Temporal Dynamics of Neuronal Activation by Channelrhodopsin-2 and TRPA1 Determine Behavioral Output in Drosophila Larvae

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Pulver SR, Pashkovski SL, Hornstein NJ, Garrity PA, Griffith LC. Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRPA1 determine behavioral output in Drosophila larvae. J Neurophysiol 101: 3075–3088, 2009. First published April 1, 2009; doi:10.1152/jn.00071.2009. In recent years, a number of tools have become available for remotely activating neural circuits in Drosophila. Despite widespread and growing use, very little work has been done to characterize exactly how these tools affect activity in identified fly neurons. Using the GAL4-UAS system, we expressed blue light–gated Channelrhodopsin-2 (ChR2) and a mutated form of ChR2 (H134R-ChR2) in motor and sensory neurons of the Drosophila third-instar locomotor circuit. Neurons expressing H134R-ChR2 show enhanced responses to blue light pulses and less spike frequency adaptation than neurons expressing ChR2. Although H134R-ChR2 was more effective at manipulating behavior than ChR2, the behavioral consequences of firing rate adaptation were different in sensory and motor neurons. For comparison, we examined the effects of ectopic expression of the warmth-activated cation channel Drosophila TRPA1 (dTRPA1). When dTRPA1 was expressed in larval motor neurons, heat ramps from 21 to 27°C evoked tonic spiking at ~25°C that showed little adaptation over many minutes. dTRPA1 activation had stronger and longer-lasting effects on behavior than ChR2 variants. These results suggest that dTRPA1 may be particularly useful for researchers interested in activating fly neural circuits over long time scales. Overall, this work suggests that understanding the cellular effects of these genetic tools and their temporal dynamics is important for the design and interpretation of behavioral experiments.

INTRODUCTION

Drosophila researchers have developed a rich array of molecular genetic tools for studying the genetic and neural bases of animal behavior. Most recently, tissue specific gene expression tools, particularly the GAL4-UAS system (Brand and Perrimon 1993), have been used to ectopically express transgenes designed to manipulate neural activity. Genetically encoded tools for inhibiting synaptic release (Kitamoto 2001; Sweeney et al. 1995), hyperpolarizing neurons (Nitabach et al. 2002; Paradis et al. 2001), and hyperexciting neurons (Broughton et al. 2004; Nitabach et al. 2006; Parisky et al. 2008) have all been used to examine how activity in specified neural circuits shapes behavioral output.

The main limitation of these genetic manipulations is that they chronically modify activity throughout the entire life of the animal. However, a new generation of genetically encoded tools that allow acute and reversible manipulation of neuronal activity has become available. These new tools exploit ion channels and vesicle trafficking proteins that can be regulated by light or temperature to alter a neuron’s membrane potential or secretion of neurotransmitter (Boyd et al. 2005; Kitamoto 2001; Nagel et al. 2003, 2005). Such tools offer the potential for noninvasive and conditional manipulation of neural circuit activity in intact animals at any developmental stage.

Two promising genetically encoded tools for inducibly altering neuronal activity are Channelrhodopsin-2 (ChR2) and Drosophila TRPA1 (dTRPA1). ChR2 is a blue light sensitive cation channel isolated from the green algae, Chlamydomonas reinhardtii (Nagel et al. 2003). Neurons expressing ChR2 depolarize and fire spikes when exposed to blue light, and ChR2 has been used to manipulate neural circuits in multiple organisms (Arenkiel et al. 2007; Crisp et al. 2008; Douglass et al. 2008; Nagel et al. 2005; Schroll et al. 2006; Zhang et al. 2007). dTRPA1 is the Drosophila ortholog of the mammalian transient receptor potential channel TRPA1. dTRPA1 is a temperature- and voltage-gated cation channel (Hamada et al. 2008; Viswanath et al. 2003) that regulates Drosophila thermotactic behavior (Hamada et al. 2008; Rosenzweig et al. 2005, 2008). Although dTRPA1 is normally expressed in a small subset of fly neurons, previous work has shown that when dTRPA1 is expressed ectopically throughout the nervous system, dTRPA1 triggers paralysis and barages of excitatory junctional potential (EJP) at the fly’s neuromuscular junction (NMJ) in response to modest temperature increases (Hamada et al. 2008). Recently, dTRPA1 has been used as a tool to remotely activate neural circuits in freely behaving flies using temperature shifts (Parisky et al. 2008; Shang et al. 2008).

Although tools such as ChR2 and dTRPA1 have been increasingly used to manipulate circuit activity, little is known about the electrophysiological properties, operational ranges, and adaptation properties of single, identified Drosophila neurons expressing such channels. In this study, we use the Drosophila third-instar larval preparation to provide baseline physiology data for animal behavior researchers interested in using ChR2 and dTRPA1 for manipulating circuit activity in flies. We also use these new tools to examine how acutely activating motor and sensory circuits affects locomotor behavior in freely moving larval animals.

As part of this effort, we generated and tested a UAS line expressing a mutated form of the ChR2 channel (H134R-ChR2). Work in other systems has shown that this point mutation causes an increase in light evoked ChR2 current amplitude (Nagel et al. 2005). We show that neurons expressing this mutated H134R-ChR2 channel are more responsive to blue light and more resistant to spike frequency adaptation than cells expressing wild-type ChR2 channels.
METHODS

Transgenic lines

UAS-H134R-Chr2 was generated by site-directed mutagenesis (QuickChange, Stratagene, La Jolla, CA) of mCherry-Chr2 (a gift from Karl Deisseroth) and subcloning into pUAST (Brand and Perrimon 1993). Transgenic lines were made by standard P element insertion. Both the UAS-Chr2 (Schroll et al. 2006) and UAS-H134R-Chr2 transgenes are on the second chromosome. For dTRPA1 experiments, we used a UAS line (also on the second chromosome) generated as part of a previous study (Hamada et al. 2008).

