RESEARCH NOTE

A novel educational module to teach neural circuits for college and high school students: NGSS-neurons, genetics, and selective stimulations [version 1; referees: 3 approved with reservations]

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Abstract

This report introduces various approaches to target defined neural pathways for stimulation and to address the effect of particular neural circuits on behavior in a model animal, the fruit fly (*Drosophila melanogaster*). The objective of this novel educational module described can be used to explain and address principle concepts in neurobiology for high school and college level students. A goal of neurobiology is to show how neural circuit activity controls corresponding behavior in animals. The fruit fly model system provides powerful genetic tools, such as the UAS-Gal4 system, to manipulate expression of non-native proteins in various populations of defined neurons: glutamergic, serotonergic, GABAergic, and cholinergic. The exhibited behaviors in the examples we provide allows teachers and students to address questions from behaviors to details at a cellular level. We provided example sets of data, obtained in a research lab, as well as ideas on ways to present data for participants and instructors. The optogenetic tool, channelrhodpsin 2 (ChR2), is employed to increase the activity of each population of neurons in a spatiotemporal controlled manner in behaving larvae and adult flies. Various behavioral assays are used to observe the effect of a specific neuron population activation on crawling behavior in larvae and climbing behavior in adult flies. Participants using this module become acquainted with the actions of different neurotransmitters in the nervous system. A pre- and post-assessment survey on the content is provided for teachers, as templates, to address learning of content and concepts.

Open Peer Review

Referee Status: ? ? ?

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Introduction

Controlling the activity in neural circuits, while monitoring the effects on acute and chronic behaviors, is a means of addressing the function of defined neural pathways. This concept is related to the more common pharmacological approaches but in some cases with less precision to accomplish some of the same tasks. To selectively manipulate a subset of neurons within an easy organism to rear and maintain, Drosophila combined with optogenetics is ideal. The animal model and exercises provided allows for a 'hands on' inquiry-based learning module for high school and college courses, which emphasize life science topics. The current article presents a teaching module that is designed to integrate modern genetics, engineering, physics, life sciences, modeling and experimental design for use with high school and college students. Researching the primary scientific literature and utilizing the related findings, as well as postulating the outcome for newly designed experiments based on the results one collects, allows students to test their own predictions and draw hypotheses. This approach provides autonomous learning among student groups. The measureable outcomes with obtaining quantitative data for analysis and interpretation are valuable learning experiences. Based on one's findings in the initial experiments, one can readily redesign experimental paradigms to test the formulated hypotheses utilizing one's own prior data. The integration with Arduino hardware and software opens the doors for students to a world of writing code with an experimental purpose, and independence in experimental design.

The underlying science in the module proposed by the current article focuses on neurobiology. The seminal discoveries by Hubel & Wiesel (1970) demonstrated that activity in sensory input and within the central nervous system (CNS) is indispensable in the development and maintenance of neural circuits. This concept is also essential for development and maintenance of synaptic establishment at the neuromuscular junction (NMJ) of skeletal muscles (Balice-Gordon *et al.*, 1990; Lømo, 2003). In some cases, the activity profile must occur prior to developmental time points before the neural circuits become more hardwired. After such a critical period in synaptic formation, the circuit is not as dependent on activity for competition with other neurons for the establishment of connections. This fundamental phenomenon occurs in organisms from fruit flies to humans. It is known in mice that even after established connections are made in adults, the terminals at NMJs are not fixed to one spot on a muscle fiber: The motor nerve terminals grow out and pull back over time while continuing to communicate with the muscle fibers (Lichtman & Sanes, 2003).

If motor neurons, which are normally innervating a muscle, are removed, then other motor neurons will take control of the target and innervate it. Thus, motor nerves are searching out targets not already committed by other synaptic inputs (Chang & Keshishian, 1996). This was examined in embryonic and larval *Drosophila* by laser ablating various body wall muscle fibers during development. Even pharmacologically activating or silencing neural circuits during development can have long term consequences in neural connections and overall physiological functions (Smith *et al.*, 2015). For example, exposing rodents to nicotine during development changes the dendritic morphology within the CNS, which lasts into adulthood (McDonald *et al.*, 2005). Even short exposures to nicotine in the juvenile stages have long lasting effects in adults for these mice (Ehlinger *et al.*, 2014). It is also established that collective synchronized synaptic activity is important for development of the neural structure (Winnubst *et al.*, 2015). Thus, long term consequences in the established neural circuitry within the CNS and at the NMJ can occur based on neural activity when the initial circuits are being wired.

A guided self-inquiry based approach to learning science has been demonstrated to be a very effective means for student learning in the long term (Bradforth, *et al.*, 2015; Waldrop, 2015). The engineering design with Arduino systems is a very engaging educational experience, which is sought after in many schools within the USA and abroad (see educational web pages: https://www.adafruit.com/educators; https://www.arduino.cc/en/Main/Education; Bender, 2012; Escudero *et al.*, 2013; Junior *et al.*, 2013; Maxwell & Meeden, 2000; Zalewski *et al.*, 2014). The surge in the use of the Arduino system in high school and college teaching is partly due to the low cost and ease in writing code for operating the system. Students can design experiments with various computer codes to control the duration of light on-off time period and frequency of stimulation to observe how activating or inhibiting a specific set on neurons alter development and behavior of *Drosophila* larvae or adults. Arduino and associated LED required hardware is relatively inexpensive, <$20 USD for an individual unit; however, making a series of units with one power supply is cheaper for adding additional units. Class sets can be used in subsequent years, so an initial investment has a long term use. There are dozens of demonstration videos on YouTube for a wide variety of inventions and coding using Arduino.

In the educational module presented in this article, we demonstrate an approach with optogenetics to selectively activate the neurons synthesizing the neurotransmitter GABA (glutamate, serotonin, and acetylcholine). The approach used to stimulate these selective neurons is to activate light sensitive channels expressed in these neurons. Different *Drosophila* lines are used for each type of neurotransmitter. The ability to control the stimulation with light is managed by an Arduino system or a simpler system with a 9 Volt battery and an LED source. Students can readily add single units or build parallel outputs with discrete parameters for controlling the LEDs. Thus, this allows various parameters to be tested simultaneously in the same laboratory setting. Since many of the experimental paradigms presented by the module are novel, many unanswered questions remain to be answered in neurobiology, and students may uncover unique findings worthy of publication in scientific journals.

This educational module is also designed to embrace the Next Generation Science Standards (NGSS Lead States, 2013), through approaches scientists employ in the development of scientific knowledge. NGSS recommends that models be used in *Developing, Evaluating, Using, and Revising* explanations and predictions of science phenomena. The students will be able to construct models in neural circuits to explain the observed behavioral phenomenon to make sense of what they observe. The direct real life examples

concerning how neural circuits develop in one's self, as well as in other animals, is of general interest, but also has applied implications for medicine and health. The ability to manipulate various neurotransmitter systems and stimulation paradigms promotes experimental design and redesign based on the observed findings from each experiment. This is an integral aspect of the NGSS. The approach presented herein promotes explanations of the findings in order to set a new or altered stimulation paradigm, as the students continue to study a phenomenon in different contexts. In addition, this article discusses some of the techniques used in a trial of this module for sophomore high school students in Louisville (KY, USA), senior high school students in Sommerset (KY, USA) and college level juniors and seniors at the University of Kentucky. (The outcomes of the trial are detailed further in the section *Instructor feedback*).

Module overview

Fly strains and crosses

Some of the experimental procedures require being able to make selective genetic crosses of two different *Drosophila* lines. To perform the crosses, it may be necessary to identify male and female adults and to be able to obtain virgin females (Figure 1). The instructors of the module can decide on their resources (dissecting microscopes and time management of students) for either performing the crosses themselves, or if the students should be given the time to make the crosses or be provided an explanation. As a learning experience, the teacher could allow the students to try these procedures, but have a cross already prepared for class use. A number of online resources are available to see the differences in males and female adult flies; the presence of black tuft of hairs on the forelegs indicates a male fly (Figure 1). It is good to compare the flies side by side to tell the differences.

There are some procedures where the fly lines obtained can be directly examined without having to make filial 1 (F1) generations with selective crosses. The line that expresses the light-activated channelrhodopsin-2 in motor neurons is OK371-Gal4;UAS-ChR2H134R-mcherry (homozygous line, there are two copies for each construct). This line is made by crossing w^{1118};P{GawB}VGlut^{OK371} (Bloomington Drosophila Stock Center at Indiana University (BDSC); catalog no., 26160) with w*; P{UAS-H134R-ChR2}2 (BDSC; catalog no., 28995; Pulver *et al.*, 2011). When trialing this module, we used another recently created ChR2 line, which is very sensitive to light, called y^1 w^{1118}; PBac{UAS-ChR2.XXL}VK00018 (BDSC; catalog no., 58374; Dawydow *et al.*, 2014). Virgin females from w*; P{UAS-H134R-ChR2}2 were crossed with males of D42-Gal4 (BDSC; catalog no., 8816) for also being expressed in motor neurons. Trh-Gal4 (BDSC; catalog no., 38389), Gad1-Gal4 (BDSC; catalog no., 51630), or ppk-Gal4 (BDSC; catalog no., 32078) to express ChR2-XXL variant in serotonergic neurons, GABAergic neurons or type IV sensory neurons, respectively. In the trial module, we also used UAS-H134R-ChR2;Trh-Gal4 (III) homozygous line, which was kindly provided by Dr. Andreas Schoofs (University of Bonn Life & Medical Sciences Institute (LIMES), Bonn, Germany; Schoofs *et al.*, 2014), to compare behavioral effects with the more light sensitive ChR2 line. Table 1 outlines which crosses of flies can be used from these lines mentioned above for targeting the desired neuronal subtypes.

All-trans-retinal preparation

All-trans-retinal is a cofactor for the channel rhodopsin which increases the sensitivity to light and increases single channel conductance (Dawydow *et al.*, 2014). ATR (500mg; available from Sigma-Aldrich, St. Louis, MO, USA) is dissolved in 17.6 ml absolute ethanol to make 100mM stock solutions. Then, 100µl of

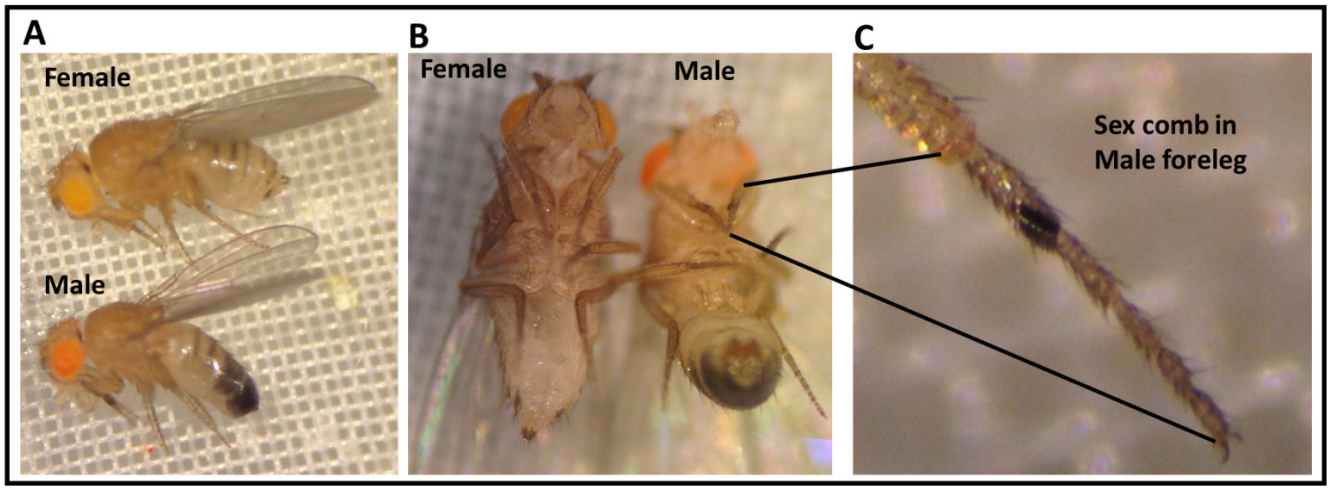

Figure 1. Determining the sex of adult fruit flies. (**A**) Morphological characteristics and sexual dimorphism of adult *Drosophila melanogaster* (lateral view). Adult female fly (top) has a light colored abdomen region; however, adult male fly (bottom) has a dark posterior abdomen region. (**B**) Morphological differences between male and female flies (ventral view). (**C**) Magnified view of the male fly foreleg shows male specific sex comb structure.

