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A Versatile Semester-Long Course-Based Undergraduate Research Experience using Optogenetics and RNAi to Identify Genes Important for Synapse Function

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Compared to traditional teaching laboratory activities, course-based undergraduate research experiences (CUREs) can increase student engagement and confidence, improve scientific literacy, enhance critical thinking, and promote accessibility in STEM. Here we describe a versatile CURE for an upper-level Neurobiology course that incorporates genetic, molecular, cellular, and behavioral experiments into a semester-long investigation to identify genes important for glutamate synapse formation or function in *C. elegans*. Following introduction to the CURE approach and basic *C. elegans* techniques, students construct their own low-cost optogenetics rigs, which we describe in detail here, to activate a mechanosensory escape reflex via photostimulation. They then perform a small-scale RNAi screen with this light-activated behavioral readout. Once a gene of interest is identified, students submit a proposal to investigate the role of this gene in nervous system function and spend the rest of the semester carrying out follow-up experiments using mutant strains. We also describe ways in which this CURE can be modified depending on the pedagogical objectives, availability of materials, or research interests of the instructor. Participating in this lab significantly enhanced students' abilities to see themselves as STEM professionals and prompted students to report substantial gains in skills critical for entry into and success in graduate and medical schools. In addition to the benefits CUREs provide to students, faculty benefit from the generation of preliminary data and training of students for potential independent research projects.

Key words: Course-Based Undergraduate Research Experience; (CURE); Caenorhabditis elegans*; optogenetics; low-cost; RNAi; behavior*

Course-based undergraduate research experiences (CUREs) are opportunities created to involve whole classes of students in collaborative and iterative explorations of the scientific process to discover new information about relevant topics (Auchincloss et al., 2014). CUREs in a variety of scientific fields can increase student confidence, improve scientific literacy, and enhance critical thinking (Thiry et al., 2012; Corwin et al., 2015; Staub et al., 2016). Moreover, the use of CUREs has been proposed as a way to promote accessibility to authentic scientific inquiry and increase student success in STEM (Thiry et al., 2012; Bangera and Brownell, 2014; Corwin et al., 2015; Staub et al., 2016).

Some recently published CUREs focus on analysis of existing datasets (Nahmani, 2019; Grove et al., 2021; Ryan and Casimo, 2021; Wickham et al., 2021). This approach is incredibly valuable for online or hybrid courses, situations where access to experimental equipment is limited, or where hands-on wet lab experimentation skills are not part of a central element of the learning objectives. Though some excellent examples exist (Kowalski et al., 2016; D'Arcy et al., 2019; Delventhal and Steinhauer, 2020; Himmel et al., 2020; Mesmer and Gaudier-Diaz, 2022), there is a need for more semester-long, hands-on CUREs designed for Neurobiology students that teach a range of applicable and interdisciplinary skills.

We set out to create a standalone, semester-long, authentic research experience that incorporates the messiness of science including troubleshooting, protocol optimization, and experiment replication, as well as data presentation that can prepare students not only with the skills, but also the mindset for future research. We also aimed to determine whether student experience in this CURE influences their attitudes about science, sense of growth in STEM areas/domains, and identity as a part of the scientific community.

The roundworm, *C. elegans,* has been extensively used as a model system in undergraduate teaching labs, and the many benefits of using *C. elegans* in these settings are well-documented (Lemons, 2016; Pokala and Glater, 2018). In particular, and of relevance to this CURE, *C. elegans* have a low operational cost, a wealth of available resources including RNAi libraries and mutant strains, and quantifiable behaviors that correlate with the activity of specific synapses. *C. elegans* is an excellent gene discovery platform for genes involved in nervous system function as they can tolerate severe reduction in nervous system activity, and many *C. elegans* genes are conserved in mammals. Consequently, *C. elegans* has been used to model human diseases for the *de novo* discovery of human disease genes (Bargmann, 1998; Apfeld and Alper, 2018). Additionally, with *C. elegans* it is simple to perform experiments that incorporate genetics, molecular and cellular biology, and behavioral testing thereby highlighting the interdisciplinary nature of Neurobiology.

One important aspect of CUREs that distinguishes them from traditional lab courses or even inquiry-based labs, is

that students are given an opportunity to contribute to new discoveries and make meaningful contributions of interest to the broader scientific community (Auchincloss et al., 2014). Robust synaptic transmission requires the proper function of presynaptic and postsynaptic proteins whose abundance, localization, and activity must be tightly regulated. While many genes are known to be important for these processes, there are undoubtedly new roles to be discovered. The majority of our approximately 100 trillion synapses use the chemical glutamate as a neurotransmitter to signal between presynaptic and postsynaptic cells (Mennerick and Zorumski, 2012), and it is difficult to overstate the importance of proper glutamate synapse function. The scientific question at the heart of student research for this CURE is: What genes contribute to the formation or function of glutamate synapses?

To address this question, students use the *C. elegans* as a model system to (1) carry out a genetic screen to reduce the expression of individual genes and (2) determine the effect on a specific glutamate-dependent behavior. Once they have identified a candidate gene, they then (3) perform follow-up experiments to learn more about how it affects the nervous system at the molecular, cellular, and organismal level.

C. elegans exhibit a glutamate-dependent sensory-motor behavior known as the nose-touch escape reflex. Gentle mechanical stimulation of the worm nose, traditionally done in the lab using a human eyelash taped to a stick, activates, among other cells, a pair of sensory neurons called ASH neurons. ASH neurons release glutamate that activates glutamate receptors including GLR-1 to cause depolarization in postsynaptic interneurons and ultimately backward movement away from the stimulus (Kaplan and Horvitz, 1993; Hart et al., 1995; Maricq et al., 1995). While eyelash-induced stimulation is effective, it can be tedious, and somewhat subjective especially for those learning the assay. On the other hand, optogenetic activation of ASH induces locomotion reversals that are less subjective to monitor (Nagel et al., 2005; Ezcurra et al., 2011). Additionally, students are excited to use cutting-edge optogenetics to activate neurons with light. Using worms that express blue light-activated channelrhodopsin in ASH neurons allows us to robustly activate ASH neurons and the escape reflex even in whole populations of worms with temporal precision. Though we present an approach here that is tailored for investigating glutamate synapse function, the overall structure is versatile and, as will be discussed later, could be adapted if other *C. elegans* strains are used.