To drive expression of UAS transgenes in larval motor neurons (MNs), we used several characterized GAL4 lines: OK371-GAL4, C380-GAL4,UAS-mCD8-GFP, and C380-GAL4,mCD8-GFP;Cha-GAL80. OK371-GAL4 drives expression in glutamatergic sensory neurons (including all MNs and glutamatergic interneurons; Mahr and Aberle 2006). C380-GAL4,mCD8-GFP expresses in cholinergic sensory neurons as well as MNs (Budnik et al. 1996). The addition of a GAL80 transgene expressed in cells containing the promoter for acetylcholine transferase (Cha-GAL80) gene limits expression primarily to all or most larval body wall sensory neurons but not in MNs or interneurons (Hughes and Thomas 2007). All expression patterns revealed that the addition of Cha-GAL80 unexpectedly abolished expression in a subset of dorsal motor neurons (data not shown). We used 5-40-GAL4 (made in the Heberlein laboratory at UCSF) to drive expression of UAS transgenes exclusively in sensory neurons. Previous work has shown that this line drives expression in all or most larval body wall sensory neurons but not in MNs or interneurons (Hughes and Thomas 2007). All expression patterns were confirmed in third instar animals; expression patterns at other stages of development were not examined.

Growth conditions

For Chr2 and H134R-Chr2 experiments, larvae were grown in constant darkness on standard fly media at 22–25°C. Fly media contained 1 mM all-trans-retinol (Toronto Research Chemicals, North York, Ontario, Canada), a cofactor necessary for proper folding and membrane insertion of Chr2. For dTRPA1 experiments, flies were grown on standard media at 20–22°C. Flies were raised on a roughly 12:12-h light:dark cycle.

Whole cell patch electrophysiology

Whole cell patch electrophysiology experiments were performed on third-instar female larvae. Animals were dissected and filled dorsal side up on Sylgard-lined dishes in Ca2+-free “A” solution [containing (in mM) 128 NaCl, 2 KCl, 4 MgCl2, 35 sucrose, 5 trehalose, and 5 HEPES, pH 7.1–7.2]. Preparations were mounted on the stage of a BX50WI compound microscope (Olympus, Center Valley, PA) and continually superfused with A solution containing 0.3 mM Ca2+. Dorsal motor neurons were identified under a ×40 water immersion lens; sheath connective tissue covering identified neurons was removed with focally applied 0.01% protease (type XIV, Sigma, St. Louis, MO) as described previously (Choi et al. 2004). Both MNSN-I and MNIB-30 were targeted for recording (Choi et al. 2004). Pipette resistances were 5–10 MΩ, and pipette solution contained (in mM) 130 potassium gluconate, 10 HEPES, 0.5 EGTA, 2 MgCl2, 0.05 CaCl2, 2 NaCl, and 10 KOH, with pH adjusted to 7.2 with KOH. We used an MP285 micromanipulator (Sutter Instruments, Novato, CA) to maneuver microelectrodes and an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) to perform whole cell recordings. Data were digitized with a Powerlab 4/30 (ADInstruments, Colorado Springs, CO) and recorded in Chart 5.1 (ADInstruments) on a desktop PC. Data were analyzed using custom scripts in Spike2 (version 5, Cambridge Electronic Design, Cambridge, UK) and standard features in Excel (Microsoft). Spike2 analysis scripts used are freely available at www.whitney.ufl.edu/BucherLab. Statistical tests were performed in Excel and JMP (version 5, JMP, Cary, NC).

For Chr2 stimulation during patch experiments, we attached (via electrical tape) an ultrabright blue LED with collimating lens and heat sink (Thorlabs, Newton, NJ) to the camera port of the Olympus scope used for desheathing. Timing and light intensity was controlled by feeding voltage steps from an output channel on the Powerlab into an LED controller box (Thorlabs). Small DC current injections were used to hold MN resting potential (Vrest) at −50 mV in all experiments. Light intensity at various voltages was calibrated with a PM100 optical power meter with an S130A slim sensor (Thorlabs). With the camera port fully open, we were able to deliver temporally precise, reproducible 470-nm light pulses up to ~1.000 μW/mm² in intensity.

For dTRPA1 stimulation, we ramped bath temperature from 22 to 28°C and then back to 22°C using a SC-20 inline heater/cooler under control of a CL-100 bipolar temperature controller (Warner Instruments, Hamden, CT). Bath temperature was monitored with the same device and recorded in Chart 5.1. Bath temperature ramp time was typically ~1 min on up and down swings. Cell patching, data acquisition, analysis, and statistical tests were performed as above.

NMJ electrophysiology

Female third-instar larvae were dissected and pinned out as above in HL3.1 saline containing (in mM) 70 NaCl, 5 KCl, 0.8 CaCl2, 4 MgCl2, 10 NaHCO3, 5 trehalose, 115 sucrose, and 5 HEPES, pH 7.1–7.2. The anterior lobes of the larval brain were removed, and nerves leading to the posterior-most tail region were cut; the ventral ganglion and body wall muscles were left undisturbed. This dissection silenced crawling rhythms (after ~10–15 min) but preserved the cell bodies of motor neurons innervating body wall muscles.

Intracellular recordings were performed from larval muscle 6 (m6). This muscle is innervated by two motor neurons: one with large boutons (MN67-Ib) and one with small boutons (MNSNb/d-Is) (Hoang and Chiba 2001). MNSNb/d-Is neuron responses are easily distinguishable from MN67-Ib responses on the basis of EJP amplitude; MNSNb/d-Is neurons give rise to large EJPs, and MN67-Ib neurons give rise to small EJPs (Hoang and Chiba 2001; Lennicka and Keshishian 2000). MNSNb/d-Is EJP frequency in m6 was measured in response to blue light pulses and/or temperature ramps in control and experimental animals.

Light stimuli were delivered using two rig configurations: the first was identical to that used for whole cell patch work. In the second setup, we taped a ×10 dissecting scope eye piece (Carl Zeiss, Thornwood, NY) in front of an ultrabright LED and positioned a larval preparation at the focal point of the eye piece (Hornstein et al. 2009). Timing and light intensity were controlled as above. This second arrangement was able to deliver light pulses with precision equivalent to that seen in whole cell patch work but at considerably less expense (it eliminated the need for a compound microscope). Heat stimuli were delivered as described above.

