ARTICLE
Demonstrating the Temperature Sensitivity of Synaptic Transmission in a *Drosophila* Mutant

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We describe exercises that illustrate the temperature sensitivity of synaptic transmission. The temperature dependence of synaptic transmission is demonstrated by cooling the larval *Drosophila melanogaster* preparation and recording excitatory junction potentials. Vesicle recycling is explored by utilizing a mutation of the *shibire* gene. This *shibire* mutant shows a robust reduction in synaptic vesicle recycling when temperature exceeds a known threshold (~29° C). Students gain proficiency with the *Drosophila* larval neuromuscular junction preparation while investigating principles of vesicle release, vesicle recycling, synaptic facilitation and synaptic depression. We show that the viability of the larval preparation is prolonged *in vitro* with moderate cooling, which is particularly important when introducing the preparation as a novel exercise.

Key words: neuromuscular junction (NMJ); excitatory junction potential (ejp); Drosophila; shibire^{TS1} *mutant*

We are developing undergraduate laboratory exercises that illustrate the neural and behavioral implication of genetic mutations. The fruit fly (*Drosophila melanogaster*) preparation was chosen for this project because of the flexibility that genetic manipulation offers and the sizable literature characterizing mutants from gene to behavior. Many *Drosophila* mutations have known homologues in human genes and are well understood at the genomic, molecular, physiological and behavioral levels. Our objective is to introduce a novel series of laboratory modules, based on the growing *Drosophila* literature, which is sufficient for course design and is similar in scope to Project Crawdad (Wyttenbach et al., 1999).

One module that is in development explores the temperature sensitivity of synaptic transmission. In addition to illustrating the temperature-dependent change in vesicle kinetics, the module utilizes *shibire*^{ts1}, a temperature sensitive paralytic mutant. Adult *shibire*^{ts1} flies paralyze at temperatures ≥ 29° C because of failed vesicle recycling (Poodry and Edgar, 1979; Koenig and Ikeda, 1983). Temperature sensitive (ts) mutations like *shibire*^{ts1} have received considerable attention (Suzuki, 1970; Palladino et al., 2002) but haven't been widely introduced as neuroscience teaching exercises. Several ts mutants have been described (*nap*^{ts} & *para*^{ts}: Suzuki, 1970; Siddiqi and Benzer, 1976; *shibire*$^{ts1/2}$: Grigliatti et al., 1973; *SNAP-25*^{ts}: Rao et al., 2001). The *shibire* and *paralytic* mutants are available through a public stock center at nominal cost, and others may be specifically requested from individual labs. The availability of *shibire* ts mutants and the mechanism of its primary phenotype make it a good candidate for investigating vesicle recycling.

In general, synaptic transmission is sensitive to changes in temperature (Charlton and Atwood, 1979). In many organisms the duration of excitatory junction potentials (ejps) increases as temperature decreases (Eccles et al., 1941; Katz and Miledi, 1965). This principle is demonstrated in a robust fashion with the *Drosophila* larval NMJ preparation. Additionally, the *shibire* mutant illustrates the importance of vesicle recycling to synaptic transmission. In *shibire*^{ts1}, vesicle recycling is attenuated at elevated temperature, which depresses and ultimately abolishes synaptic transmission. The phenotype is fully reversible upon return to room temperature. Students have the opportunity to explore the increased duration of synaptic currents that occurs with cooling, and the gradual cessation of transmission that occurs with reduced vesicle recycling due to heating.

