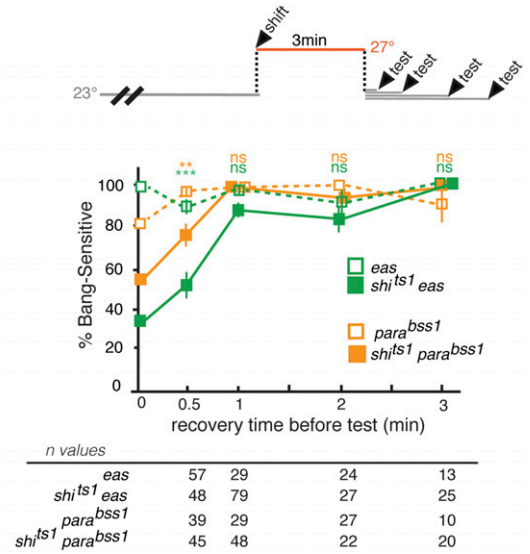

Figure 2 The effect of seizure suppression is correlated with the timescale of synaptic vesicle recycling. (A) Flies were heat shocked at 27° for 3 min to disrupt endocytosis; then flies were rested at room temperature for various recovery times before bang testing. Data from time point 0 are from Figure 1D for comparison. Flies continue to experience seizure suppression for a minimum of 30 sec after return to permissive temperatures. Longer periods of recovery no longer suppress seizures. Each time point was analyzed by Fisher's exact test between control and experimental flies for each genotype.

indicate that seizures were suppressed only when flies were tested very soon after the initial disruption of endocytosis. Flies that had rested at room temperature for 1 min after exposure to restrictive temperature were already found to have BS behaviors, similar to controls, while flies rested for only 30 sec still showed moderate levels of seizure suppression. Therefore, the observed time length of seizure suppression generally correlates with the timescale for synaptic vesicle endocytosis and mobilization of newly formed vesicles to the readily releasable pool (RRP), allowing for seizure-like activity to propagate.

Electrophysiological deficits in shi^{ts1} mutants indicate synaptic transmission defects

In previous studies, chemical synaptic function was examined electrophysiologically by stimulating the GF neural circuit and recording from the DLM. In particular, the GF circuit pathway to the DLM contains a prominent cholinergic synapse connecting the peripherally synapsing interneuron (PSI) with the DLM motoneuron, which is often monitored for synaptic function (Figure 3A) (Tanouye and Wyman 1980; Gorczyca and Hall 1984; Pavlidis and Tanouye 1995; Kuebler and Tanouye 2000; Kuebler *et al*. 2001; Allen *et al.* 2006; Fayyazuddin *et al*. 2006). Defects in synaptic transmission are indicated as a failure to follow, with fidelity, moderate stimulation frequencies. When delivering stimuli to the brain and recording from the DLM in mutant *shi^{ts1}* flies, we detected synaptic transmission defects not seen in control fly strains, even at permissive temperatures. We recorded DLM responses to a 1-Hz pulse delivered to the brain for 60 sec. We found that mutant combinations containing the *shi^{ts1}* mutation were much more likely to fail to respond to the test pulse (Figure 3, B and C). When *shi^{ts1}* flies did experience a failure, often a rest period of several seconds before stimulating was needed for the response to recover. Additionally, the latency of the GF response was much more varied in *shi^{ts1}* flies, often showing significant delays from the expected latency period (Figure 3D).

When stimulated in the brain, the wild-type DLM can maintain responses to 100-Hz stimulation (Tanouye and Wyman 1980). While control flies had high rates of successful DLM response at this high stimulation frequency, *shi^{ts1}* flies had high failure rates, usually only responding to the first stimulus in the train (Figure 3, E and F). The fact that *shi^{ts1}* synapses show failures in following frequency within the first 10 msec of electrical stimulation suggests that the mutation can exert an effect on the release or efficiency of release of synaptic vesicles in addition to its role in synaptic vesicle endocytosis. This indicates that in addition to the roles in classical endocytosis, dynamin could have roles in other processes at the synapse, especially with high-frequency synaptic transmission, similar to previous observations (Kawasaki *et al.* 2000). Overall, the *shi^{ts1}* mutation limits the ability of the GF system to reliably carry a neural signal across the synapses between the brain and the DLM at both high and low frequencies. This may be an important feature in *shi^{ts}* seizure suppression, especially for *sda* and *eas* BS mutants. The issue may be more complicated for seizure suppression in the double heterozygous flies *para^{bss1}/shi^{ts1}* (*i.e.*, genotype *shi^{+}para^{bss1}/shi^{ts}para^{+}*). The *para^{bss1}/shi^{ts1}* flies have a following frequency that is close to normal, not different from *para^{bss1}/+*. Nevertheless, *shi^{ts1}* (in this case the heterozygous *shi^{ts1}/+*) appears to make a contribution to seizure suppression. This resembles previous reports on seizure suppression and following frequency, although not specifically examining *shi^{ts}* double mutants (Kuebler *et al.* 2001). According to Kuebler *et al.* (2001) reductions in GF→DLM following frequency often was correlated with seizure suppression but was not an absolute predictor of seizure susceptibility: a low following frequency did not correspond with a high seizure threshold, indicating that other factors are involved.

Little change in seizure threshold at permissive temperatures

Seizure susceptibility and changes to it by the *shi^{ts1}* mutation can be assessed using electrophysiological recordings of neuronal seizure-like activity (Pavlidis and Tanouye 1995; Kuebler and Tanouye 2000; Lee and Wu 2002). Seizure-like activity may be evoked by HFS of the brain (0.4-msec pulses delivered at 200 Hz for 300 msec). Seizure-like activity is manifest as aberrant high-frequency firing (>80 Hz) usually recorded from the DLM but also present in all other muscle fibers and motoneurons (Figure 4A). The electrophysiology phenotype is an initial discharge of aberrant seizure-like neuronal firing followed by a period of synaptic failure and then

