Optogenetics in the teaching laboratory: using Channelrhodopsin2 to study the neural basis of behavior and synaptic physiology in *Drosophila*

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ABSTRACT

Here we incorporate recent advances in *Drosophila* neurogenetics and ‘optogenetics’ into neuroscience laboratory exercises. We used the light activated ion channel, Channelrhodopsin2 (ChR2) and tissue specific genetic expression techniques to study the neural basis of behavior in *Drosophila* larvae. We designed exercises using inexpensive, easy-to-use systems for delivering blue light pulses with fine temporal control. Students first examine the behavioral effects of activating glutamatergic neurons in *Drosophila* larvae, and then record excitatory junctional potentials (EJPs) mediated by ChR2 activation at the larval neuromuscular junction (NMJ). Comparison of electrically- and light-evoked EJPs demonstrate that the amplitudes and time courses of light-evoked EJPs are not significantly different from those generated by electrical nerve stimulation. These exercises introduce students to new genetic technology for remotely manipulating neural activity, and they simplify the process of recording EJPs at the *Drosophila* larval NMJ. Relatively little research work has been done using ChR2 in *Drosophila*, so students have opportunities to test novel hypotheses and make tangible contributions to the scientific record. Qualitative and quantitative assessment of student experiences suggest that these exercises help convey principles of synaptic transmission while also promoting integrative and inquiry based studies of genetics, cellular physiology and animal behavior.
INTRODUCTION

*Drosophila* neurogeneticists have developed an impressive array of tools for studying the neural basis of animal behavior. In recent years, tissue specific genetic expression systems, particularly GAL4-UAS (3) have been used to ectopically express transgenes that allow for acute, reversible manipulation of neural activity. These new techniques exploit ion channels and vesicle trafficking proteins that are gated by light and temperature (1, 9, 15, 25, 28). This allows researchers to remotely control neural activity in selected cells simply by raising the ambient temperature or shining light on behaving flies.

One powerful new tool for acutely activating neurons is the light gated ion channel, Channelrhodopsin-2 (ChR2). Originally isolated from the green algae, *Chlamydomonas reinhardtii*, the channel is directly activated by blue light (24). When expressed in neurons, channel opening causes depolarization through a non-specific cation conductance (2, 23) which leads to action potential generation. This technique has been used to depolarize excitable cells in invertebrate (22, 24, 27) and vertebrate (2, 5, 8, 24) preparations for research purposes.

‘Optogenetic’ methods for activating neurons offer attractive options for physiology educators. With the range of genetic tools available in *Drosophila*, teachers can potentially design exercises that explore the neural basis of animal behavior in ways that are not possible in traditional laboratory preparations. These new tools can also be used to make technically difficult preparations more accessible to students. Our goal here is to outline one potential use of *Drosophila* neurogenetics and ChR2 in neuroscience education. Specifically, we show how to use ChR2 to 1) promote quantitative analysis of animal behavior, 2) teach principles of synaptic transmission, and 3) help students learn how to formulate and test their own research hypotheses.

Previous work has proposed using the *Drosophila* larval neuromuscular junction (NMJ) to teach students synaptic physiology (16, 33). This glutamatergic synapse yields large excitatory junctional potentials (EJPs) that can be recorded with basic electrophysiology equipment (13,14). However, to successfully record
EJPs, students must precisely maneuver both an intracellular electrode and a stimulating (suction) electrode in a very small area. Here, we present inexpensive laboratory exercises that use targeted expression of ChR2 in motor neurons, instead of direct electrical nerve stimulation to activate larval NMJs. Students are exposed to newly developed Drosophila neurogenetic tools and learn synaptic neurophysiology. We report feedback on the exercises from two student cohorts across two different years in a neurophysiology laboratory course at Cornell University. Overall, this work and a companion publication (1) lay the foundation for wider use of Drosophila neurogenetics in teaching principles of neurobiology and animal behavior.

**MATERIALS AND METHODS**

**Fly lines and animal care**

We used a GAL4 driver (OK371-GAL4) that drives expression exclusively in glutamatergic neurons (22) and a UAS construct (UAS-H134R-ChR2-mcherry) from a previous larval locomotion study (25). Virgin OK371-GAL4 females were crossed to UAS-H134R-ChR2-mcherry males. The resulting larvae were grown in darkness at 23-25°C on standard fly media containing 1 mM all-trans retinal (ATR, Toronto Research Chemicals, North York, CA). ATR is a co-factor allowing proper folding and membrane insertion of ChR2. Supplementing fly food with ATR is essential for functional ChR2 expression. We previously described the preparation of ATR-containing fly food in video form (10).