The larval NMJ preparation is particularly well-suited to illustrating principles of neuromuscular transmission for several reasons: (1) it is established as a model for the study of synaptic transmission and development (Keshishian et al., 1996; Budnik and Gramates, 1999). (2) Ejps of the larval preparation can exceed 40 mV, which is larger than most crustacean preparations used in teaching neuroscience (*Drosophila*: Feng et al., 2004; Kurdyak et al., 1994; crayfish: Dunn and Mercier, 2003; Futamachi, 1972). Large amplitude events are favorable for student investigation; imperfect recordings yield large enough ejps for further quantitative analysis. (3) The cost and simplicity of *Drosophila* maintenance are conducive to student involvement. (4) Students gain experience data-mining the considerable amount of information from genomics to behavior using database sites such as FlyBase (flybase.org) and the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

In order to utilize the larval preparation in undergraduate teaching, more than the standard lifespan of about one hour *in vitro* (Stewart et al., 1994) is necessary. We reference several modified salines that may prolong preparation lifespan. We also describe increased lifespan that is achieved with moderate cooling alone. Cooling the larva doubled the time during which students could make recordings from abdominal muscles. The additional time was sufficient for teaching the methods of the larval preparation and for student exercises.

METHODS

Dissection

In its simplest form, the larval *D. melanogaster* dissection consists of three steps: (1) pinning, (2) a single shallow incision, and (3) the removal of internal organs (gut, fat body, trachea, etc.). Details of the dissection are provided in an instructional video as supplemental material (www.funjournal.org/materialsKrans.asp). We believe that video is an unmatched medium for teaching the dissection.

We use a standard preparation dish for *Drosophila* larvae, the production of which is described in Bellen and Budnik (2000). Two reflective tracheas (Figure 1*A*) extend longitudinally just superficial to the dorsal body wall and are useful in positioning the larva dorsal side up. The first two pins are placed at the anterior and posterior ends of the larva. The ventral ganglion of the central nervous system is at the anterior end of the larva. Placing the anterior pin between the mouthparts - darkly pigmented structures at the anterior end of the larva - will assure that the ganglion is not damaged. A shallow incision is made from the posterior to the anterior of the larva. Moderate tension between the pins may ease this cut and help with placement of future pins. Ultimately, six pins will be oriented radially around the preparation (Figure 1*B*). The orientation of the pins is important to the placement of stimulating and recording electrodes, described below. Removing the internal organs improves visualization of the muscles. Grasping and pulling trachea from posterior to anterior usually removes most of the guts and fat body.

Recording

Placement of electrodes for stimulating nerves and recording muscle potentials is shown in a video that is available as supplemental material (www.funjournal.org/materialsKrans.asp). Physiological recordings were made from third instar wandering larvae. Intracellular recordings of ventral-longitudinal muscle were made with 20 MΩ glass sharp electrodes filled with either K-Cl or K-acetate. Both sides of the abdominal musculature (Figure 1*B* and 1*C*) in segments 2 through 5 were used. Only muscles 6 and 12 were recorded from. A custom-built stimulus isolation unit described previously (Land et al., 2004) was used to stimulate motoneurons. One of two stimulation paradigms was used: (1) segmental nerves were drawn into a glass suction electrode as a bundle (Figure 1*C* and video, supplemental material). The tip of the stimulating electrode was fire polished to reduce its diameter and make a tighter fit around the neurons. The ventral ganglion was severed just posterior (i.e. through the posterior ventral ganglion) to the cerebral ganglia to attenuate spontaneous neural discharge (Cattaert and Birman, 2001). (2) The segmental nerves were cut near the ventral ganglion and stimulated individually. We saw no difference in ejps collected using either method. In order to compare our results to those published previously, it was important to evoke single neural events via electrical stimulation, rather than a burst of neural spikes. This was confirmed empirically: in most trials - all of those used for analysis - no additional potentials occurred during a one second window following stimulus (e.g. 500 ms post-stimulus is shown in Figure 1*C*, bottom).

D. melanogaster saline was made as in Jan and Jan (1976), standard saline A. It contained, in mM concentration: 128, Na^+; 141.6, Cl^-; 2, K^+; 4, Mg; 1.8, Ca^{++}; 35.5, Sucrose; and 5, HEPES to buffer to pH 7.1.

Fly Stocks and Genetics

Some experience with fly crosses - “fly pushing” - would be advantageous, but is not strictly necessary to generate the *shibire*^{ts1} strain described here (see appendix for more information on the cross). Ralph Greenspan (2004) provides a fairly comprehensive reference in his guide: *Fly Pushing: The theory and practice of* Drosophila *genetics*.