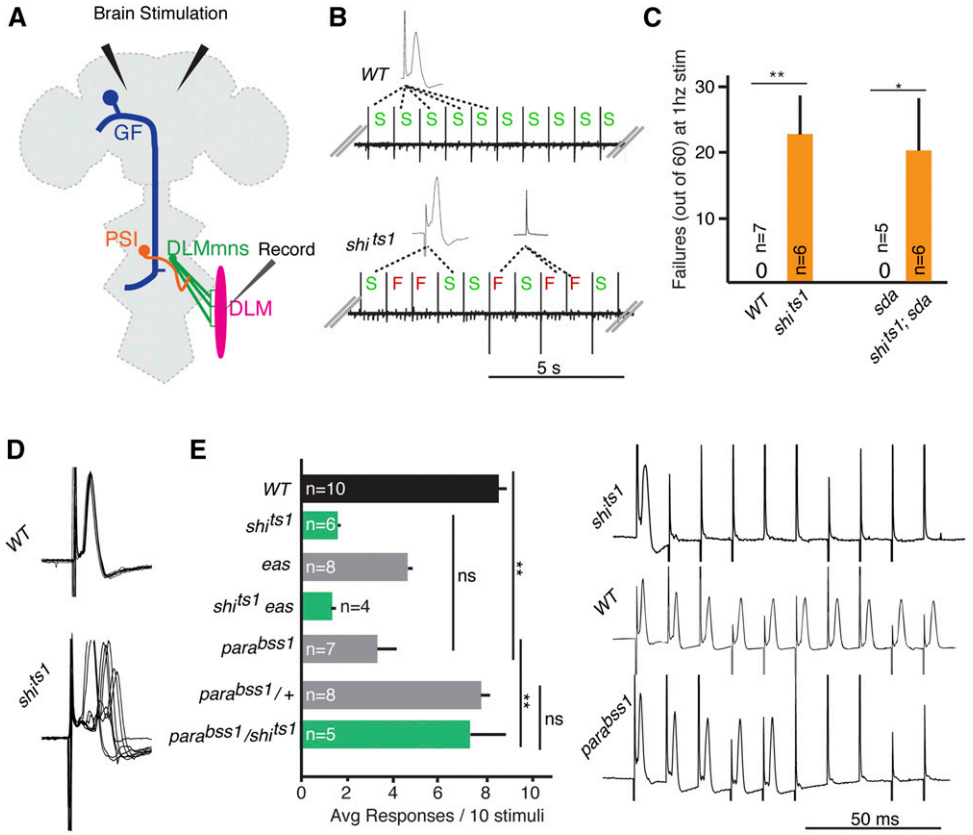

Figure 3 Electrophysiological phenotypes in *shi*^{ts}. (A) Cartoon overview of the recording and stimulation paradigm of the GF circuit in *Drosophila*. Stimulation to the brain activates the GF, which synapses to the excitatory peripherally synapsing interneuron, which then synapses to the dorsal longitudinal muscle (DLM) motoneurons (DLMmns), which results in all-or-none activation of the DLM. Latency from stimulation to DLM response is approximately 1.4 msec. (B) Examples of DLM responses to a series of 1-Hz test pulses for a total of 60 stimuli. Flies were tested at a voltage 1.5 times higher than their GF threshold to ensure consistent activation of the pathway. No differences in GF threshold voltages were observed. For the wild-type recording (WT), each stimulus was successful in evoking a DLM response, as indicated by the green S. A high-sweep-speed example of a successful stimulus and evoked DLM response is shown above the WT trace. For the *shi*^{ts1} trace, 10 stimuli were delivered: 5 stimuli were successful in evoking DLM responses (green S), and 5 stimuli were unsuccessful in evoking responses (red F). High-sweep-speed examples of a successful stimulus and evoked DLM response and an unsuccessful stimulus are shown above the *shi*^{ts1} trace. (C) While wild-type and *sda* control flies never failed to respond to a 1-Hz test pulse (0 failures of 60 stimuli for each), *shi*^{ts1} flies averaged 23 failures, and *shi*^{ts1}; *sda* flies averaged 21 failures over the course of 60 stimuli, indicating deficiencies in responding to evoked stimuli (P = 0.0012 and P = 0.03, Student's t-test). (D) Ten consecutive DLM responses from brain stimulation overlaid. Wild-type Canton S flies show extreme fidelity and precision, while *shi*^{ts1} flies initially show wild-type responses but then start to show varied latencies in their responses. (E) Tests with 100-Hz stimuli were performed on different genotypes. Ten trains of 10 100-Hz spikes were delivered to the flies with a 4-sec rest period between each train, totaling 100 stimuli. The percentage of successful responses was calculated for each animal and averaged for each genotype. The *shi*^{ts1} mutant was completely unable to respond at this frequency, with usually only the first response of each train eliciting a response. In contrast, wild-type flies were able to follow over 80% of the responses. Example traces show one train of the 10 presented to the fly. Statistical analysis was performed with one-way ANOVA followed by a post-hoc Tukey's HSD test.

a delayed discharge of aberrant firing (Pavlidis and Tanouye 1995; Kuebler and Tanouye 2000; Lee and Wu 2002; Parker *et al.* 2011b). The seizure threshold is the particular HFS voltage that triggers the seizure in the fly, with seizure-sensitive flies showing evoked seizure-like activity at relatively lower voltages. For example, wild-type flies have thresholds of ~30 V, and bang-sensitive flies have thresholds of ~3–10 V depending on the genotype (Parker *et al.* 2011a).

Interestingly, the seizure threshold of the *shi*^{ts1} mutant at room temperature was the same as that of the wild-type fly, indicating similar seizure susceptibilities (Figure 4B). Similarly, at room temperature, the *shi*^{ts1} *eas* and *shi*^{ts1} *bss* double mutants showed no significant differences in seizure threshold compared with their *eas* and *bss* single-mutant BS controls (Figure 4B). Seizure thresholds in *para*^{bss1}/+ heterozygotes with increasing levels of *shi*^{ts1} mutant alleles appeared to show a potential small increase in seizure thresholds at room temperature, but the variation is substantial, and this apparent increase was not significant (Figure 4C). Taken together, the lack of differences in the seizure threshold between BS single mutants and *shi*^{ts1} double mutants matches the behavioral response: both behavior and electrophysiology indicate that seizure susceptibility is unchanged by *shi*^{ts1} at room temperature.

The form of *shi*^{ts1} seizure-like neuronal firing resembles that of other mutant and wild-type seizures: an initial discharge, a period of synaptic failure, and then a delayed discharge. During initial and delayed discharges, the aberrant spikes observed in *shi*^{ts1} DLMs show firing frequencies of about 80 Hz, similar to that of other mutant and wild-type flies. This is considerably different from for the *shi*^{ts1} evoked DLM responses described previously that failed at about 1 Hz. Apparently, the nervous system has some capacity for high-frequency activity when it is triggered by HFS but is limited in its response to evoked activity probably because of transmission limitations in the PSI→DLM motoneuron synapse.

We used a chromosomal deficiency to investigate further the effect of *shi* loss of function on seizure thresholds. Because null alleles of *shi* are lethal, we made transheterozygotes to generate the weakest possible *shi* genotype that would still be

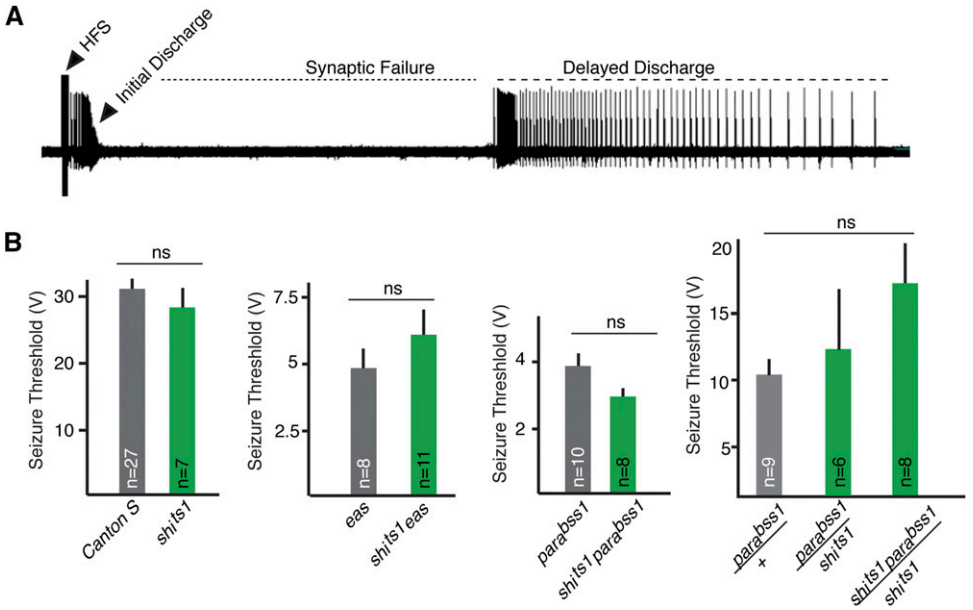

Figure 4 Seizure thresholds at room temperature are unchanged. (A) Seizure-like activity recorded from the DLM of a *para*^{bss1} fly evoked by high-frequency electrical stimulation (HFS) delivered to the brain by stimulating electrodes. The HFS is a short wave train consisting of 0.4-msec stimulus pulses delivered at 200 Hz for 300 msec. The HFS triggers a seizure-like episode when voltage is sufficient, in this instance for *para*^{bss1}, 4-V HFS. The threshold voltage for evoking a seizure-like episode is called the *seizure threshold* and is a quantitative measure of seizure susceptibility: genotypes with a lower seizure threshold voltage value are more seizure sensitive. HFS-evoked seizure-like activity is composed of a stereotypic sequence of neuronal activities in the DLM, each muscle potential representing the one-to-one firing of the DLM motoneuron. An initial discharge is a burst of uncontrolled high-frequency (~80 Hz) DLM potentials lasting 1–2 sec. The initial discharge is followed by a period of synaptic failure: the DLM does not respond to test pulses that stimulate the GF circuit because of failure of transmission of the PSI→DLM motoneuron synapse (Pavlidis and Tanouye 1995). The last event in the electrically triggered seizure-like response is a delayed discharge that resembles the initial discharge in some aspects of high-frequency spiking and is accompanied by the recovery of evoked DLM response. Seizure-like electrical activity is recorded in these experiments as aberrant DLM activity, but many other muscles and motoneurons also participate in the seizure (Kuebler and Tanouye 2000). (B) Seizure thresholds were measured at room temperature and compared between the BS mutant alone and in double-mutant combinations with the *shi*^{ts1}. At room temperature, no significant differences in seizure thresholds were detected, similar to the observed behavioral responses (Student' *t*-test and one-way ANOVA).

viable. As previous reports indicated, the *Df(1)/shi*^{ts1} transheterozygote was lethal, and no progeny could be collected, while a few *Df(1)/shi*^{ts2} flies were produced from crosses (Kim and Wu 1990). Over 16 *Df(1)/shi*^{ts2} flies were tested for seizure thresholds, but no seizure could be obtained from this genotype at any voltage range, suggesting that mutants with more severe dynamin defects may have very high seizure thresholds.