All fly lines are freely available from S.R. Pulver at the University of Cambridge and/or the Bloomington Drosophila Stock Center (OK371-GAL4: http://flybase.org/reports/FBst0026160.html; UAS-H134R-ChR2-mcherry: http://flybase.org/reports/FBst0028995.html). Detailed guidelines on rearing fruit flies and making genetic crosses are available in previous publications (10, 16).

**Blue LED control system**

Commercially available systems for controlling blue light emitting diodes (LEDs) typically cost >US $300. This could be prohibitively expensive for many
teaching laboratories, so we designed two simple, inexpensive alternatives. First we connected an ultrabright blue LED (Luxeon V star, LED Supply, Randolph, VT) to a 700 mA ‘BuckPuck’ power converter (LuxDrive 3021, LED Supply). When the BuckPuck is directly connected to the analog output from an analog-to-digital converter, light intensity and duration can be controlled with 0-5 V pulses from an external voltage source (10, 25). We attached a small heat sink to each LED (e.g. TO220, Radio Shack) to dissipate heat. To ensure good heat transfer, we placed thermal paste in between the LED and heat sink and glued only the edges of the LED to the bare metal of the heat sink. Total cost of all components is under US$50. A basic wiring plan for this LED controller is shown in Figure 1A. A typical controller is shown in Figure 1C, and an LED mounted on a heat sink is shown in Figure 1D. We controlled timing and light intensity with two commonly available analog-to-digital conversion systems. For demonstration here, we delivered 0-5 V pulses through a Powerlab 4/30 (AD Instruments, Colorado Springs) with Chart 5 data acquisition software (AD Instruments). In the teaching exercises reported below, students controlled the LED through the analog output of a NIDAQ BNC-2110 A-D board (National Instruments, Austin, TX) with the free data acquisition software ‘g-PRIME’ (21). Both systems were able to control timing and intensity equally well.

As an alternative to the above, we also designed a second simple control circuit that can be driven by analog pulse stimulators with low current output. Figure 1B shows a wiring plan for this type of control system. A 74HC04 hex inverter and a 5 KΩ resistor are used to ensure that a standard TTL signal will trigger light pulses. An input protection circuit consisting of two 1N914 diodes protects the hex inverter from a reversed connection and/or electrostatic discharge. The primary advantage of this control circuit is that it does not require analog-to-digital converters and/or data acquisition software. Total cost of all components is under US$70.

Unfocused LEDs are not able to deliver the light intensities needed to activate ChR2 in fly neurons. To focus LEDs, we placed a Carl Zeiss 10X dissecting scope eyepiece in front of the LED, and mounted both the light source
and eyepiece on magnetized bases suitable for electrophysiology ‘rig’ tables. The
make and model of eyepiece is not critical; any removable eyepiece that can
cover the LED is suitable. An LED and heat sink coupled to an eyepiece and
attached to a magnetized base is shown in Figure 1E. The complete LED setup
on a working electrophysiology rig is shown in Figure 1F. Additional views of LED
system components are shown in video form in (10). It is important to note that
the light emerging from the LED system outlined here is high intensity and very
focused, so it is imperative that students do not look directly into active LEDs.

Larval behavior

Animals with ChR2 in motor neurons (OK371-GAL4 / UAS-H134R-ChR2)
were grown in two batches: one group was raised on normal fly food, the other
on food containing 1mM ATR. We selected 3rd instar individuals from each group
and observed behavioral responses to blue light pulses. For demonstration,
larval behavior was filmed with a Leica DFC 420 C camera mounted on a Leica
MZ16 F Fluorescence Stereomicroscope (Leica Camera AG, Solms, Germany).
Blue light pulses (5 s) were delivered by manual control of shutter timing.
In classroom exercises, students placed larvae in dissection dishes and
delivered light pulses using a mounted LED. Students observed larval responses
to blue light and scored responses manually. We did not require students to
analyze larval behavior in any particular way. Instead, we encouraged students
to devise their own methods for quantifying the effects of blue light stimulation on
larval behavior in experimental and control animals

Larval dissection

For NMJ electrophysiology, third instar larvae were dissected in a clear
Sylgard (Dow Corning, Midland, MI) lined dish containing chilled ‘HL3.1’
physiological saline (6). HL3.1 consisted of (in mM): NaCl 70, KCl 5, CaCl2 0.8,
MgCl2 4, NaHC03 10, trehalose 5, sucrose 115, HEPES 5, pH 7.15. In this saline,
preparations typically remained viable for 1-2 hours at room temperature.
Each larva was positioned dorsal side up and 0.1 mm insect pins were placed in the head and tail. Using a pair of microscissors, we made a shallow incision from the posterior pin to the anterior pin. After making the initial cut, we placed one pin into each corner of the animal's body wall and stretched each corner taut. Next, we removed fat bodies and digestive organs, exposing the anterior brain lobes, ventral ganglion, segmental nerves and body wall muscles. In some experiments we removed the central nervous system (CNS), leaving only motor axons and nerve terminals. In other preparations, we dissected away the brain lobes and cut the posterior-most nerves leaving the ventral ganglion. In tightly pinned preparations, this reduces locomotor rhythms, but leaves motor neuron cell bodies, axons and nerve terminals intact (25). See (10) for videos describing the larval dissection.