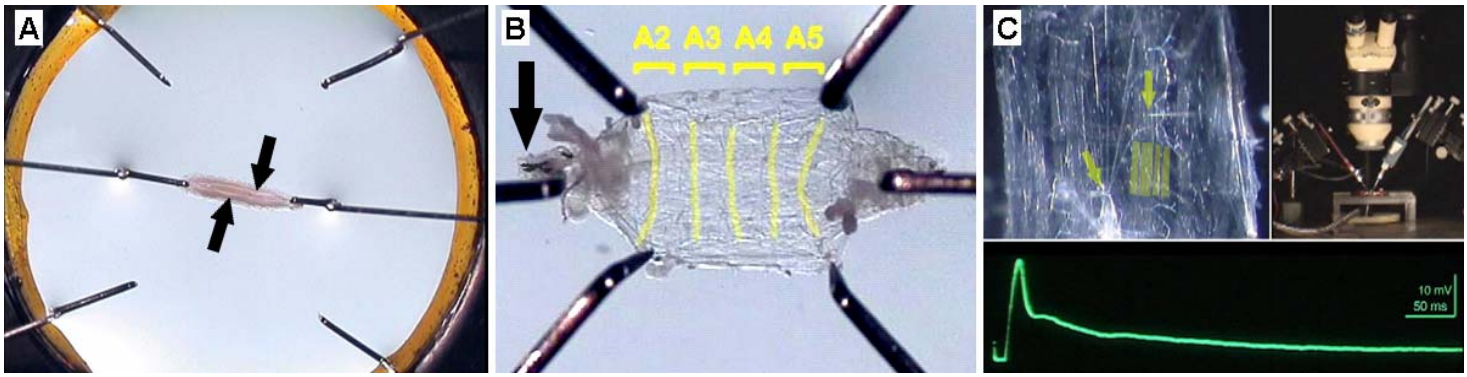

Figure 1. Still images captured from instructional videos of the larval dissection and electrode placement and stimulation. Screen shots are taken from videos that are available for download (Supplemental Material, www.funjournal.org/materialsKrans.asp). *A*: Two pins are placed at the anterior and posterior ends of the larva. Arrows indicate reflective tracheas that span the dorsal larva. Panel *B* shows all six pins in place and four abdominal segments have been outlined (A2:A5). The anterior end is to the left as evidenced by the pigmented mouth parts (arrow). The upper left portion of panel *C* shows the filleted larval preparation through a dissection microscope with transverse illumination (anterior at bottom). Arrows indicate the extracellular electrode – for stimulation (bottom arrow) - and the intracellular electrode, for muscle recording. Muscles 7, 6, 13, and 12 are outlined in yellow boxes (left to right). The upper right portion of panel *C* shows the physiology rig with two electrodes positioned around the preparation stand and dish. A compound ejp is depicted in the bottom of panel *C*.

Flies were obtained from the Bloomington Stock Center (flystocks.bio.indiana.edu). The Bloomington Stock Center collects, maintains, and distributes *Drosophila melanogaster* stocks for research. Stocks can be ordered online and currently cost $3.50 per strain. Flies were raised on standard *Drosophila* diet at 25° C. The following stocks were used: *Gal4-elav^{C155}*, *UAS-shi^{ts1},* and the wildtype strain, *Canton-S.* Briefly, we used the Gal4/UAS system (reviewed in Duffy, 2002) to drive expression of *shibire^{ts1}* in the nervous system. *shibire^{ts1}* is a temperature-sensitive mutation in *shibire*, a gene that encodes the fly homolog of dynamin (van der Bliek and Meyerowitz, 1991). Dynamin is a GTPase that is essential for synaptic vesicle recycling. At > 29° C, *shibire^{ts1}* results in a rapid and reversible inhibition of synaptic transmission and adult paralysis (Grigliatti et al., 1973; Poodry and Edgar 1979; Koenig and Ikeda, 1983). Specifically, the *shibire^{ts1}* phenotype is one of progressive, activity-dependent attenuation of vesicle recycling (Wu et al., 2005). Gal4 is a yeast transcription factor that drives transcription of genes downstream of a UAS element. *Gal4-elav^{c155}* is a neuron-specific Gal4 (Lin and Goodman, 1994). To activate transcription of *shibire^{ts1}* in the nervous system, we mated *Gal4-elav^{C155}* virgin females with *UAS-shi^{ts1}* males. The resulting male progeny possessed the following genotype, *Gal4-elav^{C155}/Y; UAS-shi^{ts1}/+* (abbreviated *C155; UAS-shi^{ts1}* throughout).

