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Using Neurogenetics and the Warmth-Gated Ion Channel TRPA1 to Study the Neural Basis of Behavior in *Drosophila*

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Here we describe a set of straightforward laboratory exercises that integrate the study of genetics, neuroanatomy, cellular physiology and animal behavior. We use genetic tools in *Drosophila* for visualizing and remotely activating ensembles of neurons with heat pulses. First, we show how to examine the anatomy of several neuronal populations using genetically encoded green fluorescent protein. Next we demonstrate how to use the warmth gated *Drosophila* TRPA1 (dTRPA1) cation channel to remotely activate neural circuits in flies. To demonstrate the cellular effects of dTRPA1 activation, we expressed dTRPA1 panneurally and recorded excitatory junctional potentials in muscles in response to warmed (29°C) saline. Finally, we present inexpensive techniques for delivering heat pulses to activate dTRPA1 in the neuronal groups we observed previously while flies are freely behaving. We suggest how to film and quantify resulting behavioral phenotypes with limited resources. Activating all neurons

with dTRPA1 caused tetanic paralysis in larvae, while in adults it led to paralysis in males and continuous uncoordinated leg and wing movements in females. Activation of cholinergic neurons produced spasms and writhing in larvae while causing paralysis in adults. When a single class of nociceptive sensory neurons was activated, it caused lateral rolling in larvae, but no discernable effects in adults. Overall, these exercises illustrate principles of modern genetics, neuroanatomy, the ionic basis of neuronal excitability, and quantitative methods in neuroethology. Relatively few research studies have used dTRPA1 to activate neural circuits, so these exercises give students opportunities to test novel hypotheses and make actual contributions to the scientific record.

Keywords: *neurogenetics; TRPA1; Behavior; Neuromuscular Junction (NMJ); Drosophila; GAL4-UAS system*

INTRODUCTION

Understanding the general principles of brain organization and function is a major challenge in biological research. To address this question, neuroscientists have developed powerful genetic tools that provide ways of both visualizing neurons (Timmons et al., 1997; Sun et al., 1999; Wang et al., 2002) and conditionally manipulating their activity (Kitamoto, 2001; Schroll et al., 2006; Hamada et al., 2008; Pulver et al., 2009). These techniques open up exciting new opportunities for neuroscience researchers; however, they also provide attractive new options for neuroscience educators. In classrooms, these new tools could be used to simultaneously teach genetics, cellular physiology and animal behavior. Unfortunately, to date, few attempts have been made to incorporate state-of-the-art neurogenetic tools into neuroscience classrooms.

Drosophila researchers have been at the forefront of generating neurogenetic tools for analyzing nervous system function. Critically, drosophilists have developed methods for controlling gene expression in restricted groups of neurons. The GAL4-UAS system (Brand and Perrimon, 1993) is the tool of choice for this purpose. In this two-part system, a “driver line” that expresses the yeast transcriptional activator (GAL4) is combined with a “target line” that contains the promoter region (Upstream Activating Sequence, UAS) specifically activated by GAL4. Transgenes under UAS control are only expressed in cells containing GAL4. Multiple GAL4 lines are now available specifically targeting GAL4 expression to cells of various neurotransmitter systems, neuropeptides, receptors, and

transcription factors (Meinertzhagen et al., 2009). To examine the expression patterns of GAL4 lines, researchers have developed an array of genetically encoded fluorescent proteins under UAS control (Wang et al., 2002; Hazelrigg and Mansfield, 2006; Timmons et al., 1997). These genetic tools offer neuroscience educators an opportunity to visualize the anatomy of distinct neuronal populations without the use of dyes or antibody staining.

To understand the function of particular neurons, drosophilists have also developed a new generation of genetically encoded tools that can be conditionally activated by light or temperature. These tools exploit light- and temperature-sensitive ion channels and vesicle trafficking proteins to conditionally alter a neuron's membrane potential or secretion of neurotransmitter (Kitamoto, 2001; Boyden et al., 2005; Schroll et al., 2006; Zhang et al., 2007; Clyne and Miesenbock, 2008; Hamada et al., 2008; Peabody et al., 2009). These new tools give researchers (and potentially educators) the ability to turn on and off selected neurons as flies freely behave.

One promising new tool for remotely activating neurons is the *Drosophila* Transient Receptor Potential channel (dTRPA1). dTRPA1 (an ortholog of the mammalian TRPA1 channel) is a temperature and voltage-gated cation channel that regulates *Drosophila* thermotactic behavior (Rosenzweig et al., 2005; Hamada et al., 2008; Rosenzweig et al., 2008). Previous work has shown that neurons expressing dTRPA1 begin firing action potentials when ambient temperatures rise above 25°C (Hamada et al., 2008). By ectopically expressing TRPA1 (using the

GAL4-UAS system), and then delivering modest heat pulses, sets of neurons can be remotely activated in freely behaving animals (Hamada et al., 2008; Parisky et al., 2008; Shang et al., 2008; Pulver et al., 2009).

Visualization of neural networks with GFP and activation of neurons with dTPRA1 in *Drosophila* is simple and inexpensive. As a result, these techniques present unique opportunities for educators as well as researchers. The goal of the present work is to propose a series of laboratory exercises that use current neurogenetic tools (i.e., the GAL4-UAS system, genetically encoded Green Fluorescent Protein (GFP) and dTPRA1) to explore the neural basis of behavior in *Drosophila*. First, we show how to visualize neural anatomy using ectopically expressed GFP. Then we show how to examine the effects of TRPA1 activation on neuronal physiology. Finally, we demonstrate how to activate neural circuits with TRPA1 and quantify resulting effects on behavior. The experiments are inexpensive to set up and generate robust, reproducible effects appropriate for teaching labs. Overall, this work provides neuroscience educators with novel exercises that illustrate principles of genetics, cellular physiology, and animal behavior.