Sharp glass electrodes (12–18 MΩ) filled with 3 M KCl were used to record membrane potentials in m6. We used either an Axopatch 200B (Molecular Devices) or a Neuroprobe model 1600 (A-M Systems, Carlsborg, WA) for amplifying intracellular voltage signals. In situations involving high firing rates (and, subsequently, summation), individual EJPs were time marked by detecting changes in the slope of the trace with standard analysis tools in Spike 2. All other data acquisition, analysis, and statistical tests were performed as above.
Behavior

To measure ChR2-mediated changes in crawling behavior, we filmed third-instar female larvae crawling on 1% Bacto agar (dissolved in water) in a 25 × 25-cm plexiglass arena. The arena was placed on a light box to increase contrast. A VL3-ZU camcorder (Sharp Electronics, Mahwah, NJ) was positioned directly above and an ultrabright LED with focusing lens was directed into the center of the field of view. To prevent glare from obscuring the crawling larvae, we mounted a red filter from HOYA (HOYA Filters, Long Beach, CA) on the camcorder lens. We placed individual larvae in the center of the arena, recorded crawling for ~10 s, and projected either constant or rhythmic (1 Hz, 0.5-s duration) blue light onto the animal. Light intensity was ~300 μW/mm². Movies were recorded using Windows Movie Maker on a desktop PC. Larval crawling speed was computed from crawling traces created using Dynamic Image Analysis Software (DIAS, Iowa City, IA) and analyzed in Microsoft Excel. Statistical tests were performed in Excel and JMP.

To measure dTRPA1-mediated changes in crawling behavior, we poured 1% Bacto agar onto the surface of a black-colored 5 × 5-cm Peltier device (TE Technology, Traverse City, MI). With this setup, we were able to raise the surface temperature of the crawling arena to 27°C using a DC voltage source. Surface temperature was measured with a Fluke 51 (Fluke, Everett, WA) thermocouple probe. Larval behavior was filmed with a VL3-ZU camera mounted above the arena; the digital display on the thermometer was positioned under the camera so that surface temperatures could be recorded in parallel.

RESULTS

Larval motor neurons expressing ChR2-H134R show enhanced responsiveness to blue light pulses

To assess the relative abilities of standard and H134R mutant ChR2 (H134R-ChR2) to activate neurons, we examined the light responsiveness of identified third-instar larval motor neurons expressing these proteins by performing whole cell patch recordings in animals with fully intact nervous systems. Figure 1A shows a schematic of the larval whole cell patch preparation. Figure 1B shows representative MN30-Ib cells expressing ChR2 (left) and H134R-ChR2 (right) responding to 1-s light pulses (intensity: 245 μW/mm²; wavelength: 470 nm). MNs expressing H134R-ChR2 (n = 8) responded with higher firing rates than cells expressing ChR2 (n = 8) at all intensities (Fig. 1C). This was not because of differences in intrinsic membrane properties between the two groups. In the absence of blue light, average response to depolarizing current injection when voltage was held at −50 mV (Fig. 1D), as well as average input resistance (ChR2: 1.1 ± 0.2 GΩ; H134R-ChR2: 1.1 ± 0.2 GΩ) and resting membrane potential (ChR2: −51.3 ± 1.0 mV; H134R-ChR2: −52 ± 1.7 mV), were not significantly different between genotypes (Student’s t-test; P = 0.68 and 0.65, respectively). Similar trends were observed in patch recordings of MNISN-Is motor neurons (data not shown).

To test the generality of the H134R-ChR2 enhancement, we measured ChR2- and H134R-ChR2-mediated responses in a separate set of MNs by recording light-evoked EJPs in body wall muscles. Figure 2A shows a schematic of the preparation. MN cell bodies were intact in these experiments, and intrinsic rhythmic motor patterns were silenced by ablating descending and ascending inputs to the ventral ganglion (see METHODS). UAS transgene expression was limited to glutamatergic neurons using the OK371-GAL4 driver. Paired recordings have not shown any evidence for chemical and/or electrical synapses between dorsal MNs (J. C. Choi, unpublished data). As a result, light-evoked EJPs in m6 are likely the result of direct depolarization of motor neurons. Figure 2B shows light evoked...
EJPs in response to 200-ms blue light pulses of increasing intensity; traces from a ChR2 preparation are shown on top, traces from a H134R-ChR2 animal are shown on bottom. Note the high degree of summation at high light intensities in the H134R-ChR2 trace. In these situations, individual EJPs were still reliably detected by determining slope changes in the trace (see METHODS). Previous studies indicated that two classes of EJPs are observed at m6: smaller EJPs generated by MN6/7-Ib activation and larger EJPs generated by MNSNb/d-Is activation (Hoang and Chiba 2001). In some preparations, both small and large EJPs were observed (data not shown). We used the large EJPs for quantitation because they obscure the small EJPs at higher light intensities. Pooled data from these experiments are shown in Fig. 2 C. MNSNb/d-Is cells expressing H134R-ChR2 (n/H11005 35) showed significantly higher firing rates than cells expressing ChR2 (n/H11005 21) at all light intensities (P/H11021 0.05, Student’s t-test). Homozygous genetic controls (UAS-ChR2 and UAS-H134R-ChR2) showed no responses to blue light at any light intensity (n = 2 for both).

In a second set of experiments, we assayed stimulus thresholds for evoking single EJPs in ChR2- and H134R-ChR2–expressing MNs. Figure 3A shows m6 recordings from two animals: one expressing ChR2 in MNs (top) and the other expressing H134R-ChR2 in MNs (bottom). At a light intensity of \(545 \, \mu W/mm^2\), 10-ms pulses reliably evoked EJPs in H134R-ChR2–expressing cells but not in ChR2 cells. Ten-millisecond light pulses at 1,000 \(\mu W/mm^2\) evoked EJPs in both preparations. In Fig. 3B, the percentages of successful EJPs in five low-frequency pulse trains are plotted as a function of light intensity, pulse duration, and genotype. H134R-ChR2 cells evoked EJPs at lower light intensities and shorter pulse durations than ChR2-expressing cells (*P < 0.05, Student’s t-test).

Finally, to assay the distribution of functional H134R-ChR2 within motor neurons, we delivered light pulses to preparations in which the ventral ganglion had been dissected away, leaving only axons and nerve terminals. We were able to evoke EJPs in axons expressing H134R-ChR2 (n = 3 of 4 preparations). Because the H134R-ChR2 construct also contains the fluorophore mCherry, we were also able to obtain visual confirmation of H134R-ChR2 expression. Strong mCherry labeling was present in the motor neuron axons and nerve terminals as well as cell bodies (data not shown).