Preparation Longevity

An important consideration in teaching the larval preparation is its lifespan *in vitro*. In our experience, the larval preparation lasts about an hour at room temperature (22-24° C) in standard saline A (Jan and Jan, 1976). That is sufficient for students who have mastered the dissection and electrode placement, but insufficient to teach those methods. Several salines have been developed with the objective of lengthening the period during which the larval preparation remains physiologically viable (Jan and Jan, 1976; Stewart et al., 1994; Macleod et al., 2002; Ball et al., 2003; Feng et al., 2004). Some of these salines may mask mutant phenotypes at the neuromuscular junction, and thus may not be useful for physiological investigations (Ball et al., 2003; Feng et al., 2004). The most recent of these, by Feng et al. (2004), offers both preparation longevity and physiologically relevant NMJ behavior. The use of a modified saline may further extend lifespan, but may also mask some phenotypes. Muscle recordings can be held during the application and wash-out of saline. Students could thus address "phenotype masking" by simply changing salines, which would promote student exposure to the primary literature. Our objective is to make fruit fly exercises widely accessible (Rivlin et al., 2004), so we describe moderate cooling as a means of providing more than one hour of preparation health without using modified salines. Detailed information about an inexpensive temperature control system is available in a companion paper, in this issue (Krans and Hoy, 2005).

Maintaining the larval preparation at 16-18° C extended lifespan from about one hour to about two hours (Figure 2). This and lower temperature ranges have been used in reports on neuromuscular physiology of *D. melanogaster* larvae (Haugland and Wu, 1990; Zhong and Wu, 1991). The ratio of saline surface area to volume when using the fruit fly dish is high; temperatures return to ambient relatively quickly upon cessation of cooling. Raising temperature from 16-18° C to ambient is thus a simple endeavor. The extra time during which students can acquire data and perform manipulations of the preparation is critical.

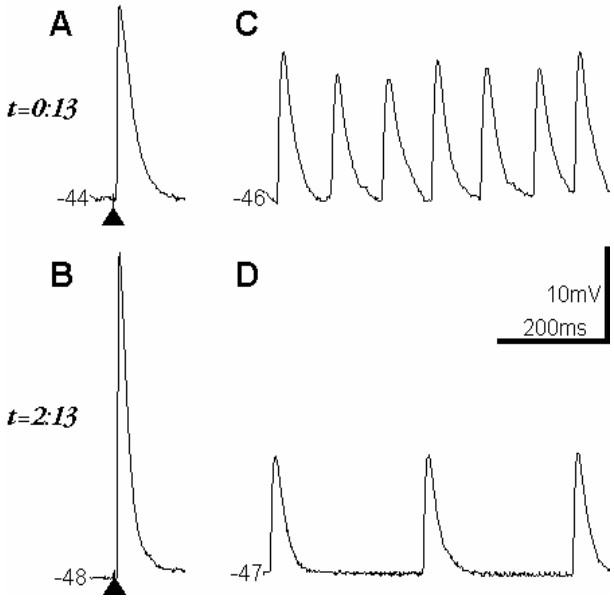

Figure 2. Junction potentials of the larval *D. melanogaster* preparation over time, with cooling. Excitatory potentials recorded in muscle 12 of the fifth abdominal segment at two different times at ~18° C. *A* and *C* were recorded upon initial setup of the preparation. *B* and *D* were recorded two hours later (times given at far left). Spontaneous potentials (*C* and *D*) maintained their monophasic shape and amplitude, though evoked potentials (stimulus = *arrowhead*) increased in amplitude after about two hours *in vitro*. Resting potential is shown at the left of each trace. Signal to noise values are typical of the larval preparation and suitable for further analysis of these data.