Discharges and hyperactivity associated with synaptic depletion in shi^{ts1} *flies*

To determine the electrophysiological correlates and activity of the DLM during heat exposure, *shi*^{ts1} flies were exposed to restrictive temperature (29°–30°) while recording from the DLM. Immediately following the temperature shift, spontaneous seizure-like activity was observed in the DLM. This preceded a loss of evoked transmission from the GF to the DLM (Figure 5), observations similarly reported previously for *shi*^{ts1} (Salkoff and Kelly 1978). With a return to permissive temperature, *shi*^{ts1} flies again displayed spontaneous DLM seizure-like activity, followed by a recovery of the evoked GF response to the DLM. We are struck by similarities between this temperature-induced sequence of events for *shi*^{ts1}, on the one hand, and for BS mutants, events evoked by HFS and mechanical stimulation, on the other. That is, for *shi*^{ts1}, the temperature-induced events of spontaneous seizures and transmission loss resemble DLM responses during a HFS electrically induced BS mutant seizure (*i.e.*, initial discharge, synaptic failure, and delayed discharge) and BS behavioral events following mechanical stimulation (*i.e.*, initial seizure, paralysis, and recovery seizure). The temperature-induced electrical events in the DLM were seen only in flies with the *shi*^{ts1} mutation, suggesting that these particular spontaneous discharges are a consequence of GF circuit–DLM activity related to the disruption of endocytosis. The similarities of the BS mutant responses could indicate similarly that this seizure-like activity also might be related to some type of synaptic depletion. After ~20–30 sec of paralysis, in which enough time has elapsed for newly recycled synaptic vesicles to regenerate, BS flies regain the ability to walk, maintain posture, and respond to evoked stimuli in the GF circuit.

Overexpression of shi^{ts1} *in neurons suppresses seizures in para*^{bss1} *and eas*

Endocytosis can be reversibly impaired using *shi*^{ts1} transgenes owing to the dominant-negative functioning of the Shi^{ts1} product (Pfeiffer *et al.* 2012). In combination with *GAL4/UAS*, *shi*^{ts1} transgenes have been used to inhibit synaptic transmission in neuronal subsets to determine functional involvement in specific behaviors (Brand and Perrimon 1993; Kitamoto 2001). Here we tested *UAS-shi*^{ts1} for its ability to suppress BS seizures initially using the broadly expressed neuronal *GAL4* line *R57C10-GAL4*, which is based on an *N-Syb* enhancer (Pfeiffer *et al.* 2012). Conditional BS suppression by *R57C10-GAL4/UAS-shi*$^{ts1\text{-}J100}$ was observed in hemizygous *para*^{bss1}/Y and *eas*/Y flies. BS suppression was observed when flies

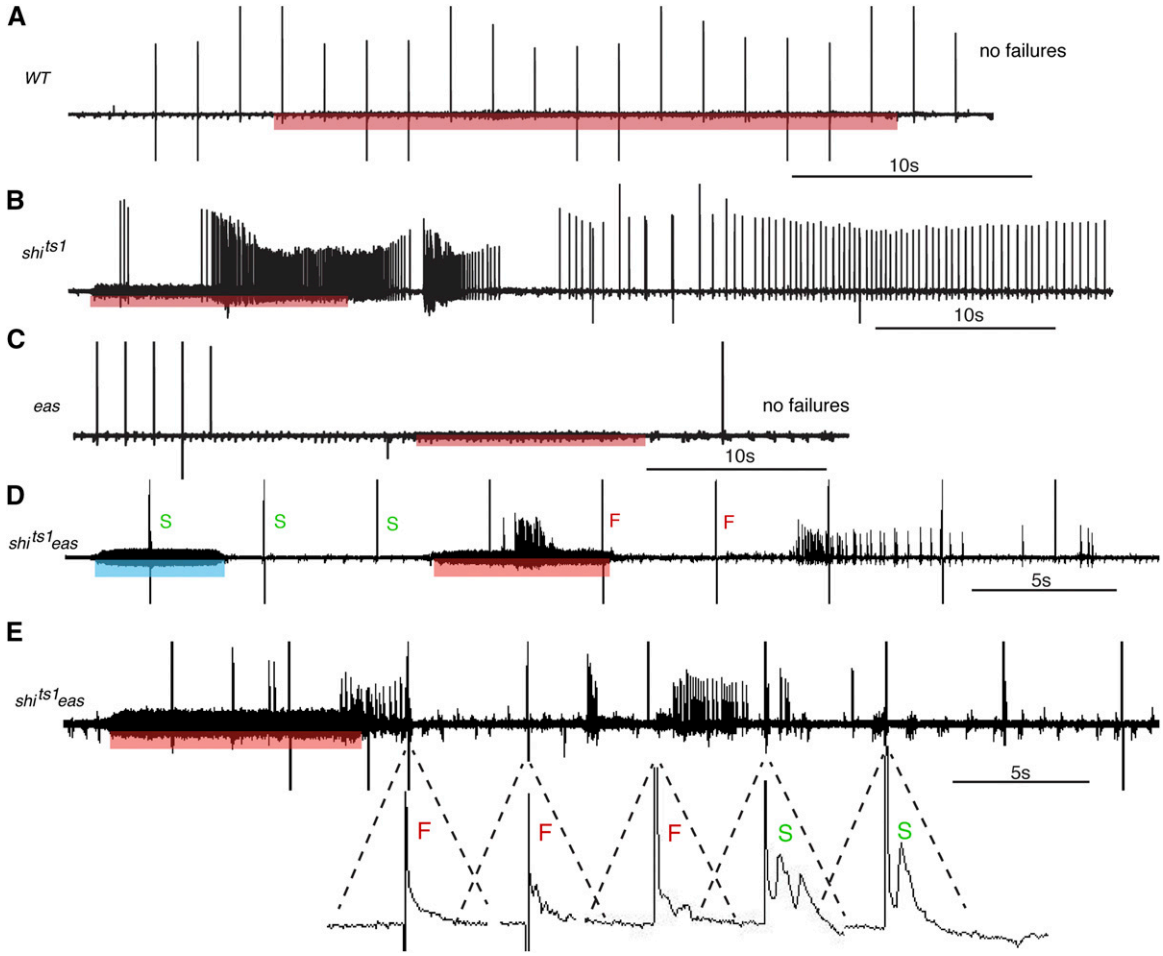

Figure 5 DLM recordings showing discharges and failures in *shi*^{ts1} heated flies. (A) A typical DLM recording from a wild-type Canton S fly. The fly was heated to 30° for 25 sec by blown air from a hair dryer, as indicated by the horizontal red bar. During the course of the experiment, the GF was activated continuously by single-pulse stimuli (0.2-msec duration, 0.5 Hz) delivered to the brain. Observed most saliently in this trace are the stimulus artifacts occurring at about 2-sec intervals (note: in this particular recording, there is some regularly occurring amplitude variation in the stimulus artifacts from an unknown source). Associated with every stimulus artifact is an evoked DLM muscle potential (not resolved at this sweep speed). The temperature shift causes no loss of evoked DLM response. Also, there is little evidence of temperature-induced discharges in the DLM. Spontaneous activity is limited to three spikes that occur about 5 sec following the return to room temperature. (B) A typical DLM recording from a *shi*^{ts1} fly. The fly was heated to 29° for 15 sec, as indicated by the horizontal red bar. Observed most saliently in this trace is seizure-like DLM spiking that begins about midway through the temperature shift and, in this instance, continues throughout the course of the recording. Some interruptions in the discharge and changes in frequency are observed. For purposes of clarity, the GF neural circuit was not stimulated in this example. (C) A typical DLM recording from an *eas* mutant fly. The fly was heated to 30° for about 15 sec, as indicated by the horizontal red bar. At the start of the recording, the GF was stimulated by single-pulse stimuli at 0.5 Hz (0.2-msec stimulus pulses). The recording shows the delivery of five stimuli, with each stimulus evoking a DLM potential (not resolved at this sweep speed). GF stimulation was turned off prior to the temperature shift. The temperature shift causes no seizure-like DLM spiking; a single spontaneous DLM spike is observed near the end of the recording showing that the DLM remained responsive throughout the course of the recording. (D) A DLM recording from a *shi*^{ts1} *eas* double-mutant fly. Room-temperature air (23°, horizontal blue bar) was blown on the fly, followed by heated air that caused a temperature shift to 29° (horizontal red bar). Seizure-like discharges were triggered by heat, beginning at about the middle of the temperature shift. Seizure-like discharges are not triggered by the unheated blown air. GF stimulation showed that evoked DLM responses were unaffected by unheated blown air but were lost by the high-temperature shift (DLM potentials are not resolved at this sweep speed). A red F indicates examples of GF stimulations that failed to evoke a DLM response; a green S indicates examples of recovering evoked DLM responses. (E) A DLM recording from a *shi*^{ts1} *eas* double-mutant fly. The fly was heated to 29° for 10 sec, as indicated by the horizontal red bar. During the course of the experiment, the GF was activated continuously by single-pulse stimuli (0.2-msec duration, 0.5 Hz) delivered to the brain. Seizure-like discharges were triggered by heat, beginning near the end of the temperature-shift period. GF stimulation showed that evoked DLM responses were lost after the temperature shift. A red F indicates examples of GF stimulations that failed to evoke a DLM response, displayed at high sweep speed; a green S indicates examples of recovering evoked DLM responses, displayed at high sweep speed.

were preincubated for 3 min at elevated temperature (Figure 6A). In contrast, at room temperature, without elevated temperature preincubation, no BS suppression was observed for *para*^{bss1}/Y and *eas*/Y flies. Thus, for this *GAL4/UAS* suppression, elevated-temperature preincubation is required, resembling the suppression observed for *shi*^{ts1} BS double mutants.