**Intracellular recording**

Dishes with dissected preparations were first fixed to a plexiglass stage with artist's clay and viewed through a dissecting microscope on a standard electrophysiology 'rig'. We targeted larval muscle 6 (m6; see Fig. 3A) for all intracellular recordings. Recordings were made with sharp glass electrodes (10-20 MΩ, filled with 3M KCl). For the demonstration electrophysiology data presented here, the electrode and headstage were maneuvered with a MP285 micromanipulator (Sutter Instruments, Novato, CA). Voltage signals were amplified with a Neuroprobe amplifier (A-M systems, Sequim, WA). Data were digitized using a Power lab 4/30 and recorded in Chart 5 (ADinstruments, Colorado Springs, CO). Data were analyzed in Spike2 (Cambridge Electronic Design, Cambridge, UK) using custom made analysis scripts (www.whitney.ufl.edu/BucherLab). EJPs were evoked in ChR2 expressing animals with 1, 2.5, 5, and 10 ms pulses (25) to examine the effects of light pulse duration on light-evoked EJPs (lEJPs). We also compared lEJPs to electrically evoked EJPs (eEJPs) by attaching a suction electrode to segmental nerves and delivering 1 ms duration electrical shocks with a model 2100 isolated pulse stimulator (A-M Systems) (see Figure 3A).
In teaching exercises, students used Narishige MM-333 micromanipulators (Narishige, East Meadow, NY) to maneuver recording electrodes. These micromanipulators offer enough precision to record from larval NMJs, and are substantially less expensive than other research-grade manipulators. Students also used Neuroprobe amplifiers to amplify voltage signals, but used g-PRIME for LED control, data acquisition and analysis (21). The quality of data recorded with teaching lab equipment was equivalent to the demonstration data we present here. In teaching laboratory exercises, students began by giving light pulse durations (10 ms) and intensities (5V into control circuit, ~1 mW / mm$^2$) that reliably evoked at least 1 IEJP with pulse stimulation in previous work (25). Students were encouraged to design their own experiments and explore the effects of varying intensity, duration and frequency of light pulses on synaptic transmission.

Analysis of student evaluations

We test ran these exercises with two different student cohorts in two successive years (Spring semesters, 2009 and 2010) of an undergraduate neurophysiology course (BIONB/BME 4910) at Cornell University. The 2009 students completed the exercise in 1 laboratory session; they were undergraduate students from Biology (11), Biological Engineering (2), and one each from Psychology, Mathematic and Human Ecology majors, and first year graduate students from Neurobiology and Behavior (6), and Biomedical (2) and Electrical/Computer (2) Engineering. In 2010 we spread the exercise over two weeks; undergraduate students were from Biology (11), and one each from Biology and Society, Psychology, Biological and Electrical/Computer Engineering majors, and first year graduate students from Neurobiology and Behavior (4) Biomedical Engineering (6) and one each from Electrical/Computer Engineering, Entomology and Psychology. Students worked in groups of 2 or 3 at each physiology rig. Their background in neuroscience ranged from very little (the Engineering students) to a sophomore level class in Neuroscience (biology students), which used the Purves et al. (26) textbook. Student experiences were
evaluated qualitatively in 2009; we asked for a 1 page informal opinion on the exercise from each student. In the second year, we quantified student experiences by asking them 12 questions designed to evaluate various technical and conceptual aspects of the exercise. Student responses were measured on a Likert scale (19). All students had previous electrophysiological experience earlier in the semester with exercises from the Crawdad CD (32), including recording synaptic potentials from the crayfish NMJ. NJH and SRP presented background lectures on fly genetics and *Drosophila* NMJ electrophysiology prior to students starting the lab exercises.

**RESULTS**

**Behavioral responses to blue light**

Previous work has demonstrated that larval locomotion is inhibited when motor neurons are depolarized with ChR2 activation to fire action potentials (25). To assess whether these effects are robust enough for use in teaching laboratories, we expressed ChR2 in motor neurons (Fig. 2A), then filmed behavioral responses to blue light. OK371-GAL4 x UAS-H134R-ChR2 animals raised on normal fly food were not affected by blue light pulses (Fig. 2B, left and right; Fig. 2D; n = 10). In contrast, genetically identical animals reared on food containing ATR showed immediate, obvious responses to blue light. In ambient light or green light, these larvae usually crawled normally, showing well-coordinated posterior to anterior peristaltic waves of muscle contractions (Fig. 2C, left; Supplemental movie 1). In blue light, all body segments contracted at once and peristaltic waves stopped (Fig. 2C, right; Supplemental movie 1). 100% of all animals raised on ATR food showed immediate, strong contraction of all body segments (Fig. 2D, left). Over 90% of these animals were completely paralyzed for the duration of a 5 s light pulse (Fig. 2D, right; n = 12). Paralyzed animals recovered within 5 s following a 5 s light pulse (Supplemental movie 1). In demonstration experiments (shown here), we delivered blue light pulses through a dissecting microscope equipped for fluorescence microscopy. In
classroom exercises, we obtained similar results using the LED control system described above.