Lab modules have been developed that use optogenetics to investigate *C. elegans* behavior and synapse function (Pokala and Glater, 2018; Rose, 2018). Other CUREs or lab activities use RNAi in *C. elegans* to teach about the connection between gene expression and phenotype (Andersen et al., 2008; Kitt, 2023). The semester-long experience we present here combines those approaches in an authentic research format in which neither the student nor the instructor knows how the experiments will turn out. Conducting such novel experiments has been shown to increase student engagement in teaching laboratories (Wiseman et al., 2020). Only by enhancing engagement and exposing new populations of students to authentic research can we hope to create a more diverse and inclusive scientific community (Bangera and Brownell, 2014).

CURE DESIGN AND IMPLEMENTATION

Course Context

BIOL 334 Neurobiology is an in-person undergraduate course for juniors and seniors taught at Simmons University by author ESL. The class typically enrolls 32 students and is divided into two lab sections of 16 that meet weekly. Each lab of 16 students is divided into groups of 4 that work together throughout the semester.

Student Participants and Data Collection/Analysis

This pedagogical study was reviewed by the Simmons University Institutional Review Board and was granted exemption. Data for this study was collected during the Spring 2022 semester. Thirty-one (31) students were enrolled in the course for the entirety of that semester, but only 20 students completed both the pre- and post-lab surveys (see below). Those students were working to complete the following majors: Neuroscience and Behavior (16/20), Biochemistry (2/20), Biology (1/20) and Exercise Science (1/20). Fifty-five percent (55%) of students were in their junior year, and 45% were in their senior year. Simmons University is a women-centered institution: nineteen (19) students identified as female, and one (1) identified as non-binary. In terms of race or ethnicity, 55% identified as white and 35% identified as belonging to a PEER (Persons Excluded due to Ethnicity or Race) group (African American: 10%, Asian American: 10%, Hispanic or Latinx: 10%, two or more races: 5%). Additionally, one (1) student identified as a foreign national, and another preferred not to answer.

At the beginning and end of the semester, students were asked to complete surveys via Google Forms. Student-generated anonymizing codes were used to match pre- and post-lab responses (Audette et al., 2020). Surveys contained a modified version (Wickham et al., 2021) of the CURE survey for which students rate their attitudes towards science and their perception of their research experiences and skills (Lopatto, 2004; Lopatto et al., 2008). In addition, students completed a single multiple-choice STEM professional identity overlap measure in which students select the image that best represents how they view their own identity overlaps with the identity of a STEM professional (McDonald et al., 2019). We compared the results from this CURE to an aggregated set of 18,062 pre/post-CURE responses collected from multiple institutions between 2015 and 2018 (Lopatto and Jowarski, 2018). Statistical analysis of anonymized student responses was performed using Prism 9 software (GraphPad).

Learning Objectives

Students are often used to lab classes in which each week is associated with a new topic and set of skills. They may initially be skeptical of a course in which one project is meant to last the entire semester and have the misconception that they will only cover one topic or learn one set of skills. In our experience it is beneficial to explicitly address at the start of

Domain	Learning Objective
Neurobiology Content	Use RNAi to discover functions of neuronal genes
	Explore the molecular basis of behavior
	Perform behavioral tests to assess the function of neurotransmitter systems
	Activate neural circuits using optogenetics
	Visualize neuronal morphology and sensory structures
	Quantify synaptic protein levels in living animals
	Understand how genetics and molecular biology contribute to neurobiology research
Scientific Processes	Read and analyze primary literature
	Generate hypotheses and the means to test them
	Determine appropriate controls for a given experiment
	Display and satistically analyze collected data
	Develop concise oral and written communication skills

Table 1. Learning Objectives. This CURE covers a range of discipline-specific Neurobiology Content and broadly applicable skills related to the Scientific Process.

the semester the range of concepts and skills students are expected to learn in order to maximize student buy-in early on. Some learning objectives pertain to discipline-specific neurobiology content, but our primary objectives for this course relate to scientific processes more broadly (Table 1), We focused our analysis here on these process-related skills, as done previously using the CURE survey (Lopatto, 2004; Lopatto et al., 2008), so we could also compare our CURE to others (Lopatto and Jowarski, 2018).

CURE Description and Student Research Activities

The research objective of this semester-long laboratory was to identify and characterize new roles for evolutionarily conserved genes in synapse function and behavior. This CURE was divided into three (3) phases: Introduction and Training, RNAi Screening, and Follow-up Experiments. Each of these phases consists of Discussion Topics, Activities, Associated Material (either readings or videos) and Assessments (Table 4). Based on the scope of the experimentation, students must collaborate and divide up the work, though how they do so is up to them. Students are instructed to try each procedure at least once, after which they can specialize in different techniques for more efficient data collection, similar to how such collaborations would play out in a research environment. All techniques used throughout the CURE are introduced in some form during the first two phases, and only after this point are students able to specialize.

Phase One: Introduction and Training

In this first phase, the instructor introduces the overarching plan for the semester from both a scientific and pedagogical perspective. The scientific portion includes background on RNAi, optogenetics, and genetic screens (and their use in *C. elegans*) as well as the glutamate-dependent behavioral reflex circuit students will be investigating.

This phase also includes an introduction to the theory and techniques involved in *C. elegans* husbandry including worm picking (see www.wormbook.org and Lemons, 2016 for detailed information on picking), an example of the effects of systemic RNAi using *dpy-11* RNAi (Ko and Chow, 2002), and training in how to perform the optogenetic behavioral assay of ASH-mediated locomotor reversals. As part of this phase, students are expected to read a brief introduction to feeding-induced RNAi by Timmons and Fire (1998), as well as excerpts from review articles on genetic screens by Jorgensen and Mango (2002) and Sin et al. (2014) and excerpts from primary resources on enhanced neuronal RNAi (Calixto et al., 2010) and optogenetic activation of ASH neurons (Ezcurra et al., 2011).

Phase Two: RNAi Screening

In this second phase, students perform a small scale, one generation RNAi screen (Figure 1) by feeding adult worms bacteria expressing double stranded RNA (dsRNA) targeting genes of interest and assessing their offspring for phenotypes. In this screen they knock down the expression of eight (8) different genes and compare to both an empty vector negative control in which bacteria do not express dsRNA and an *eat-4* positive control. The *eat-4* gene encodes a vesicular glutamate transporter necessary for glutamate packaging and release from synaptic vesicles (Lee et al., 1999). For a detailed RNAi protocol, see Supplement 1. Students examine how gene knockdown affects ASH-mediated, glutamate-dependent reversal behavior and glutamate-independent neuromuscular junction activity as measured by worm thrashing (Figure 1).

In preparation for the third phase of the CURE, students practice preparing slides of immobilized worms, performing fluorescence microscopy and using FiJi software (Schindelin et al., 2012) to analyze images, Toward the end of this phase, the list of genes they have been testing is revealed and the class explores the online *C. elegans* database Wormbase.org to learn more about their genes. Students are introduced to the molecular biology software SnapGene Viewer (Dotmatics) and are taught to import and annotate gene sequences and design PCR primers flanking a mutation to be used when conducting genetic crosses.