100mM stock solution is transferred to small tubes, wrapped with aluminum foil and kept in a -20°C freezer. The ATR should be kept away from light, since it is light sensitive; it would be degraded and become ineffective if it is exposed to light for a long time.

Preparation of fly food supplemented with ATR. In order to prepare fly food supplemented with 1mM ATR, 10ml fly food is dissolved in the microwave. The food is left to cool, then 100µl of 100mM ATR is mixed well with the fly food, or 100µl of absolute ethanol is mixed with food as a control. The food vial should be wrapped in aluminum foil and the food left until well solidified (flies may stick to wet food). The larvae or adult flies for whichever experimental lines to be tested are then transferred from their vial to an ATR-food-containing vial and are kept in a dark place (to keep the ATR from degradation) at room temperature (22-23°C).

Larval locomotion behavior

Locomotion behavior of larvae is assessed by placing a single larva on an apple-juice agar plate. The larva is left for one minute to acclimate to the new environment. Having the room lights off or very dim while the students work might be difficult to achieve in some classrooms. The body wall contractions (BWCs) are counted for one minute (BWCs/min) while the larva is exposed to regular white light. Then the body wall contractions are counted for one minute while the larvae is exposed to blue light (470nm wavelength; a dispersed-soda-can device can be used, see Figure 2). Also, body wall contractions are counted while the larva is exposed to focused

Table 1. *Drosophila melanogaster* lines.

Line	Sex	Outcome
w^{1118};P{GawB}VGlut^{OK371} Cross with line below opposite sex	Male or Female	ChR expressed in neurons which express vesicular transporter for glutamate (motor neurons).
w*; P{UAS-H134R-ChR2}2	Male or Female	
y^{1} w^{1118}; PBac{UAS-ChR2.XXL}VK00018 Cross with below lines	Virgin Female	
D42-Gal4	Male	ChR2 expressed in motor neurons.
Trh-Gal4	Male	ChR2 expressed in serotonergic neurons
Gad1-Gal4	Male	ChR2 expressed in GABAergic neurons
ppk-Gal4	Male	ChR2 expressed in Type IV sensory neurons

w*; P{UAS-H134R-ChR2}2; Trh-Gal4 (homozygous line) ChR2 expressed in serotonergic neurons. There is no need to make crosses as this line is homozygous. The larvae or adults should be raised on food supplemented with all trans retinal (ATR), which is a cofactor essential for ChR2 function, since unlike mammals the flies cannot synthesize sufficient amount of ATR for ChR2 function) and a control group without ATR (use ethanol (EtOH) as a vehicle since ATR is dissolved inside absolute ethanol).

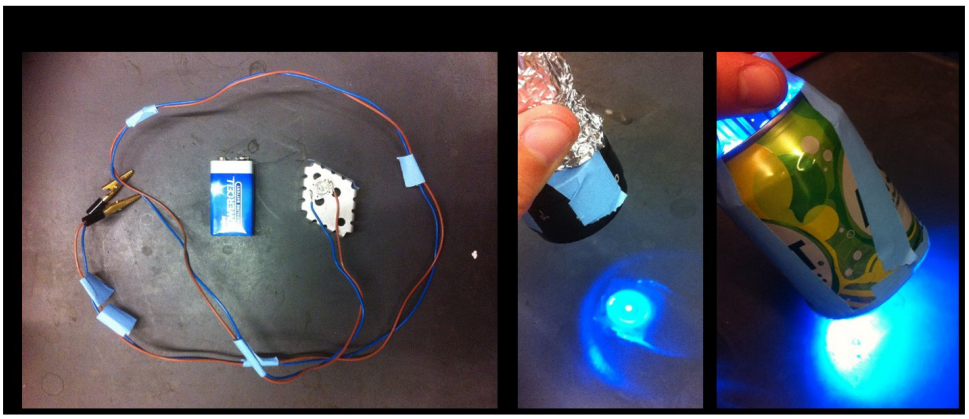

Figure 2. Approaches to use in delivering the LED blue light to the larvae as well as adult *Drosophila*. (**A**) A blue light emitting diode (LED; wavelength = 470nm) is glued on a cooler plate with a temperature resistant glue. The LED light is connected to a 9V battery. Various intensities of LED light can be used by attaching the LED to (**B**) a microscope ocular lens (x10), which gives off high intensity light or (**C**) a soda can with the bottom removed and the LED placed though the top, which gives a low intensity diffuse light.

focal blue light (a focused light through a microscope eyepiece with a mounted LED can be used, see Figure 2). This assay can be performed for first, second and third instar larva. In the module trial, the typical behaviors of third instar larvae are shown in Figure 3 for flies fed and not fed ATR, as well as for dim white light, diffuse blue light delivered by a soda can set up and a focused blue light with a microscope eyepiece objective. Notice in Figure 3B3 the contracted larvae. The microscope eyepiece can be bought on Amazon.com as 10X eyepieces; a wide base type is most useful, so the LED can fit inside. Table 2 provides a template in which students can record the type of behaviors observed with this experimental paradigm.

Rolling behavior in larvae. Assessing rolling behavior is performed by placing a single larva on the surface of an apple-juice agar plate. The occurrence of rolling behavior can be counted for the 1st and 2nd minute. The percentage of larvae that show rolling behavior can be presented in graphical form, as shown in Figure 4 (module trial results), for a ChR2 being expressed in type IV sensory neurons in third instar larvae and stimulated with blue light. The fly lines crossed for this experiment are the UAS-ChR2-XXL and ppk-Gal4 and the food was without ATR or supplemented with ATR (1mM). A sample size of 20 larvae were tested for this data set

Figure 3. Module trial results: Activation of ChR2 channels in the motor neuron of third instar larva. OK371-Gal4 (Gal4 driver specific for motor neurons) is crossed with UAS-ChR2H134R-mcherry *Drosophila* line (this line is homozygous foe both Gal4 and UAS constructs). The progeny expresses ChR2 in motor neurons. (**A**) The larvae were raised in fly food, which was not supplemented with all-trans-retinal (ATR), a cofactor important for ChR2 membrane integration and function. (**A1**) The body wall contractions (BWCs) are counted on an apple juice agar plate for 1min when the larva is exposed to regular light. (**A2**) The larvae is exposed to low intensity blue LED light (470nm) for 1min while the BWCs are counted. (**A3**) The crawling behavior of larva is observed when it is exposed to intense blue light for 1min. (**B**) The larva was fed ATR (1mM), which was mixed with fly food. The body wall contractions are counted when the lava is being exposed to regular light (**B1**), low intensity blue light (**B2**), or high intensity blue light (**B3).** The larva does not respond to the low intensity light although when it is being exposed to high intensity blue light, the BWCs contract, which can be observed by shortened body length (**B3**).

Table 2. Larval behaviors with exciting the various neurons expressing different types of neurotransmitters. The participants can fill in a data table such as this one presented.

Neuron type	Dim or regular light /1min	Low intensity blue light /1 min	Dim or regular light /1min	High intensity blue light /1min	Dim or regular light /1min
OK371-Gal4 (appropriate neurotransmitter line) and UAS-ChR2H134					
Soda can					
motor neurons	?	?	?	?	?
Focuses light					
motor neurons	?	?	?	?	?
y^1 w^{1118}; PBac{UAS-ChR2.XXL} VK00018 crossed with appropriate neurotransmitter line					
Soda can					
motor neurons	?	?	?	?	?
serotonergic neurons	?	?	?	?	?
GABAergic neurons	?	?	?	?	?
Type IV sensory neurons	?	?	?	?	?
Focuses light					
motor neurons	?	?	?	?	?
serotonergic neurons	?	?	?	?	?
GABAergic neurons	?	?	?	?	?
Type IV sensory neurons	?	?	?	?	?
UAS-ChR2H134R-mcherry;					
Soda can					
Serotonergic neurons	?	?	?	?	?
Focused light					
Serotonergic neurons	?	?	?	?	?

Coded behavior can be used. Type of behavior coding used: continue crawling forward (CC), crawling backward (CB), stop (S), head wagging (HW), rolling (R) , keeps turning left or right while crawling (T).

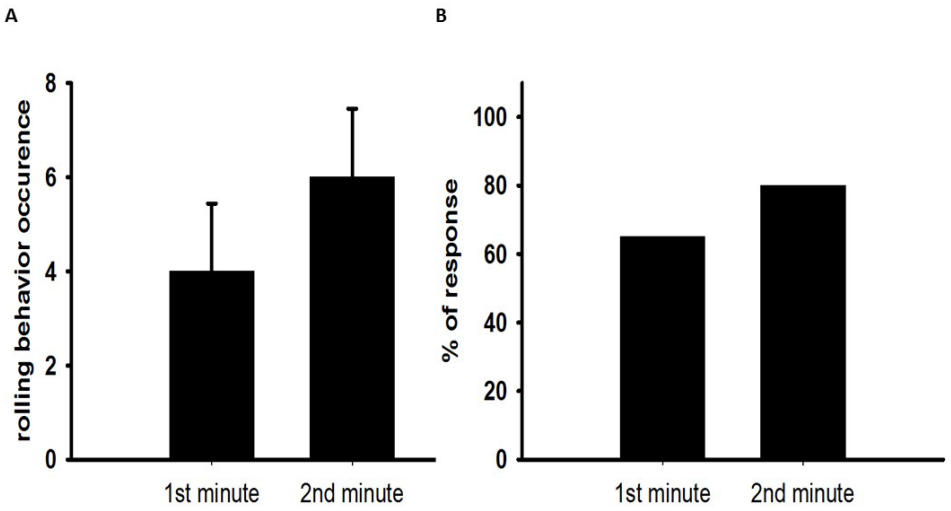

Figure 4. Module trial results: Rolling behavior is induced by activating ChR2 in type IV sensory neurons in third instar larvae (UAS-ChR2-XXL; ppk-Gal4 (n=20), food supplemented with ATR 1mM). (**A**) Shows the occurrence of rolling behavior in the 1st and 2nd minute of light exposure (normal scope light). (**B**) Most of the larvae showed rolling behavior when they are exposed to light.