Spike frequency adaptation in cells expressing ChR2 and H134R-ChR2

Light-activation of ChR2 generates large transient inward currents, and neurons expressing ChR2 in other systems have shown rapid spike frequency adaptation (Liewald et al. 2008). Because ChR2 is often used in Drosophila to manipulate behavior over long time scales (i.e., minutes; Schroll et al. 2006; Zhang et al. 2007), adaptation could complicate the interpretation of behavioral experiments. This critical issue has not been examined in previous work in Drosophila, so we were curious to know how the dynamics of blue light stimulation affected firing rate in larval neurons. To address this, we...
firing rates were approximately halved and continued to decline further over the next 20 min (Fig. 4B, blue bars). The long-term decline in firing rate in ChR2-expressing cells could be alleviated using a simple pulsed light protocol (Fig. 4B, light blue; \( P < 0.05 \) for \( t = 300–1,200 \) s). However, rhythmic pulses did not alleviate the firing rate decay in the first 30 s of blue light stimulation.

Cells expressing H134R-ChR2 also showed a sharp drop in firing rate within the first 30 s of both constant and pulsed stimulation (Fig. 4B). However, over longer time scales, H134R-ChR2–expressing cells maintained significantly higher overall firing rates than MNs containing ChR2 under both stimulation conditions (constant: \( P < 0.05 \) for \( t = 300–1,200 \) s; pulsed: \( P < 0.05 \) for \( t = 30–1,200 \) s). Thus while rapid spike frequency adaptation was observed using both forms of ChR2, the H134R-ChR2 form supported higher levels of sustained MN spiking over the 20-min time course.

How does motor neuron activation with ChR2 and H134R-ChR2 affect locomotor behavior?

To compare the effectiveness of the two ChR2 isoforms in activating circuits in behaving animals and examine possible behavioral correlates of adaptation, we examined how light activation of MNs expressing these ChR2 isoforms affected crawling. We expressed ChR2 or H134R-ChR2 in MNs using the OK371-GAL4 driver and then delivered blue light pulses at intensities equivalent to those used in spike frequency adaptation experiments (300 \( \mu \text{W/mm}^2 \)). Larvae expressing ChR2 in MNs (\( n = 10 \)) were initially immobilized with the onset of constant illumination and began crawling away within 10 s (Fig. 5A, blue line). However, both GAL4 (\( n = 10 \)) and UAS (\( n = 10 \)) controls showed similar decreases in mobility at the onset of illumination. When assessed over the entire crawling trial, crawling speeds of experimental and control animals were not significantly different.

In contrast, animals expressing H134R-ChR2 (\( n = 10 \)) had significantly slower crawling speeds throughout the entire illumination period compared with animals expressing ChR2 (cf. Fig. 5B, red line, to 5A, blue line; \( P < 0.05 \) for \( t = 20–130 \) s). H134R-ChR2 animals were also significantly slower than both UAS-H134R-ChR2 (\( n = 10 \)) and OK371-GAL4 controls (\( n = 10 \)) (\( P < 0.05 \) for \( t = 10–70 \) s). The decreased speed of OK371-GAL4/UAS-H134R-ChR2 was not simply a result of genetic background influence because UAS-H134R-ChR2 control animals actually crawled faster than UAS-ChR2 controls, (Fig. 5, A and B; \( P < 0.05 \) for \( t = 30–40, 60–80, 90–110, \) and \( 120–140 \) s). UAS-H134R-ChR2 controls also crawled faster than OK371-GAL4 controls (Fig. 5B; \( P < 0.05 \) for \( t = 20–40, 50–150 \)). Together these data indicate that, similar to what was observed for EJP spike adaptation, H134R-ChR2 activation of MNs causes more prolonged alterations in behavior than ChR2 activation.

Interestingly, even though pulsed light maintained highest overall firing rates in MNs, the same stimulation protocol failed to yield significant behavioral effects in animals expressing either type of ChR2 in MNs (cf. Fig. 5, C and D). Crawling speeds for ChR2-expressing animals exposed to pulsed light were not significantly different from either UAS or GAL4 controls (Fig. 5C; \( P > 0.05 \) for all time points).

Video examination of larvae indicated that pulsed illumina-
tion of both CHR2 and H134R-CHR2 animals induced pulsatile alterations in body posture (Supplemental Movie S1). However, at no point in time under pulsed blue light were crawling speeds of H134R-CHR2 animals significantly different from those of either UAS or GAL4 control flies. Thus pulsed excitation of motor neurons could alter behavioral patterns but had no significant effect on the parameter of crawling speed.

Sensory neuron activation with ChR2 and H134R-ChR2

Feedback from sensory neurons is critical to rhythmic locomotion in larvae (Hughes and Thomas 2007; Song et al. 2007). To address how activation of sensory neurons changes crawling behavior, we expressed ChR2 and H134R-ChR2 in body wall sensory neurons using 5-40-GAL4 (Hughes and Thomas 2007). ChR2-expressing animals initially stopped crawling and contracted along the A–P body axis in response to constant blue light (Fig. 6A, blue line; n = 10). However, by 30 s after onset of illumination, crawling speeds were not different from those of UAS controls (P < 0.05 for t = 10–30 s) or GAL4 controls (P < 0.05 for t = 10–40 s) (n = 10 for both). In contrast, animals expressing H134R-ChR2 were almost completely immobilized under constant illumination for the entire 2-min stimulation period (Fig. 6B, red line; P < 0.05 for t = 10–130 s; n = 10), whereas UAS and GAL4 control animals were relatively unaffected (n = 11 and 10, respectively). Under constant light, H134R-ChR2 animals were immobilized more effectively than their ChR2 counterparts (Fig. 6, A and B; P < 0.05 for t = 30–130 s). Under constant illumination, homozygous UAS-H134R-ChR2 control animals crawled faster than 5-40-GAL4 homozygous controls (P < 0.05 for t = 50–100 and 120–150 s), suggesting that the enhanced immobilization seen in H134-ChR2 animals was not simply a result of genetic background.
In contrast to the results when motor neurons were stimulated, rhythmic stimulation of ChR2-expressing sensory cells (Fig. 6C, light blue line, \( n = 9 \)) was more effective than constant illumination, significantly inhibiting crawling for the entire 2-min trial relative to both UAS (black line, \( n = 11 \)) and GAL4 controls (gray line, \( n = 10 \); Fig. 6C; \( P < 0.05 \) for \( t = 10–70 \text{ s} \)). Animals expressing H134R-ChR2 (Fig. 6D, orange line; \( n = 9 \); Supplemental Movie S2) also responded strongly to rhythmic stimulation and were completely paralyzed throughout the entire trial in comparison with both UAS (black line, \( n = 11 \)) and GAL4 (gray line, \( n = 10 \)) genetic controls (\( P < 0.05 \) for \( t = 10–130 \text{ s} \) for GAL4 controls; \( P < 0.05 \) for \( t = 10–110 \text{ s} \) for UAS controls). H134R-ChR2-expressing animals responded more strongly to blue light than ChR2 animals (cf. Fig. 6, D and C, purple asterisks; \( P < 0.05 \) for \( t = 60–140 \text{ s} \)). After illumination, crawling speed failed to recover completely and remained significantly slower than that of GAL4 genetic controls (\( P < 0.05 \) for \( t = 130–150 \text{ s} \)). These results indicate that activation of sensory neurons has profound effects on locomotion and that the firing rate/firing pattern requirements for disruption of crawling are different in motor and sensory neurons.