The *para*^{bss1}/+ mutant had a surprising and unusual response to the suppressive effects of *UAS-shi*^{ts1}: BS suppression was found to be constitutive (Figure 6B). That is, we found

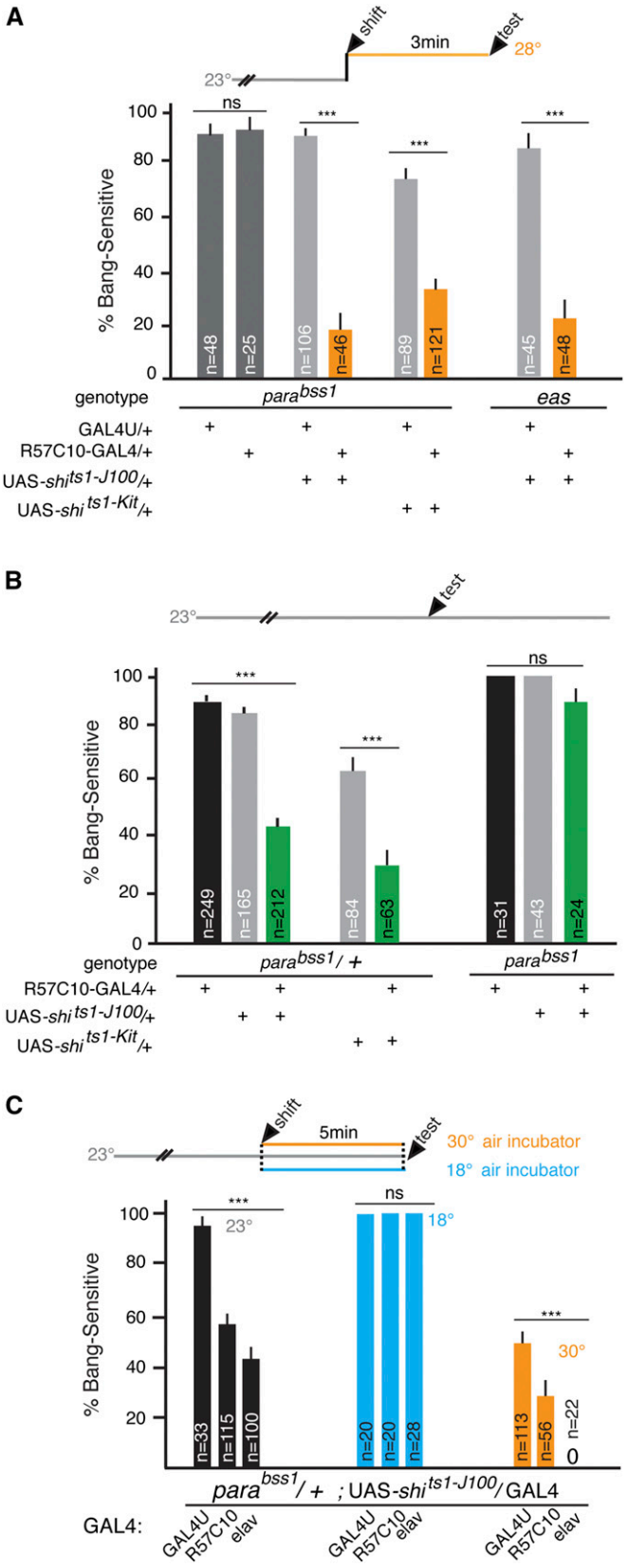

Figure 6 Overexpression of *shi*^{ts1} with the *GAL4/UAS* system suppresses seizures at permissive and near-restrictive temperatures depending on the bang-sensitive mutant. (A) Seizures in *para*^{bss1} and *eas* flies are suppressed with overexpression of *shi*^{ts1}. The *GAL4* driver *R57C10* was combined with different *UAS-shi*^{ts1} transgenes to express the mutant gene product. Preincubation temperature shifts were made to 28° for 3 min. Note that preincubation is carried out at a little higher temperature for the transgene-carrying flies than for *shi*^{ts1} mutant flies because of the differences in restrictive temperature: 29° for *shi*^{ts1} mutant flies, 30.6° for *UAS-shi*^{ts1} flies (Pfeiffer *et al.* 2012). Mutants were *para*^{bss1} and *eas* hemizgotes expressing *UAS-shi*$^{ts1\text{-}J100}$ or *UAS-shi*$^{ts1\text{-}Kit}$ with the *57C10-GAL4* line, which expresses pan-neuronally, or *GAL4U*, which does not have nervous system expression. Both genotypes showed BS levels around 20% with the *UAS-shi*$^{ts1\text{-}J100}$ transgene ($P < 0.0001$, Fisher's exact test). (B) Seizures in *para*^{bss1}/+ flies are suppressed at room temperature (23°) using *GAL4/UAS* to express *shi*^{ts1}. At room temperature, there was a reduction in the proportion of *para*^{bss1}/+ flies experiencing a seizure when both the *UAS-shi*$^{ts1\text{-}J100}$ transgene and the *GAL4* line were combined, with 43% of the flies experiencing seizures compared to 88 and 84% for controls ($P < 0.0001$, chi-square test). The Kitamoto line (*UAS-shi*$^{ts1\text{-}Kit}$) also had significant effects at room temperature ($P < 0.0001$, Fisher's exact test). There was no effect on hemizygous *para*^{bss1} males at 23°, indicating that a stronger blockade of synaptic vesicle recycling than can be achieved at room temperature is required to suppress seizures in that mutant. (C) Temperature shifts were performed on *para*^{bss1}/+ flies with two broadly expressed neuronal *GAL4* drivers and *UAS-shi*$^{ts1\text{-}J100}$ transgenes compared to the *GAL4* control line. The *para*^{bss1}/+ mutants showed increased bang sensitivity when shifted to 18°. In contrast, flies that were shifted to a 30° air incubator all experienced fewer seizures. While controls also were affected by temperature, making it difficult to determine the magnitude of the effects, they were less affected than any lines expressing *shi*^{ts1} (***$P < 0.001$, chi-square test).

that expression of *UAS-shi*^{ts1} transgenes suppressed BS phenotypes in *para*^{bss1}/+ heterozygous flies at room temperature (permissive temperature). Control flies with either the transgene or *GAL4* alone were still seizure sensitive at all temperatures (Figure 6B). We used two different *UAS-shi*^{ts1} lines (*UAS-shi*$^{ts1\text{-}J100}$ and *UAS-shi*$^{ts1\text{-}Kit}$), and both were effective at suppressing *para*^{bss1}/+ at room temperature (Figure 6B). Both of these *UAS-shi*^{ts1} constructs have been described previously as strongly expressing Shi^{ts1} dominant-negative protein because they both contain multiple copies of the *UAS-shi*^{ts1} transgene (Pfeiffer *et al.* 2012). Also, we used two different *GAL4* drivers, with *elav*^{c155}*-GAL4* a little more effective at BS suppression than *R57C10-GAL4* (Figure 6C). Because seizure suppression was not observed in *para*^{bss1}/*shi*^{ts1} mutants at room temperature, increased expression of mutant dynamin is likely responsible for the observed differences. The distinct effect of *shi*^{ts1} at room temperature when overexpressed, despite no apparent major impairment or depletion of synaptic vesicles through the use of elevated temperatures, also has been observed by another group when they observed changes in the length of circadian rhythms in flies expressing *shi*^{ts1} (Kilman *et al.* 2009). Additionally, they found no changes when expressing wild-type Shibire protein, suggesting that only the mutant Shibire protein caused the observed changes at room temperature (Kilman *et al.* 2009).