Adult fly behavior assays

For adult behaviors, left over larvae from conducting the larval behaviors can be used, the 1st crosses should be saved and grown to adults. Thus, the differences between the larval and the adult lines can be compared with the same crosses. Also, if ATR-tainted food from the larval assays is saved, this can be used to feed the adults. The adults should be a few days old before conducting the following behavioral experiments to insure they have built back up the levels of ATR in the body. There are a number of behavioral assays that are commonly used for adult Drosophila (Badre & Cooper, 2008; Nichols *et al.*, 2012; http://www.sdbonline.org/sites/fly/aimain/6behavior.htm). For some of the assays, the separation of males and females should be considered, as there are differences in the size and weight of the adult flies. Also, as the adults age there may be differences in their behaviors.

The two commonly used behavioral assays that are relatively easy to implement, but informative for the biological concepts, are the negative geotaxic and phototaxic assays, which are described below. These assays can be expanded on for deeper investigation into the neurobiology of the flies. Also, these behavioral assays allow for data gathering, redesign and vivid discussion for inquiry based labs.

***Negative geotaxic assay*.** Adult flies aged 2–8 days can be anesthetized with CO_2. Males and females are sorted and transferred into separate vials, containing food, in cohorts of 10–14 flies. The flies should be left to recover for 24h before running the experiments. A plastic vial (Drosophila culture cylindrical vial 1-1/4" diameter x 4" tall; http://www.enasco.com/product/SB11136M) can be marked at 8cm length, and the 10–14 fly cohort transferred to an empty marked vial. Another plastic vial can be placed on top of the marked one (Figure 5). The flies should be left for one minute. The vials can be tapped on a table to knock down the flies to the bottom of the tube. Then the number of flies that climb across the 8cm mark within 10sec can be recorded, as shown in Figure 5. This procedure can be repeated a few times with tapping to knock the flies down to the bottom of the vial each time. Table 3 provides a template in which students can record the data for this assay of adult behaviors.

During the module trial, we used flies that were expressing ChR2 in motor neurons and fed ATR 1mM (UAS-CHR2H134R-mcherry;OK371-Gal4). This particular line (ChR2H134R) is a strain where the protein (the channel rhodopsin) has been altered with different amino acids and is not as sensitive as the ChR-XXL line. This line did not show a large difference from the 1st min of

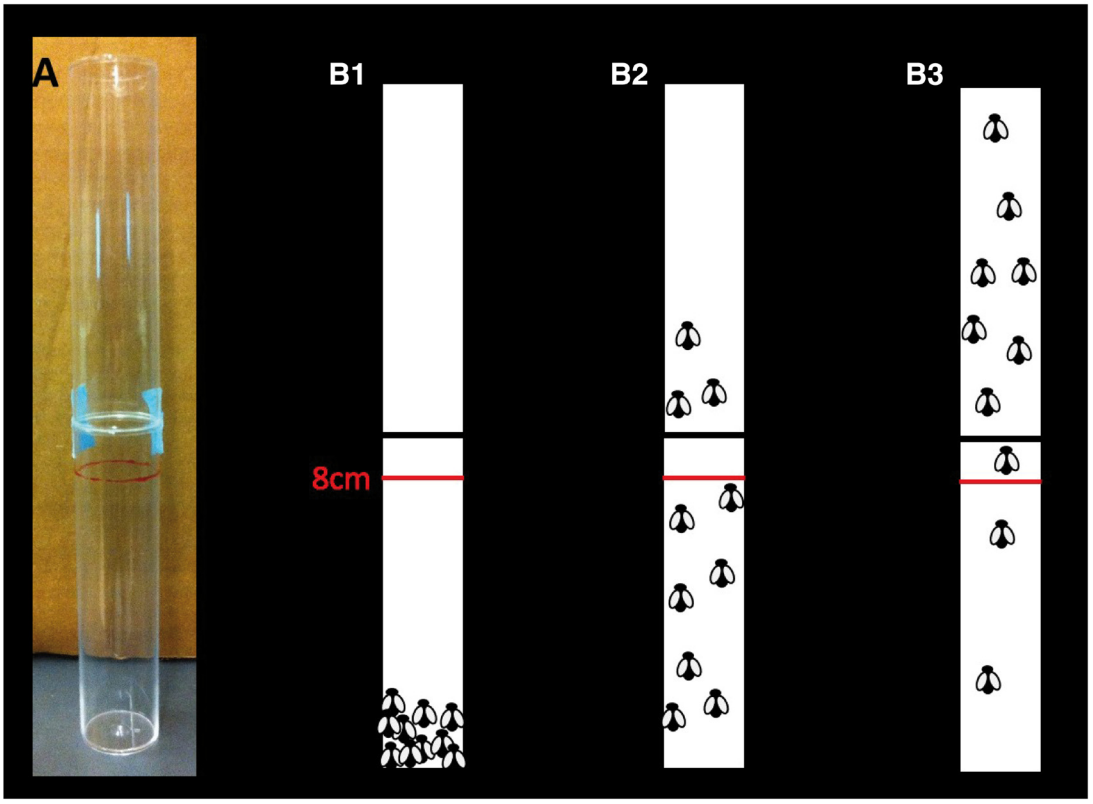

Figure 5. Negative geotaxic assay in adult flies. (**A**) Two plastic tubes are put together for this assay. 10–14 adult male or females flies are transferred to an empty plastic tube which is marked at 8cm length. Second plastic tube is put on the top of the first marked plastic tube and sealed with tape. (**B1**–**B3**) The tube is tapped until all the flies fall into the bottom of the first tube (**B1**). The flies start climbing up on the wall of the plastic tubes. After 10 seconds the number of the flies that cross the 8cm red line is counted which shows the percentage of the flies that are crossed the line in 10sec. (**B1**) 0%, (**B2**) 30%, (**B3**) 80% of the flies are crossed the red line.

Table 3. Representative sample data table for the negative geotaxic assay with various neuron population activation by light.

Neuron type	Time taken to cross 8 cm line (10 trials before blue light exposure, 10 trials after blue light exposure)
OK371-Gal4 (appropriate neurotransmitter line) and UAS-ChR2H134	
Soda can	
motor neurons	?
Focuses light	
motor neurons	?
y^1 w^{1118}; PBac{UAS-ChR2.XXL} VK00018 crossed with appropriate neurotransmitter line	
Soda can	
motor neurons	?
serotonergic neurons	?
GABAergic neurons	?
Type IV sensory neurons	?
Focuses light	
motor neurons	?
serotonergic neurons	?
GABAergic neurons	?
Type IV sensory neurons	?
UAS-ChR2H134R-mcherry;	
Soda can	
Serotonergic neurons	?
Focused light	
Serotonergic neurons	?

recovery time after the blue light was turned off to the 3rd minute of recovery time for the percent number of flies passing the 8 cm mark. The bars that are labeled 'crawl' represents the flies that are crawling around the bottom of the vial (Figure 6). Using the very sensitive strain of flies (ChR2-XXL), where the channel demonstrates high sensitivity to blue light, the percentage of flies recovering took much longer than for the UAS-CHR2H134R-mcherry;OK371-Gal4 cross (Figure 7). Also, the UAS-ChR2-XXL/+;D42-Gal4/+ cross targeted motor neurons, which express D42. The recovery time of paralyzed flies for this fly strain was not even fully recovered after 14 minutes after the blue light exposure.

Extra details on this behavioral assay is found in Ali *et al.* (2011). When using a similar assay, one can also measure the percent of flies which start to crawl as an index. This can be tried with motor neurons drivers or other types of neuronal drivers. In the module trial, we used a subset of sensory neurons, referred to as Type VI sensory or pickpocket neurons, which had ChR2-XXL expressed (Figure 8). It is obvious in the first experiment (by observing how many flies crawled or moved up the tube) that it was difficult for the flies to walk up the walls of the tube, but in subsequent experiments more were able to walk up the tube.

***Phototaxic assay*.** To conduct this assay, a device with a 25cm long plastic tube and light source at one end in a dimly lit room is used to assess the phototaxic behavior of the adult flies. The tube is narrow enough not to allow the adults to fly, but only walk up the tube. Also a standard small LED maglight fits snuggly in one end (Figure 9). The male or female flies can be anesthetized by a quick exposure to CO_2 or by placing a vial in ice for 25–30sec. Individual flies are placed in each apparatus. The flies need to recover for at least 10min. Each apparatus with an individual fly should be positioned vertically and tapped until the fly falls to the bottom of the tube, which is closed by a rubber stopper. The time the fly crosses a 10cm line and a 20cm line can be recorded as a measure. This apparatus could be positioned horizontally or vertically, but vertical placement examines both geotaxic, as well as light sensitivity. A sample table to enter student data is presented as Table 4.

Data collection and interpretation

The results from the various experiments can be tabulated or graphed in various ways, depending on the variables the instructor and students wish to investigate. Data that can be plotted over time, such as time for the adults to cross the 10 and 20 cm line, can be graphed using free web based graphing software Joinpoint, (http://joinpoint.software.informer.com/), which allows students to work at home or at school. Also, graphing the values for the different experimental lines of flies allows for discussion of the data in relation to biological significance. For high school teachers focusing on The Next Generation Science Standards (NGSS Lead States, 2013), or college instructors wanting more of an inquiry based experience in real life topics for students, the exercises provided can be varied or expanded.

For example, different instar stages can be compared for a particular strain. In the module trial, we used the ChR2 channels in motor neurons with a less sensitive strain (UAS-ChR2H134R-mcherry;OK371-Gal4) which showed different responses between the various instars and measured body wall contractions for one minute (BWCs/min). These larvae were fed ATR 1mM (Figure 10). Also central neurons that utilize different neurotransmitters can be examined for changes in larval, as well as adult, behaviors. We used a line that results in activation of the GABAergic neurons and measured locomotor activity in third instar larvae fed ATR (UAS-ChR2-XXL;Gad1-Gal4). The blue light stimulation resulted in a substantial decrease in body wall movements (Figure 11). This same line can be used for measuring adult behaviors and measuring climbing. A sample of such responses is shown in Figure 12. The adults were fed ATR (1mM) and they showed a reduced ability to climb.