We evaluated the physiological viability of the preparation using metrics that we believe are reasonable for undergraduate observation. Three simple metrics can be assessed via recordings from the larval NMJ: (1) the shape and amplitude of ejps, (2) the resting membrane potential of the muscle, and (3) the presence or absence of spontaneous neural activity recorded post-synaptically at the muscle. Figure 2 shows intracellular recordings from muscle of healthy wildtype larvae. The left column shows evoked potentials and the right column shows spontaneous events. Figures 2*B* and 2*D* were recorded more than two hours after the dissection began and about two hours after Figures 2*A* and 2*C*.

Activity depicted in Figure 2 suggests that the preparation remained physiologically viable for more than two hours. The slight hyperpolarization from Figure 2*A* to 2*B* (-44 to -48 mV) is consistent with the muscle being healthy at the time of recording. Ejp amplitude increased

with time, possibly because of improved electrode sealing in the muscle or recovery from activity dependent synaptic depression. There was little change in spontaneous potential amplitude (Figures 2*C* and 2*D*) with time. In contrast, the rate of spontaneous discharge decreased with time.

RESULTS

Learning Objectives

Students explore the temperature dependence of synaptic transmission kinetics by comparing evoked ejps recorded at room temperature with those recorded at reduced temperature. As temperature decreases, ejp duration and time-to-peak amplitude increase, while the rising and falling phases of the ejp show decreased slopes (Figure 3; Eccles et al., 1941; Katz and Miledi, 1965). Students can quantify changes in ejp waveform across a temperature series and statistically evaluate their observations. Putative metrics that can be quantified include: latency from stimulus artifact to ejp peak, time constant of decay (τ), duration of the potential at 50% of its peak amplitude, and time to the inflection between 1b and 1s potentials (Figure 3, arrows; morphology and nomenclature are described in Kurdyak et al., 1994). Muscle 6 is innervated by two motoneurons with two types of terminals: 1s and 1b. Ejps recorded in muscle 6 are most often compound potentials, as depicted in Figure 3.

Using the *shibire* ts mutant, students learn that vesicle recycling is essential for long term changes in synaptic efficacy. This is evidenced by the switch from synaptic facilitation that occurs at room temperature to the depression that is characteristic of vesicle recycling failure at elevated temperature. This experiment introduces principles of synaptic facilitation and depression, which presents a useful segue into topics of long term potentiation and depression. Students can compute facilitation or depression indexes and compare these at temperatures above and below that which is critical for presentation of the mutant phenotype (Zucker and Regehr, 2002, offer a review of these and other metrics of synaptic plasticity).

General Temperature Sensitivity

Figure 3 illustrates the central result of these exercises: that synaptic transmission is sensitive to temperature. Ejps recorded at reduced temperature (blue) are much longer than ejps recorded at room temperature (black). Time to peak, half width, rise time and rate of decay are all considerably slower at 14° than 23° C. Peak ejp amplitude and resting potential do not change; these metrics are less sensitive to temperature change.

Shibire Exercises

Results shown in Figure 4 are consistent with those reported in the primary literature (as in Figure 3 of Delgado et al., 2000; reviewed in Kidokoro et al., 2004). A nearly complete depletion of synaptic vesicles is reported to occur in *shibire*^{ts1} larvae with tetanic stimulation. We found that muscle contraction resulting from tetanic stimulus can cause a loss of intracellular recording. Though this is a readily surmountable problem in the hands of a trained investigator, comparable results are available with lesser stimulus duration and rates. This made for more easily attainable results; perhaps more appropriate in the undergraduate setting. We used two stimulus paradigms to illustrate two temperature sensitive phenomena of the *C155; UAS-shi*^{ts1} mutant. Both paradigms involved stimulation at 25 Hz. The first was a one second long train, and the second was 200 ms. The former illustrates progressive vesicle depletion whereas the latter permits a more focused investigation of underlying changes in membrane potential.

