ARTICLE
An Inquiry-Based Approach to Study the Synapse: Student-Driven Experiments Using *C. elegans*

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Inquiry-based instruction has been well demonstrated to enhance long term retention and to improve application and synthesis of knowledge. Here we describe an inquiry-based teaching module that trains undergraduates as scientists who pose questions, design and execute hypothesis-driven experiments, analyze data and communicate their research findings. Before students design their research projects, they learn and practice several research techniques with the model organism, *Caenorhabditis elegans*. This nematode is an ideal choice for experimentation in an undergraduate lab due to its powerful genetics, ease and low cost of maintenance, and amenability for undergraduate training. Students are challenged to characterize an instructor-assigned "mystery mutant" *C. elegans* strain. The "mystery mutant" strain has a defect in cholinergic synaptic transmission. Students are well poised to experimentally test *how* the mutation impacts synaptic transmission. For example, students design experiments that address questions including: Does the effected gene influence acetylcholine neurotransmitter release? Does it inhibit postsynaptic cholinergic receptors? Students must apply their understanding of the synapse while using their recently acquired research skills (including aldicarb and levamisole assays) to successfully design, execute and analyze their experiments. Students prepare an experimental plan and a timeline for proposed experiments. Undergraduates work collaboratively in pairs and share their research findings in oral and written formats. Modifications to suit instructor-specific goals and courses with limited or no lab time are provided. Students have anonymously reported their surprise regarding how much can be learned from a worm and feelings of satisfaction from conducting research experiments of their own design.

Key words: neurobiology, Caenorhabditis elegans, hypothesis-driven, neuroscience education, nematode, worm, aldicarb, levamisole, active learning, open-ended project, inquiry-based instruction

Inquiry-based and active learning approaches have been well demonstrated to enhance learning and retention (Handelsman et al., 2004; DeHaan, 2005; Wood, 2009; Goldey et al., 2012). Consistent with these findings, training students as scientists is an effective method to enhance student learning (Handelsman et al., 2004; Wood, 2009). Training student scientists to pose questions, design and execute experiments to answer questions, and subsequently draw inferences from data, is a productive example of active and inquiry-based learning.

Here, we describe a student-driven research project module executed within the context of an undergraduate teaching lab. This series of lab exercises employs active and student-centered learning. This inquiry-based lab has two overarching goals: 1) train students as scientists to design, execute and analyze hypothesis-focused research projects of their own design and 2) challenge students to gain a deep, genuine understanding of synapse biology by applying their knowledge to interpret the meaning of experimental data. This module trains undergraduates to first learn, and then subsequently use, a set of research techniques to execute student-designed and hypothesis-driven experiments.

Using C. elegans to study the synapse

This lab offers a unique approach to learn about synapse biology. Rather than a traditional information-transfer style lecture, this module uses a problem-based approach to encourage students to think more deeply about synaptic events, including how drugs impact the synapse and animal behavior. In many traditional lectures, a step-by-step explanation is often used to explain how information is transferred from a presynaptic neuron to a postsynaptic cell. Here, students use scientific experimentation to answer questions about how a drug, such as an acetylcholinesterase inhibitor, aldicarb, impacts synapse function and animal behavior. Typically, this would be challenging to do in a time-restricted and resource-limited undergraduate teaching lab, however students can observe the effects of drugs on animal behavior rapidly (within two hours) and reliably using the nematode, *Caenorhabditis elegans*.

C. elegans is a non-parasitic worm that is easily maintained in small petri dishes in the lab. The genome of these animals has been fully sequenced and the nervous system wiring diagram is fully mapped. In experiments described here, we also take advantage of anthelmintic drugs, such as aldicarb, that pass through the cuticle of living worms and impact synaptic function. Aldicarb's impact on synaptic function is rapidly and clearly detected by a change in animal behavior; the wildtype worm paralyzes within two hours due to overstimulation of the neuromuscular synapse.

Students design, execute and analyze experiments using anthelmintic drugs and *C. elegans* that reveal the site of action of a genetic mutation in an instructor-provided

"mystery mutant" worm strain. Students generate data that are used to determine if the mutation impacts the presynaptic or postsynaptic compartment. For example, students solve questions such as: Does the mutation enhance neurotransmitter release (e.g., a presynaptic effect)? Does the mutation decrease function of acetylcholine receptors (e.g., a postsynaptic effect)? Undergraduates present their findings in both oral and written formats, similar to a seminar presentation and a primary science article.

This module would be effective for several undergraduate courses such as neurobiology, physiology, pharmacology and physiological psychology. Modifications described below can adapt this inquiry-based module for courses with restricted lab time or courses without a lab component.