The temperature dependence of BS suppression of *para*^{bss1}/+ by *UAS-shi*^{ts1} is complex, as shown by preincubation experiments (Figure 6C). Preincubation at an elevated temperature (30° for 5 min) appears to show greater suppression than for room temperature, although it is difficult to be

completely sure about this because control flies with *GAL4U* (nonneural driver) were reduced in their BS paralysis. A surprising and unusual finding occurred with flies that were preincubated at a lowered temperature (18° for 5 min). For these flies, the lowered temperature appeared to completely abolish suppression of $para^{bss1}$/+ BS phenotypes; 100% of flies showed BS paralysis regardless of *UAS-*$shi^{ts1\text{-}J100}$ driven by *GAL4U*, *elav*^{c155}*-GAL4*, or *R57C10-GAL4* (Figure 6C). Taken together, the observations at different temperatures suggest that the BS phenotype seen in $para^{bss1}$/+ flies is directly related to the severity of the endocytosis block. BS suppression is observed at 23°, it may be increased at 30°, and suppression appears to be completely absent at 18°. Because the more severe BS mutants need a stronger disruption of dynamin and endocytosis to suppress the seizures compared to the $para^{bss1}$/+ heterozygote, this suggests that more synaptic vesicles are used or released during the seizure in *eas* and $para^{bss1}$ flies compared to $para^{bss1}$/+ flies.

Mapping a seizure circuit: disruption of endocytosis in excitatory neurons or the GF neuron suppresses seizures

Since shi^{ts1} was effective at suppressing seizures when expressed in all neurons, we used it to investigate the contribution of different neuronal subsets on the BS phenotype and map out a preliminary seizure circuit. Different *GAL4* drivers were tested for their ability to suppress the BS phenotype and increase seizure thresholds in mutant flies. We first performed a small preliminary screen to identify the types of *GAL4* lines that might produce a seizure-suppression effect. A number of *GAL4* lines were selected and then crossed to $para^{bss1}$; *UAS-*$shi^{ts1\text{-}J100}$ virgin flies, and the $para^{bss1}$/+ heterozygous progeny were tested. For this initial screen, flies were raised at both 23° and 25°, and no significant differences were detected between them (supporting information, Figure S1A). We found that most lines tested in this initial screen had no effect on seizures, and lines that had more expansive expression patterns were more likely to have reduced seizure sensitivity (Figure S1B).

We also narrowed our focus on neurons within the GF circuit upstream of the DLM. Hemizygous $para^{bss1}$ and *eas* flies were tested after preincubation at an elevated temperature, while $para^{bss1}$/+ flies were tested at room temperature. We first tested whether disruption of the neuromuscular junction directly upstream of the DLM was sufficient to suppress bang sensitivity. We found that *OK6-GAL4*, which expresses in motoneurons, had little or no effect on the behavioral BS phenotype of any of the flies. Matching the behavioral response, the seizure thresholds were not increased. This indicates that the suppression of action potentials associated with the seizure mainly occurs upstream of the motoneuron within the seizure circuit (Figure 7).

Upstream of the DLM motoneuron in the GF circuit is the PSI neuron, an excitatory cholinergic interneuron that forms a chemical synapse to the DLM motoneuron. Using *Cha-GAL4*, which expresses in cholinergic neurons in addition to the PSI neuron, we observed that suppression of bang sensitivity and seizure thresholds was increased (Figure 7, A and B). Given the widespread expression of the *Cha-GAL4* pattern, it is difficult to determine the specific contribution of the PSI neuron itself. However, these results fit the model that an overall disruption of synaptic transmission in excitatory neurons would reduce overall neuronal excitability and limit the spread of the action potentials associated with the seizure throughout the animal.

Flies also were tested for suppression using *a307-GAL4*, a line expressed strongly and specifically in the GF neurons (Allen *et al.* 2007). While no behavioral effect was observed in the $para^{bss1}$/+ mutant flies, nor were seizure thresholds increased at room temperature, we saw strong suppression with *a307-GAL4* in $para^{bss1}$ and *eas* genotypes that were preincubated at 28° before bang testing (Figure 7C). Inhibition of the GF neurons was sufficient to block bang sensitivity, implicating them as an important pathway for the seizure to spread from the brain; however, it is difficult to determine in the remaining flies that were still BS whether another potential seizure circuit was used or there was insufficient disruption of dynamin in the GF neuron. The strikingly similar responses to the different *GAL4* lines between the $para^{bss1}$ and *eas* mutants suggest that the same seizure circuit is used between the different BS mutants during their responses to mechanical or electrical stimuli.

Additionally, expression of shi^{ts1} in the GF neurons was sufficient to copy the phenotype of heated shi^{ts1} flies. Heated *eas* and $para^{bss1}$ flies with *a307-GAL4/UAS-*$shi^{ts1\text{-}J100}$ showed DLM responses that were similar to heated shi^{ts1} mutant flies (Figure 7, D and E). Therefore, expression of shi^{ts1} in the GF neuron is sufficient to produce the similar sequence of discharges and failures associated with synaptic depletion, and the GF could be the responsible neuron for the discharges seen in the mutant.

Finally, flies were given a HFS to attempt to induce seizure-like activity after being heated. While seven of nine control $para^{bss1}$; *a307-GAL4*/+ flies experienced a seizure at 5.6 V after being heated and did not show characteristic discharges of the DLM in response to heat, only one of nine experimental $para^{bss1}$; *a307-GAL4/UAS-*$shi^{ts1\text{-}J100}$ flies experienced a seizure at 5.6 V, and eight showed discharges of the DLM before the seizure, indicating that the experimental flies had a higher seizure threshold than controls ($P = 0.015$, Fisher's exact test) (Figure 7, D and E). Mutant *eas*; *a307-GAL4/UAS-*$shi^{ts1\text{-}J100}$ flies also showed similar responses to heat and HFS as the $para^{bss1}$ mutant, indicating that seizure thresholds are increased and bang sensitivity is reduced when endocytosis is disrupted in the GF neuron (Figure 7F).

Beyond dynamin: Rab mutations also suppress $para^{bss1}$/+ *bang sensitivity*

To more broadly determine whether the suppression of bang sensitivity is due to the specific role of dynamin itself or is more generally related to disruption or alteration of endocytic processes, we tested additional mutants involved in endocytosis and vesicle trafficking but that did not interact directly