Based on their preliminary RNAi screen data, students choose a gene of interest and as a small group submit a proposal for the second half of this semester (see *Assessments*). In this proposal, groups plan specific follow-up experiments to be completed using a loss of function

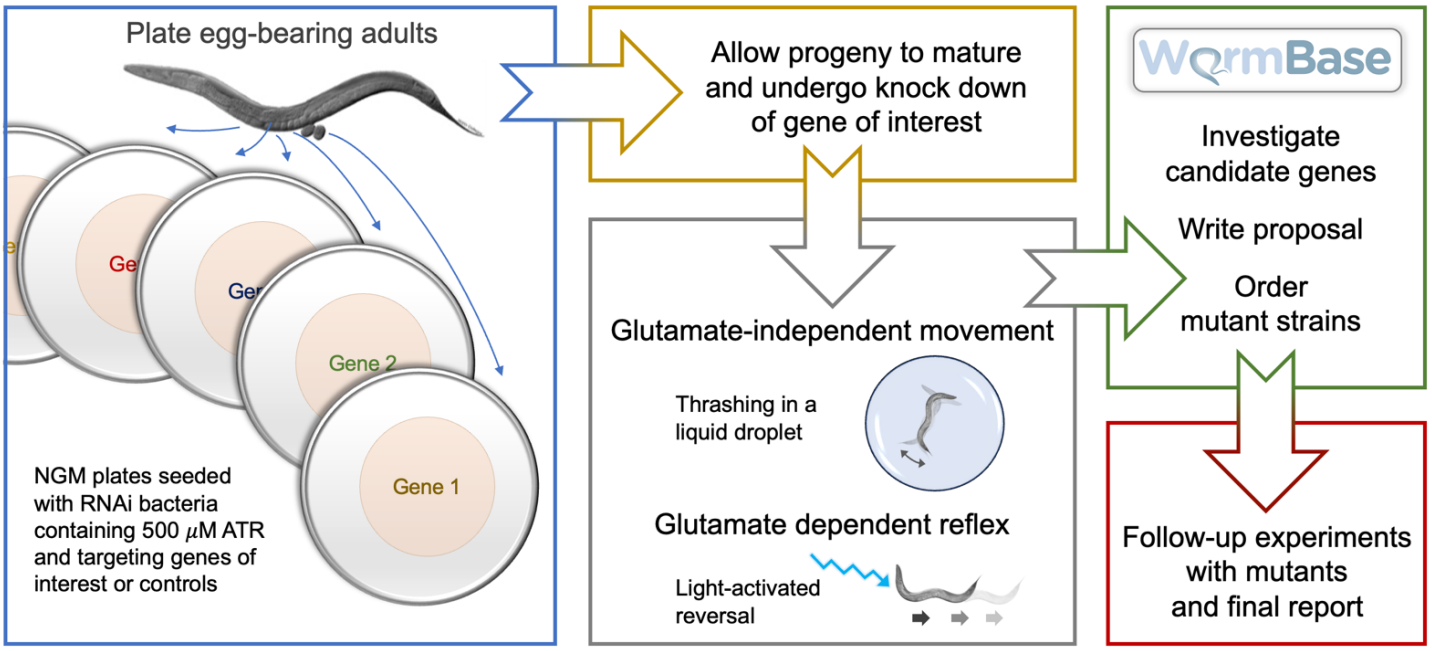

Figure 1. RNAi screen overview. Egg-bearing worms are added to standard worm growth plates containing RNAi bacteria and all-trans retinal (ATR), a necessary cofactor for the light-responsive channelrhodopsin (ChR2) protein. One week later, offspring with genes knocked down are scored for glutamate-independent gross locomotor activity (thrashing) and glutamate-dependent reflex behavior, which is triggered by blue light activation of ChR2 in sensory neurons. Based on the results of these behavioral tests, students identify and investigate a candidate gene for which a mutant strain is then ordered from the Caenorhabditis Genetics Center.

mutant strain that is available from the Caenorhabditis Genetics Center (CGC). In some cases, genes of interest may need to be selected based on strain availability. Groups are given a list of possible follow-up experiments from which they are instructed to choose a "now" experiment and a "later" experiment. The "now" experiments are typically behavioral tests that can be performed without the need for a genetic cross. The "later" experiments involve either optogenetics or measurement of a fluorescent marker of choice. To determine the effect of a mutation on the light activated reversal response or on the expression of a fluorescent protein, students must perform a genetic cross between worms with their mutation of interest and transgenic worms with light-activatable or fluorescent proteins. This generates worms with both a transgene and a mutation, which can then be compared to transgenic worms without the mutation.

Phase Three: Follow-Up Experiments

In this third phase, groups work to complete their proposed experiments and present their findings. This may include performing a genetic cross and genotyping using PCR/electrophoresis, additional behavioral assays chosen by groups, microscopy of fluorescent transgenes, or even additional RNAi experiments. Students also generate figures and tables for their final paper (see *Assessments*). During the final session of the semester, students participate in a jigsaw-style presentation activity (Baken et al., 2022) in which student groups are shuffled so that each new "Expert Group" contains one member of each lab group. After students take turns presenting individually on their lab group's findings, lab groups are reassembled (now considered the "Learning Group"), and students share what they learned from their peers' presentations. Though we have not implemented it thus far, a peer review rubric could be used in this step to assess student presentations.

Because this third phase begins after students return from Spring Break, it is helpful to start with a discussion reviewing the overall lab objectives, the biological questions addressed, and approaches used for individual experiments. In this instructor's experience, it is also clarifying for students to discuss the distinction between working with RNAi vs. mutant strains including the advantages and disadvantages of each approach. Following a tutorial on how to perform a genetic cross, students are given a virtual cross worksheet to apply their knowledge and identify any areas of confusion. We also include discussions related to PCR theory and practice, data visualization, and statistical analysis during weeks aligning with student progress. No additional readings have been assigned during this phase due to the emphasis on primary literature in the lecture section at this time.

Assessments

Most assessments are in the form of group laboratory notebook checks (shared Google Doc), but groups also submit a mid-semester proposal and final paper. Using Google Docs has several advantages over traditional lab notebooks for students and instructors. For students, it provides a way for all group members to update the same notebook simultaneously while collecting data for different experiments, allows for simple incorporation of images (ex: from fluorescence microscopy or of DNA gels), and acts as an easily searchable record of their experimentation.