MATERIALS AND METHODS

Genetics and Fly Husbandry

Fly strains were obtained from Bloomington Stock Center <http://flystocks.bio.indiana.edu/>. To order them it is necessary to register and obtain a Bloomington User Number (BUN), this can be done online following the instructions. *ppk*-GAL4 were obtained from Wayne A. Johnson (Ainsley et al., 2003). It is customary in the *Drosophila* community to freely distribute published genetic lines. We can alternatively provide all stocks on request.

A complete and simple guideline explaining how to maintain flies stocks, collect virgins and prepare crosses in a laboratory can be found at <http://biology.arizona.edu/sciconn/lessons2/Geiger/intro2.htm#ane>. Alternatively educators can find detailed videos explaining how to select virgins and set up fly crosses in Hornstein et al. (2009).

The flies used in the experiments are described in Table 1. For anatomy experiments, we crossed virgin females of

the driver lines A with males of the target line A'. For larval behavior, we crossed virgin females of the driver lines B with males B'. For adult behavior we crossed virgin females of the driver line C with males of the target line C' and we added the wild type line E as a control. For electrophysiology, we crossed virgin females D with males D'.

Neuroanatomy

To image overall patterns of GFP expression, 3rd instar larvae were immobilized between a slide and a coverslip in a drop of water. Images were taken with a DFC420 C digital camera under a Leica MZ16 F Fluorescence Stereomicroscope (Leica Camera AG, Solms, Germany). Alternatively, educators can use any stereo or compound microscope fitted for GFP fluorescence microscopy to visualize expression patterns.

Dissection for electrophysiology

We dissected 3rd instar larvae following a protocol outlined in previous studies (Hornstein et al., 2009; Pulver et al., 2009). Larvae were placed in a clear Sylgard (Dow Corning, Midland, MI, USA) lined dish filled with 'HL3.1' physiological saline. Saline consisted of (in mM): NaCl 70, KCl 5, CaCl₂ 2.4, MgCl₂ 4, NaHCO₃ 10, trehalose 5, sucrose 115, HEPES 5, pH 7.15. Insect pins (0.1 mm) were placed in the head and tail, then an incision was made along the length of the dorsum with fine dissection scissors. The body wall was then pinned flat. Fat bodies and digestive organs were carefully removed with forceps, uncovering the larval nervous system and body wall muscles. Finally, we dissected away the central nervous system (brain lobes and ventral ganglion), leaving only axons and motor neuron terminals.

The best way to learn the larval dissection is to watch someone do it. Detailed, narrated movies showing how to dissect and pin out 3rd instar are available in Hornstein et al. (2009), Brent et al. (2009), and Imlach and McCabe (2009). An alternative method of securing larvae with magnet clips is shown in a movie at: <http://hoylab.cornell.edu/fruitfly/shaker/physiology/>. Both techniques for securing larvae produce equivalent results.

Anatomy	Larval behavior	Adult behavior	Electrophysiology	Driver lines	name	stock number	chromosome	generated by
A	B	D		panneural	<i>elav</i> -GAL4	8760	II	S. Robinow and K. White, 1991
		C		panneural	<i>elav</i> -GAL4	458	X	
A	B	C		cholinergic neurons	<i>cha7.4</i> -GAL4	6793	II	T. Kitamoto, 2001
A	B	C		<i>pickpocket</i> class IV	<i>ppk1.9</i> -GAL4		II	J. A. Ainsley et al., 2003
				multidendritic sensory neurons				
				Target lines	name	stock number	chromosome	generated by
A'				green fluorescent protein	UAS- <i>CD8-GFP</i>	5137	II	J. Wang et al., 2002
	B'	C'	D'	<i>Drosophila</i> Transient Receptor Potential channel	UAS- <i>dTPRA1</i>	26263	II	F. N. Hamada et al., 2008
				Wildtype line	Canton-S	1		

Table1. Fly strains description.

Intracellular recordings

After dissection and pinning, preparations were placed on the stage of a BX50WI compound microscope (Olympus USA, Center Valley, PA, USA). Preparations were maintained at room temperature (22-24°C) in HL3.1 physiological saline. Using HL3.1 saline, neuromuscular junction (NMJ) preparations typically remained viable for ~1-2 hours at 22-23 °C without cooling and/or superfusion.

At 23-24°C, axons expressing dTRPA1 do not generate action potentials; however, once the surrounding bath temperature reaches 26-29 °C, dTRPA1 expressing axons fire spikes tonically (Hamada et al., 2008; Pulver et al., 2009). To activate dTRPA1 channels in dissected preparations, we raised the bath temperature by replacing the bath solution with pre-heated saline. With 2-3 careful washes (using a glass pipette filled with 40°C saline), we could quickly shift the bath temperature to ~30°C. Similar results were obtained by feeding ~50°C saline into a gravity-fed perfusion system and slowly exchanging the bath solution with a peristaltic pump.

Previous work has shown that dTRPA1-evoked action potentials in motor neurons give rise to EJPs at the larval NMJ (Pulver et al., 2009). We used sharp electrodes filled with 3M KCl (10-20 MΩ) to record intracellularly from muscle 6 (m6, also known as VL3) during temperature ramps from 23-30°C. We maneuvered electrodes with a MP225 motorized micromanipulator (Sutter Instruments, Novato CA). Voltage signals were amplified with a model 2400 patch clamp amplifier (A-M Systems, Sequim, WA, USA) and digitized at 10KHz with a Powerlab 8/30 data acquisition system (ADInstruments, Colorado Springs, CO, USA). Data were recorded in Chart 7 (ADInstruments).