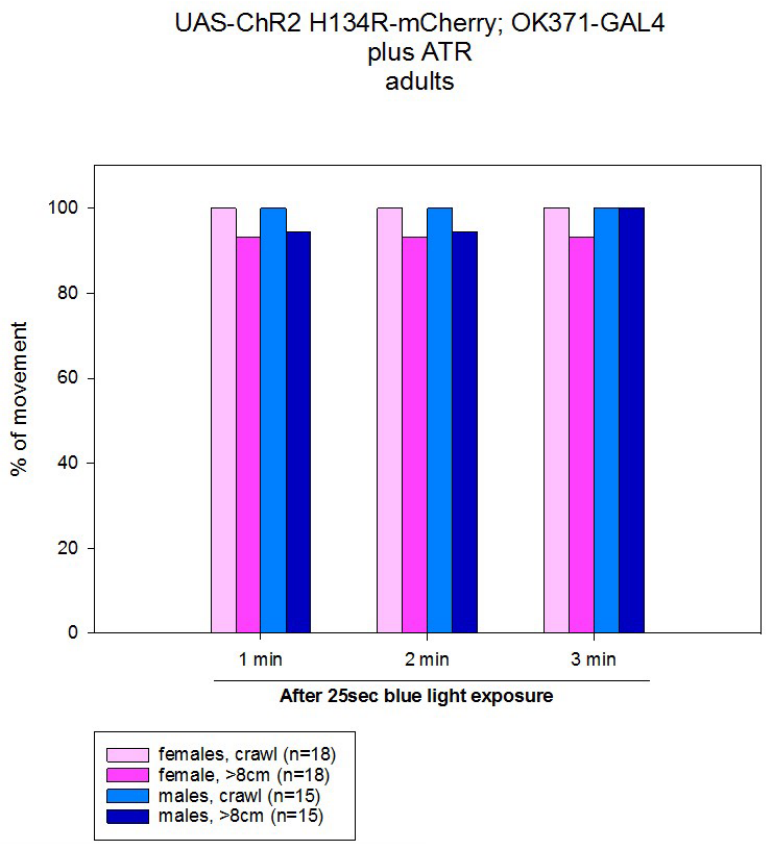

Figure 6. Module trial: Locomotor activity in adult male and female flies after exposure to blue light. These flies are expressing ChR2 in motor neurons and also are fed ATR 1mM (UAS-CHR2H134R-mcherry;OK371-Gal4). The blue light does not exert influence on the negative geotaxic assay since the blue light cannot penetrate well the thick dark adult cuticle.

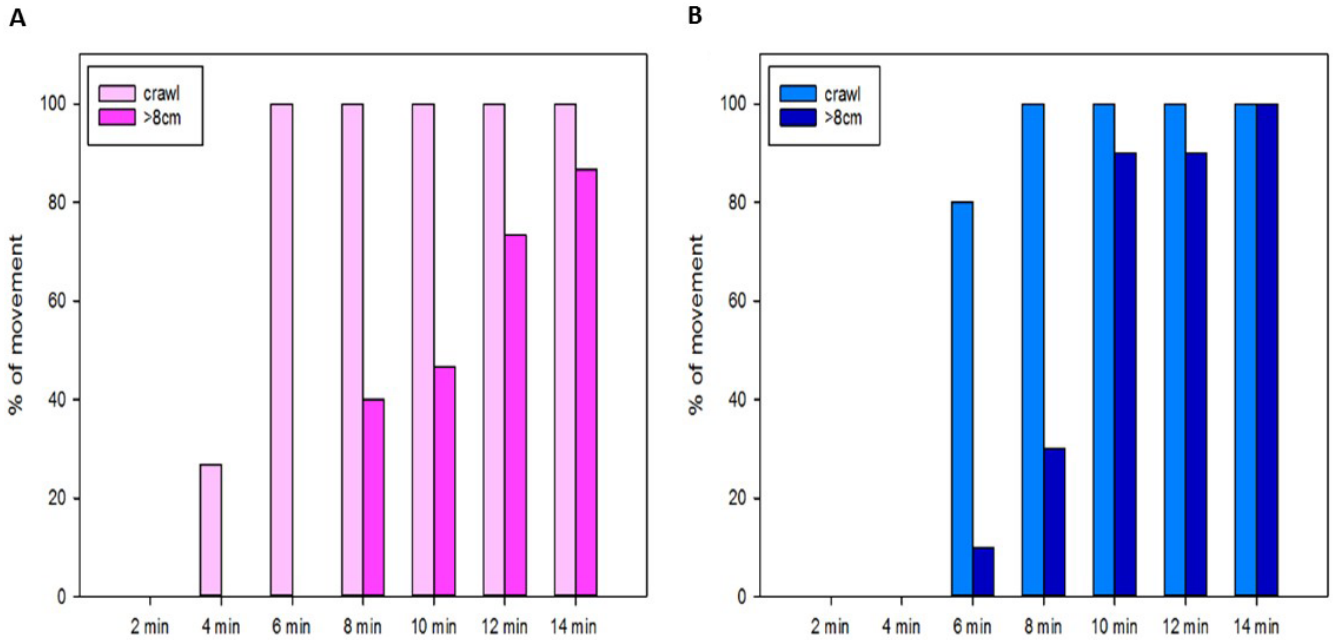

Figure 7. Module trial results: Activation of highly sensitive ChR2 in motor neurons (UAS-CHR2-XXL;D42-Gal4). (**A**) The crawling and negative geotaxic behavior of adult female flies is decreased after 25 second blue light (low intensity) exposure. After 14 minutes the flies restored their normal climbing ability. (**B**) The ability to crawl and climb was markedly compromised in adult male flies being exposed to blue light for 25 seconds. The crawling ability restored after 6min of paralysis although the climbing behavior went back to normal after 12 minutes of paralysis. These flies were raised of food supplemented with ATR 1mM.

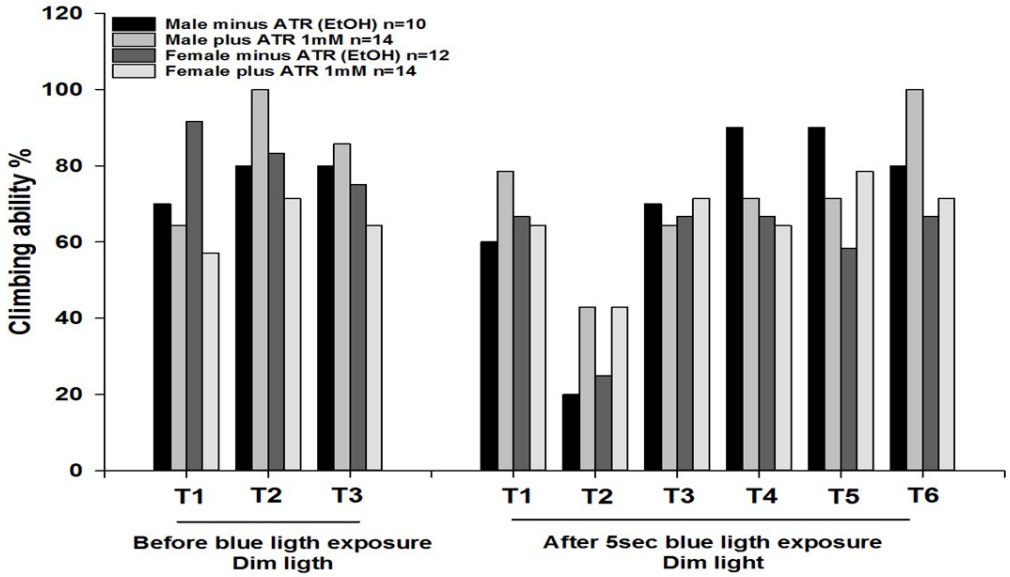

Figure 8. Module trial results: Type VI sensory neuron activity modulation affects climbing ability in flies expressing ChR2 in pickpocket neurons (UAS-ChR2-XXI; ppk-Gal4). After 5 sec blue light exposure, some of the flies were paralyzed for 1–2 seconds then they recovered well. As it is shown that the first trial (T1) after blue light exposure, the flies do well in climbing assay; although, in the second trial (T2 after blue light exposure), the flies climb the middle of the bottom tube then they stop climbing further. They recover quickly in the following trials.

Instructors can also use published literature and standard textbooks to explain to students that the locomotor behaviors are driven by motor neurons, which activate body wall muscles (Marieb & Hoehn, 2013; Sherwood, 2001). In addition, an instructor can use the illustrations in Figure 13 to examine how the electrical responses are monitored in the muscles and the effect of stimulating various types of neurons. In addition, there are published studies and figures readily found on the web that highlight the various neuronal types within the CNS and ventral cord of larval, as well as adult, *Drosophila*, which contain different types of transmitters, such as serotonin (as shown in Figure 14). In the module trial, we have shown the effect of activating serotonin producing neurons on behaviors for body wall movements of larvae with a line less sensitive to blue light (Figure 14B; UAS-mCherry-ChR2 H134R; Trh-GAL4, homozygous for both constructs), as well as a line very sensitive to blue light (Figure 15; UAS-ChR2-XXL;Trh-Gal4). The two levels of blue light sensitive lines can also be examined as adults, as we demonstrate in Figure 16.

It may be confusing for students to understand that sensory neurons can present a behavior similar to that of stimulating motor neurons. Instructors should help students to understand neural circuits and that activating sensory neurons can lead to motor neuron activation. The activation of type IV sensory neurons with blue light and then recording in motor neurons can help in explanation. A representative intracellular recording in muscles with sensory neurons stimulation is shown and can be used for instructive purposes. This line is UAS-ChR2-XXL;ppk-Gal4, and supplemented with ATR (1mM) produces robust responses in the muscle fibers (Figure 17).

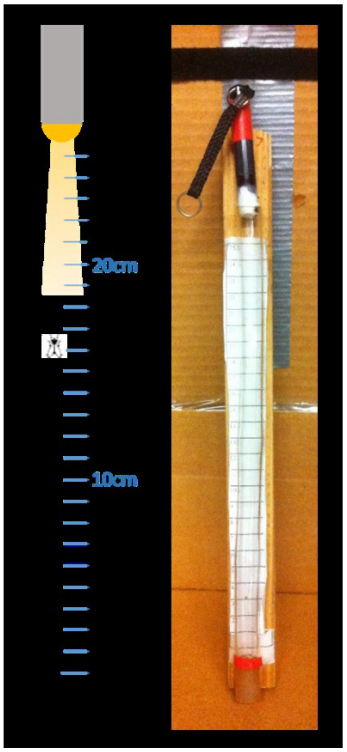

Figure 9. Experimental set up for adult phototaxic assay in a narrow tube. A single male or female fly is transferred into the tube. The tube is tapped until the fly falls into the bottom of the tube. The time that the fly takes to reach 10cm and 20cm is recorded.

Table 4. Representative sample data table for the phototaxic assay with various neuron population activation by light.

Neuron type	Time taken to cross 10 cm line	Time taken to cross 20 cm line
OK371-Gal4 (appropriate neurotransmitter line) and UAS-ChR2H134		
Soda can		
motor neurons	?	?
Focuses light		
motor neurons	?	?
y^1 w^{1118}; PBac{UAS-ChR2.XXL} VK00018 crossed with appropriate neurotransmitter line		
Soda can		
motor neurons	?	?
serotonergic neurons	?	?
GABAergic neurons	?	?
Type IV sensory neurons	?	?
Focuses light		
motor neurons	?	?
serotonergic neurons	?	?
GABAergic neurons	?	?
Type IV sensory neurons	?	?
UAS-ChR2H134R-mcherry;		
Soda can		
Serotonergic neurons	?	?
Focused light		
Serotonergic neurons	?	?