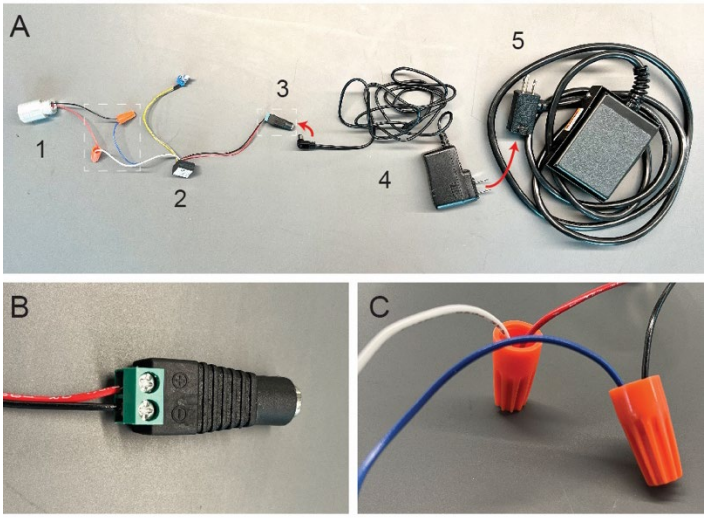

Figure 2. Assembly of the LED illuminator. *A)* LED components: *1.* LED light kit *2.* LED driver *3.* Power adapter *4.* Wall mount switch *5.* Foot switch. Boxed regions are represented in B and C. Red arrows indicate additional connection sites. *B)* Red and black wires from the LED driver should be connected to the + and – terminals, respectively, of the power adapter. *C)* LED driver and light kit should be wired so that black is connected to blue and red to white.

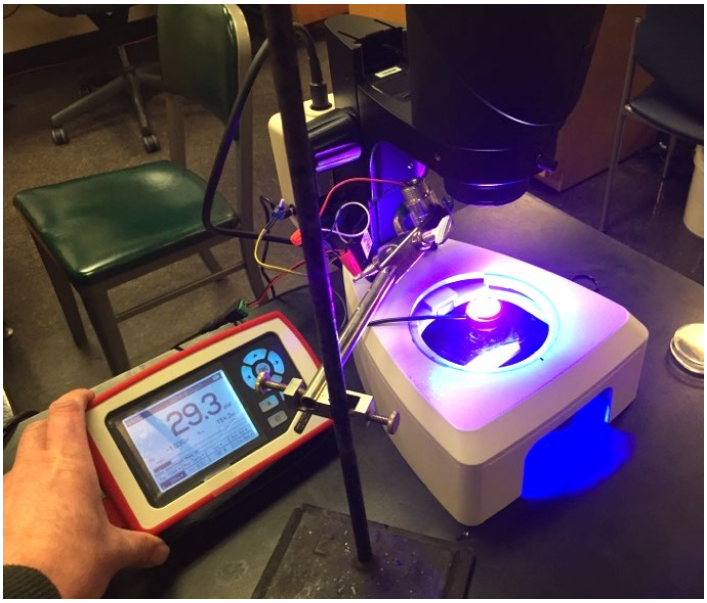

Figure 3. Measuring power output of a student-assembled LED illuminator. Assembled LED rig is positioned approximately 10 cm above the stage of a dissecting microscope. Light intensity at the point of observation is measured using a silicon photodiode sensor connected to a digital power and energy console.

Instructors can review the Google Doc notebook for assessment at convenient times or locations, track the contributions of different students, and easily provide in-line comments or questions.

The mid-semester proposal should summarize their group's preliminary data (including narrative and graphs/tables) in the context of the overall research question, justify their choice of a gene of interest, provide brief background on that gene, present a hypothesis as to how the gene may influence synaptic transmission, and present a plan to execute controlled experiments to test this hypothesis. The final paper is written in the style of a MicroPublication Biology journal article.

SUDENT ASSEMBLY OF SIMPLE LOW-COST OPTOGENETICS RIGS

The optogenetics rig used here was inspired by the elegant model described by Pulver et al (2011), though ours is simpler to assemble, does not require computer control and can be triggered with an inexpensive foot pedal. Rose (2018) also described a low-cost optogenetics set up using a high-intensity blue flashlight. The set-up we describe below has the advantage of using a low-profile LED light source that can be situated between the objective lens and the stage without interfering with the line of sight so that the worms can be stimulated while being observed under the microscope.

Students assembled LED rigs for use throughout the semester from the following components: Dynamic LED light kit with the following configuration: Royal Blue 1 Up Cree XTE LED with Carclo 20 mm narrow spot optic, (LED Supply # DLK-xUP-EH-KIT), a BuckPuck DC LED driver with the following configuration: wired connection, with a 5K potentiometer dimming switch and 700 mA output current (LED Supply # 03023-D-E-700P), screw-in terminal power adapter plug with a female plug size of 2.1 mm (LED Supply # DC-PA-2.1-F), a 12 V, 12 W Phihong wall mount switch (LED Supply, #12V-WM-1A), and S-series light duty foot switch with piggyback plug (SSC Controls #S100-1501) (Figure 2A). At the time of publication, these materials suitable for complete, foot pedal-controlled LED illumination of *C. elegans* can be purchased for less than $80.

To assemble the rigs, students first fed the bare portion of the red wire from the BuckPuck LED driver into the positive (+) terminal of the power adapter and the bare portion of the black wire from the LED driver into the negative (-) terminal of the power adapter (Figure 2B). They then used a screwdriver to tighten the connections. To connect the LED light kit to the LED driver, students connected the exposed portion of the blue wire from the LED driver to the exposed portion of the black wire coming from the cylindrical metal LED light kit and the exposed portion of the red wire from the driver to the white wire from the LED light kit. Students placed wire nuts over the wire junctions and twisted clockwise to tighten and seal the connections. (Figure 2C). The adapter plug was inserted into to the wall power supply, which was then plugged into the piggyback outlet on the foot switch (Figure 2A, red arrows). To provide power to the LED, students then plugged the foot switch into a standard outlet, Plugging the wall power supply directly to an outlet would provide continuous power to the LED. By positioning the foot switch between the outlet and the power supply, the LED remains off until students provide power by depressing the foot switch.