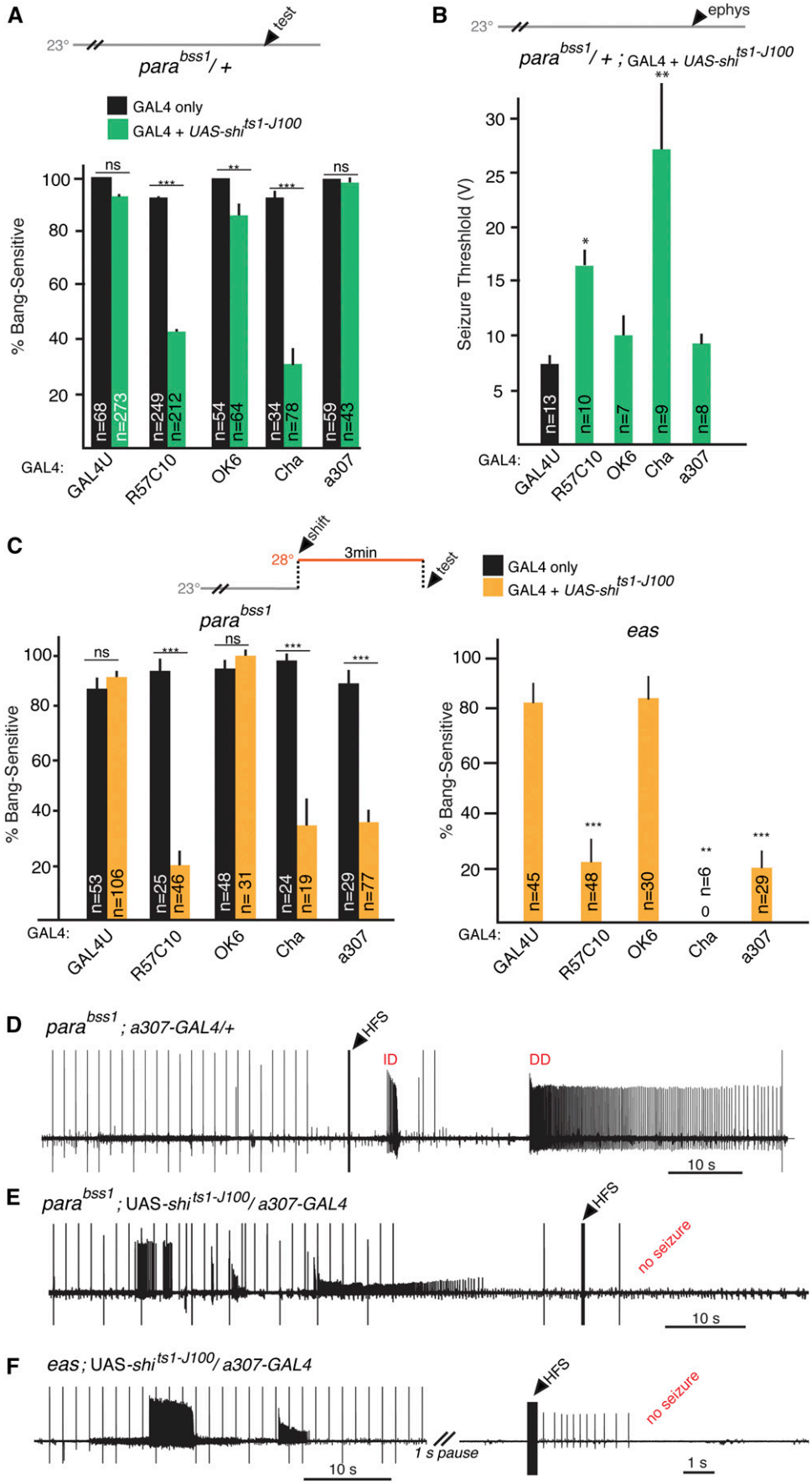

Figure 7 Involvement of the GF circuit in suppression of seizure-like activity in the DLM. (A) Heterozygous *para*^{bss1}/+ flies expressing *UAS-shi*$^{ts1-J100}$ with different *GAL4* lines were tested at room temperature for BS paralysis. Using the *GAL4/UAS* system, we expressed *shi*^{ts1} in different neurons of the GF circuit. No effect was seen with *a307-GAL4*, which expresses in GF neurons; a small effect was seen with *OK6-GAL4*, which expresses in motoneurons. However, there were large, significant effects with *Cha-GAL4*, with only 30% of the flies experiencing seizures (***$P < 0.0001$, **$P < 0.001$, Fisher's exact test). For comparison, experiments with *R57C10-GAL4* produced the data in Figure 6. (B) Heterozygous *para*^{bss1}/+ flies expressing *UAS-shi*$^{ts1-J100}$ with different *GAL4* lines were tested at room temperature for seizure thresholds. Similar to behavioral results, the *57C10-GAL4* and *Cha-GAL4* lines increased seizure thresholds to 17 and 29 V compared to 7.5 V for the control [**$P \leq 0.01$, *$P < 0.05$ by one-way ANOVA followed by post-hoc Bonferroni and Holm correction (groups *vs.* control)]. (C) Hemizygous *para*^{bss1} and *eas* flies were tested for bang sensitivity after a 3-min incubation at 28° while expressing *UAS-shi*$^{ts1-J100}$ with different *GAL4* lines. Strong effects were seen with *R57C10-GAL4*, *Cha-GAL4*, and *a307-GAL4* lines (***$P < 0.0001$, **$P < 0.001$, Fisher's exact tests). (D) A *para*^{bss1}; *a307-GAL4*/+ control fly was heated and stimulated with a HFS at 5.6 V to attempt to trigger a seizure. The control fly had a stereotypic seizure, with an initial discharge (ID), followed by synaptic failure, followed by a delayed discharge (DD). (E) A *para*^{bss1}; *a307-GAL4/UAS-shi*$^{ts1-J100}$ fly was heated and stimulated with a HFS at 5.6 V to attempt to trigger a seizure. The fly did not experience any seizure or failures in response to the 5.6-V stimulus. (F) Similar to E, an *eas*; *a307-GAL4/UAS-shi*$^{ts1-J100}$ fly was heated and stimulated with a HFS at 5.6 V to attempt to trigger a seizure. The fly did not experience any seizure or failures in response to the 5.6-V stimulus.

Table 1 Suppression of *para*^{bss1}/+ bang-sensitivity (BS) by mutant Rab proteins

Drosophila gene	Human ortholog(s)[a]		*57C10 BS (%)*	*57C10 n*	*GAL4U BS (%)*	*GAL4U n*	*P-* value[b]	Stock no.[c]
Rab3	*Rab3a, 3b,3c, 3d*	CA	100	45	100	35	1	9764
		DN	93	41	94	36	1	9766
Rab5	*Rab5a, 5b, 5c, 17*	CA	60	45	96	28	0.0004*	9773
		DN	85	124	84	107	1	9771
Rab8	*Rab8a, 8b, 13, 12*	CA	97	32	86	28	0.174	9781
		DN	81	101	92	97	0.038*	9780
Rab9	*Rab9a, 9b*	CA	78	99	90	99	0.033*	9785
		DN	74	66	92	71	0.011*	23642
Rab23	*Rab23*	CA	98	95	98	46	1	9806
		DN	97	36	100	41	1	9804
Rab27	*Rab27a, 27b*	CA	88	69	100	17	0.347	23266
		DN	92	25	100	47	0.117	23267
Rab30	*Rab30*	CA	65	110	78	115	0.027*	9814
		DN	75	99	89	84	0.011*	9813
Rab32	*Rab32*	CA	95	44	95	22	1	23280
		DN	76	87	98	45	0.001*	23281
Rab35	*Rab35*	CA	91	150	94	180	0.206	9818
		DN	92	112	100	66	0.027*	9819

Summary of Rab GTPases used in this study and their effects on suppression of *para*^{bss1}/+ bang sensitivity. The nine Rab GTPases used in the screen were chosen because of evidence indicating colocalization with CSP, a synaptic vesicle marker (Jin *et al.* 2012).

[a] Human orthologs were determined from Gillingham *et al.* (2014).

[b] *P*-values obtained from a Fisher's exact test comparing the bang sensitivity of the *para*^{bss1}/+; *GAL4U/UAS-Rab* (control) compared to the *para*^{bss1}/+; *R57C10/UAS-Rab* (experimental) genotype.

[c] Bloomington Stock Center number for the *UAS-Rab* transgene used in the experiment (Zhang *et al.*, 2006).

with dynamin. Rab GTPases are part of a large class of eukaryotic proteins that act as key regulators of vesicle trafficking, formation, fusion, transport, and endocytosis (Somsel Rodman and Wandinger-Ness 2000; Deinhardt *et al.* 2006; Chan *et al.* 2011). While the exact function of each Rab is still unclear, they are known to play a significant role in neuronal function (Ng and Tang 2008). Additionally, many Rabs express solely in neurons, and of those, many display strong localization at synapses (Chan *et al.* 2011). We hypothesized that altering Rab GTPase activity would either directly disrupt or interfere with endocytosis, synaptic vesicle availability, or the efficiency of the synapses and lead to suppression of seizure phenotypes, similar to the effects of dynamin. While there are approximately 31 distinct *Rab* genes in *Drosophila*, we focused our efforts on nine that have been shown previously to colocalize with synaptic vesicles (Jin *et al.* 2012; Gillingham *et al.* 2014). To alter Rab GTPase activity, we expressed dominant-negative (DN) or constitutively active (CA) mutant Rab proteins in *para*^{bss1}/+ flies and scored them for bang sensitivity (Zhang *et al.* 2006). We used the pan-neuronal *R57C10-GAL4* driver. Of a total of 18 *Rab* mutant combinations tested (representing nine genes, each gene with a DN and CA mutation), we found eight *Rab* combinations that caused significant reductions of bang sensitivity ($P < 0.05$) compared to control flies containing the *Rab* transgene with the control *GAL4U* line (Table 1). In contrast to the effect on *para*^{bss1}/+, no reduction in bang sensitivity was seen when testing the *eas* mutant or the *para*^{bss1} hemizygote, implying that the *Rab* mutants are weaker seizure suppressors than *shi*^{ts1} (data not shown). Overall, the reduction in seizure susceptibility by multiple Rab GTPases suggests that a general interference or disruption of synaptic vesicle trafficking and regulation may be sufficient to suppress some seizure disorders and that the effect is not limited only to dynamin.