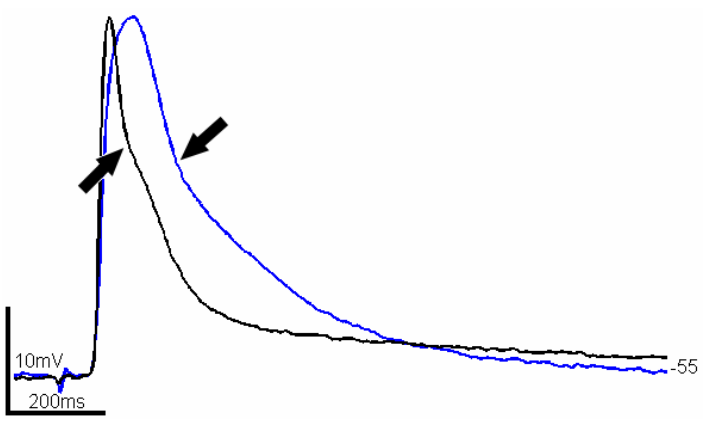

Figure 3. Larval ejps at two temperatures. Both traces were recorded from muscle 6 of the fourth abdominal segment. Each trace is the average of 10 ejps (a single, cut segmental nerve was stimulated). Black = 23° C, blue = 14° C. Arrows show the inflection between 1b (big) and 1s (small) nerve potentials (Kurdyak et al., 1994). Traces are aligned by onset of depolarization to best illustrate change in duration.

At room temperature, *C155; UAS-shi*^{ts1} larvae showed only slight depression in ejp amplitude that reached sustainable amplitude rapidly (black, Figure 4*A*). After one second of stimulation at 34° C, this decrease was much greater: ~40% of initial amplitude (Figure 4*C*). Unlike the *shibire* mutant, wildtype flies (CS) show nominal facilitation of ejp amplitude at 34° C (data not shown). When the preparation is cooled to a temperature below ambient, ejp duration increases dramatically and the inflection between falling and rising phases is indistinct. At about 12° C, temporal summation led to ~20 mV increase in underlying membrane potential (blue, Figure 4*D*). Upon activation of the *C155; UAS-shi*^{ts1} mutation at ~34° C, temporal summation during the stimulus trains was almost entirely abolished (red, Figure 4*B* and 4*D*). Ejps were much shorter in duration at these temperatures.

DISCUSSION

To our knowledge, this is the first mainstream effort to bring larval fruit fly physiology to undergraduate teaching. The preparation is notably smaller than many of its counterparts in neuroscience teaching labs. This presents students and teachers with a learning curve that requires finer motor skills than other common preparations. We