To orient the LED for proper illumination of the microscope stage, students secured the assembled LED unit into a clamp attached to a ring stand and positioned it so that it could illuminate the center of the microscope

Strain	Purpose	Genotype	Availability	Reference
N2	Picking practice and systemic RNAi testing	wildtype	purchase from CGC	Brenner, 1974
FJ1300	RNAi screening. This strain expresses light activatable channelrhodopsin in ASH sensory neurons and is engineered for enhanced RNAi in neurons and reduced RNAi in other tissues	*lin-35 (n745) nuIs25 (glr-1p* :: GLR-1::GFP, *lin-15)* I, *sid-1 (pk3321)* , *uIs69 (myo-2p* :: mCherry, *unc-119p* ::*sid-1)* V; *lite-1 (ce314)* , *ljIs114 (gpa-13p* ::FLPase, *sra-6p* :: *FTP* ::ChR2::YFP) X	Peter Juo lab	Luth et al., 2021
AQ2235	Control for light-induced reversals. This strain expresses light activatable channelrhodopsin in ASH sensory neurons	*lite-1 (ce314)* , *ljIs114 (gpa-13p* :: FLPase, *sra-6p* ::*FTP* ::ChR2::YFP) X	William Schafer lab	Ezcurra et al., 2011
FJ1282	Control for defective light-induced reversals. This strain expresses light activatable channelrhodopsin in ASH sensory neurons and contains a mutation in the gene *eat-4* , which is required for glutamate neurotransmitter release from ASH sensory neurons	*eat-4 (n2474)* III ;*lite-1 (ce314)* , *ljIs114 (gpa-13p* ::FLPase, *sra-6p ::FTP* ::ChR2::YFP) X	Peter Juo lab	Luth et al., 2021
CB1265	Control for defective thrashing behavior. This strain contains a mutation in the *unc-104* gene, which is critical for synaptic vesicle trafficking and gross neuromuscular junction activity.	*unc-104 (e1265) II*	purchase from CGC	Kumar et al., 2010
Mutants of interest	Validation of RNAi phentypes	varies	purchase from CGC	varies

Table 2: Strains used in this study. Each strain that was used by all groups, the purpose of the strain, and from where they can be obtained is listed.

stage/field of view from above at a distance of approximately 10-15 cm. For more targeted illumination, students aligned the LED as close to directly above the stage as possible, keeping in mind that it should not interfere with their ability to see through the eyepieces (Figure 3). Finally, students stepped on the foot switch, and made any necessary small adjustments so that the light hit the center of their field of view. Instructors should remind students not to look directly at the LED when it is on, since it is very bright.

LED output intensity was measured with a digital power and energy meter console with Silicon Photodiode Sensor with a power range of 50 nW - 50 mW and an absorbance range of 400-1100 nm. (Thorlabs #PM120D). While the light is shining, students turned the dial on the potentiometer switch (small white dial attached to the LED driver) to adjust the intensity of the LED until the power meter read approximately 30 mW at 488 nm (0.47 mW/mm^2) at the point where the worms would be positioned on the stage (Figure 3). Alternatively, if a power and energy meter is not available, one can calibrate by finding an intensity at which control AQ2235 worms (Ezcurra et al., 2011) grown in the presence of 500 μM all-*trans* retinal (ATR) (MilliporeSigma #R2500), a necessary cofactor for ChR2 activation, move backwards approximately 80-90% of the time after 1 second of illumination. LED intensity is quite stable over time, and if the set-up is not disturbed and intensity is not adjusted with the potentiometer dial, there should be no need for recalibration over the course of this CURE.

COMMON EXPERIMENTAL PROCEDURES

A series of common experiments was performed by all groups as part of the Introduction and RNAi Screen phases. Optional procedures carried out by individual groups in the Follow-up phase based on their initial observations will be noted, but the procedures will not be explained in detail.

C. elegans Strains and Maintenance

Unless otherwise specified, *C. elegans* were maintained on 60 mm plates containing solid nematode growth medium (NGM) seeded with OP50 *E. coli* according to standard cultivation practices (Brenner, 1974) except that worms were generally housed at 15˚C instead of 20˚C to extend the generation time to one week which better suits students' laboratory schedules. The following strains were used by all student groups in this study (see also Table 2): N2 Bristol wildtype, FJ1300: *lin-35 (n745) nuIs25 (glr-1p*::GLR-1::GFP, *lin-15)* I, *sid-1(pk3321)*, *uIs69 (myo-2p*:: mCherry, *unc-119p*::*sid-1)* V; *lite-1 (ce314)*, *ljIs114 (gpa-13p*::FLPase, *sra-6p*::*FTP*::ChR2::YFP) X (Luth et al., 2021) ; AQ2235: *lite-1 (ce314)*, *ljIs114 (gpa-13p*::FLPase, *sra-6p*::*FTP*::ChR2::YFP) X (Ezcurra et al., 2011); FJ1282: *eat-4 (n2474)* III; *lite-1 (ce314)*, *ljIs114 (gpa-13p*::FLPase, *sra-6p*::*FTP*::ChR2::YFP) X (Luth et al., 2021); CB1265: *unc-104 (e1265)* II (Kumar et al., 2010). Additional mutant strains corresponding to genes of interest that were identified through RNAi screening were purchased for individual groups from the CGC. Strains FJ1282 and FJ1300 are available from the authors upon request.

RNAi Screening

RNAi can be achieved in *C. elegans* by feeding worms bacteria containing double-stranded RNA targeting a gene of interest (RNAi clones). RNAi clones used in this lab were selected from a genome-wide library consisting of *E. coli HT115 (DE3*) expressing RNAi constructs (Kamath and Ahringer, 2003; Rual et al., 2004), which is available for purchase from Horizon Discovery (Catalog ID: RCE1181). For the purpose of identifying new functions of neuronal genes that play a conserved role in synaptic transmission, we prepared a sub-library from a cross-referenced list of *C. elegans* genes that are orthologous to human genes (Shaye

Score	General Activity	Reversal Response
3	Most worms are moving	Most moving worms reverse with each flash
2.5	Few worms are moving	Some moving worms reverse with each flash
2	Worms are moving heads only	Few moving worms reverse with each flash
1.5	Little head motion	Few worms reverse across all flashes
1	No movement	No reversals

Table 3. Scoring system for populations of worms after RNAi knockdown. Worms were rated 1-3 with regard to their glutamate-independent general activity and glutamate-dependent reversal response following optogenetic activation of ASH sensory neurons.

and Greenwald, 2011) and are expressed in neurons (Spencer and Zeller et al., 2011). Such a sub-library is not a requirement.

Screening was carried out with FJ1300 worms that express light-responsive channelrhodopsin2 (ChR2) in ASH sensory neurons and are optimized for enhanced neuronal sensitivity to RNAi. RNAi screening was performed as described previously (Luth et al., 2021) except that standard 60 mm dishes were used instead of 24-well plates. An earlier version of this CURE was attempted with a 24-well format and a greater number of RNAi clones to screen. This was quickly abandoned as picking to and from 24-well plates (especially when done with halocarbon oil to minimize food transfer) proved too challenging for many students with the limited practice time afforded. We refer the reader to the Supplemental Material for complete methods details related to growing RNAi clones and preparing knockdown plates.