The electrophysiology equipment we used is designed for research purposes (and is therefore expensive). To minimize expense, educators can: 1) use classical stereomicroscopes (e.g., Wild Heerbrugg M3, M5, Leica MZ6, Nikon SMZ-2B: all listed at Vermont Optec, Charlotte, VT, www.scopeshop.com) in place of a compound microscope. 2) use the Neuroprobe Model 1600 intracellular amplifier (A-M Systems) in place of the Model 2400 patch clamp amplifier (A-M Systems). 3) use Narishege MM-333 mechanical manipulators (Narishege, East Meadow, NY, USA) in place of motorized manipulators. 4) use NIDAQ BNC-2110 A-D boards (National Instruments, Austin, TX, USA) with the free software 'g-PRIME' (Lott et al., 2009) in place of Powerlab (A-D Instruments) for data acquisition and analysis. Previous work has shown the feasibility of gathering high quality electrophysiological data from larval NMJs with this less expensive equipment (Krans et al., 2005; Hornstein et al., 2009; Pulver et al., 2009).

We chose to present the NMJ preparation to students as a demonstration on a single electrophysiology rig. Small groups of two to three rotated between doing behavior experiments and electrophysiology.

Crawling behavior analysis

For monitoring larval locomotion, we built a crawling 'arena' using simple equipment (Fig. 3A and B). To perform temperature manipulations a blackened 5 X 5 cm Peltier

device (TE Technology, Traverse City, MI, USA) was mounted on a manipulator arm and driven by a 0-30V variable DC Power Supply Unit (PSU) (Rapid Electronics Ltd., Essex, UK). To better dissipate the temperature gradient between both sides of the Peltier device, a CPU heatsink (Akasa AK-786, Greenford, UK) was fixed under it (facultative), driven by a secondary 12V DC output from the PSU. A thermocouple probe attached to a multimeter (Uni-Trend UT60-E, Uni-Trend Group Limited, Hong Kong, China) was added to monitor the temperature during the experiment. A consumer-grade VGA webcam (Logitech, Logitech Europe S.A., EEU) was used to capture movies with VIRTUALDUB (www.virtualdub.org) at a rate of 1 frame per second. The surfaces of the camera were darkened and wrapped with black tape around the edge of the lens to reduce reflections off the camera surface. Four fiber-optic light sources (Microtec MFO-90, Micro instrument Limited, UK) were arranged at right angles to each edge of the arena. Light sources were carefully positioned so that larvae were lit evenly from all sides. The movies were filmed in a dark room.

To provide a soft and humid surface for crawling, we poured 2 ml heated 0.9% agarose with 0.6% black Indian ink directly onto the Peltier device (2-3mm thick). We left an outer perimeter (~0.5cm) free of agarose to prevent larvae escaping from the camera's field of view (larvae avoided crawling on bare peltier surfaces). The substrate was changed if it became visibly dessicated. A 5 x 5 mm piece of paper was placed in the field of view to calibrate the scale for all videos.

In each trial, four 3rd instar larvae were placed in the arena and recorded for 30s at 24°C. The PSU was then turned on and the temperature was monitored. When substrate temperature reached 29°C, a new 30s movie was recorded.

To reduce expense, crawling surface temperatures can also be manipulated simply by placing agarose-lined petri-dishes in a hot water bath maintained at 29-30°C. Thin agar surfaces on plastic petri-dishes at 23-24°C typically equilibrate to a 29-30°C water bath in 1-3 minutes. Students can observe larval behavior at 23-24°C before the petri-dish is placed in the hot water bath, then observe the same animals after moving the petri-dish to the water bath, and again after returning larvae to 22-23°C. A very inexpensive device to modulate surface temperatures in stationary petri-dishes can also be built as described in Krans and Hoy (2005).

Data analysis was performed using either the free software Image J (<http://rsbweb.nih.gov/ij/>) with the plugin "Manual Tracking" (<http://rsb.info.nih.gov/ij/plugins/track/track.html>), or Dynamic Image Analysis Software (DIAS) 3.4.2 (Sholl technologies, Iowa city, USA). Clear documentation about how to use the tracker is available at the same link. To quantify larval speed and distance, the posterior end of the animal was tracked. For direction changes, larval head movements were tracked. Here we present the Dynamic image analysis results that analyze centroid movements. Both DIAS and the free Manual Tracking system produce equivalent results (Fig. 3C).

Adult locomotor analysis

Groups of five male or female flies were selected under anesthesia (CO₂ or cooling), then placed in separate vials containing food for ~24 hours. Approximately one hour before the exercise, groups were transferred to clear, UV graded disposable spectroscopy cuvettes (Kartell, Australia). Each cuvette was covered with a cotton lid.

Groups of four cuvettes were introduced simultaneously for 65 seconds in a water bath at 35°C; resulting behaviors were recorded with a VGA webcam (Logitech) (Fig. 4A). For all genotypes, we measured the time it took for flies to fall to the bottom of the cuvette. We also quantified time to paralysis (paralysis was defined as a condition in which the animal lies on its back with little effective movement of the legs and wings). Students can also use stopwatches and/or hand tally counters to manually score adult behaviors without the use of a camera.