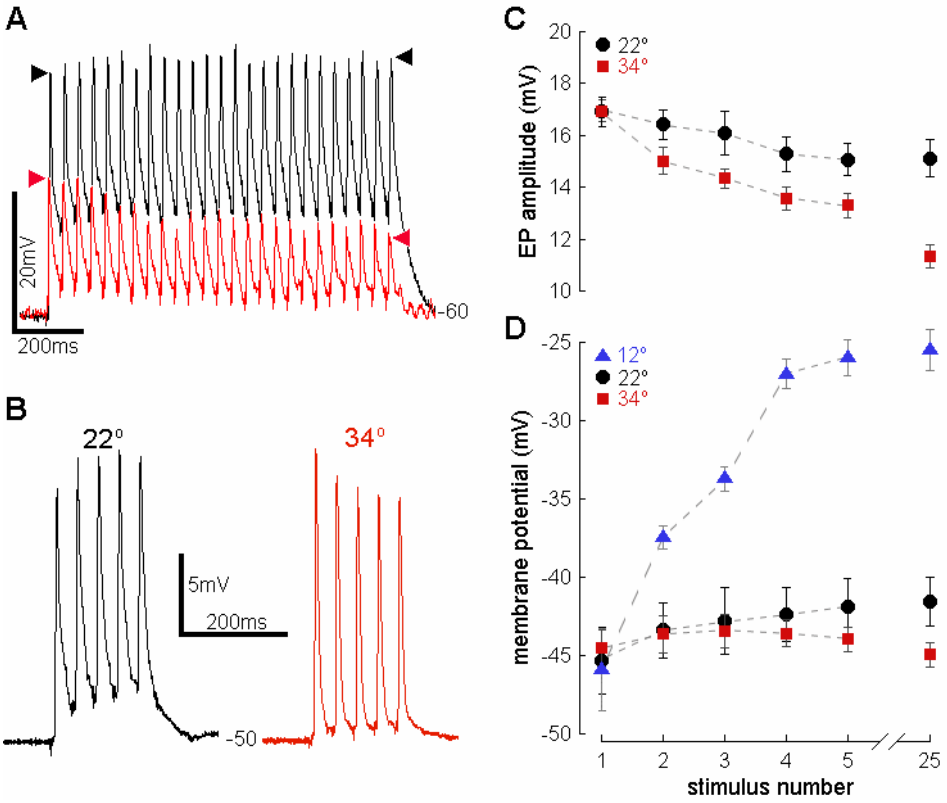

Figure 4. Temperature dependent change in synaptic depression at the neuromuscular junction in the *C155; UAS-shi^{ts1}* mutant. *A,* 25 Hz, 1s train of stimuli. At 22° C (black, throughout) there is little change in evoked potentials after the second stimulus. At 34° C (red, throughout), there is notable depression that progresses with time and stimulus number (arrowheads indicate initial and terminal ejp peak). *B,* 200 ms of 25 Hz stimuli illustrates temperature sensitivity of underlying temporal summation. At room temperature, ejp amplitude is relatively constant, but depresses at 34° C. Temporal summation and ejp depression are quantified in *C* and *D*. *C,* Synaptic depression over repetitive stimuli increases at ~34° C in the mutant. (Below room temperature, temporal summation and progressive elongation of the evoked potentials mask rising phase of ejps; data not shown.) *D,* Temporal summation increases as temperature decreases. At 12° C (blue triangles), ejps are longer in duration than at 34° C and thus temporally summate. Membrane potential, measured at the onset of each stimulus is shown. Summation at 22° C is less than at 12° but was always present (n=7 muscles, 3 animals in *C* and *D*).

assert that the gains from using the preparation are great and that the learning curve is worthwhile. The preparation exposes students to standards of contemporary scientific exploration: data-mining, genomics, and interdisciplinary investigation. The flexibility of *Drosophila* genetics makes possible the hands-on exploration of protein function and biochemical cascades in the context of physiology and behavior.

The use of temperature sensitive mutants in the undergraduate setting is an exciting application of genetics within physiology. At least two temperature sensitive mutants are obtainable from the Bloomington Stock Center: *shibire* (*C155; UAS-shi^{ts1}*) and *paralytic* (*para^{ts}*). These offer easily observable neuromuscular phenotypes. Students can mine for information from genetics to associated dysfunction in human mutations regarding both mutants using FlyBase (flybase.bio.indiana.edu) and the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The amount of genomic, mechanistic, and behavioral information available regarding fruit fly mutants is remarkable and ever-expanding (Matthews et al., 2005). These exercises provide students the opportunity and context with which to approach such vast quantities of information.

One concern when performing these experiments is providing a measure of preparation temperature. Reasonably accurate temperature control is essential to these exercises. However, absolute control of temperature is unnecessary. We describe the resolution of a simple temperature control system in a companion paper, about ±2° C, which is sufficient for these exercises (Krans and Hoy, 2005). It is possible that the volume of saline surrounding the larva may not have equilibrated to the temperature we measured. We thus used a slightly higher temperature than was necessary (29° C) that did not impede synaptic transmission: 34° C.