Discussion

Disruption of synaptic transmission vs. restoration of ionic current

Our goal was to determine whether mutations affecting synaptic transmission that would perturb synaptic transmission at chemical synapses would be sufficient to suppress seizures. Through use of the temperature-sensitive paralytic *shi*^{ts1} mutation, we introduced a reversible block of endocytosis into the flies and observed seizure suppression in multiple bang-sensitive genotypes, including a model of epilepsy that resembles aspects of intractable *SCN1A* gain-of-function mutations in humans. Our approach to suppress seizures and the spread of action potentials at chemical synapses by disrupting synaptic vesicle availability and recycling bypasses the requirement to overcome the difficult-to-suppress ionic or molecular changes that may be introduced by epilepsy mutations, many of which are *de novo*. For example, AEDs such as phenytoin or carbamazepine bind to Na^+ channels and block high-frequency firing, decreasing the amount of neurotransmitter released at synapses (Rogawski and Löscher 2004). In cases of *SCN1A* epilepsy mutations in which there is a truncation or other disruption, all AEDs that target Na^+ channels likely would be ineffective.

The role of dynamin in synaptic transmission

Robust synaptic transmission requires recycling of vesicles at the plasma membrane via endocytosis to maintain efficient and accurate synaptic transmission. Endocytosis is especially

critical for replenishment of the synaptic vesicle pools during high-frequency stimulation, which would be present during a seizure-like episode or discharge. If the synapse were unable to maintain the rate of endocytosis and replenish the population of vesicles via introduction of the shi^{ts1} mutation, for example, synaptic transmission would be affected as a result of the unavailability of docked vesicles to release neurotransmitter. In addition to this cumulative effect, evidence suggests that dynamin also has a role in normal synaptic transmission and maintenance of the RRP of vesicles independent of the depletion of synaptic vesicles achieved with a preincubation at restrictive temperatures: the following frequency of the DLM in shi^{ts1} mutant flies is extremely low even at permissive temperatures, and the onset of the shi^{ts1} phenotype can occur within ~20 msec, much quicker than the estimates of synaptic vesicle recycling time (Kawasaki *et al.* 2000). One potential mechanism for this observation is that the trapped endocytic intermediates formed by the mutation accumulate in the active zone, inhibiting further rounds of exocytosis and ultimately reducing the synaptic efficiency or potential of the synapse (Kawasaki *et al.* 2000). Therefore, seizures and other high-frequency evoked stimuli would be impeded or blocked because of both the reduction of synaptic vesicles over time and the formation of intermediates accumulating in the active zone.

Synaptic vesicle pools

In general, three pools of vesicles can be found at the synapse: the readily releasable pool, the recycling pool, and the reserve pool (Rizzoli and Betz 2005). First, the RRP contains the docked vesicles primed for release and contains only a small percentage of the total amount of vesicles in the cell. Estimates suggest that in the wild-type DLM motoneuron neuromuscular junction, there are only ~11 docked vesicles per individual active zone (Koenig *et al.* 1989). Owing to their limited number, vesicles considered part of the RRP will rapidly deplete from single shocks of high-frequency stimulation or a few milliseconds of depolarization (Delgado *et al.* 2000; Rizzoli and Betz 2005). Replacement of docked vesicles at the membrane and refilling of the RRP are accomplished by mobilization of vesicles from the recycling pool (or exo/endo recycling pool), a pool composed of continuously recycling vesicles that are used to maintain moderate physiological stimulation frequencies (Rizzoli and Betz 2005). These vesicles would mostly be retrieved by endocytosis from the plasma membrane via classical clathrin-mediated endocytosis (Kuromi and Kidokoro 1998). The last pool of vesicles, the reserve pool, holds the highest percentage of vesicles and is used only in extreme circumstances or intense stimulation, and mixing between the reserve and recycling pools is slow. It is thought that vesicles for this pool are mostly generated via bulk endocytosis, a noncanonical membrane recycling pathway (Akbergenova and Bykhovskaia 2009). It is unclear at this moment whether the stimulation needed to trigger a seizure also triggers mobilization of the reserve pool of vesicles, but some behavioral observations, such as the refractory period following a bang-sensitive episode, in which for ~10 min seizure thresholds are highly increased and flies are no longer bang sensitive, suggest that those vesicles may be mobilized and partially responsible for the lack of seizures following the initial seizure. Dynamin mutations did not alter the refractory period of the flies (data not shown), consistent with the mobilization of preformed vesicles from one pool to another, which would occur independent of dynamin function.

Overexpression of mutant dynamin

Overexpression of mutant Shibire protein caused suppression of bang sensitivity at permissive temperatures in the $para^{bss1}/+$ mutant. How is seizure sensitivity in this mutant different from other types of behaviors that require a preincubation at restrictive temperatures? First, seizure threshold may be a behavioral phenotype that is more sensitive to modification than other types of behaviors, and it could be easier to detect smaller and subtler changes to the phenotype. Second, ultrastructural defects have even been observed in flies raised even at 18°, indicating that Shi^{ts1} protein is defective at a larger range of temperatures than what is observable with the behavioral paralysis phenotype (Masur *et al.* 1990; Gonzalez-Bellido *et al.* 2009). Additionally, the $para^{bss1}/+$ seizure threshold is much higher than the $para^{bss1}$/Y and *eas*/Y thresholds, so smaller changes will be more likely to cross the threshold of bang sensitivity and rescue the phenotype. Finally, in contrast to many behavioral studies in which the Shi^{ts1} protein is expressed in small subsets of neurons, we expressed the Shi^{ts1} pan-neuronally in a broad expression pattern. When restricting the expression pattern to smaller subsets of neurons, the permissive temperature effect was no longer apparent. Therefore, the combined effect of the Shi^{ts1} protein on many neurons within the nervous system may reduce the temperature at which a change in behavior is detected.

Dual roles of dynamin and other epilepsy-related genes in neuronal excitability

The homology of the dynamin protein is highly conserved between *Drosophila* and mammals despite three separate dynamin genes in mammals (Praefcke and McMahon 2004). For example, homologous mutations of shi^{ts1} in mammalian dynamin-1 also causes temperature-sensitive disruption of endocytosis (Damke *et al.* 1995).

Interestingly, studies in mice have shown that the *fitful* mutant, caused by a missense mutation in *Dynamin-1*, exhibits spontaneous limbic and generalized tonic-clonic seizures on routine handling (Boumil *et al.* 2010). The seizure-like activity detected in the DLM from heated shi^{ts1} flies could resemble aspects of the *fitful* mutant. In fact, the dynamin mutant shi^{ts1} was characterized early as a "temperature-sensitive induced seizure mutant" before it was later described more often as a temperature-sensitive paralytic mutant (Salkoff and Kelly 1978). Why there is excitatory activity is unclear, but one possible explanation is that different neurons within the fly have different thresholds or numbers of vesicles in the RRP. Therefore, inhibitory neurons that

keep the activity the DLM quiet are inhibited first by *shi*^{ts1}, leading to spontaneous discharges of the DLM when that tonic inhibition is removed. The somewhat paradoxical role of dynamin in either promoting seizures or suppressing seizures indicates the importance of proper regulation of synaptic vesicles on overall excitability within the animal.

Other genes linked to epilepsy have different alleles that can drastically alter the balance of neural excitability. For example, in mice and humans, missense mutations of the *Cacna1a* calcium channel have been linked to absence seizures, while in *Drosophila*, mutations in the homologous *cac*^{TS2} channel result in heat-activated seizures but are also a suppressor of bang-sensitive mutants (Zamponi *et al.* 2009). Additionally, there are different *para* alleles in *Drosophila* that can cause either bang sensitivity, seizure suppression, heat-activated seizure sensitivity, cold sensitivity, or temperature-sensitive paralysis, all extremes of either hyper- or hypoactivity of the sodium channel (Lindsay *et al.* 2008; Parker *et al.* 2011b; Sun *et al.* 2012; Schutte *et al.* 2014).

Seizure suppression by Rab GTPases

Our results suggest that specific mutations in Rab GTPases have minor effects on seizure susceptibility compared to the relatively strong effects of mutant dynamin. The strong seizure suppression seen by the *Rab5*^{CA} mutant is especially interesting because of the proposed role of *Rab5* in early stages of endocytosis and membrane fusion (Wucherpfennig *et al.* 2003; Shimizu 2003). While the activation of *Rab5* seems counterintuitive to the inhibition of endocytosis brought about by dynamin, one possibility is that the activated *Rab5* sequesters other proteins or vesicles that normally would be involved with endocytosis, therefore disrupting the normal functioning of endocytosis at the synapse. Additionally, the functional difference between DN and CA mutations appears complex. For example, both *Rab5*^{CA} and *Rab5*^{DN} mutations were shown to inhibit vesicle mobility compared to wild-type *Rab5* expression, suggesting that the ability to switch between active and inactive states may be especially important for their proper function (Potokar *et al.* 2012). While there was no observed effect with the *Rab5*^{DN} mutation, the degree of inhibition by the transgene may not be strong enough for any detectable changes in seizure threshold, especially since strong transgene expression is necessary for seizure suppression by dynamin. Additionally, it has been shown previously that *Rab5* is not strictly required for vesicle formation following synaptic depletion, even when inhibitor is fully expressed (Shimizu 2003). In another mutant, *Rab35*^{DN}, previous studies have shown that the mutant exhibits diminished excitatory junctional currents (EJCs), suggesting that decreased synaptic transmission in that mutant could contribute to the suppression of bang sensitivity (Uytterhoeven *et al.* 2011).