Each student group was encouraged to devise their own methods for measuring ChR2-mediated behavioral effects. One example of a student conceived analysis is shown in Table 1. This student group compared crawling behavior in control and ChR2 expressing animals under ambient and blue light. They measured the frequency of forward peristaltic waves by counting number of waves in a 30 s trial. They also estimated total distance traveled by placing a grid of 1cm x 1cm squares beneath each larva, and measuring the number of squares traveled during the same 30 s trial. Under ambient light, both genotypes showed similar crawling parameters. In the presence of rhythmic blue light pulses (1 sec duration, 0.5 Hz cycle period), controls continued to crawl, whereas animals expressing ChR2 showed no forward peristalsis. Consistent with previous work, behavioral effects were strong at first, but gradually wore off after 20-30 s under constant illumination (Data not shown; 25). Several student groups noted that high intensity white light could also elicit behavioral responses in ChR2 expressing animals. Students were therefore encouraged to minimize the intensity of dissection scope lamps during experiments.

Light-evoked EJPs at the larval NMJ

Previous work has shown that the LED system presented here can reliably generate single IEJPs at the larval NMJ (10, 25). We asked students to first apply light pulses of varying durations to the larval preparation (Fig. 3A) and record IEJPS to ensure that they had a working preparation (demonstration examples in Fig. 3C). Next we encouraged them to formulate and investigate their own research questions. Several groups chose to examine how these IEJPs compared to eEJPs at the larval NMJ. They easily recorded IEJPs, but had difficulty successfully stimulating motor nerves to record eEJPs. For demonstration purposes, we repeated this experiment. In the preparation shown in Figure 3A, the central nervous system (CNS) was removed and a suction electrode was placed on a single segmental nerve. Nerve shocks (1 ms) reliably
evoked single eEJPs. Consistent with previous work, as stimulus intensity increased, a second motor unit innervating muscle m6 was recruited, leading to a stepwise increase in eEJP amplitude (Fig. 3B). One ms blue light pulses failed to evoke IEJPs in 7 out of 7 preparations, but 2.5, 5, and 10 ms light pulses evoked IEJPs in most preparations (2.5 ms: 5 / 7; 5 ms: 7 / 7; 10 ms: 7 / 7 experiments). IEJP and low threshold eEJP amplitudes and time courses were not significantly different (Fig. 3B-F; p > 0.05, One way ANOVA with Tukey-Kramer post-hoc test).

In previous work, IEJPs have been measured in preparations in which motor neuron cell bodies were present and ventral ganglion circuitry was intact (25). Several student groups chose to study IEJPs in this type of preparation (a schematic is shown in Fig. 4A). In demonstration experiments, 1 ms electrical pulses recruited both motor units with amplitudes and time courses similar to those seen in reduced nerve-muscle preparations (data not shown). With the ventral ganglion intact, we reliably evoked single low threshold IEJPs with light pulse durations as short as 1 ms (Fig. 4B). Longer light pulses evoked summating trains of EJPs (Figure 4B,C). Increasing light pulse duration did not affect the amplitudes of leading eEJPs (Fig. 4D).

In several preparations with intact ventral ganglia, (3 / 7), short light pulses evoked a single IEJP, followed by a long (1 - 5 s) train of spontaneously generated EJPs (Fig. 4E). In these experiments, IEJPs were similar in amplitude and time course to spontaneous EJPs (Figure 4F). Trains of spontaneously generated EJPs were not seen in preparations in which the ventral ganglion had been removed. In classroom experiments, several groups noted that in dissection lamps with intact ventral ganglia, high intensity white light pulses from dissection lamps could trigger trains of IEJPs.

An example of data collected during a student initiated classroom project is shown in Figure 5. This particular group recorded IEJPs in response to paired pulses of blue light (Fig. 5A). They then calculated facilitation ratios (EJP$_2$ amplitude / EJP$_1$ amplitude) at various stimulation intervals (Fig. 5B) to compare with published descriptions of short-term plasticity at the larval NMJ. The
students used off-line analysis tools in G-Prime to compensate for summation at short stimulus intervals. Specifically, they fit an exponential curve to the repolarizing phase of leading EJPs and used that as a baseline to estimate trailing EJP amplitudes. This allowed them to accurately estimate facilitation ratios even at stimulus intervals where summation dominated in the synaptic responses. The students’ results suggest the presence of short-term facilitation at stimulus intervals <1 s.