**Temperature activation threshold for larval neurons expressing dTRPA1**

For some applications, such as the excitation of structures deep in the fly brain or the manipulation of behaviors affected by light, light-activated channels are not ideal. Recent work has shown that dTRPA1 is a warmth-activated ion channel and suggests that neurons ectopically expressing dTRPA1 begin firing action potentials around 25°C (Hamada et al. 2008). To directly evaluate the efficacy and dynamics of dTRPA1 as a modulator of neuronal activity, we performed whole cell patch recordings from MNIS-Ns neurons expressing dTRPA1 channels. Figure 7A shows a representative whole cell patch recording from a dTRPA1-expressing MNIS-N cell as the bath temperature was raised from 22 to 27°C and subsequently lowered. The cell was initially silent, but depolarized as the temperature rose, initiated spiking at \( \sim 26°C \), fired action potentials tonically at 27°C, and went silent as bath temperature fell back below 26°C. Figure 7B shows firing rates as a function of temperature and genotype during the up and down phases of temperature ramps. Results from whole cell patch experiments (\( n = 6 \)) are shown in purple. Unlike previous work, the GAL4 driver combination (C380-GAL4, UAS-md8-GFP; Cha-GAL80) used in these experiments drives dTRPA1 expression specifically in MNs, suggesting that the observed spiking reflects dTRPA1 function within the MNs. To test the generality of these results, we also measured responses of a different identified neuron, MNSNh/d-Is. In this case, responses were measured via the EJPs evoked in the target muscle m6 rather than whole cell patch. Green bars in Fig. 7B show pooled data from preparations in which OK371-GAL4 is used to drive dTRPA1 expression. MN cell bodies...
were intact, but crawling rhythms had been silenced (as for ChR2 NMJ work shown above). Blue asterisks indicate where ChR2 animals crawled significantly slower than both GAL4 and UAS controls \((P < 0.05 \text{ for } t = 10–30 \text{ s})\). B: same experiment as in A, but with H134R-ChR2 expressed in sensory neurons (red). Red asterisks indicate times when H134R-ChR2–expressing animals crawled significantly more slowly than both controls \((P < 0.05 \text{ for } t = 30–130 \text{ s})\). Pink # symbols indicate times when H134R-ChR2 animals are slower than animals expressing ChR2 (cf. B to A; \(P < 0.05 \text{ for } t = 10–130 \text{ s}\)). C: response to rhythmic (500 ms on, 500 ms off) illumination in animals with ChR2 expressed in sensory neurons (light blue). Genetic controls are shown in black and gray. Blue asterisks represent times when ChR2-expressing animals crawl significantly slower than both GAL4 and UAS controls \((P < 0.05 \text{ for } t = 10–70 \text{ and } 90–130 \text{ s})\). D: same experiment as in C, but with H134R-ChR2 expressed in sensory neurons (orange). Orange asterisks represent times when H134R-ChR2 animals are slower than both genetic controls \((P < 0.05 \text{ for } t = 110 \text{ s})\). #When H134R-ChR2 animals are slower than animals expressing ChR2 (cf. D to C; \(P < 0.05 \text{ for } t = 60–140 \text{ s}\)). UAS control data end abruptly at 100 s because animals crawled out of the filming arena. Average crawling speeds were binned as in Fig. 5. In A and B and again in C and D, GAL4 control data are shown twice, to facilitate comparison. Blue bars represent duration of stimulus. Data are presented as means ± SE. *\(P < 0.05\) (ANOVA with Bonferroni post hoc test).

FIG. 6. Acute activation of sensory neurons with ChR2 and H134R-ChR2 affects locomotion. A: response to constant illumination in animals expressing ChR2 in sensory neurons (blue) and in genetic controls (black and gray). Blue asterisks indicate where ChR2 animals crawled significantly slower than both GAL4 and UAS controls \((P < 0.05 \text{ for } t = 10–30 \text{ s})\). B: same experiment as in A, but with H134R-ChR2 expressed in sensory neurons (red). Red asterisks indicate times when H134R-ChR2–expressing animals crawled significantly more slowly than both controls \((P < 0.05 \text{ for } t = 30–130 \text{ s})\). C: response to rhythmic (500 ms on, 500 ms off) illumination in animals with ChR2 expressed in sensory neurons (light blue). Genetic controls are shown in black and gray. Blue asterisks represent times when ChR2-expressing animals crawl significantly slower than both GAL4 and UAS controls \((P < 0.05 \text{ for } t = 10–70 \text{ and } 90–130 \text{ s})\). D: same experiment as in C, but with H134R-ChR2 expressed in sensory neurons (orange). Orange asterisks represent times when H134R-ChR2 animals are slower than both genetic controls \((P < 0.05 \text{ for } t = 110 \text{ s})\). #When H134R-ChR2 animals are slower than animals expressing ChR2 (cf. D to C; \(P < 0.05 \text{ for } t = 60–140 \text{ s}\)). UAS control data end abruptly at 100 s because animals crawled out of the filming arena. Average crawling speeds were binned as in Fig. 5. In A and B and again in C and D, GAL4 control data are shown twice, to facilitate comparison. Blue bars represent duration of stimulus. Data are presented as means ± SE. *\(P < 0.05\) (ANOVA with Bonferroni post hoc test).