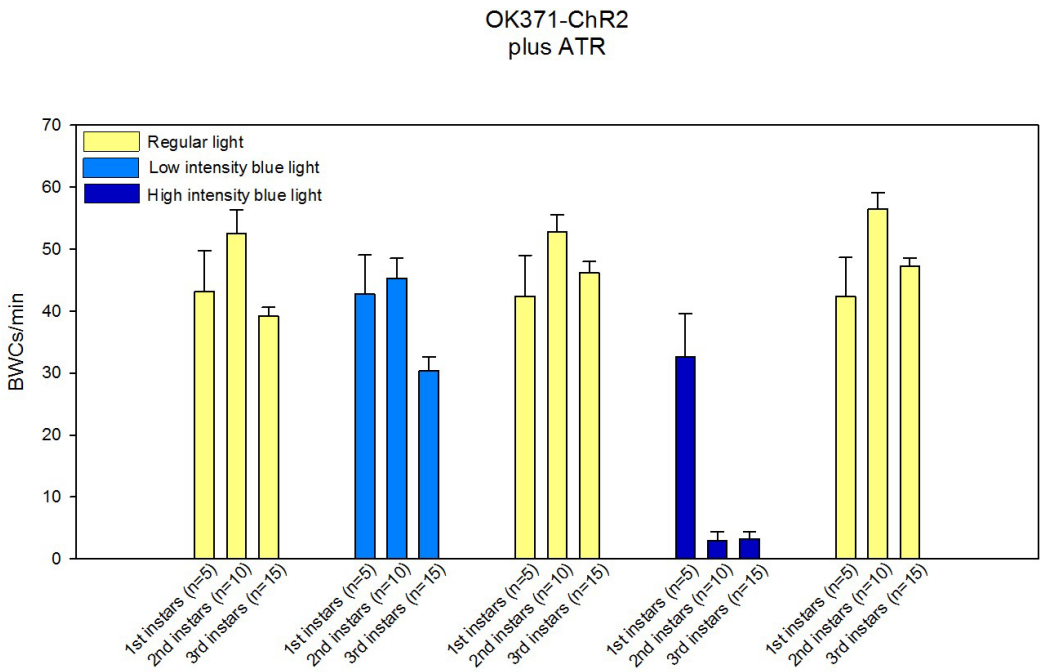

Figure 10. Module trial results: Activation of ChR2 channels in motor neurons (UAS-ChR2H134R-mcherry;OK371-Gal4) produces different responses in various instar larvae (The larvae were fed ATR 1mM). The body wall contractions for one minute (BWCs/min) are being counted while the larva is being exposed to regular light, low intensity blue light or high intensity blue light. The data shows that the first instar larvae do not respond well to even high intensity blue light.

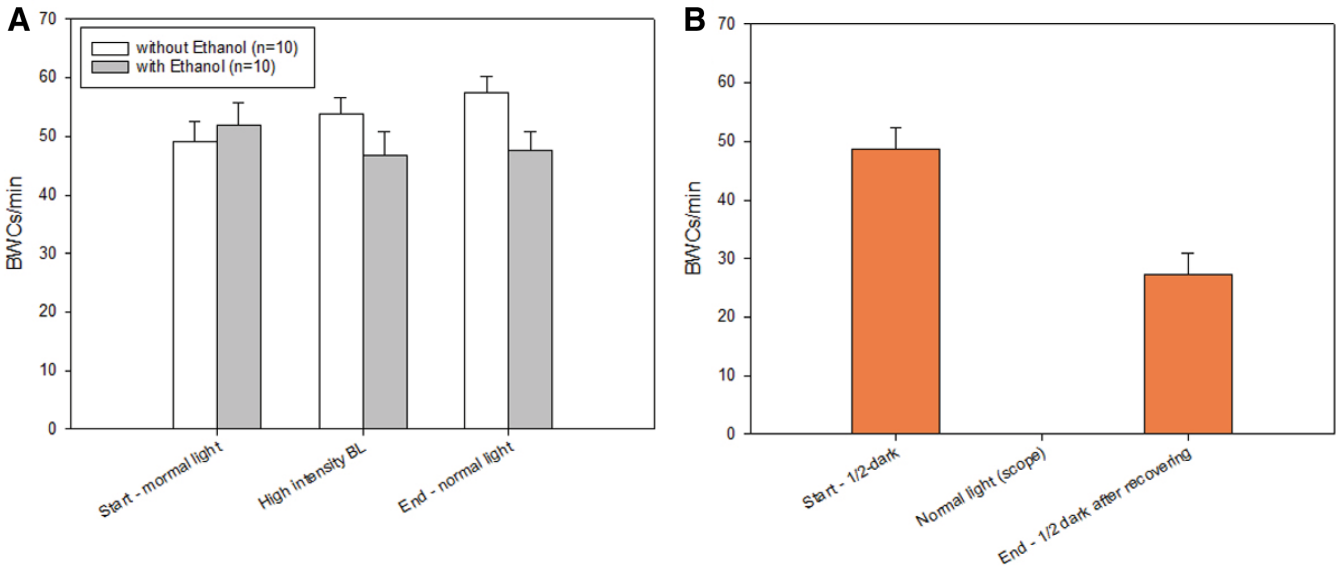

Figure 11. Module trial results: Manipulation of GABAergic neuron electrical activity diminishes locomotor activity in third instar larvae (UAS-ChR2-XXL;Gad1-Gal4). (**A**) The locomotor activity in larvae fed ethanol (vehicle) does not change with either normal light or blue light exposure. (**B**) Although, when the larvae which were raised on food supplemented with ATR 1mM and exposed to white light, the locomotor activity significantly decreased. During the exposure to light the larvae first started to contract which was followed by body muscle relaxation.

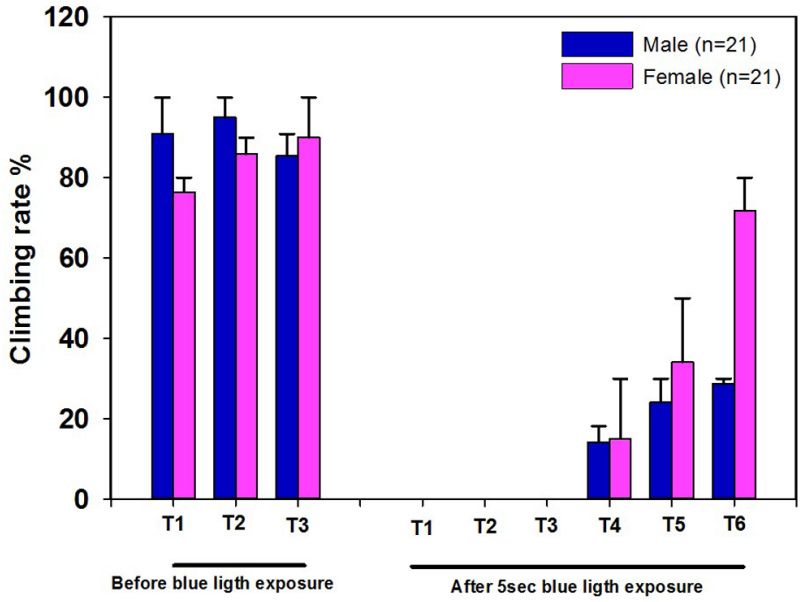

Figure 12. Module trial results: Climbing ability in adult flies expressing ChR2 in GABAergic neurons (UAS-ChR2-XXL; Gad1-Gal4) is compromised after 5sec blue light exposure. The climbing ability was measured in three different trials before the blue light exposure. After 5sec blue light exposure, the climbing ability was performed in six different trials by knocking the flies down to the bottom of the vial without blue light exposure and measuring how many could crawl. ChR2-XXL activation significantly reduced climbing ability. Flies were fed food supplemented with ATR 1mM. The climbing assay was carried out a dim light room since the bright light might also activate ChR2 channels which make it difficult to perform the assay in a well-lighted classroom.

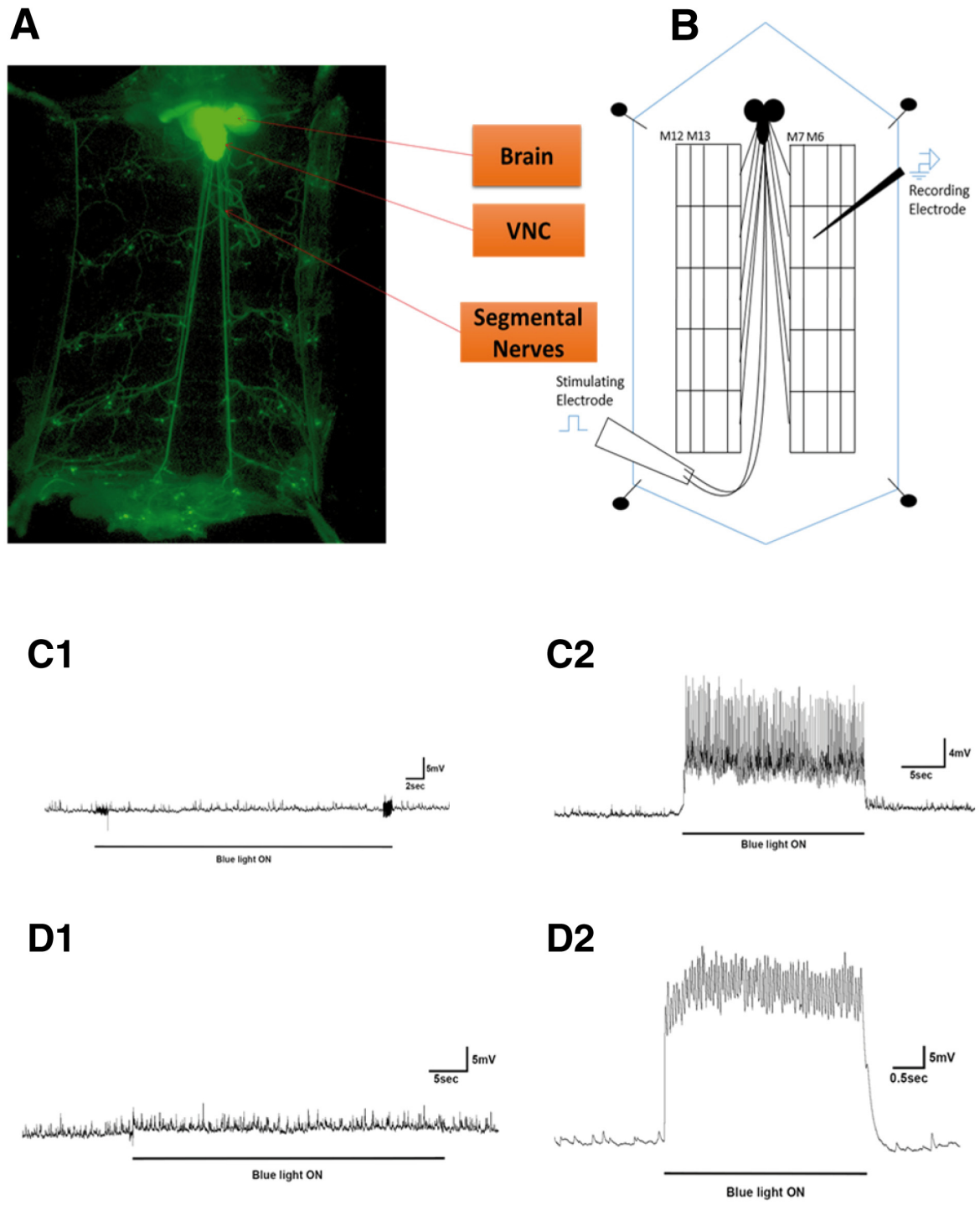

Figure 13. The approach used to stimulate sensory neurons and record synaptic responses from motor neurons on the body wall muscles of a larvae. (**A**) The nervous system in Drosophila melanogaster third instar larvae expression green fluorescent protein (GFP) in the whole nervous system. (**B**) Dissected third instar larvae shows m6 muscle fibers and intracellular microelectrode to record excitatory postsynaptic potentials (EPSPs) while the ChR2 in motor neurons are being activated by blue light exposure. (**C1**) Shows the intracellular recording in OK371-ChR2 minus ATR (CNS intact) third instar larvae. The blue light (low intensity) exposure does not produce any postsynaptic responses in muscle fiber M6 since the larvae is not fed ATR, which is a required supplementation for the action of ChR2. (**C2**) Shows the intracellular recording in OK371-ChR2 plus ATR 1mM (CNS intact) third instar larvae. Blue light (low intensity) exposure produces responses in M6 muscle fiber which is presented as EPSPs. (**D1**) Intracellular recording from M6 fiber muscle in OK371-ChR2 minus ATR (CNS intact) third instar larva. Blue light (high intensity) exposure does not activate motor neurons. No EPSPs are seen in this trace although the miniature EPSPs are still present. (**D2**) the evoked response is being recorded in OK371-ChR2 plus ATR 1mM (CNS intact) third instar larvae while it is being exposed to blue light (high intensity).