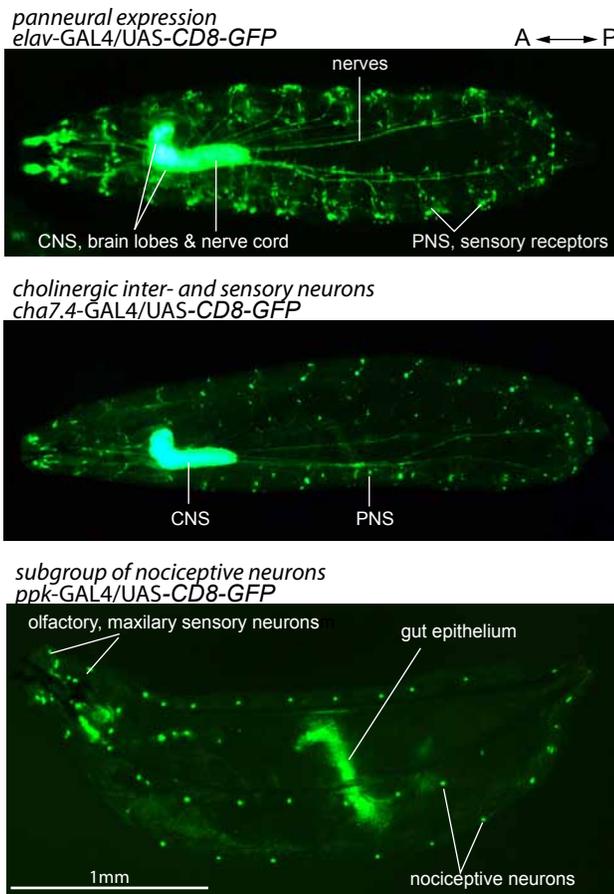


Figure 1. Expression pattern of the selected neuronal driver lines. Each of the driver lines was crossed to UAS-CD8-GFP and larvae were observed under the dissection scope. *elav-GAL4/UAS-CD8-GFP* labeled all the neurons in the Central Nervous System (CNS) and Peripheral Nervous System (PNS). The pattern of expression in *cha7.4-GAL4/UAS-CD8-GFP* resembles the previous one but only the cholinergic fraction of neurons was labeled. The *ppk-GAL4* expression can be observed in a cluster of 6 nociceptive neurons per segment, some maxillary and olfactory sensory neurons and the epithelium of the midgut.

Statistical analysis

Statistical analysis was performed employing Prism Graphpad 4.0 software package (2003). In larvae, average speed and number of turns were evaluated employing a one-way ANOVA with a Bonferroni post hoc test (Fig. 3C, D, E and Fig. 4C). In Fig. 4B, a linear regression analysis was carried out with pair comparisons. The corresponding *p*-values are included in the legend.

RESULTS AND DISCUSSION

Visualizing neural circuit anatomy

To illustrate the gross anatomy of the central and peripheral nervous system in larvae, we crossed males from several GAL4 driver lines to virgin UAS-CD8-GFP females. Resulting larvae were observed under a fluorescent dissection microscope (Fig. 1). *elav-GAL4* is expressed in all neurons, so in *elav-GAL4/UAS-CD8-GFP* animals, membrane bound GFP expression was visible in the brain lobes, nerve cord and all sensory neurons. Nerves containing both motor and sensory axons were labeled as well. *cha7.4-GAL4* is expressed in cholinergic neurons in the central and peripheral nervous system. As a result, GFP expression in *cha7.4-GAL4/UAS-CD8-GFP* animals was more restricted than that observed in *elav-GAL4/UAS-CD8-GFP*. Expression was present in the CNS and PNS, but was largely absent from motor axons (motor neurons in *Drosophila* are primarily glutamatergic). In contrast to *elav-GAL4* and *cha7.4-GAL4*, *ppk-GAL4* targeted a relatively small number of neurons, namely neurons expressing the *pickpocket* gene. *ppk-GAL4* labeled 6 sensory neurons per hemi-segment as well as sensory neurons in the antennal maxillary complex. The line also drives GFP expression in a portion of the gut epithelium and in four bipolar neurons in each brain lobe (Ainsley et al., 2003).

The objective of this part of the exercise was to show how the nervous system of *Drosophila* is organized, while also highlighting the neurons being manipulated in the exercises. These experiments also illustrate how genetic labeling tools can be used to identify and describe the cellular elements of neural circuits.

Observation with a dissection microscope does not allow detailed description of patterns of expression at the cellular level. However, these microscopes do provide enough resolution to observe gross patterns of GAL4 expression while sparing students from having to perform intricate dissections.

Using dTRPA1 to activate synapses

In addition to expressing GFP, the GAL4-UAS system can also be used to ectopically express tools for acutely manipulating neural activity. Our second exercise was designed to help students understand the cellular basis of dTRPA1 mediated activation of neural circuits. We recorded from larval muscles and used heat pulses to activate dTRPA1 in the motor axons of *elav-GAL4/UAS-dTRPA1* animals. We were able to evoke Excitatory Junctional Potentials (EJPs) in these animals by using a simple heating protocol. Figure 2A shows a schematic of the larval NMJ preparation. Figure 2B shows an

intracellular recording from m6 in a 3rd instar larva during a temperature ramp. At 23°C, no EJPs were present in m6. Exchanging the bath saline with heated saline caused a barrage of action potentials in motor neurons, leading to trains of EJPs in m6. At 26°C, EJPs began to appear in m6 (Figure 2C). The frequency of warmth-evoked EJPs increased as the temperature rose to 29°C (Figure 2D), then decreased as bath temperature fell (Figure 2E). Similar temperature responses were obtained in three other NMJ preparations. No temperature responses were seen in control animals (*UAS-dTRPA1/UAS-dTRPA1*, n = 2). All data were collected by students during a 3-4 hour teaching session and are consistent with previous work (Pulver et al., 2009).

m6 is innervated by two motor neurons with functionally distinct NMJs (Jan and Jan, 1976). As a result, two different EJP amplitudes were visible at all temperatures (asterisks indicate large amplitude EJPs in Figure 2C-E). m6 resting membrane potential depolarized substantially during the heat pulses (due to contraction artifacts). However, even at more depolarized voltages, both EJP

amplitudes were still visible (Figure 2E). Since larval muscles do not generate action potentials upon depolarization, educators and students can be sure that the events observed at more depolarized muscle membrane potentials are caused solely by motor neuron activity.