As a final test of the activation properties of dTRPA1, we completely removed the ventral ganglion (containing the MN cell bodies) and measured MNSNb/d-Is axonal responses to heat ramps in OK371-GAL4; UAS-dTRPA1 animals. Even without cell bodies, MNSNb/d-Is axons fired tonic action potentials in response to heating. The threshold temperature for evoking EJPs in axons/terminals alone \((26 ± 0.2°C, n = 9)\) was only \(~1°C\) higher than in experiments with MN cell bodies were intact (patch, \(25 ± 0.3°C, n = 6\); NMJ, \(25 ± 0.3°C, n = 9\); 1-way ANOVA with Bonferroni post hoc test, \(P < 0.05\) for cell body vs. no cell body preparations). Overall, these results show that modest warming can directly evoke action potentials in dTRPA1-expressing MNs.

Does dTRPA1 expression affect motor neuron intrinsic properties in the absence of warming?

Previous work has shown that ectopic expression of voltage-gated ion channels can have unpredicted effects on the intrinsic properties of fly neurons (Sheeba et al. 2008). Because dTRPA1 channels are voltage dependent and temperature activated (Hamada et al. 2008), we wanted to see whether dTRPA1 expression might affect intrinsic membrane properties even in the absence of heating. To test this, we measured resting membrane potential, input resistance, and response to current injection in cells expressing dTRPA1 and in genetic control animals. Figure 8A shows MNISN-Is responses to...
60-pA DC current injection in genetic controls (left, center) and in a cell expressing dTRPA1. Resting membrane potential was not significantly different among the genotypes (Fig. 8B). Resting input resistance was significantly higher in cells expressing dTRPA1 (Fig. 8C); however, there were no significant differences in MN firing rate in response to depolarizing current injection (Fig. 8D). Taken together, these results show that, although dTRPA1 expression does have some subtle effects in the absence of warming, it does not cause any changes in MN excitability, suggesting that at low temperatures, the response to synaptic inputs should be the same as in wild-type animals.

**Spike frequency adaptation in cells expressing dTRPA1**

In our initial experiments activating dTRPA1 with heat ramps, we noticed that, unlike ChR2, MNs expressing dTRPA1 showed little spike frequency adaptation (Fig. 7A). To test this further, we measured the decay of EJP frequency in constantly activated dTRPA1-expressing cells. Figure 9A shows large EJPs in m6 in response to a bath temperature increase to 27°C when dTRPA1 is expressed in MNs. For comparison, EJP frequency decay in a ChR2 activated neuron at 27°C is shown in Fig. 9A (bottom). Instantaneous EJP frequencies are shown above each trace. Pooled data for these experiments are shown in Fig. 9B. EJP frequency in dTRPA1-expressing animals does not decay significantly over 20 min of constant activation. At 27°C, EJP firing rates in ChR2-expressing animals decay drastically over the same time frame. As at 22°C, rhythmic light pulses were initially able to alleviate some but not all of the decay, especially after the first 1–2 min of stimulation (data not shown). In aggregate, the ChR2 and dTRPA1 data indicate that the spike frequency adaptation we see in fly neurons is dictated by properties of the spike-generating channel and is not a feature of the neuron itself.

**Effects of dTRPA1-mediated activation of motor and sensory neurons on locomotor behavior**

Neurons expressing dTRPA1 show little spike frequency adaptation in response to constant warming. Does this translate into stronger, longer lasting, behavioral phenotypes than those seen in ChR2 and H134R-ChR2 experiments? To address this question, we activated dTRPA1 in MNs and sensory neurons and measured the resulting effects on crawling behavior. Figure 10 shows a representative temperature curve for a typical set of crawling trials (A) and the corresponding effect on crawling speed in animals expressing dTRPA1 in MNs (B). Surface temperature ramps from 22 to 27°C caused complete paralysis for the entire 2-min trial in these animals (Supplemental Movie S3). As surface temperatures gradually fell back to 22°C, larvae resumed normal crawling. Crawling speeds in genetic controls were not affected by equivalent temperature ramps (Fig. 10B, black lines; P < 0.05 for t = 0–120 s).

Sensory neuron activation did not fully immobilize larvae (Fig. 10C), although it clearly disrupted locomotor rhythms for the entire 2-min trial (Supplementary Movie S2). As surface temperatures rose and held, average crawling speed decreased and stayed low throughout the entire trial. As surface temperatures fell, normal crawling speeds did not resume until ~50 s after surface temperatures had returned to 22°C. Equivalent surface temperature protocols in genetic controls had no effect on crawling speed (Fig. 10C, black lines; GAL4 controls: P < 0.05 for t = 30–190 s; for UAS controls: P < 0.05 for t = 10–230 and 240–260 s). Thus dTRPA1 is able to elicit strong and sustained behavioral effects when used to activate either motor or sensory neurons.

**Discussion**

In this study, we investigated the cellular consequences of using the genetic tools ChR2, H134R-ChR2, and dTRPA1 to manipulate neuronal activity. We showed that, for ChR2, mutation of histidine 134 to arginine enhances light activation and decreases spike frequency adaptation. We also found that dTRPA1 shows less adaptation than either form of ChR2, providing stable spiking over long (20 min) time scales. These cellular properties seem to be important determinants of the...
time course of behavioral effects seen with channel activation. In larval sensory neurons, limiting spike frequency adaptation either by use of pulsed light stimulation of ChR2 or by using the nonadapting channel dTRPA1 resulted in stronger locomotor effects. In motor neurons, adaptation was also detrimental to locomotor manipulations because dTRPA1 was more effective than ChR2 over long time scales. Interestingly, however, in this type of neuron, pulsed light could not be used to enhance the ChR2 phenotype because the recovery of neurons during the off-periods in the pulsed light protocol appeared fast enough to reverse the firing-induced paralysis. In aggregate, our results suggest that a good understanding of the acute and chronic cellular effects of these genetic tools is critical to both designing and interpreting behavioral experiments.