D. melanogaster offers unparalleled opportunities to undergraduate neuroscience courses by introducing a new – and we think superior – means of exploring the genetics that underlie physiology and behavior. Exercises described here illustrate the importance of vesicle recycling to synaptic transmission and the general impact of temperature on synaptic kinetics. These exercises could also be used to introduce principles of synaptic potentiation and depression. Moreover, data from these experiments are suitable for a number of elementary statistics and quantification procedures. Further computational exercises might include line fitting and significance equations, which can be applied to the rates of decay shown in Figure 3 as well as stimulus number data in Figures 4*C* and 4*D*. We endeavor to harness the excitement of fruit fly genetics to introduce concepts in the teaching environment that are otherwise difficult to visualize.

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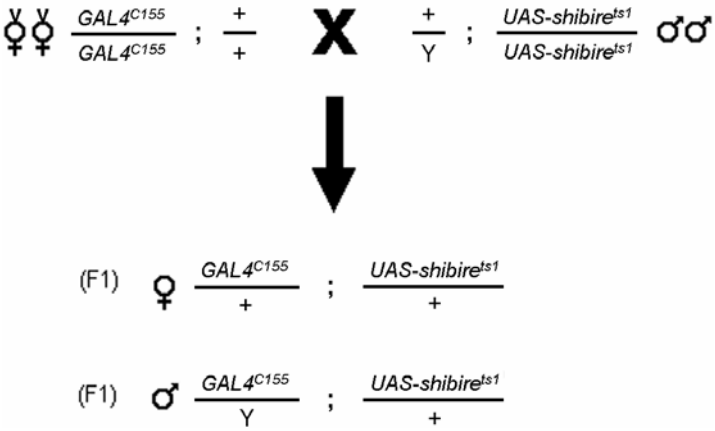
APPENDIX

The Bloomington Stock Center distributes and maintains stocks of *Cha-GAL4*, $GAL4^{C155}$, $UAS\text{-}shibire^{ts1}$, and the $shibire^{ts1}$. We used the GAL4/UAS system to target expression of $shibire^{ts1}$ to neurons. This eliminates any potential contribution from other tissues to the phenotypes examined. In addition, this lab exercise introduces students to the utility of the GAL4/UAS system for gene expression and the possibility of designing additional experiments. Results shown in Figure 4 can be replicated using the $shibire^{ts1}$ mutant itself.

$GAL4^{C155}$ virgin females were crossed with $UAS\text{-}shibire^{ts1}$ males. F1 larvae are the first generation of progeny and were used in this lab exercise. F1 larvae were also reared to adulthood and tested for temperature-sensitive paralysis. Since dynamin probably functions as a multimer (Kim and Wu, 1990), adults require high temperatures to paralyze, but the larval phenotype is as described in the primary literature (Figure 3 of Delgado et al., 2000). $shibire^{ts1}$ allele is semidominant which means that even heterozygotes ($shibire^{ts1}$ /+) will display a temperature-sensitive

paralytic phenotype. Similarly, expression of *UAS-shibire^{ts1}* can induce a mutant phenotype even in the presence of the wildtype gene. Kitamoto (2001) used a cholinergic neuron specific-Gal4 to drive expression of UAS-*shibire^{ts1}* and found that *Cha-GAL4*/*UAS-shibire^{ts1}* paralyze at temperatures ≥ 30° C. We used *Gal4^{C155}* in this study. This driver utilizes the *elav*-promotor (called C155) to drive expression in all neurons.



Genetic Cross. *GAL4^{C155}* virgin females were crossed *UAS-shibire^{ts1}* males. *GAL4^{C155}* is located on the X chromosome. *UAS-shibire^{ts1}* is located on the third chromosome. A *UAS-shibire^{ts1}* located on the X chromosome is also available from the stock center (not shown here), and will produce the central results described here.

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