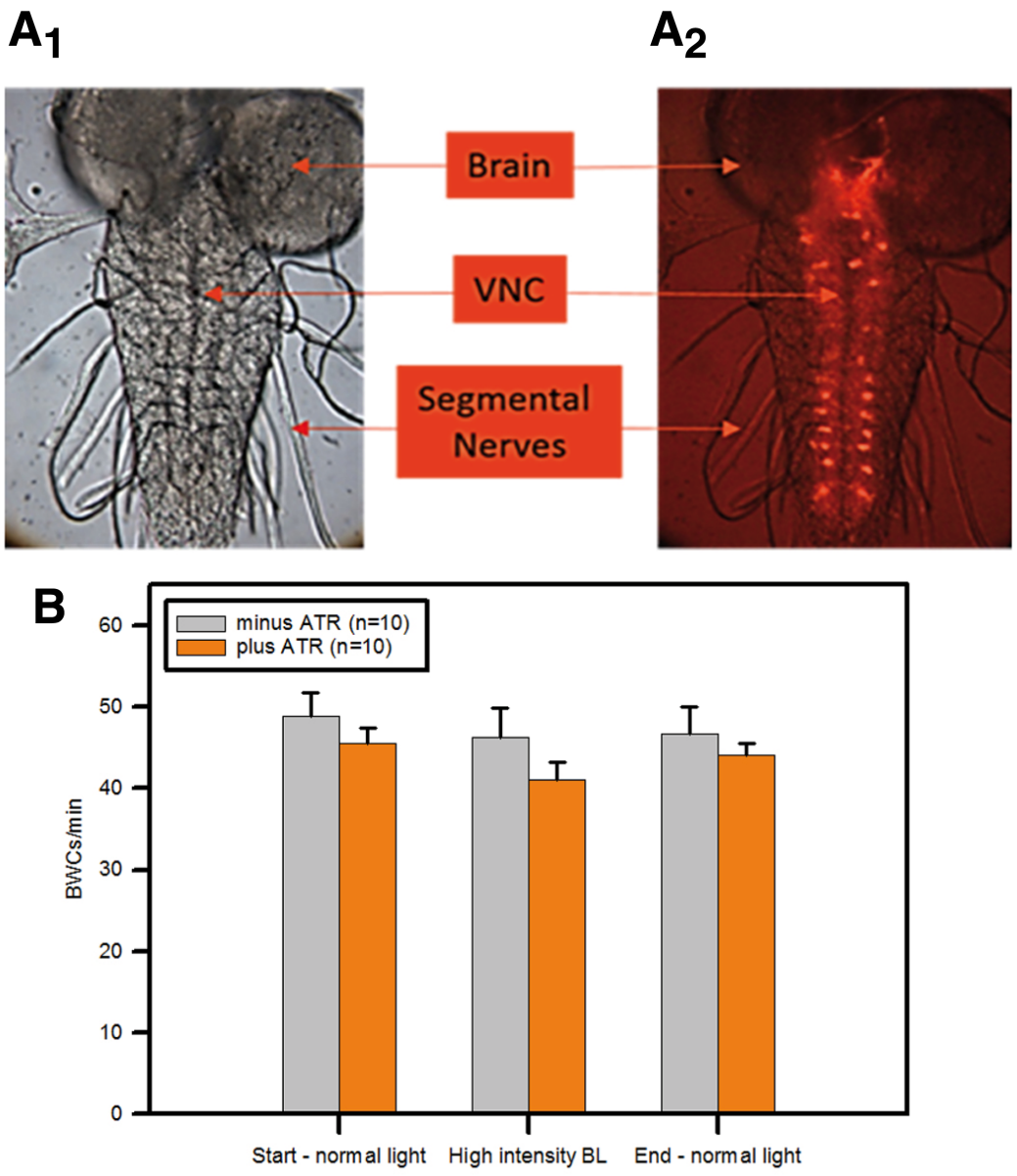

Figure 14. Module trial results of activating serotonin producing neurons with a low sensitivity channel rhodopsin construct. (**A1**) Central nervous system in third instar larva. (**A2**) Serotonergic neurons expressing mcherry fluorescent protein (UAS-mCherry-ChR2 H134R; Trh-GAL4, homozygous for both constructs). (**B**) Activation of 5-HTergic neurons did not produce a significant effect on locomotor activity in third instar larvae.

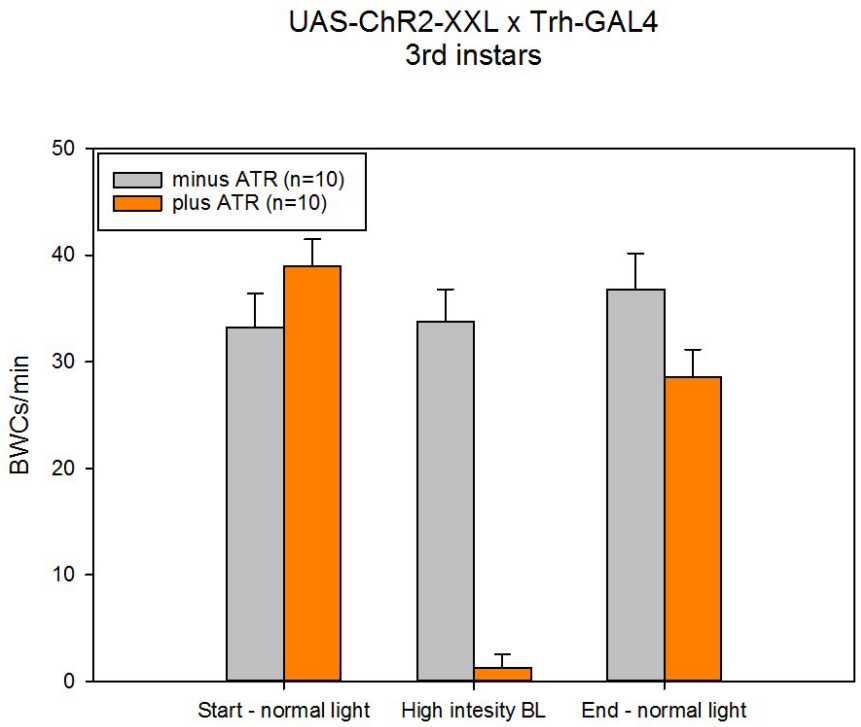

Figure 15. Module trial results: Serotonergic neuron activation modulates locomotor behavior in third instar larvae. To change serotonergic neuron activity, CHR2 were expressed in serotonergic neuron population (UAS-ChR2-XXL;Trh-Gal4). The body wall contractions were counted in third instar larvae fed on food supplemented with ATR 1mM or ethanol (vehicle). When the larvae, which were fed on ATR (1mM) were exposed to blue light (high intensity), the locomotor activity significantly compromised. However, when the larvae fed on a food without ATR supplementation were exposed to blue light (high intensity), the locomotor activity were not affected (as shown in Majeed *et al.*, 2016).

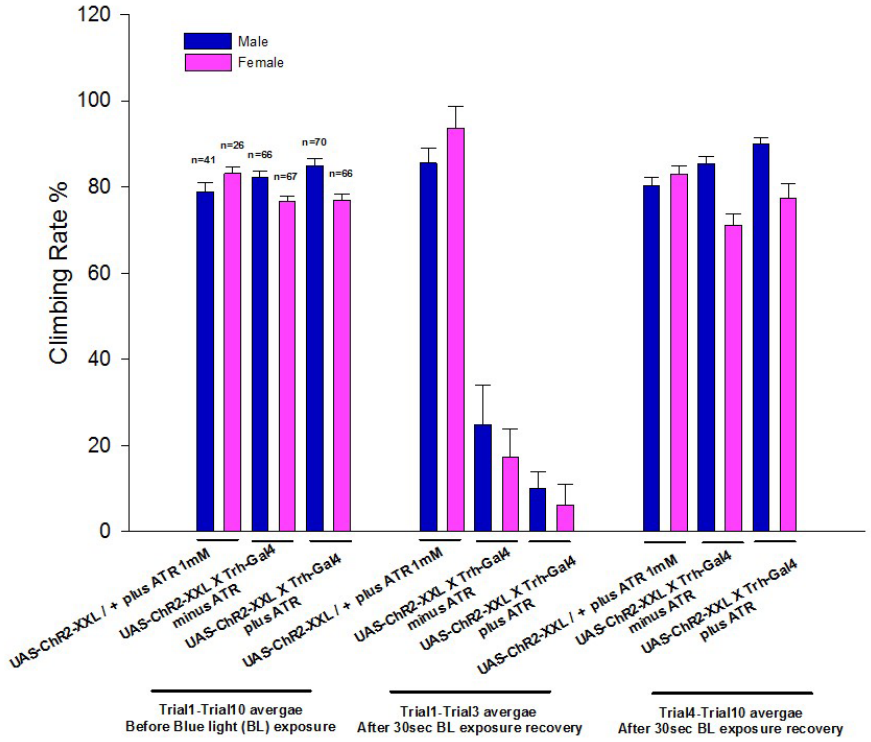

Figure 16. Module trial results: Activation of serotonergic neurons in adult flies decreases climbing ability. The electrical activity in serotonergic neuros is increased by expressing ChR2. When the adult flies were exposed to blue light (low intensity), the climbing ability significantly reduced. Both flies groups (UAS-ChR2-XXL;Trh-Gal4) which were fed supplemented with ATR (1mM) or ethanol (vehicle) were affected by the blue light exposure. However, blue light did not have effect on the control lines (UAS-ChR2-XXl/+) (As shown in Majeed *et al.*, 2016).