**H134R-ChR2: a multiply enhanced ChR2**

The H134R point mutation has been shown to increase both transient and steady-state photocurrents in oocyte experiments compared with ChR2 (Nagel et al. 2005). However, to our knowledge, no previous work has directly compared neuronal firing properties in neurons expressing H134R-ChR2 and ChR2. Here, we confirmed that previous non-neuronal work translates well into fly neurons; larval MNs expressing H134R-ChR2 have a larger response to blue light than MNs expressing ChR2. We also detect neuronal activation at lower levels of light when using H134R-ChR2 than ChR2. This increased sensitivity could reflect increases in expression, conductance, and/or light sensitivity with the mutant channel.

In addition to the anticipated effects on light responsiveness, the H134R mutation also had an unexpected effect on spike frequency. With constant illumination over a relatively long time scale, firing rates in neurons expressing H134R-ChR2 remained significantly higher than in ChR2-expressing cells. What could underlie the long, slow firing rate decay in ChR2-activated cells? Both fast transient and steady-state ChR2 photocurrents have been reported in nonneuronal cell types over the course of several seconds (Nagel et al. 2003, 2005). However, the stability of the steady-state current over longer time scales (i.e., 5–20 s) remains unclear. A slowly decaying current response to constant light is visible in published oocyte recordings (Nagel et al. 2003). Such a long, slow decay in the steady state photocurrent could account for the slow, continuous drop in firing rate we see in ChR2 expressing MNs.

Although definitive experiments remain to be done, there are several ways in which mutating histidine 134 could alter channel properties. In bacteriorhodopsin, the aspartate residue found at the position analogous to histidine 134 typically exchanges protons with the retinal Schiff base and participates in generating the various conformational intermediates present in a single photocycle (Lanyi 1998). Replacing channelrhodopsin’s native histidine residue with a more basic arginine may bias the channels’ intermediate conformations in favor of the open state, either by altering the pKₐ of the protonated Schiff base or by disrupting its interaction with its counter ion. This could shift the activation curve, reducing inactivation and resulting in higher steady-state currents.

**Interpretation of negative results in behavioral work using ChR2**

The strong adaptation we saw with ChR2 is an important caveat for interpretation of negative behavioral results. ChR2 has been used in a growing number of studies to remotely monitor cell activity in the peripheral nervous system.
activate neural circuits in *Drosophila* larvae and in adult animals. (cf. Hwang et al. 2007; Zhang et al. 2007). Hwang et al. used ChR2 to activate specific body wall sensory neurons. They saw strong behavioral responses when they activated some subsets, but little or no effect when activating other subsets. These results could very well reflect subtle modulatory roles that certain sensory neurons play in coordinating crawling; however, these findings could also be the result of ineffectual activation of the neurons in question. Schroll et al. (2006) used ChR2 to study the roles of aminergic modulatory neurons in larval locomotor behavior and appetitive learning. When they activated either dopaminergic cells or octopaminergic cells, they saw no obvious effects on larval locomotion or chemotaxis. This is somewhat surprising given that mutants deficient in biogenic amine transmission have significant locomotor defects (Fox et al. 2006; Saraswati et al. 2004) and activation of dopaminergic neurons is known to alter locomotion in adult animals (Zhang et al. 2007; A. Ghezzi and L. C. Griffith, unpublished data). Clearly, Schroll et al. (2006) were activating the neurons in question because they could induce aversive and appetitive learning with blue light pulses. The lack of locomotor effects could be caused by differential thresholds or timing requirements for locomotor and learning effects. Revisiting such experiments using dynamic pulse protocols and/or H134-ChR2 or dTRPA1 could help investigate these issues. Ultimately, it is important to have some independent measure of the activity of a neuron over the time course of a given experiment to confirm a negative result.

**Comparison of the effects of dTRPA1 and ChR2 activation**

There is a growing body of evidence that dTRPA1 channels are gated by temperature and function as molecular warmth detectors in flies (Hamada et al. 2008; Rosenzweig et al. 2005, 2008). Here, we added to this by showing that larval neurons ectopically expressing dTRPA1 are directly activated by modest increases in temperature. Furthermore, we repeated these results in a variety of recording conditions and in conditions where the effects of circuit activation were eliminated.

We found that activation of neurons with dTRPA1 and ChR2 are similar in many respects. In the intensity ranges tested here, both manipulations induce physiological depolarizations and normal spiking. However, dTRPA1 and ChR2 differed in some respects. dTRPA1 activation of motor neurons resulted in significantly less adaptation than either ChR2 or H134-ChR2, and dTRPA1 activation of both motor and sensory neurons yielded more robust and sustained behavioral effects. While the underlying reasons for these differences are not yet certain, the biophysical properties of the two channels may be an important contributing factor. First, the single
channel conductances of TRPA family channels are much greater than of ChR2 channels. Although there is no single channel data for dTRPA1, the single channel maximal conductance ($g_{\text{max}}$) of mammalian TRPA1 is $\sim 100$ pS (Nagel et al. 2005). Similar results have been found for the divergent Drosophila TRPA channel Painless, where $g_{\text{max}}$ is $\sim 50$ pS (Sokabe et al. 2008). Such single-channel conductances are $\geq 1,000$-fold higher than the $50–60$ fS estimated for single ChR2 channels (Nagel et al. 2003). Because the amount of current that a single ChR2 channel can pass is so small, relatively high expression levels are likely required to strongly depolarize neurons. This could be a limiting factor in many systems, because high expression levels of foreign proteins may have detrimental effects on cell health. The much higher single channel conductance of TRPA1 family channels is a distinct advantage in this context. Because each channel is able to pass more current, relatively low expression levels should suffice to engineer temperature-responsive neurons.

Another important difference lies in the voltage sensitivity of the two channels. dTRPA1 channels are minimally voltage gated at low temperatures (20–22°C) but become increasingly voltage dependent as temperature rises (Hamada et al. 2008). In contrast, ChR2 variants show no voltage sensitivity (Nagel et al. 2003). As a result, the potential for ChR2 modifying intrinsic neuronal properties in the absence of light is low. Our results suggest that dTRPA1 voltage sensitivity does not radically alter the intrinsic properties of motor neurons at 20–22°C. However, it is important to note that we did see a significant difference in one cellular parameter of dTRPA1-expressing cells: resting input resistance. The ability of dTRPA1 to be gated by voltage or second messenger activation (Bandell et al. 2004; Hamada et al. 2008) means that endogenous activity could activate this channel at some level, engaging activity-dependent regulatory pathways. Interestingly, the change in input resistance did not alter how cells responded to depolarizing current injection (and consequently, is probably not affecting behavior at temperatures below the dTRPA1 threshold). One possible explanation for the failure of increased input resistance to alter the response to depolarization is that dTRPA1 expression up-regulates the expression of voltage gated K+ channels in motor neurons. Homeostatic regulation of one or more channels could conceivably counteract the effect of higher input resistance in motor neuron cell bodies. Overall, these results show that dTRPA1 expression can have subtle effects on neuronal properties (and possibly engage cellular homeostatic mechanisms) even in the absence of heat pulses.