Neuroscience educators have traditionally used electrical stimulation to evoke EJPs at NMJs during teaching exercises. One important practical advantage of our preparation is that it does not require the use of external electrical stimulation to evoke EJPs. This reduces the workload for students and reduces the need for isolated pulse stimulators, suction electrodes and additional micromanipulators. One disadvantage of our preparation is that there is currently no way to evoke single EJPs using *dTRPA1*. This limits the preparation's usefulness in studies of synaptic transmission. However, alternative means for 'optogenetically' evoking single EJPs with blue light pulses are now available for educators. (Hornstein et al., 2009; Pulver et al., 2009).

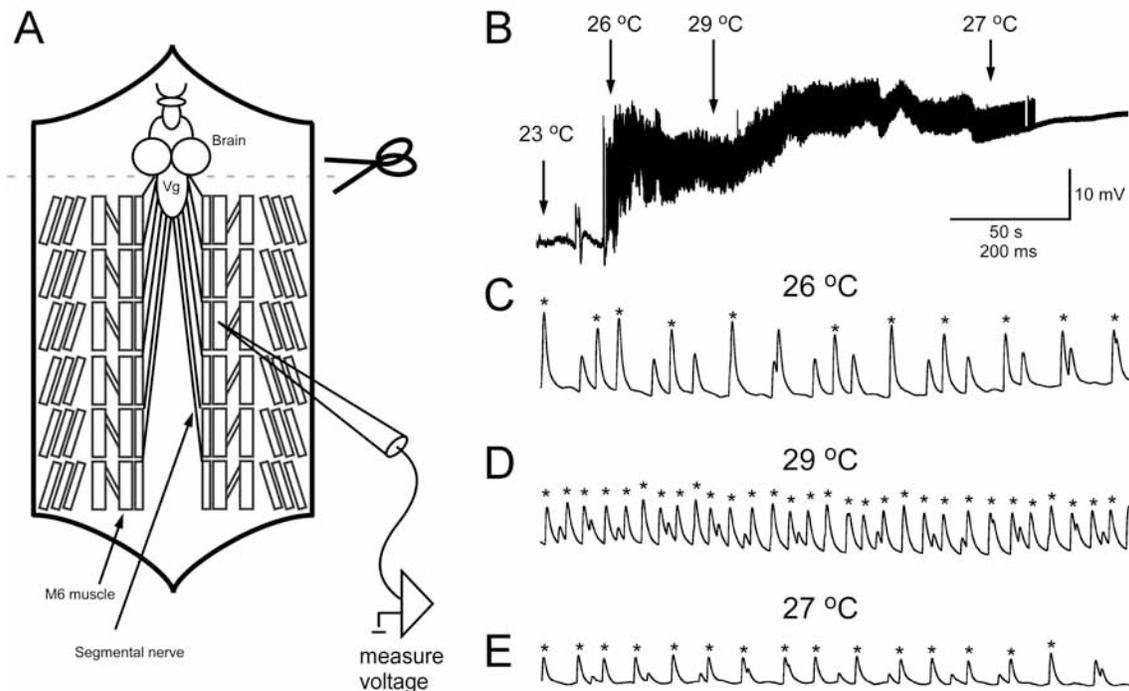


Figure 2. Warming evokes EJPs in larvae expressing TRPA1 in motor neurons. A) Schematic of larval NMJ preparation. Motor neuron cell bodies are located in the ventral ganglion (Vg) and project axons through segmental nerves. Removing the brain lobes inhibits endogenous locomotor rhythms; removing the ventral ganglion eliminates spontaneous motor neuron activity. B) Intracellular recording from a 3rd instar m6 muscle during warming and cooling. *dTRPA1* is expressed in all neurons, Brain and Vg have been removed. At 25-26 °C, *dTRPA1* motor axons begin to fire action potentials, causing EJPs at the NMJ. C-E) Expanded time-scale view of warmth-evoked EJPs in m6 at 26°C, 29°C, and 27°C, respectively. Two separate EJP amplitudes are visible after warming; asterisks indicate large amplitude EJPs. Resting membrane potentials at 23°C, 26°C, 29°C and 27°C, were -56 mV, -42 mV, -43 mV, and -32 mV, respectively. The schematic in (A) has been modified from Pulver et al. (2009).