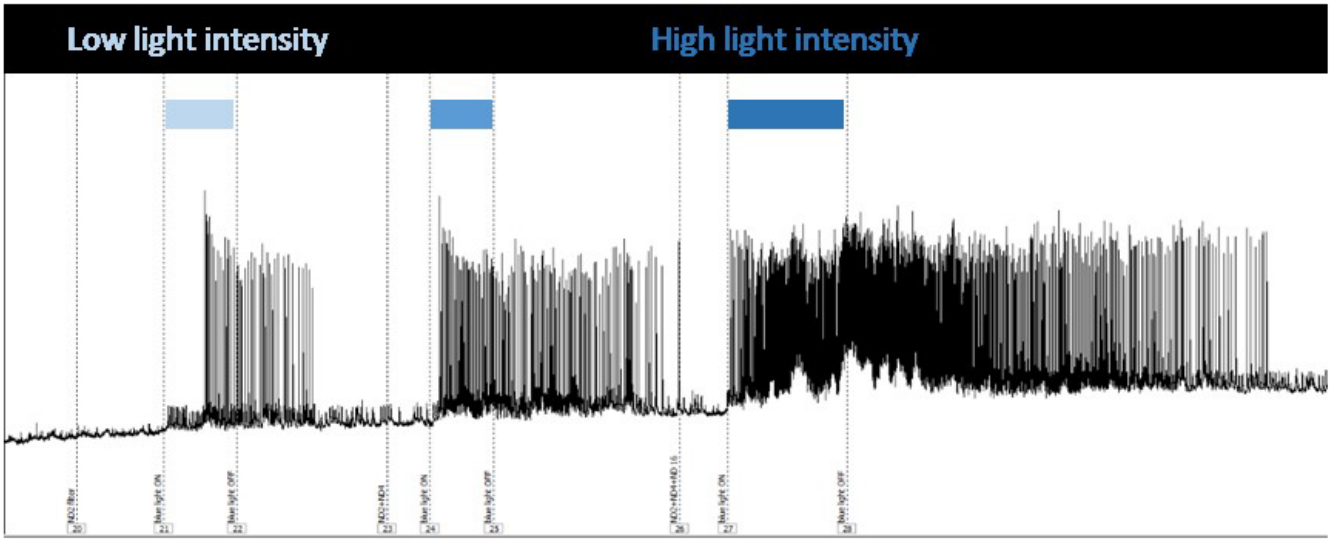

Figure 17. Intracellular EPSPs recording from body wall muscle fiber in third instar larva expressing ChR2 in type IV sensory neurons (UAS-ChR2-XXL;ppk-Gal4, food supplemented with ATR 1mM). Activation of ChR2 in type IV sensory neurons makes motor neurons to fire action potentials which in turn depolarize muscle fibers. The motor output (EPSPs traces) is being recorded while the third instar larva is being exposed to various intensity of blue light (see shading of blue light as intensity).

There are many additional types of behavioral assays that students may develop and try out. Another fun larval behavior is one in which the larvae are lined up on an agar dish and exposed to blue or white light and then the dish is placed in a dark spot or left exposed to dim room light. The larvae with ChR expressed in sensory or motor neurons demonstrate a paralyzed stance, which takes time to recover, as we illustrated in the module trial (Figure 18). The recovery time can be viewed by the movement away from the original line over time. Snap shots with a cell phone camera is an easy way for students to document movements over time.

Electronics and code overview for using the Arduino control

The use of the Arduino hardware and sample codes for flashing the LED light on and off is provided in Supplementary File 1. If instructors or students were so inclined to design experiments using the automated light controls for longer term studies on the effects of pulsing the lights on and off, this system is ideal, due to the low cost and ease in programing various codes. We are now using the system to teach subsets of freshman biology majors concepts of integrating engineering design with biological application at the University of Kentucky in the Department of Biology.

Dataset 1. Raw data for all module trial results

http://dx.doi.org/10.5256/f1000research.10632.d150960

Sigma Plot version 13 files for Figure 4, Figure 6–Figure 8, Figure 10–Figure 12, Figure 14–Figure 16.

Discussion

The exercises presented here promote investigations of practical neurobiological phenomenon in relation to human disorders (Parkinson's, Stiff man syndrome, epilepsy, and autism), as well as promote discussion of potential medical interventions by pharmacological agents on these various neurotransmitter systems. An instructor might even have participants conduct a literature research and make predictions of the behavioral outcomes when stimulating the particular subsets of neurotransmitter systems for the larva and adult *Drosophila* before conducting the experiments on the flies. Establishing a conceptual model of the neurotransmitter and the neural circuits related to the mammalian behavior, and then testing if the model holds for *Drosophila,* is an important concept of the NGSS in the use of models and redesigning to observations (Krajcik & Merritt, 2012; NGSS Lead States, 2013). Titlow *et al*. (2015) and Pulver *et al*. (2011) used optogenetics and neurophysiological recordings with *Drosophila* for a college level educational activity with a similar context, which focused on neural circuits and synaptic function. Furthermore, body wall movements and adult behaviors can be recorded with a webcam (for example, WEBCAM HD4110, Hewlett-Packard Company, Palo Alto, CA, USA) connected to a computer, with a rate of 25 frames per second, for analysis outside of class time. See Titlow *et al*. (2014) for details in recording and analysis from captured data files.

Analysis of the learning and understanding of the module content

Module instructors might wish to conduct pre- and post-assessment surveys, for students to provide their views of the exercises.

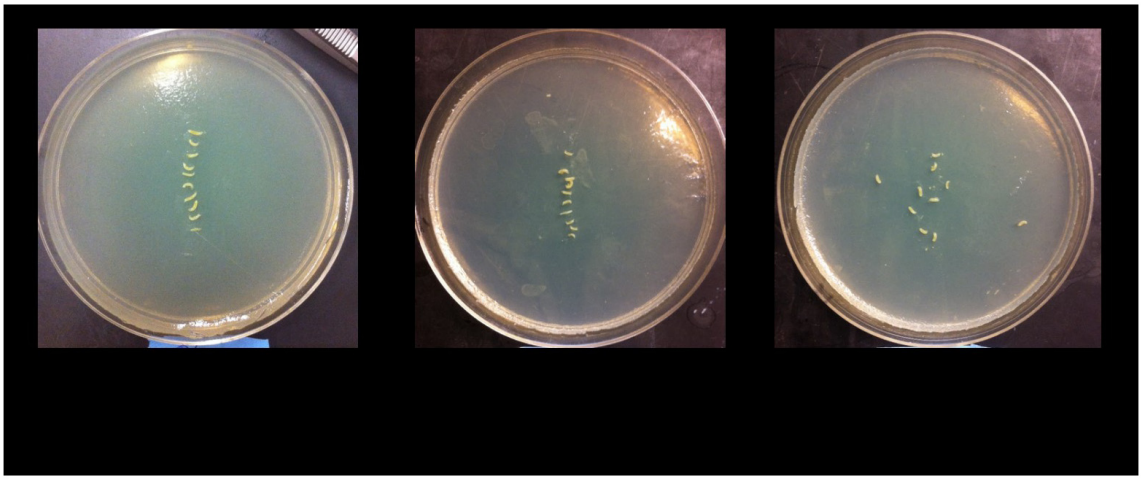

Figure 18. Module trial results: Locomotor activity in third instar larvae expressing ChR2 in motor neurons (UAS-ChR2-XXL;D42-Gal4). When the larvae were exposed to regular light, they were all contracted and did not move (n=30, 10 larvae per agar plate per condition). 10 third instar larvae were placed on an apple juice agar plate. The larvae were exposed to regular light for 2 hours. The larvae stayed in their location without any movement. Three different conditions were used to show how much time it would take for the larvae to start moving again after 2 hour regular light exposure. The data shows that it takes about 15min for the larvae to restore their locomotor activity.

The results of this brief survey is helpful for instructors to know what the students would likely know before the module starts and what might be gained from these exercises, since the module is intended to be an educational activity with in-depth content. A pre-assessment given a day before the laboratory experiments and the post-assessment given a few days after the exercises would be informative for instructors.

The pre- and post- assessment questions in Supplementary File 2 could be useful to future instructors.

In both high school and college settings, a very similar power point introduction of the lab exercises can be shown to students. This introduction should be given after the pre-assessment survey, so the presentation is part of the educational module. Instructors can decide on their own if they wish to use the power point content provided or modify for the level of the participants. The power point presentation we used for the trial module in a high school and a college class are provided as Supplementary File 3.

Instructor feedback after implementing the module

A high school teacher, who has >10 years of experience teaching high school Introductory Biology, Anatomy and Physiology, and Advanced Biology and has level one Biology certification, taught this module to sophomores to about 30 students high school students in Louisville (KY, USA). In addition, the teacher had received a MS in entomology prior to beginning a teaching career. These classes were 50 minutes in length and the class was divided into groups to work with different subsets of the exercises. After the data collection was completed, the various groups shared out the results with other class mates. Groups were divided into a pair of students and each group was given a different line of flies. Some were provided with the sensory lines while others with the motor drivers or serotonin lines. Each group conducted a larval body wall movement assay and an adult climbing assay. A second cohort of 15 senior high school students in Sommerset (KY, USA) was introduced to this educational module. The high school instructors were pleased to expose students to molecular biology in how the fly lines were produced with genetic manipulation, addressing neural circuits and allowing the students to produce various behavioral assays while collected data which later was graphed and discussed as a class. The teachers integrated this content along with teaching the nervous system, which was part of the normal curriculum.

The high school teacher's comments were:

"Sometimes it is hard to focus the light on the larvae crawling on the dish."

"Having the room lights off or very dim while the students work might be difficult to achieve in some classrooms."

"Students might not understand the physiological concepts of how nerves and muscles work until they [have] covered this concept in a biology class"."

These modules were taught in the beginning of the school year in a college level biology major class with juniors and seniors with about 120 students. The college teacher's comments were as follows:

"Implementing this module for a senior college level biology class, which has a laboratory component, provided a different perspective than for high school students. The teaching instructors had a three hour period in one setting to explain and conduct the experiments. An aggregate of instructors' comments were:

"A three hour lab is just about right for this series of experiments if various groups work on different fly lines."

Conclusions

In summary, the presented exercises have been beta tested with students at different educational levels, and these students appear to be learning novel content and have an interest in learning. However, we have not quantified student learning assessments beyond the causal discussion with students. The instructors have provided informative feedback after implementing the activities, so modifications can be made for future classes such as addressing if the room can be dark enough to conduct the assays and if dissecting scopes are available to students to examine the 1st and 2nd instar larvae. The topics presented are rich in physiological history and show how the current state of biotechnology, engineering and science have merged into the ability of controlling the development of defined neural circuits that regulate animal behavior. The future applications for human disease states are just now being probed with this technology of optogenetics, so these exercises should be exciting to students and teachers, if they are made aware in the beauty in the integration of computer coding, biotechnology, and implications for neurobiology by embracing the content presented.

Data availability

Dataset 1: Raw data for all module trial results. Sigma Plot version 13 files for Figure 4, Figure 6–Figure 8, Figure 10–Figure 12, Figure 14–Figure 16. doi, 10.5256/f1000research.10632.d150960 (Majeed, 2017).

Author contributions

ZM, FK, RLC conceived the study. ZM, FK, JM, HA, JW, RLC designed the experiments. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supplementary material

Supplementary File 1: Use of the Arduino hardware and sample codes for flashing LED lights.
Click here to access the data.

Supplementary File 2: Pre and post-test educational sample assessment is provided.
Click here to access the data.