**Student evaluations**

In the first year qualitative evaluation, student reviews of the exercises were generally favorable. The students were excited to be working with a novel research preparation, they enjoyed the integration of behavior and physiology, and they seemed to be inspired by the idea of using genetics to remotely control neural activity. From a practical point of view, students liked being able to see light-evoked muscle contractions in dissected preparations; it helped them target healthy muscle cells for intracellular recording. In the first year, students complained that 1) the LED control system was not 100% reliable, 2) 1 week was too short to complete the exercise, and 3) there was not enough time allocated for exploring their own research questions.

Before running the exercises in year two, we corrected problems with the LED control system and allocated a second week for student exploration. After the exercises, we quantitatively evaluated student reactions. Figure 6 shows student responses (n = 21) to 6 questions designed to rank technical features of the exercises. While some students had difficulty clearly seeing muscle fibers for electrode penetration (Fig. 6D), on the whole, students were satisfied with the technical features of the exercises (Fig. 6 A, C, E). The students also liked starting the lab with behavioral analysis (Fig. 6B), and appeared to understand and be excited about what they were doing (Fig. 6F). Figure 7 shows student responses to an additional 6 questions aimed at evaluating how effective these exercises were at conveying biological concepts and promoting interest in biological research. Students indicated that these exercises helped them
understand principles of synaptic transmission (Fig. 7A) while also stimulating
interest in studying neural mechanisms of behavior and genetics (Figs. 7B, C).
Students were extremely excited about using new optogenetic technology and
doing experiments that have not yet been done by researchers (Fig. 7D). Overall,
the exercises helped students learn how to implement the scientific method and
heightened student interest in pursuing careers as research scientists (Fig. 7E,
F).

DISCUSSION

Behavior experiments
In a teaching exercise, it is important that any behavior phenotypes being studied are robust. We reasoned that activating glutamatergic neurons with ChR2 might produce phenotypes appropriate for teaching labs. Glutamate is the primary neurotransmitter at neuromuscular junctions in Drosophila (13, 14).

Demonstration and student data (Fig. 2; Table 1) show clearly that despite longer-term adaptation (25), activation of glutamatergic neurons with ChR2 leads to an immediate and dramatic decrease in larval locomotion. Quantification of student feedback suggests that it was instructive to start the exercise by examining ChR2 mediated behavioral responses (Fig. 5B), thus providing a behavioral context for the following physiology. This is probably because the behavior responses are so unambiguous; they produce immediate positive reinforcement for students early on in the exercise.

Activating glutamatergic neurons provides a reliable and easily interpretable phenotype (motor neuron activation = muscle contraction = tetanic paralysis). However, these experiments also provide a solid jumping off point for additional behavioral studies aimed at analysis of other ensembles of fly neurons. With the genetic tools currently available in Drosophila, students can remotely stimulate a variety of transmitter systems and neuronal subpopulations. For example, GAL4 drivers currently exist for labeling various aminergic systems (28); peptidergic cells, (31) and cholinergic neurons (27). Other drivers target the peripheral nervous system and identified sensory cells (11, 30). To date, the
functions of some identified neuronal populations have been examined with ChR2 (12, 25, 28, 29, 34), but a large and ever growing number of GAL4 lines (and by extension, hypotheses) remain to be tested.

Electrophysiology experiments

Consistent with previous work (25), in demonstration experiments, we reliably evoked IEJPs in reduced preparations that consisted only of motor axons, nerve terminals and muscles with stimulus durations of 2.5-10 ms. When evoking EJPs with electrical stimulation, researchers typically use 100 µs to 1 ms duration stimuli (14, 33). Critically, the IEJPs recorded with longer stimulation times were essentially identical to those evoked by 1 ms electrical stimulation of a single low threshold motor unit innervating m6 (most likely the ‘RP3’ motor neuron, 18, 20). Furthermore, increasing light pulse duration did not affect single IEJP parameters. These results suggest that EJPs resulting from ChR2 initiated action potentials (APs) are not essentially different from EJPs evoked by traditional nerve stimulation. There was only one obvious difference between the two methods of evoking EJPs: using ChR2, we were not able to recruit both motor units innervating m6. One possible explanation for this result is simply that our LED system cannot generate high enough intensity blue light to trigger an AP in the motor unit with the higher threshold. A second possibility is that ChR2 expression in the two motor neurons is not uniform. The strength of GAL4 expression often varies among cell types within an expression pattern (S. R. Pulver, personal observations). If GAL4 expression is relatively weak in high threshold motor neurons, then those cells would have fewer functional ChR2 channels and would, in turn be less responsive to blue light than other ChR2 containing motor neurons. The use of higher power LEDs and/or alternative motor neuron GAL4 drivers could help resolve this issue.