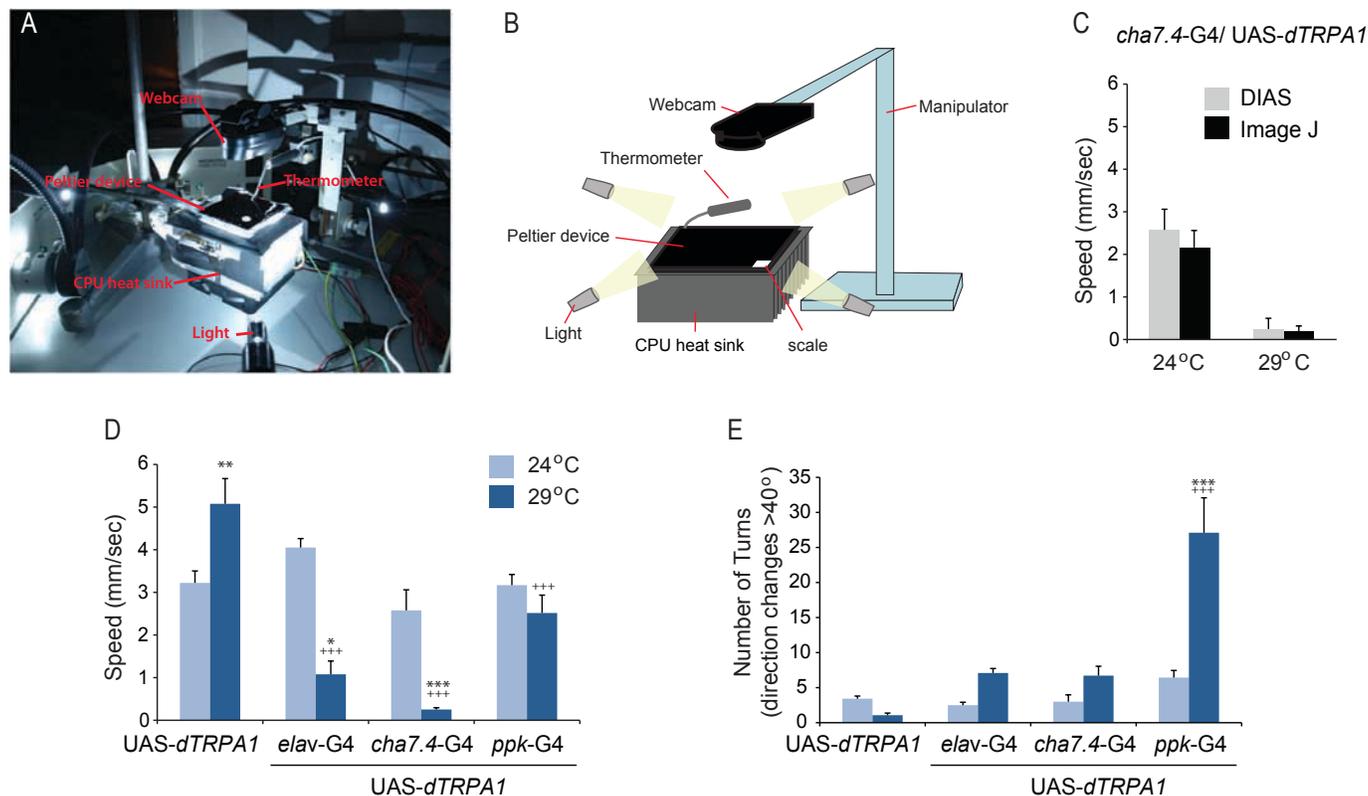


Figure 3. Effects of *dTRPA1* mis-expression on crawling behavior. A and B) Picture and diagram of the crawling arena respectively. Larvae were placed onto a homogenous lightened agarose surface that covered a Peltier device. Animals subjected to different temperatures were recorded with a webcam (more details in Mat. & Met and movie1). C) Comparison of the results obtained with DIAS and with the Manual Tracking plugin developed by F. Cordelières for Image J. The speed is not significantly different between the two methods. D) Average mean speed (\pm SEM) for the different genotypes and treatments. At 24°C none of the genotypes was significantly different. The activation of *dTRPA1* at 29°C induced highly significant behavioral changes in all genotypes compare to the control UAS-*dTRPA1*/+ (+++ stands for $p < 0.001$). The difference between 24°C and 29°C for the same genotype was evaluated and significant differences are indicated with asterisks. *ppk-GAL4/UAS-dTRPA1* animals speed did not change while the activation of the channel induced opposing significant phenotypes in controls and the other genotypes (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$). E) Average number of turns (\pm SEM) for the different genotypes and treatments. A turn was counted if the angle change was over 40°. Comparisons between genotypes and temperatures were performed. The remote thermo-activation of the *ppk* neurons was the only treatment that produced a significant difference compare to the same genotype at 24°C (*** $p < 0.001$) and to the control, UAS-*dTRPA1*/+, at 29°C (+++ $p < 0.001$). At least 12 animals were analyzed for each group.

Despite the lack of fine temporal control, students can still see a synapse ‘come alive’ simply by adding hot saline to an axon expressing *dTRPA1*. Our work suggests that unambiguous responses to temperature are possible with a very simple means of controlling bath temperature. In a previous study, Krans and collaborators (Krans et al., 2005) explored the temperature sensitivity of synaptic transmission at the larval NMJ. They also incorporated *Drosophila* temperature sensitive synaptic transmission mutants into classroom exercises. The authors were able to see effects suitable for teaching labs; however, reasonably accurate control of bath temperature was essential. One advantage of the preparation presented here is that students can see robust warmth-gated changes in synaptic activity without any kind of temperature control system. This increases the chances of students being able to see the predicted effects, while reducing overall costs to educators.

Overall, our cellular physiology exercises demonstrate

basic principles of how changes in ion channel function lead to changes in cellular excitability, and how those, in turn, change synaptic function. These exercises also demonstrate the concept that genetics can be used to install ways of controlling the excitability of selected neurons. This is a new exciting trend in neuroscience, but one with potential pitfalls. For example, neuroscientists might be tempted to make assumptions about how genetic manipulations affect cellular physiology. Our exercises are designed to drive home to students the importance of not making such assumptions. If one wants to use a new genetic tool to control neural activity in behaving animals, then it is crucial to confirm electrophysiologically that the tool in question is actually doing what it is predicted to do.

Behavioral effects of TRPA1 neural activation in larvae

To understand how neural activity within an animal's nervous system contributes to behavioral output, one