Briefly, three FJ1300 worms were transferred using halocarbon oil (MilliporeSigma #H8773) to NGM plates containing 50 μg/ml carbenicillin (to select for plasmid-containing bacteria) and 5 mM isopropyl-β-D-thiogalactopyraniside (IPTG) (to induce dsRNA expression) and spotted at least one day prior with an RNAi clone containing 500 μM ATR (a necessary cofactor for ChR2). Halocarbon oil was used to minimize the transfer of standard bacterial food and maximize consumption of RNAi bacteria. Plates were stored at 15˚C for one week in aluminum foil to protect light-sensitive IPTG and ATR, and the resulting offspring were then scored for reversal response after optogenetic stimulation of ASH neurons and gross locomotor activity (see below).

Optogenetic Stimulation of ASH Neurons (optoASH)

After setting up their optogenetics rig, students first practiced the optoASH assay with control strains, a "wildtype" control that expresses light-activated ChR2 in ASH sensory neurons (AQ2235) (Ezcurra et al., 2011) and a mutant strain in which ASH sensory neurons cannot release glutamate to activate downstream interneurons (FJ1282) (Luth et al., 2021). In addition to light-reactive ChR2, this strain carries a loss-of-function mutation in the vesicular glutamate transporter *eat-4* (Table 2)**.** *eat-4* mutant worms do not release glutamate from ASH sensory neurons and are therefore unresponsive to photostimulation. In the case of these control "wildtype" and mutant strains for which RNAi was not needed, worms were grown for one generation on standard NGM that was spotted with OP50 containing 500 μM ATR and wrapped in foil.

Because the stated goal of the screen was to identify genes important for glutamate synapse function (as opposed to neuronal function in general) students scored both glutamate-independent general locomotion and glutamate-dependent blue light stimulated reversals according to the system in Table 3.

Response rates of individual worms were assayed on plates containing OP50 without ATR, as described (Durbeck et al., 2021; Luth et al., 2021). Briefly, control or RNAi treated worms were transferred from plates containing ATR to fresh NGM plates with OP50 but in the absence of ATR and illuminated for 1 sec with 0.47 mW/ mm^2 LED light with a minimum interstimulus interval of 10 sec (Schmitt et al., 2012). Backward locomotion occurring during or immediately after illumination was scored as a response.

Quantification of Thrashing/Body Bends

Thrashing assays to assess neuromuscular junction activity were performed essentially as described (Miller et al., 1996). Briefly, worms were picked with a minimal amount of food into a 5 μl droplet of M9 buffer (KH_2PO_4, 22.0 mM; Na_2HPO_4, 42.3 mM; NaCl, 85.6 mM autoclaved, plus 1 mM sterile $MgSO_4$) on a coverslip. After an acclimation period of 1 minute, students recorded the number of thrashes (movements of the head and tail from one side of the worm to the other and back) in 30 sec.

Fluorescence Microscopy of GLR-1::GFP

FJ1300 worms that were used for RNAi screening also carry a GLR-1::GFP transgene such that synaptic clusters of GLR-1 glutamate receptors can be visualized in the ventral nerve cord (Rongo et al., 1998; Burbea et al., 2002). Though this worm strain was used to teach students how to acquire and quantify images of synaptic puncta, groups were free to image other transgenic strains later in the semester.

Worms were immobilized on a coverslip in a 5 μl droplet of M9 containing 30 mg/ml 2,3-butanedione monoxime (MilliporeSigma #B0753) and mounted on a slide with a 2% agarose pad. Images of the ventral nerve cord were acquired on an Axio Observer 7 inverted compound microscope (Carl Zeiss) using a 63x objective.

FiJi (Schindelin et al., 2012) was used to create line scans from TIFF files and quantify puncta parameters. The segmented line tool was used to trace the ventral nerve cord and the *Plot Profile* function (*Analyze* > *Plot Profile)* was used to generate a graph of the pixel intensities along the line. The default settings of the *Find Peaks* function of the BAR plugin (Ferreira et al., 2015) (*BAR* > *Data Analysis* > *Find Peaks*) was used to identify puncta from background

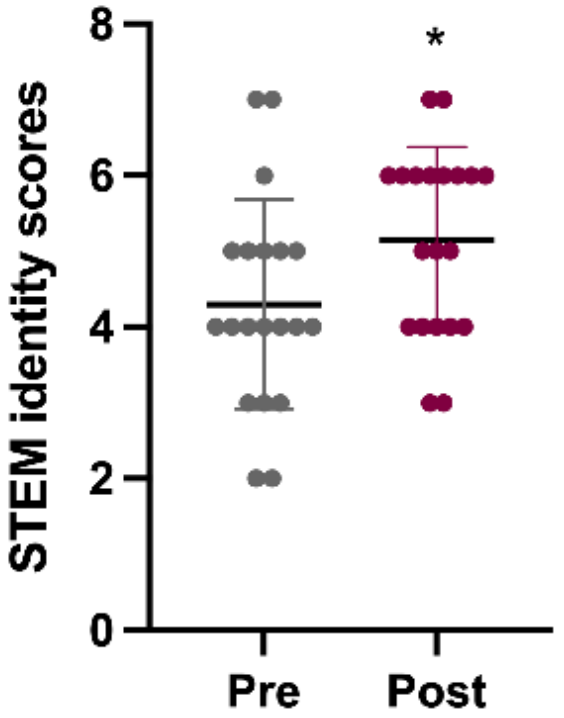

Figure 4. Student STEM Identity scores before and after the CURE. In response to a multiple-choice item, students selected the option illustrating how much their identity overlaps with their idea of a "STEM professional". * p = 0.02 Paired T-test

fluorescence. Data from the resultant table were used to calculate mean puncta intensity and puncta density along the ventral nerve cord.

Additional Follow-up Assays

Examples of potential follow-up assays include measurements of egg laying (Carnell et al., 2005), chemotaxis (Bargmann et al., 1993), exploration behavior (Pokala and Glater, 2018), or aldicarb- or levamisole-induced paralysis (Lemons, 2016). Students have also performed fluorescence microscopy of DiI-labeled sensory neurons (Shaham, 2006), or of GFP-tagged presynaptic synaptobrevin in cholinergic motor neurons that innervate body wall muscle (Ch'ng et al., 2008).

RESULTS

At the conclusion of the semester, students used a Likert scale to rate the degree of experience they gained, ranging from 1 ("None") to 5 ("Extensive"), on several skill elements that were incorporated into the course (Table 5). We selected the elements in question from the CURE survey (Lopatto, 2004; Lopatto et al., 2008), and compared student ratings on the amount of experience gained to aggregate CURE data (Lopatto and Jowarski, 2018). Students reported substantial gains on all elements, in most cases significantly higher than the aggregate CURE data (Table 5).