Course Background: providing context for project

This student-driven research module is implemented in an upper level neurobiology course entitled *Principles of Neuroscience* that is taught at a small liberal arts college that has ~2,000 undergraduates. The *Principles of Neuroscience* course serves as a required capstone course for Biology Majors earning a Concentration in Neuroscience and Behavior, and for Psychology Majors earning a Concentration in Brain, Behavior and Cognition. This course also serves as an upper level elective for biology majors.

All students, regardless of their major, must take *Concepts in Biology* and *Genetics* prior to this course. In *Genetics*, students use the genetically powerful model organism *Drosophila* to conduct research experiments, which serves them well as they learn about another genetically powerful model organism, *C. elegans,* in the *Principles of Neuroscience* course. In addition, biology majors must take at least 1 of 2 courses: *Cellular and Molecular Biology* or *General Physiology*; whereas Psychology majors must take *Physiological Psychology* prior to entry into the *Principles of Neuroscience* course. Therefore, all students entering this class have completed at least three prerequisite courses and have developed a firm grasp of key biological concepts and fundamental lab skills.

The entire course runs for 15 weeks. Lecture runs for 50 minutes, 3 times per week. Lab is scheduled to meet once a week, for a 3-hour time block, and is limited to 12 students per section. This entire module lasts 5-6 weeks. Three weeks are devoted to "technique labs" that are designed to train students the necessary techniques to conduct their projects. Two-three weeks are designated for student-driven independent research, depending on the time restrictions of the instructor.

Students work in groups of 2 or 3 for this lab exercise. The instructor intentionally selects the groups to ensure that a combination of academic backgrounds is represented in each group, if possible. For example, a biology major is often paired with a psychology major, or a biology major who has taken *Cellular and Molecular Biology* is paired with a biology major who has taken *General Physiology*. The instructor shares the rationale for group selection with students and encourages students to capitalize on the diverse strengths and background of each group.

Another lab exercise used in this same course has been previously described (Lemons, 2012). This published lab exercise challenges students to design and execute experiments, employing cell culture and immunocytochemistry techniques, to determine if their "mystery" cell line is derived from a neuronal or glial origin (Lemons, 2012). In contrast, the lab exercise described here focuses on synaptic transmission and uses *C. elegans* as a research model. More specifically, this synapse-focused research project challenges students to design, execute and analyze experiments to determine if their assigned worm strain has a genetic mutation that impacts presynaptic or postsynaptic function. Both of these lab exercises (cell culture based and the *C. elegans* based projects) can be used in the same course during the same semester.

C. elegans is a practical and powerful model to use in an undergraduate teaching lab

C. elegans has numerous benefits for undergraduate experimentation including the ease and relative low cost of worm maintenance, short life cycle, transparency and powerful genetics. Undergraduates are able to rapidly grow worms and quickly assess behavior within time restrictions common to an undergraduate teaching lab. Using a dissecting microscope, students can view through the transparent worm and see eggs prior to hatching, as well as age-specific anatomical landmarks. There is a wealth of excellent websites that students can use to study this model organism, including: wormbook (http://www.wormbook.org/), wormbase (http://www.wormbase.org/) and the *Caenorhabditis* Genetics Center (CGC; http://cbs.umn.edu/cgc/home).

Growing or maintaining these nematodes is straight-forward, relatively inexpensive and does not require a lot of time. This is beneficial for this instructor, who prepares these worms for undergraduates. There are numerous options and methods to grow these worms (see Materials and Methods and "Instructor Preparation"). The instructor can choose the method that best suits their needs and schedule.

Lastly, numerous mutant and wild type worm strains can be purchased from the CGC and are inexpensive ($7 per strain for academic/non-profit institutions). These features make this model organism a practical choice for instructors or persons responsible for preparing the worms for student labs.

INSTRUCTOR PREPARATION FOR TEACHING LABS

To prepare for this lab module, the instructor: 1) prepares OP50 seeded NGM agar plates, 2) gathers equipment needed for students, 3) prepares necessary solutions and 4) grows wildtype and mutant *C. elegans* worm strains.

Worm plate preparation

In the lab, *C. elegans* are grown on nematode growth medium (NGM) agar plates that have been seeded with a

lawn of OP50, a mutated strain of *Escherichia coli*. OP50 serves as a food source for these nematodes, and a small aliquot of OP50 is placed (e.g., seeded) onto the middle of the NGM agar plates. NGM plates are easily made (see methods) and can be kept at 4^0C for months, thus providing flexibility for lab preparation. This instructor begins pouring plates ~2-3 months prior to lab execution. As an alternative, instructors can purchase NGM plates from vendors such as Tecknova or LabExpress.