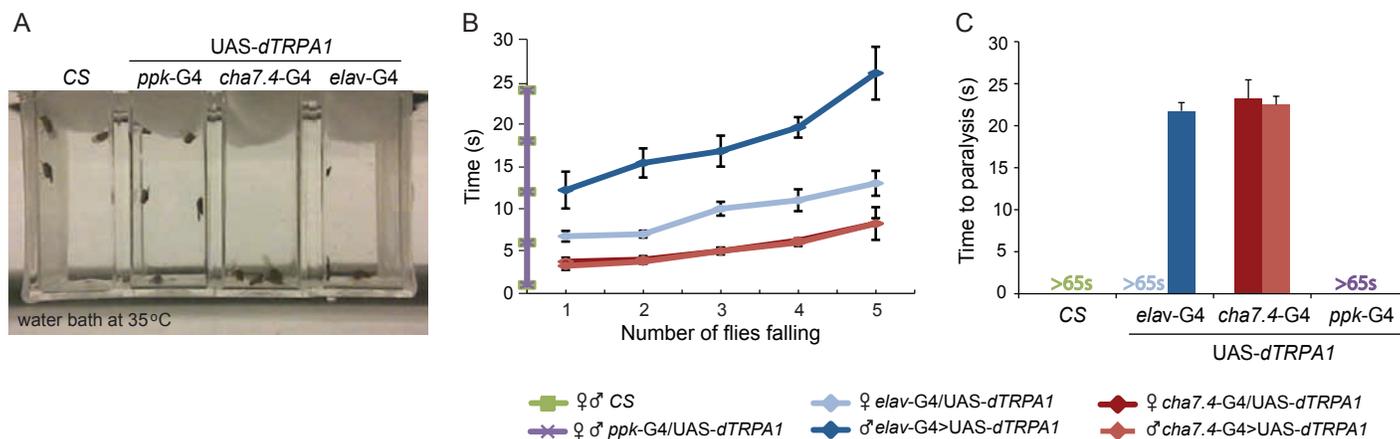


Figure 4. Effects of dTRPA1-mediated activation on adult locomotion. A) Picture of the experimental setup. Five male or female flies were put in a electrophoresis cuvette and immersed in a 35°C water bath while recorded (more details in Mat. & Met and movie 6). B) The time it takes the flies to fall to the bottom of the cuvette was measured. Control (CS) and *ppk-GAL4/UAS-dTRPA1* animals never fall while the other genotypes fell in a time dependent manner. The slopes calculated with a linear regression analysis were for *cha7.4-GAL4/UAS-dTRPA1* females 1.13 ± 0.18 , $r^2=0.93$; males 1.23 ± 0.17 , $r^2=0.95$. They were not significantly different ($F=0.158938$ DFn=1 Dfd=6 $p=0.70$). *elav-GAL4/UAS-dTRPA1* males had a slope of 3.18 ± 0.49 , $r^2=0.93$ that was significantly different from the females slope 1.65 ± 0.21 , $r^2=0.95$: $F=8.16498$ Dfn=1 Dfd=6 $p=0.02$. C) The time to paralysis was measured. The cholinergic and panneural male activation induced paralysis while in the other genotypes it was not observed during the 65s the experiment lasted. At least 15 animals were evaluated for each experimental group.

needs to be able to modify the activity of neuronal ensembles and to evaluate the behavioral outcome of such treatment. To illustrate how this can be done, we evaluated the impact of activating different neuronal groups with dTRPA1 in freely behaving larvae. We analyzed two salient, but simple to measure, larval behaviors: overall speed and number of turns.

To provide positive controls for students, we first analyzed the behavioral effects of activating large neuronal populations (e.g., all neurons and all cholinergic neurons). In control larvae, raising surface temperatures to 29°C, caused an increase in crawling speed, but no change in the number of turning events (first pair of columns Fig. 3D and E). In contrast, elevating surface temperatures slowed crawling in *elav-GAL4/UAS-dTRPA1* and *cha7.4-GAL4/UAS-dTRPA1* animals without significantly affecting turn number. To better appreciate larval phenotypes, we filmed animals at high magnification. At 29°C, panneural activation induced concomitant contraction of multiple larval segments. During the occasional peristaltic wave propagation, the pre-contracted muscles within each segment were not able to undergo normal contraction-relaxation cycles resulting in decreased effectiveness of peristalsis (movies 2 and 3). On the other hand, activation of cholinergic neurons induced a completely different behavior; the larvae presented a high level of uncoordinated 'seizure-like' activity with spasms and waves of contractions that inhibited movement (movie 4).

These first two treatments show how acute activation of neuronal groups can impact behavior. In both treatments, activation was widespread; as a result, it was not possible to clearly ascribe function to distinct, small groups of

neurons. Nevertheless, these experiments show the importance of segmentally coordinated neuronal activity for larval locomotion. They also demonstrate how seizure-like behaviors can result from abnormal activation of excitatory (i.e., cholinergic) neural networks. Importantly, these experiments provide solid positive controls for further behavioral experiments in which dTRPA1 is restricted to smaller neuronal populations.

After manipulating the activity of large ensembles of neurons, we used dTRPA1 to explore the function of a restricted group of sensory neurons. When compared to controls, activation of *pickpocket* neurons (*ppk-GAL4/UAS-dTRPA1*) caused an increase in speed ($p<0.001$), and significantly incremented the number of direction changes. High magnification videography showed that *ppk-GAL4/UAS-dTRPA1* animals rolled sideways in a corkscrew-like motion at elevated temperatures (Fig. 3D, E and movie 5).

Remote activation of neuronal circuits is a powerful way to elucidate the function of candidate neurons for a specific behavioral response. In particular, if triggering activity in a group of neurons is adequate to recapitulate a behavior, these neurons can be considered *sufficient* to induce it (Ainsley et al., 2003; Schroll et al., 2006; Hwang et al., 2007). The activation of *ppk* neurons with TRPA1 illustrates this concept beautifully, since as soon as the larvae were exposed to activating temperature (29°C), they started to roll laterally. This behavior phenocopied the stereotypical defensive response observed in response to thermal, mechanical or chemical noxious stimulation (Tracey et al., 2003; Al-Anzi et al., 2006) and demonstrate that the sensory signal initiated in the *ppk* neurons is

sufficient to induce the activation of a certain pattern of locomotion.

Behavioral effects of activating neurons with TRPA1 in adult flies

In a second set of experiments, we used dTRPA1 to activate neural circuits in adult animals. We raised ambient temperatures to 35°C, then filmed and quantified resulting locomotor behaviors (movie 6). We examined the same genotypes used for larval behavior studies and added the wildtype control strain 'Canton S'.