Importantly, participating in this lab significantly enhanced students' abilities to see themselves as STEM professionals (Figure 4). When considering the image they have of themselves compared to the image of what they feel a STEM professional looks like, prior to the lab (McDonald et al., 2019), 10% of students selected the two options corresponding to the least amount of overlap of those two identities. At the end of the semester, no students selected either of these options. Along these lines, the percentage of students selecting the two options with the highest degree of overlap between how they view themselves and "STEM professionals" rose from 15% to 50% after taking this course.

Responses related to the benefits that this CURE imparted support the increased sense of belonging in the scientific community. Students ranked each benefit on a scale from 1 ("No gain or very small gain") to 5 ("Very large gain"). The vast majority of students reported "Large gains" or "Very large gains" in "Understanding of how scientists work on real problems" (79%), "Becoming part of a learning community" (79%) and "Confidence in my potential to be a teacher of science" (89%).

While the career plans for greater than half of the students did not change as a result of this lab, the gains achieved during this CURE did influence many students. Thirty percent (30%) said they now plan to pursue a doctoral degree in a science-related field, and 10% indicated they now plan to pursue a Master's degree in a science-related field. In support of this, most students reported "Large gain" or "Very large gains" in skills critical for entry into and success in graduate and medical schools: "Tolerance for obstacles faced in the research process" (74%), "Readiness for more demanding research" (74%), "Learning laboratory techniques" (74%), "Understanding of the research process in your field" (63%), "The ability to read and understand primary literature" (79%), and "Skill in science writing" (84%).

Student Experience

It's important to note that the overall structure of this lab was not the preferred structure for 50% of students, who would have preferred trying something different each week even if it meant performing lab with predetermined outcomes. Despite that, almost all students agreed or strongly agreed that, "This lab was a good way of learning about the process of research" (95%) and that the "Lab had a positive impact on their interest in science: (89%).

In their semester course evaluations, students wrote:

"(The lab) allows the students to be like real scientists,"

"By continuing the work from week to week, it really made me more comfortable with the work we did in lab."

"I loved the independent aspect of lab. We all learned a lot! I also appreciated the midsemester write up. It was a great way to practice scientific writing and getting great feedback."

"Even though it was a bit confusing in the beginning, by the end of the semester, it helped me build a solid foundation of understanding not only neurobiology in action but also research approaches and new techniques."

Overall, these outcomes hold promise for experiences

like this to increase student desire and preparedness to continue in the STEM pipeline.

DISCUSSION

Here we present a CURE for a Neurobiology course that incorporates genetic, molecular, cellular, and behavioral experiments into a semester-long investigation using RNAi and optogenetics to identify genes important for synapse formation or function in *C. elegans*. This CURE is effective at increasing student engagement, readiness for additional research, and sense of belonging in the scientific community. It should be noted that these analyses were completed with a limited number of students as well as the time restrictions of a standard academic semester.

Challenges

It should be noted that this lab involves a considerable amount of preparation time by the instructor. It could be

	Week(s)	Discussion Topic(s)	Activities	Associated Material	Assessment
Phase 1: Introduction and Training	1	Introduction to research topic and working with *C. elegans* (including nomenclature, life cycle and stages), CURE structure overview	• Choose lab groups, observe worms and identify stages • Make worm picks, practice transferring worms • Maintain AQ2235 and FJ1282 worms to plates with ATR, maintain N2 worms to plates with *dpy-11* RNAi bacteria • Add RNAi bacteria corresponding to assigned genes to growth plates	Timmons and Fire, 1998 Excerpts from Sin et al 2014; Jorgensen and Mango 2002; Wormbook chapter on *C. elegans* maintenance	Lab notebook
	2	Working with RNAi and optogenetics, genetic screens	• Assemble optogenetics rigs and test reversal responses of control AQ2235 and FJ1282 worms • Examine plate phenotype of N2 worms with and without *dpy-11* RNAi • Maintain FJ1300 screening strain and pick to each designated RNAi plate (10 per group)	Excerpts from: Calixto et al., 2010; Excurra et al., 2011	Lab notebook
Phase 2: RNAi Screen	3		• Perform initial RNAi screen using the optoASH assay on populations of worms • Practice thrashing assay of neuromuscular junction function with control strains • Maintain FJ1300 worms and prepare RNAi plates for next week's experiments		Lab notebook
	4	Fluorescence microscopy of *C. elegans*, using ImageJ/FiJi for image analysis and quantification	• Continue RNAi screen, using quantitative single-worm optoASH assays • Groups can also gather initial data for a gene (ex: if that plate was contaminated) in the previous week • Practice preparing slides of immobilized worms		Lab notebook
	5	Introduction to Wormbase.org, sequence annotation and PCR primer design tutorial using SnapGene Viewer, options for follow-up experiments	• Additional practice making slides with immobilized worms • Learn about genes/strains/alleles of interest identified via RNAi screening • Discuss your plan for follow-up experiments with your group and instructor.	"Downloading a sequence from WormBase" video "Getting Started with SnapGene" Videos 1-3	Lab notebook
	6		• Ventral nerve cord fluorescence microscopy and image quantification practice • Discuss proposal assignment with your group • Collect additional RNAi screen data if necessary		Proposal with preliminary data and hypothesis
	7	*Spring Break*			
Phase 3: Follow-up Experiments	8	Review overall lab objectives, approach for individual experiments, and pros and cons of RNAi and mutants. Discuss how to perform a genetic cross	• Draw out your planned genetic cross • Maintain mutant and transgenic worms (from now on) • Prepare worm lysate and PCR primers	Virtual genetic cross worksheet	Lab notebook
	9	PCR theory and practice	• Perform PCR on control and mutant worm lysates • Begin your genetic cross • Begin any experiments on mutant worms that do not require a genetic cross • Practice fluorescence microscopy on control strains (optional)		Lab notebook
	10		• Analyze PCR results using gel electrophoresis and discuss any necessary troubleshooting • Continue your genetic cross and experiments started last week		Lab notebook
	11-13	Data visualization and statistical analysis	• Continue your genetic cross and experiments started • Generate graphs and/or tables for final paper • Freeze down your mutant worms and newly-generated strain when available		Lab notebook
	14	Jigsaw style oral presentations	• Wrap up any remaining experiments • Participate in presentations		MicroPublication-style paper

Table 4. Overview of RNAi and Optogenetics CURE. The CURE is divided into three Phases, each with associated discussion topics, activities, associated material, and assessments. This schedule should remain flexible to allow for variation in group progress, potential delay in primer or worm strain delivery, weather-related campus closures, etc.