One technical limitation of light as an activating signal is its ability to penetrate animal tissue. In particular, blue light does not penetrate tissue well (Eichler et al. 1977). As a result, deep brain regions may not be accessible to ChR2 stimulation in Drosophila. Because fruit flies are effectively isothermal (Heinrich 1993; Stevenson 1985), temperature-regulated channels should allow researchers to reliably activate deep brain regions in Drosophila, even when animals are maximally active (e.g., running, flying, crawling).

Although increased tissue penetration is a clear advantage of using dTRPA1 over ChR2 for activating fly neurons, light-activated channels have the advantage of being easily controlled temporally (Boyden et al. 2005; Nagel et al. 2003, 2005) and to some extent, spatially (Wang et al. 2007). Heat is more difficult to control both temporally and spatially. Fine control of neuronal excitability may not be possible with dTRPA1; nonetheless, the penetrative abilities and lack of densensitization still make dTRPA1 useful for longer time scale work in Drosophila and possibly other poikilothermic animals. Recent work suggests that dTRPA1 can continuously activate neurons and have an impact on behavior over hours and even days (Parisky et al. 2008; Shang et al. 2008). This type of conditional, but relatively long-lasting, activation could be particularly useful for activating neural circuits that control...
behaviors that take place over longer time scales, such as courtship and mating, eclosion, circadian rhythms, and sleep.

Why are dTRPA1 expressing cells resistant to spike frequency adaptation?

One striking and unexpected feature of dTRPA1 expressing neurons was the lack of appreciable spike frequency adaptation during heat treatments. Clearly, this is not just a feature of larval MN intrinsic properties; with constant activation with ChR2, MNs showed a ~75% decrease in firing rate over the same time frame. This could not have been caused by differential initial firing rates because, in both ChR2 and dTRPA1 experiments, starting spike frequency was almost identical. The most straightforward interpretation is that the differences in spike frequency adaptation that we see are a function of the particular channel being used to activate the neuron. The most probable explanation for the lack of spike adaptation with dTRPA1 is simply that dTRPA1-evoked currents are more stable than those evoked by ChR2 over long times scales. In oocytes, dTRPA1 channels show no discernible inactivation at 32°C (K. Kang and P. A. Garrity, unpublished data). To our knowledge, the long-term stability of light-evoked currents has yet to be fully studied, but it is clear that even over short time scales there is a decay in steady state current levels with constant stimulation that could be caused by inactivation (Nagel et al. 2003).

Activation of circuits controlling larval crawling

In our behavior experiments, we found that H134R-ChR2 activation of MNs had larger and longer lasting effects on crawling than ChR2 activation. This indicates that the enhancement mediated by H134R-ChR2 seen in physiology experiments is big enough to have a significant impact on behavior. This is supported by our finding that activation of the same neurons with dTRPA1, which has little spike adaptation, was also more effective.

Interestingly, strong activation of glutamatergic MNs lead to slower crawling speeds. This could be caused in part by coincident activation of inhibitory glutamatergic interneurons (they are also targeted by the GAL4 driver used). Activating inhibitory interneurons could disrupt central locomotor rhythms and lead to slower crawling. Because we did not see any obvious light- or heat-evoked inhibitory activity during our patch experiments, such interneuron effects are likely to be subtle. A more likely explanation is that the slowed crawling is the result of coincident motor neuron activation (and muscle contraction) in multiple body segments. This probably disrupts normal progression of contraction waves from segment to segment during peristaltic movements. This idea is supported by the fact that dTRPA1 activation of MNs results in visible anterior to posterior shortening of the whole animal.

Interestingly, rhythmic activation of MNs with light was less effective at inhibiting crawling than constant activation. This was surprising, given that rhythmic light pulses were more effective at maintaining high overall firing rates. One possible explanation involves quick recovery after acute activation of motor neurons and not sensory neurons. Acute activation of sensory neurons in larvae keeps them immobilized during the OFF phase of intermittent illumination. In contrast, larvae expressing ChR2 in motor neurons were able to crawl away during this time. These results suggest that, under certain circumstances, maximum overall activation of a population of neurons does not always translate into a maximum impact on behavior, although our lack of electrophysiological data from sensory neurons precludes making any strong conclusions about that subset of cells. Our results do, however, clearly show that the dynamics of activation can have differential impacts on behavior depending on the subset of cells activated.

In previous reports, inactivation of synaptic output from sensory neurons using shibire<sup>23</sup> inhibited locomotion by disrupting patterned motor neuron activity in the CNS (Hughes and Thomas 2007; Song et al. 2007). Interestingly, we found that strongly activating peripheral sensory neurons with either dTRPA1 or H134R-ChR2 similarly inhibits crawling. The elimination of locomotor activity by both over stimulation and inactivation of sensory feedback suggests that sensory modulation must remain in a tightly controlled activity range to maintain appropriate motor output from the larval locomotor central pattern generator.

Broader implications for animal behaviorists

In this study, we attempted to show the advantages and challenges posed by two new tools for remotely activating neural circuits in Drosophila. This work highlights the importance of carefully characterizing activity manipulations in the context of animal behavior experiments: understanding the cellular effects and temporal properties of a manipulation is critical to understanding how it affects behavior. Comparable genetic tools are becoming available in a growing number of model organisms, and though the cells and circuits will be different, the challenges are certain to be similar. Most neurons are not simply on or off; they typically exist in multiple activity states and the differences between these states can be subtle, but functionally important. Both the nature of imposed activity changes and the timing of those changes in relation to the normal activity pattern of a given neuron will have an influence on the behavioral changes elicited by the manipulation.

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