In our second set of demonstration experiments, we found that leaving the ventral ganglion intact lowered the effective stimulus duration needed to evoke EJPs. This could be a consequence of having intact motor neurons (dendritic regions, cell bodies, and initial spike generation zones) in the ventral ganglion
exposed to blue light. It could also be caused by activation of excitatory glutamatergic interneurons, which in turn, activate motor neurons through synaptic pathways. Regardless, the leading IEJPs in these CNS-nerve-muscle preparations were similar in amplitude and duration to IEJPs in experiments with only nerve and muscles present.

One prominent feature of preparations with intact ventral ganglia was that they generated multiple EJPs in response to single light pulses with durations longer than 2.5 ms. In addition, in about half the preparations, short light pulses triggered long lasting trains of spontaneously generated IEJPs. From a teaching perspective, these features provide students and educators with opportunities for further exploration. For example, students can easily examine basic synaptic integration when motor neurons fire high frequency bursts and postsynaptic potentials summate; students can also compare IEJPs and spontaneously generated EJPs without the use of stimulating electrodes.

In classroom exercises, students recorded EJPs from different body wall muscles. They were encouraged to target any muscles that contracted in response to light pulses (as opposed to specifically targeting only m6). While this resulted in heterogeneity across student results, it also increased the chances of students obtaining usable data, because many had difficulty visualizing individual muscles for electrode penetration (Fig. 6D). Opportunistically targeting muscle areas that contract with light stimulation facilitated student success. For example, all student groups (11 groups / two lab sessions) from our 2010 cohort recorded IEJPs. Once they successfully recorded EJPs, most students focused on examining short-term synaptic plasticity at the larval NMJ (Figure 5). They were aided by a suite of powerful software tools to analyze the dynamics of synaptic transmission. The data analysis program, g-PRIME (http://crawdad.cornell.edu/gprime/), has been optimized and student tested for analyzing many aspects of synaptic transmission at the crayfish NMJ (21). These freely available analysis tools can be immediately and directly applied to analyzing synaptic transmission in Drosophila.
Dissection for electrophysiology experiments: coping with small size

The largest drawback to the *Drosophila* NMJ electrophysiology preparation is its small size. Because of this, students have difficulty doing the larval dissection. In particular, they often cannot make a clean initial posterior to anterior cut with the spring scissors typically provided in teaching laboratories (10). We have found two solutions to this problem. One option is for teachers and teaching assistants (TAs) to prepare the dissections ahead of time and provide preparations ‘on the fly’ during a 3-4 hour lab class. With high quality scissors and a few practice sessions, experienced TAs (and students) can typically complete a dissection in under 5 minutes. The second approach is to follow a ‘try one, then get one free’ policy. Student groups try the dissection once, and if they do not see light evoked muscle contractions, they receive a fresh preparation from an instructor. Most preparations will provide some data unless large areas of the body wall are obviously damaged. Scotch Tape™ placed on the under surface of Sylgard lined petri-dishes diffuses transmitted light and increases contrast to more easily visualize target muscles.

Practical advantages of using ChR2

A major advantage of using ChR2 is that students are able to evoke IEJPs without the use of suction electrodes. Students (and researchers) often have difficulties maneuvering and operating suction or other stimulating electrodes in small working areas, especially with the larval fly prep. Eliminating the need for a suction electrode potentially eliminates a major source of frustration in the teaching laboratory. Before our fly laboratory sessions, the BIONB/BME 4910 students spent 2 weeks studying synaptic transmission at the crayfish NMJ. The students used the same equipment as used in our study and had the same primary instructor (BRJ); use of suction electrodes in the crayfish preparation was required. This gave us the opportunity to test the hypothesis that evoking EJPs with ChR2 in *Drosophila* was technically easier for students than traditional suction electrode stimulation in crayfish. Indeed, about 75% of the students agreed that using ChR2 to evoke IEJPs at the larval NMJ was easier than using...
a suction electrode at the crayfish NMJ (Figure 6C). This suggests that the ChR2
based exercises demonstrated here offer a technical advantage over at least one
traditional NMJ teaching preparation.

A second practical advantage of using ChR2 is that students can get
continuous feedback on the health of their preparations and where to insert
intracellular electrodes. In dissected preparations, shining blue light on a larval
CNS expressing ChR2 causes visible muscle contractions. Therefore, if students
see light-evoked contractions, they know that their preparation is healthy, and in
what muscle area to insert an electrode, even if individual muscle fibers are not
distinguishable. Since all motor neurons express ChR2, students can target
muscles in any healthy body wall segment of the larvae for intracellular
recording.