Lab Element	RNAi/Optogenetics CURE (mean ± SD)	CURE aggregate (mean ± SD)
A lab or project where no one knows the outcome	4.00 ± 1.05	3.51 ± 1.21
A project where students have input into the research process/what is studied	4.20 ± 0.95	3.92 ± 1.00
Doing research in small groups	4.55 ± 0.69	3.96 ± 0.95
Becoming responsible for part of a project	4.45 ± 0.60	4.01 ± 0.94
Reading primary scientific literature	4.35 ± 0.99	3.72 ± 1.11
Writing a research proposal	4.40 ± 0.68	3.56 ± 1.21
Collecting data	4.50 ± 0.69	4.01 ± 0.96
Analyzing data	4.35 ± 0.67	4.11 ± 0.91
Presenting results orally	4.16 ±0.76	3.70 ± 1.17
Presenting results in written papers or reports	4.55 ± 0.51	3.83 ± 1.07
Critiquing the work of others	4.11 ± 0.81	3.34 ± 1.25
Maintaining a lab notebook	4.55 ± 0.51	3.60 ± 1.25

Table 5. Lab elements and estimated student experience gains. For each element students rated experience gains on a 5-point scale from "None" (1) to "Extensive" (5). Green shading indicates significantly higher reports of student gains in these areas after the BIOL 334 RNAi/Optogenetics CURE compared to the CURE aggregate. $p \leq 0.05$, unpaired t-test with Welch correction.

beneficial to involve students more in the preparation of the materials for each week (ex: growing RNAi bacteria, pouring and seeding worm growth plates, etc.). The practicality of that approach, however, may vary depending on the student population and their ability to engage in lab work outside of their scheduled lab time. Another challenge is that occasionally a group's first choice of a gene has no available corresponding mutant strain, and students are forced to turn to their second choice. One could avoid this problem by opting to only use RNAi clones for which a mutant strain is available or by preordering sets of mutant strains (ex: for the rab or kinesin families) and building an RNAi sub-library around those genes. Students could also continue to focus on RNAi experiments in conjunction with behavioral testing or microscopy. In the future, if there is sufficient time between when the gene is identified and when a mutant strain needs to be used (see Variations below), one could generate a knockout strain using CRISPR/Cas9 gene editing. Lastly, due to a variety of factors (including potential shipping delays, PCR/genotyping difficulties, and especially the time constraints of the academic semester), students rarely get to perform all the experiments that they propose. That said, the purpose of this lab is to create an authentic research experience, and science is not beholden to the academic calendar. Students are understanding of this, especially if this is explained at the outset and they are assured that their grade is not dependent on how much data they can collect or how many experiments they carry out. On the other hand, it may be possible for students to accomplish more if the structure of the CURE is altered (see below).

Potential CURE Variations

Tag Team Approach

One way to enable students to carry out full sets of experiments with mutant strains would be to swap or partially overlap the RNAi Screen Phase with the Follow-up Experiments Phase, though in that case, students would be executing follow-up experiments proposed by a previous cohort of students. After the Introduction Phase, students would be given a proposal from the previous year. They would then start with a genetic cross and any ready-to-go behavioral experiments. Once those are underway, some members of the group would begin a new RNAi screen.

There are advantages and disadvantages to this alternative approach. The students would certainly be able to accomplish more, but there is a chance they would be less invested in their projects and take less ownership of them. Having a greater number and variety of experiments being conducted at the same time could also lead to more confusion among students and increase the amount instructor prep work. Because the works from one group would need to be picked up seamlessly and presented by another, one potential benefit of this approach is that it could provide incentive to write clearer proposals in which preliminary results are better articulated and their choice of gene, proposed experiments, and controls are better justified. Of course, when assigning the proposal, the instructor could always leave open the possibility of a future cohort following up on the current cohort's set of proposals in order to motivate groups to write as if their peers depended on it.

Other Screens

In this report we describe an RNAi screen using a worm strain in which light stimulation activates a pair of glutamate releasing neurons and triggers a glutamate-dependent reflex behavior. By using other strains in which different classes of neurons express ChR2, one can alter the neurons that are activated and the neurotransmitter system that is being studied. Several such strains are available for purchase for a nominal fee from the CGC. For example, HBR546 with ChR2 in *atpf-1*-expressing neurons (Turek et al., 2013) and ZX460, which expresses ChR2 in cholinergic motor neurons. In particular, EEG98 worms that express

ChR2 in serotonergic neurons via the *tph-1* promoter have a robust body bend phenotype (Schultheis et al., 2011) that can be exploited for RNAi screening after crossing into an RNAi sensitive strain.

It would also be possible to run a version of this CURE without RNAi. If the goal is still to identify novel genetic regulators of synapse function and an RNAi library is not available, instructors can start with a curated selection of mutant strains based on genes with reported RNAi phenotypes for which studies of loss of function mutants have not been published. If genes are not the focus, a class could perform a screen of chemical compounds to identify those that affect the light-activated behavioral response or neurotransmitter system of interest and perform follow-up experiments to explore the mechanism of such effects.

Expanded Opportunities for Research

We have shown how this CURE increased student interest in science and feelings of readiness for more demanding research. These qualities, when combined with a semester of technical experience, allow students to easily transition into independent research projects that carry on the work they began in class. In one such example, a student validated and greatly expanded on her group's preliminary data for her capstone project. It is our hope that sharing concrete examples like this with students in class may inspire other students to continue their work after the semester has ended. Furthermore, the low cost optogenetics set-up described here can be used in conjunction with recently developed inexpensive alternatives to conventional or fluorescence dissecting microscopes (Fudickar et al., 2021; Schaefer et al., 2023). When employed together, they can be used for teaching and outreach in K-12 settings or other environments where traditional setups are cost-prohibitive.

CONCLUSION

The power of CUREs to enhance engagement, learning, diversity, and inclusion in STEM is undeniable (Thiry et al., 2012; Corwin et al., 2015; Staub et al., 2016). Here we present a semester-long neurobiology CURE using *C. elegans* that is effective at enhancing learning in a variety of STEM areas and increasing student confidence as scientists. In addition to the benefits CUREs provide to students, faculty benefit from the generation of preliminary data and training of students for potential independent research projects. We hope this will help encourage other *C. elegans* researchers who may be resistant to adopting the CURE model to consider adapting their own research into semester-long or even multi-week experiences. By bringing authentic research into the classroom, instructors create more opportunities for all students, not just those who seek out extracurricular mentored experiences (Bangera and Brownell, 2014), to be inspired by and contribute to the advancement of science.

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