When the cuvettes containing wildtype flies were introduced into the 35°C water bath, the animals showed no obvious changes in behavior. They continued to move, walk on the walls, and make short flights as at room temperature. In contrast, activation of all neurons (*elav^{c155}-GAL4/UAS-dTRPA1*) affected adult behavior, but males and females responded differently. After a few seconds at 35°C, males started to lose postural control and fell to the bottom of their enclosure (cuvette tube). They presented uncoordinated bursts of fast wing beating and finally they became paralyzed; however, females were more resistant to the effects of dTRPA1 activation. They fell at a significantly slower rate and never completely stopped moving during the 65s that the experiment lasted (Fig. 4B,C). In contrast, both male and female (*cha7.4-GAL4/UAS-dTRPA1*) animals showed identical responses to warming. Activation of cholinergic neurons caused flies to fall in a linear time-dependent manner. After 22s at 35°C, *cha7.4-GAL4/UAS-dTRPA1* flies were paralyzed, lying on their back with little effective movement of legs and wings (Fig. 3B,C). Finally, activation of *ppk* neurons did not produce any clear behavioral changes when compared to controls (Fig. 3B,C).

Our behavioral experiments with adult flies were aimed to present another simple protocol to manipulate locomotor activity. This technique has the advantage of being very fast but it lacks the versatility of the larval setup since it is more difficult to evaluate speed or changes in directions in the flying animals.

Our adult experiments provide educators with the opportunity to discuss aspects of designing neurogenetic experiments in *Drosophila*. For example, both pan-neuronal and cholinergic activation affected locomotion. *cha7.4-GAL4/UAS-dTRPA1* flies and *elav-GAL4/UAS-dTRPA1* males became paralytic while females had postural defects but were flapping their wings. This gender specific behavioral effect was probably due to the fact that the *elav-GAL4* driver is inserted on the X chromosome. In males, genes on the X chromosome are often expressed at higher levels than a single copy of the same gene on the X chromosome of females. This regulatory mechanism, known as 'dosage compensation' serves to equalize expression of genes on the X chromosome (Gelbart and Kuroda, 2009). This sexual dimorphism highlights the necessity of carefully designing experiments to take into account possible behavioral differences related to gender.

In addition, behavioral differences could be observed by comparing the results of the same genotypes in the two life stages. In adult flies, activation of *ppk* neurons does not

produce any obvious change in behavior; this is in contrast to the large behavioral effects observed in larvae. This result highlights an important point for students: functional changes can occur during development or during the lifetime of individuals and this aspect should also be taken into account when conducting experiments.

Overall, our larval and adult behavior experiments show that dTRPA1 activation of ensembles of neurons can generate distinct behavioral phenotypes. The resulting behaviors are obvious and can be easily measured in teaching laboratories. Practically, the behavior experiments provide reliable results, require minimal equipment, and can be up- or down-scaled depending on the size and focus of a particular class. The exercises can stand alone, or be integrated into anatomical and/or neurophysiological investigations. It is important to note that our behavioral experiments are also very effective at generating student excitement and a sense of wonder. We have found that students (and educators) are typically fascinated by the idea of remotely controlling what other animals do. In our experience, having the ability to do this heightens student interest in studying animal behavior.

Aside from practical concerns, we feel that these exercises are powerful because students learn more than just how to measure and quantify animal behavior. The students also learn how to manipulate neural activity using genetic tools. Participants are given a chance to integrate what they know about animal behavior with what they know about anatomy, cellular physiology and genetics. To our knowledge, very few teaching lab exercises are focused on bringing together the study of these four disciplines. The experiments we propose provide one avenue for simultaneously getting students interested in all four subjects.

What principles do these exercises illustrate?

The exercises outlined here illustrate multiple important concepts in genetics, neurophysiology and behavior. First of all, students learn how modern genetic techniques (i.e., GAL4-UAS) can be used to spatially restrict gene expression. This has been a crucial advance in neurogenetics, and future work aimed at genetically manipulating neural circuits hinges critically on having this ability. Secondly, these exercises demonstrate how genetics can be used to install ways of remotely controlling activity in selected neurons. This is a relatively new concept in neuroscience, but one that is starting to revolutionize how research is being conducted. Third, the ability to control dTRPA1 conductance with heat provides an opportunity to illustrate basic principles of how changes in ion channel function translate into changes in neuronal excitability and synaptic function. Fourth, our behavior exercises show that by modulating activity in a group of neurons it is possible to gain insight into the way the brain controls behavior. Specifically, these exercises demonstrate that activation of specific subsets of neurons can be sufficient to evoke specific coordinated behaviors.

Finally, these exercises highlight the importance of quantitative methods in measuring animal behavior. At all stages, students are encouraged to come up with their own

plans for quantifying observed behaviors. This helps instill the principle that to understand animal behavior, it is necessary to do more than just qualitatively describe what animals do.

Motivating research attitude

We ran these exercises as demonstrations in an upper-level (3rd year) undergraduate neuroscience course in the Department of Zoology at Cambridge University. One of our aims was to motivate a 'research attitude' in students. We did this primarily by giving students a certain degree of freedom. They were encouraged to propose and test their own hypotheses related to the function of various neural circuits. We encouraged the students to design their own experiments, observe the effects, then decide on their own which parameters they wanted to analyze. We also made a point of stressing not how much we know about *Drosophila*, but *how little we know* about fly brains and behavior. Many of the experiments presented here have yet to be published by research scientists. We stressed this to students; we made it clear that they were breaking new ground, not just rediscovering old results. We feel that this is an important component of any *Drosophila* neurogenetics teaching lab. There is a large scope for future work in fruit flies and plenty of room for new discoveries by young scientists.

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