We noticed that many students had difficulty identifying muscle cells for
penetration with recording electrodes (Fig. 6D). Our student evaluations point to
a solution to this problem: simply being able to see light-evoked muscle
contractions in dissected preparations helped over 90% of students target
individual muscles for successful recordings (Figure 6E). We also noted that
seeing these contractions appeared to galvanize students to continue trying to
get intracellular recordings even in the face of frustration caused by technical
difficulties.

**Outlook for student-led research**

The ability to optogenetically evoke EJPs at the larval NMJ opens multiple
avenues for further exploration and independent student projects. For example,
students can explore in depth fundamental features of ChR2 mediated synaptic
transmission and its plasticity, including facilitation, summation, post-tetanic
potentiation and depression. They can also examine how these properties vary
among identified muscles in larvae (something that has never been done
systematically by researchers). Furthermore, since miniature EJPs (mEJPs) are
visible in m6 (13,14) students can estimate the quantal content of IEJPs (i.e. IEJP
amplitude / mEJP amplitude; 4). Finally, students can also examine how acute
application of neuromodulatory substances (i.e. neuropeptides and biogenic amines) affect synaptic transmission at the larval NMJ. Overall, many fundamental experiments have yet to be performed using optogenetic methods to evoke IEJPs in fly larvae; therefore, any student projects would be breaking new ground, not just repeating previous work.

Students were clearly motivated by this laboratory exercise. They felt it helped them understand communication within the nervous system, and it enhanced their interest in the intellectual background material (Fig. 7 A-C). Perhaps more importantly, almost all (94%) expressed excitement that they could potentially do novel experiments that have not yet been done by researchers (Fig. 7D). This lead most of them to express a positive interest in practicing the scientific method as students, and even to consider a career in research (Figs. 7 E, F).

CONCLUSIONS

Here we present inexpensive methods for remotely activating neural circuits in freely behaving Drosophila larvae with ChR2. We also show how to record ChR2 mediated EJPs at the larval NMJ and show that they are equivalent to EJPs evoked by traditional electrical stimulation. These teaching exercises give reliable results with minimal effort and expense. More importantly, they generate avenues for further research and give students and educators the means to explore them independently.

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REFERENCES


**FIGURE / TABLE LEGENDS**

**Figure 1:** LED control systems. A) Diagram of control system used in teaching exercises. Connections between LED, ‘Buckpuck,’ BNC connector, and power adapter are indicated. B) Equivalent diagram for control system designed for analog pulse stimulators and TTL signals with low current output. C) Typical LED control system (based on diagram in A) showing Buck Puck, BNC connector, and wiring. Housing is made from an empty pipette tip holder box. D) LED mounted on heat sink. Rolls of electrical tape are placed around the LED to prevent the microscope eyepiece from crimping wires supplying power to LED. E) LED and heat sink mounted to eyepiece and attached to magnetized base. F) LED system in place on a working electrophysiology rig.

**Figure 2:** Activation of glutamatergic neurons with ChR2 causes tetanic paralysis in larvae raised on food containing ATR. A) Schematic of genetic crossing scheme and larval rearing. B) 3rd instar larva raised on food without ATR. Locomotion and body posture under ambient light is the same as that under blue light. B) 3rd instar larva raised on food containing 1 mM ATR. Locomotion is unimpaired under ambient light. Under blue light all body segments contract and
animal stops crawling. C) Pooled data: animals raised without dietary ATR do not respond to blue light, while 100% of animals expressing ChR2 show contractile responses to blue light (left; n = 10). 92% of these animals are paralyzed for the duration of a 5 s light pulse (right; n = 12).

**Figure 3:** Comparison of light and electrically evoked EJPs (IEJPs and eEJPs, respectively) in the absence of motor neuron cell bodies and ventral ganglion circuitry. A) Schematic of a dissected larval preparation. Brain and ventral ganglion are removed. A single segmental nerve is stimulated via suction electrode. Muscle 6 (m6) is targeted for recording. B) Long time-base recording showing a typical experiment. One motor unit is recruited with the lowest stimulus voltage. An additional motor unit is recruited as electrical stimulus intensity is increased. IEJPs are evoked by 2.5-10 ms light pulses. C) Expanded time-base views of eEJPs and IEJPs shown in (B). D-F) IEJPs show amplitudes and time courses which are not statistically different from eEJPs evoked by the low threshold motor unit (F > 0.05, One-way ANOVA). Data from 1 ms light pulses are not shown because they did not evoke IEJPs in any preparations. In pooled data, resting membrane potentials were between -40 and -55 mV. Resting membrane potentials were not significantly different across stimulation types (F > 0.05, One-way ANOVA, data not shown). Pooled data are presented as mean +/- S.E.M. Asterisk indicates significant difference compared to all other conditions (p < 0.05, One-way ANOVA with Tukey-Kramer posthoc test).