The identification of 8 seizure-suppressor mutations within a sample of 18 (~40% hit rate) is very high and surpasses any previous attempts to identify seizure-suppressor mutations (forward or reverse genetics) in any bang-sensitive genotype. For example, we have tested existing *P*-element mutations by crossing them into the *para*^{bss1}/+ bang-sensitive genotype and have achieved a much lower hit rate of <5%. Identifying the exact role and function of each Rab protein and how it specifically affects seizure susceptibility and synaptic transmission will be important for determining the mechanism of suppression in each case. However, because the genes tested were chosen based on their colocalization with a synaptic vesicle marker, synaptic vesicles are likely to be affected by each of the mutations, which has been shown to be the case in other studies with DN and CA versions of *Rab5*, *Rab23*, and *Rab35* (Uytterhoeven *et al.* 2011).

Neurons and circuits involved in the bang-sensitive phenotype

A full understanding of the neurons involved in the spread of a seizure from the electrical or mechanical origin of the seizure to the DLM motor output (the entire "seizure circuit") is unknown. One difficulty is that the activity and connections of neurons that may be involved in such behavior are still relatively unknown in healthy animals, let alone how they then might function or interact in a disease state such as epilepsy. For example, expression of wild-type *eas*$^{+}$ in cholinergic neurons is sufficient to suppress bang sensitivity in *eas* mutants, while RNA interference (RNAi) of *kazachoc* (*kcc*) in neurons, glia, or the GF neuron independently caused seizure sensitivity (Kroll and Tanouye 2013; Rusan *et al.* 2014). With these results, it is difficult to determine whether the neurons targeted were actually part of the circuit within which the seizure travels or whether compensating for an ionic or molecular defect, independent of the neurons, eliminated seizures and the circuits through which seizures travel.

Our data suggest that action potentials associated with bang sensitivity originate in the brain and then spread to downstream motor outputs through the GF neuron, passing through the PSI and DLM motoneuron before being detected as the series of discharges and failures in the DLM. Overexpression of *shi*^{ts1} in all neurons, excitatory cholinergic neurons, or the GF neuron was sufficient for suppression of bang sensitivity. We also tested an assortment of ~40 other *GAL4* lines with various sparse expression patterns and found no significant seizure suppression (data not shown), indicating specific requirements for a given *GAL4* line to suppress seizures.

We cannot confirm to what extent the PSI neuron alone affects the suppression of seizures because the *GAL4* line expresses in many other cells (cholinergic) outside the circuit. However, it does appear that the PSI, in addition to the GF, has an important role in the manifestation of seizures. First, previous studies have shown that seizures and discharges are not detected in the tergortrochanteral muscle (TTM) despite the fact that both the DLM and TTM respond to input from the GF neuron (Pavlidis and Tanouye 1995). This is so because the TTM motoneuron is innervated directly by the GF, while the DLM motoneuron is innervated directly by the PSI neuron, which receives its input from the GF. Additionally, given the independent recovery of the individual DLM fibers during recovery from a seizure and the ability for DLM motoneurons to fire action potentials when directly stimulated in the thorax

during the failure period of the seizure, it was reasoned that the PSI-DLM motoneuron synapse was the site of synaptic failure following the high-frequency stimulus necessary to trigger a seizure (Pavlidis and Tanouye 1995).

Our data from this study suggest that indirectly inhibiting the PSI neuron by inhibiting the upstream GF neuron could be preventing the failure of the PSI neuron and therefore the discharges and failures observed in the DLM that are associated with the response to mechanical or electrical stimuli sufficient to trigger a seizure. More detailed and comprehensive *GAL4* lines/split-*GAL4* lines that express only in the PSI neuron will enable a more rigorous test of this hypothesis.

Synaptic vesicles and epilepsy

Synaptic vesicles are the basic unit of synaptic transmission, converting changes in ionic current and the total excitability of a particular synaptic terminal into account. Thus, synaptic vesicles could represent an important and critical target for the prevention of aberrant action potentials and hyperactivity in patients with epilepsy. Recent progress to suppress epilepsy and seizures has emphasized the use of various light- or chemical-gated channels expressed in different neurons and circuits within the brain (Tønnesen *et al.* 2009; Sukhotinsky *et al.* 2013; Kätzel *et al.* 2014). While these show great promise, the problems with these types of manipulations in human patients will be halted by the ability to safely perform genome editing in human tissues, a significant technological challenge. Our study has shown that mutant dynamin and Rab proteins can act as seizure suppressors. Therefore, we imagine that additional proteins involved with synaptic vesicle regulation, biogenesis, or mobilization could be potential new targets for AEDs without the need for novel expression of channels or pumps.

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Author contributions: J.R.K. and M.A.T. designed the study, experiments, and wrote the manuscript. J.R.K. performed the behavior and electrophysiology experiments, analyzed data, and performed statistical analysis. K.G.W. and F.M.S. performed the behavior experiments and analyzed data. All authors edited the manuscript.

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Disruption of Endocytosis with the Dynamin Mutant *shibire^{ts1}* Suppresses Seizures in *Drosophila*

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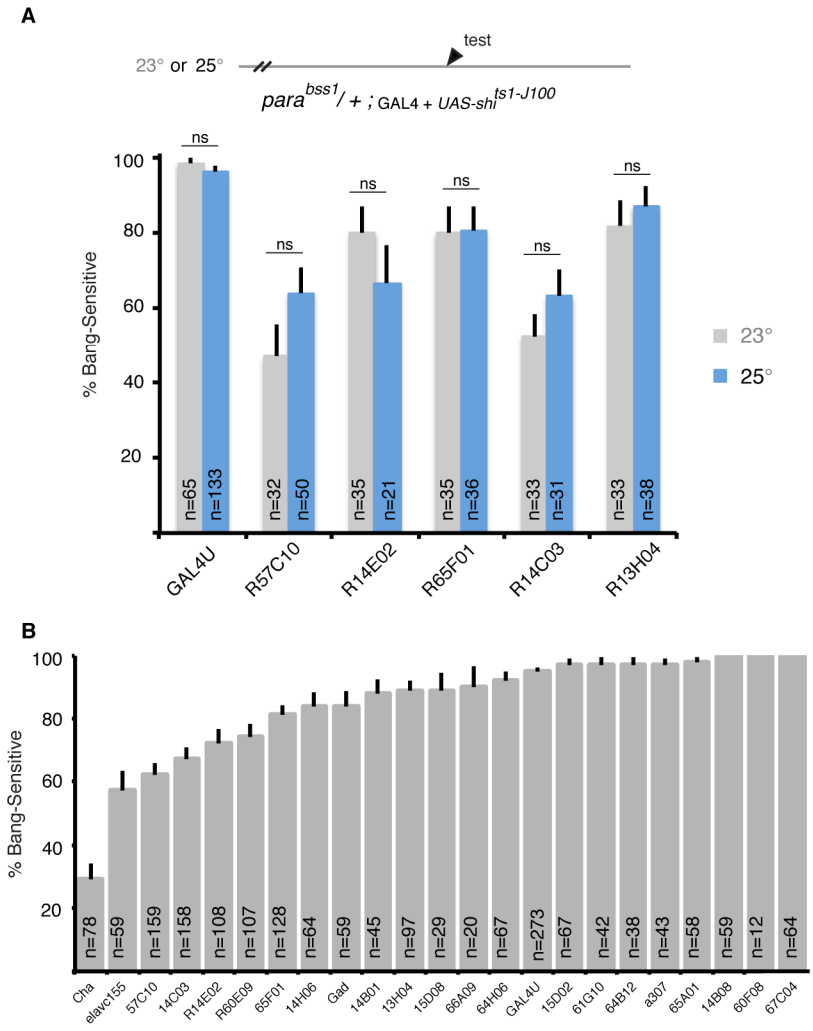

Figure S1. Preliminary GAL4 screen for seizure suppression

A) An initial test was performed to determine whether raising flies at 25° or 23° would have any significant effect on the phenotype, and no differences were detected.

B) The small initial GAL4 screen shows that most GAL4 lines did not have effects on the seizure phenotype. The three strongest hits corresponded to GAL4 drivers with wide spread expression patterns.