Supplementary File 3: Power point presentation used to introduce the exercises for the participating classes. The content can readily be modified by the instructor according to the level of instruction required.
Click here to access the data.

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Version 1

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? **Michele L. Lemons**
Department of Natural Sciences, Assumption College, Worcester, MA, USA

This manuscript describes several transgenic fly strains and numerous behavioural assays that can be used to challenge students to understand connections between cellular activity and behaviour. The strengths of this approach are numerous and include the ability to observe rapid changes in fly behaviour following exposure to blue light. Thus, students are able to observe changes in behaviour within a single lab session or class. Another advantage of this approach includes the relatively low cost associated with conducting the proposed experiments. The authors describe how to make low-cost behavioural instrumentation, which is useful. In addition to these advantages, I have several questions and concerns, which are listed below.

Title:
In the title of the manuscript, the phrase “NGSS-neurons” is unclear. It could give the impression that NGSS is a type of a neuron. The authors state in the manuscript that NGSS stands for Next Generation Science Standards.

Article content:
This manuscript mentions several fly strains and numerous behavioural techniques that could be performed under a variety of conditions (*e.g.* focused light, diffused light, pulsing light patterns) on various ages of flies. In addition, the strains listed have various subsets of neurons that are specifically stimulated by blue light, adding another possible variable. The combination of possible experiments seems nearly endless, which is exciting. While the number of possible experiments is impressive, it can also be confusing, particularly from the perspective of someone who is not familiar with *Drosophila*. Considering this is a pedagogical-themed paper, it would be very helpful if: 1) the manuscript could be re-organized with this comment in mind and 2) at least one or two specific lesson plans would be provided. The lesson plans could give the reader an example of a specific subset of techniques that could be used during one, three-hour undergraduate lab, for example. The authors state that experiments have been executed in an undergraduate teaching lab and in a high school setting. It would be extremely beneficial to share hand outs used during these lessons and/or instructors’ notes. Similar to other pedagogical papers, it would also be helpful if authors include specific learning objectives in the body of the manuscript. In this regard, some of the points addressed in the PowerPoint slides could be helpful if more detail was provided.

In the introduction, it would be very helpful to include: 1) a description of the subsets of neurons (*e.g.* type IV sensory neurons/pickpocket neurons, GABAergic neurons, etc..) that are activated in these proposed experiments, 2) diagram/explanation of the circuits that are tested in these experiments and how activation of these circuits induces behavioural changes and 3) explanation of optogenetics with a focus

on the ChR2 model used in this manuscript. Data in Figure 17 could be used to demonstrate the ability of blue light to cause EPSP in specific subsets of neurons. The reader would benefit from learning about the neurons that are excited by blue light, where these neurons project and how ChR2 works in these fly strains. Some information that is currently in the introduction could be removed.

Please provide additional explanation regarding tables, such as Table 2. There are two main points that could be clarified. First, why is “soda can” written three times in the table? What is the difference between “soda can” and the term “motor neuron” towards the top of Table 2 and the term “soda can” and “motor neurons” written approximately in the middle of Table 2? Similar questions arise for “focused light.” I understand that a soda can is used with an LED light to generate “unfocused light” while an eye piece of a microscope is used to generate “focused light.” However, I don’t understand why these terms appear multiple times in the tables, with similar subheadings, such as motor neurons. Secondly, it is not clear *how* students would complete such a table and how this data would be graphed and interpreted. For example, does a student write “CC” on the Table each time “continued crawling forward” behaviour is seen? Or is it a binary reporting system where one reports CC as occurring or not? What if multiple behaviours or seen? Are the numbers of behaviours recorded? It is not clear how data in Table 2 would be graphed, displayed or analyzed. The authors state the data could be graphed similar to that shown in Figure 4, but it is not clear where “CC” and other codes are represented in Figure 4. Is “CC” a kind of rolling behaviour? How was rolling behaviour occurrence calculated in Figure 4A? Please provide additional details. Similar questions apply to Tables 3 and 4.

Data in Figure 4 reveal behaviours of transgenic flies subjected to blue light. How do flies without blue light or without ATR behave? Controls would clarify the impact of activating type IV sensory neurons on rolling behaviour. Similar questions regarding the need for controls arise for data in other Figures (such as 6, 7, 12 and 18.) It would be helpful to use and explain the importance of controls and the variety of controls available (*e.g.* absence of ATR, vehicle (ethanol) only, absence of blue light without ATR, absence of blue light with ATR, etc…) in these experiments.

The authors mention that the Arduino system can be used to control stimulation of light. A supplemental file with sample codes is provided under supplemental material. Please provide at least one example of how the Arduino system could be used in any of the proposed techniques. What are examples of light stimulation patterns that produce distinct behaviours compared to single light exposure? Which of the sample codes would generate distinctive rolling behaviour, for example?

This manuscript could be significantly enhanced by including pre and post assessment of *students*. An assessment file is provided as a supplemental document, but was not used. In the discussion the authors state that students appear to be learning novel content, but this is not documented. An assessment piece would confirm the ability of this module to enhance student learning. The feedback from instructors was very limited. Additional feedback would prove helpful.

Overall, the manuscript could be improved by writing in an active voice and fixing several typos and grammatical errors.

Minor points:
A few examples of typing errors include on page 3…. “neurotransmitter GABA (glutamate, serotonin and acetylcholine).” Why are three neurotransmitters listed in brackets after the term “GABA”? Do the authors mean to write neurotransmitters such as GABA, glutamate, serotonin and acetylcholine?

In Table 2, the term “focuses light” and “focused light” are used. Perhaps the “s” in “focuses light” should

be a “d” instead of an “s”? The same is true for other Tables. In Figure 3, the term “foe” is written instead of the word “for”. There are additional typos in the manuscript. Please address those.

In the module overview, the description of: 1) all-trans-retinal preparation and 2) the preparation of the fly food supplemented with ATR, is very clear. However, the level of detail regarding the genetic crosses that are suggested should be elevated to match the level of detail of other techniques/procedures. References should be cited to document methods.

Under “larval locomotion behaviour”, authors mention that asking students to conduct tests in dimly lit or dark rooms could be challenging. This seems to stem from a limited set of responses from teachers. Please address how this challenge could be addressed in the classroom to help teachers who wish to use this module.

Please clarify how the % of movement was calculated in Figures 6 and 7. Is this the % of worms that were moving?

Please clarify what time points images in Figure 18 were taken. The figure caption does not specify times points across the three images.

Should all BWC data be displayed in sequential figures? For example, BWC data is shown in Figures 3, 10, 11, 14 and 15. It could be advantageous to present BWC data in sequential figures rather than in 5 figures spread throughout the paper. Please comment.

Figure 17 demonstrates that blue light induces EPSPs in type IV sensory neurons expressing ChR2. This is convincing data that demonstrates the power of optogenetics and in particular, the efficacy of ChR2 in these sensory neurons. This could be powerful to show in the beginning of the manuscript to document that the blue light is causing EPSPs within the neurons, as predicted. This is the basis of why blue light induces a behavioural change in these flies.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 02 March 2017

doi:10.5256/f1000research.11456.r20049

? **James E. Jepson**
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This Research Note by Majeed et al. is a comprehensive educational module for high school and college students, focusing on optogenetic modulation of *Drosophila* locomotor behaviour (either larval or adult) using a combination of channel-rhodopsin effectors and neuron-specific driver lines.

The methods and results are clearly presented, detailed and economically viable to initiate. Teaching

students using this approach will expose them to the scientific method and directly observe a link between neuroscience theory and its application. As such, I support this module, but have several suggestions below for how it might be improved:

1. The introduction requires re-writing. My main critique is that the topics discussed in the introduction do not match the experiments in the main text. All experiments focus on acute modulation of behaviour by activating subsets of the fly's neuronal repertoire. However, the introduction focuses more on the link between activity and neuronal development. However, neurodevelopment in *Drosophila* (e.g studying synaptic growth at the larval NMJ) is not a component of this module. It would be more appropriate to discuss how different circuits in the mammalian and fly brain regulate specific behavioural patterns, and how activity within these circuits can be manipulated by researchers using opsins etc. The sentence '...GABA (glutamate, serotonin, acetylcholine)...' in paragraph 5 might lead students to think that GABA is an acronym for 'glutamate, serotonin, acetylcholine' - this should be re-worded. The grammar in the first paragraph could also be improved.

2. The methods related to fly strains and crosses could be expanded. For example, details of the fly life cycle would be helpful, and to stress the temperature-dependence of the generation time. If an instructor or a student is setting up crosses, they need to know when the progeny will be ready to experiment on: this in turn will depend on how the flies are housed. For sexing, while testing for the presence of sex-combs is indeed reliable, memorising the differences in male/female abdominal anatomy is a much more rapid method. Will the students/examiners know the difference between a virgin and non-virgin female? Methods to discern this could be described, or at least referenced. The fly food recipe could be referenced too. Finally, two of the transgenic fly strains (58374 and 51630) have the CyO balancer floating, so it may be useful to mention that these need to be used as non-*curly* homozygotes for larval experiments when setting up crosses.

3. The authors give some nice examples of electrophysiological recordings from muscles during optogenetic stimulation of motor- or *ppk*-neurons. Are these recordings from their lab or from the literature (if so, a reference would be appropriate)? More importantly, as mentioned by the high school teacher, these results might be difficult for high school students to understand if they have not been taught the basics of neurotransmission. Could the authors more clearly delineate which data are more appropriate for high school vs. college students?

4. In the legend of Figure 8, the authors state that type VI sensory neurons were studied, but I think they mean type IV.

5. In Figure 13B, the schematic shows the stimulation pipette injecting current into the CNS, whereas it should be the other way round: the segmental nerve should be innervating the muscle and have the electrode attached at the proximal end relative to the CNS.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 13 February 2017

doi:10.5256/f1000research.11456.r20045

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This article describes an educational module that incorporates the utilization of sophisticated optogenetic techniques but employs fairly low-cost, easily obtainable subjects and apparatus. This article makes the exercises described very accessible to institutions of almost every size and budget.

Absolutely stunning details are provided from fly feeding, care, and sexing to constructing the apparatus and Arduino codes. These details not only should allow reconstruction of the experience at various high schools, colleges, and universities but also should allow students and instructors to go further and design their own questions and experiments.

Supplementary materials, including PowerPoint slides and a pre/post evaluation instrument, are provided for instructors to use. No data from this pre/post instrument are presented in the article, however, which is the article's biggest weakness. Rather, the pedagogical data provided consist of a series of qualitative statements, mostly from high school instructors.

The introduction needs to be re-written. It should more explicitly relate issues raised about the development of sensory and motor systems to the Drosophila literature rather than to the mammalian literature. Stylistic alterations, such as avoiding passive voice and multiple clauses, would render it more readable and intelligible. Similarly, the first sentence in the discussion seems inappropriate, considering the actual scope of the model.

Some figures need to be re-worked to be more informative. The rolling behavior data displayed (Figure 4) would make a more powerful point if there was some comparison with different lighting conditions or comparing different lines of flies with different constructs. Similarly, Figure 7 would make its point more forcefully if two conditions were displayed on a single graph, say with and without light exposure, such as in Figures 8 and 12.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.