**Figure 4:** Comparison of IEJPs and eEJPs with motor neuron cell bodies and ventral ganglion intact. A) Schematic of a dissected larval preparation, showing brain, ventral ganglion (Vg) segmental nerves and an intracellular electrode in the m6 muscle. The brain is removed, but ventral ganglion is intact. B) EJPs in response to a 1 ms electrical stimulus, and four different blue light pulse durations. Electrical stimulus intensity has been adjusted to activate both motor units innervating m6. Note multiple summating IEJPs after longer light pulse durations. C) Number of EJPs for each light pulse duration. D) Increasing light
pulse duration does not affect the amplitudes of leading IEJPs. E) Short light pulses can trigger long trains of spontaneous EJPs. A 1 ms light pulse (arrow) triggers a single EJP in m6 (1) followed by a train of endogenously generated EJPs (2, 3). F) The IEJP is similar in amplitude and duration to the spontaneous EJPs. Data in B and E, F are from two different animals. In pooled data, resting membrane potentials were between -40 and -55 mV. Leading EJP amplitudes and resting membrane potentials were not significantly different across stimulation types (F > 0.05, One-way ANOVA). Pooled data are presented as mean +/- S.E.M.

**Figure 5:** Example of student-initiated electrophysiology experiment: analysis of short-term plasticity at the larval NMJ. A) A pair of IEJPs evoked by 20 ms light pulses spaced 40 ms apart. Arrows indicate IEJPs. To compensate for additive summation at short stimulation intervals, an exponential curve (grey) is fit to the repolarizing phase of the first EJP. The amplitude of IEJP2 is determined by the difference between its peak voltage and the exponential fit voltage at the time of peak voltage. B) Paired pulse facilitation indices over a range of stimulation intervals (black squares). Data are fit to an exponential decay equation. Calculated long-term facilitation ratio is 0.8 +/- 4 (95% confidence interval). Data are from a single NMJ. All experimental design, data collection, analysis, and figure preparation carried out by students.

**Figure 6:** Student evaluation of the technical aspects of the ChR2 behavior and physiology exercises. A-F) Responses to 6 queries (shown above each plot) ranked on Leikert scale; n = 21 students.

**Figure 7:** Student evaluation of the conceptual and motivational aspects of ChR2 exercises. A-F) Responses to 6 queries (shown above each plot) ranked on Leikert scale; n = 21 students.
Table 1: Example of student-initiated behavioral analysis. Students counted number of peristaltic waves and distance traveled during 30 s trials in control (no ChR2 expression, n = 2) and experimental animals (ChR2 expressed in glutamatergic neurons, n = 2). In both groups, locomotion was measured in ambient light and in the presence of rhythmic (1 s pulses, 0.5 Hz) blue light pulses.

Supplemental Movie 1: Activation of glutamatergic neurons with ChR2 causes tetanic paralysis in larvae. Larvae (genotype: OK371-GAL4 / UAS-H134R-ChR2) crawl freely in ambient and green light, but stop moving in blue light.
Figure 2

A) Cross-species mating experiment involving male UAS-ChR2 and virgin female Df(3L)Gal4 flies. F1 larvae were reared on a food containing ATR, resulting in ChR2 expression in all glutamatergic neurons.

B) Behavioral response to ambient light before and after exposure to 0 mM ATR in food.

C) Behavioral response to ambient light before and after exposure to 1 mM ATR in food.

D) Graphs showing the percentage of flies that exhibited the desired response (left) and the percentage of larvae for which the desired response was obtained (right) before and after exposure to 1 mM ATR in food.
Figure 4
A. The LED control system did NOT work reliably.

B. It was helpful to start the lab by looking at behavioral responses in CHR2 expressing animals.

C. Using light to remotely activate zebrafish neuronal activity was less frustrating than using a suction electrode on the crayfish motor nerve.

D. I was able to clearly see individual muscle cells for electrode penetration.

E. Being able to see light-evoked muscle contractions in dissected preparations helped me target muscle cells for intracellular recording.

F. After the exercise, I understood how CHR2 was working to evoke EPSPs at the larval NMJ.
Figure 7

A. This exercise helped me understand basic principles of synaptic neurotransmission.

B. This exercise did NOT increase my interest in studying neurophysiology and animal behavior.

C. This exercise did NOT increase my interest in studying genetics.

D. I was motivated by the fact that the techniques used here are new and have not been extensively used in research publications.

E. This exercise encouraged me to generate and test my own research hypotheses.

F. This exercise increased my interest in becoming a research biologist.
<table>
<thead>
<tr>
<th>Group</th>
<th>Group A (No ChR2)</th>
<th>Group B (ChR2 Expression)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
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<td><strong>A: Control Condition - No Blue Light Stimulation</strong></td>
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<td>24</td>
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<td>Total Distance Traveled (# of squares)</td>
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**Table 1**