Equipment

The equipment needed for this lab module is modest. Students need a binocular dissecting microscope, a Bunsen burner, a pair of pliers (Figure 1A), a small spatula and a worm pick. A worm pick is a piece of platinum wire attached to a glass Pasteur pipette (Figure 1B,C). Students make their own worm pick, as described under "Student preparation for independent project." The microscope should be equipped with brightfield illumination (with light emitted from under the stage) and with total magnification between 8X-50X. We have used the relatively inexpensive Motic SMZ-171 with great success.

Solutions

The number of solutions needed for this exercise is limited. Worm bleach solution (see Materials and Methods) can be used for age synchronization and for removing contamination. Students also use 70% ethanol to spray down their benches to keep them clean and to keep their spatulas clean.

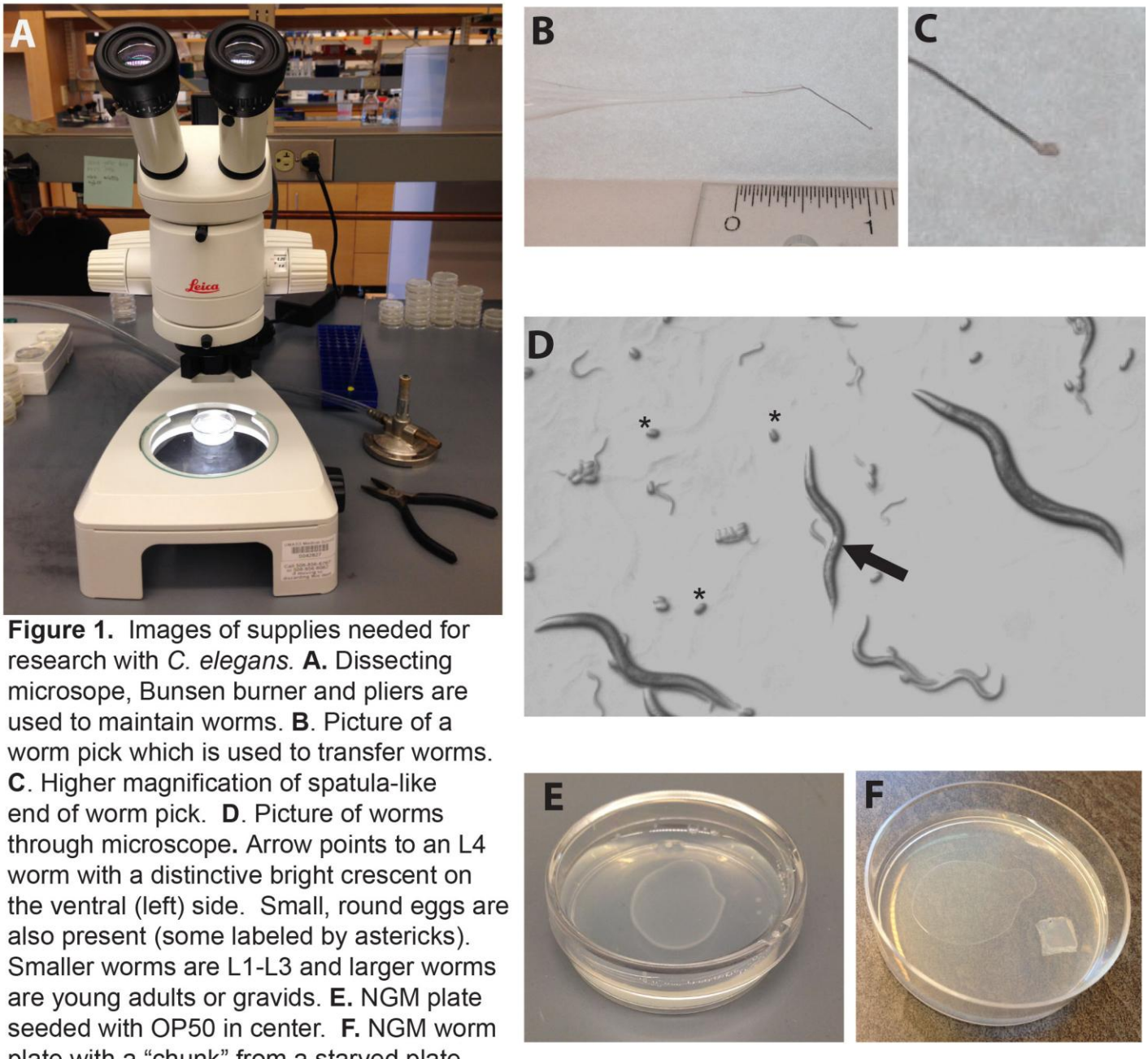

Figure 1. Images of supplies needed for research with *C. elegans.* **A.** Dissecting microsope, Bunsen burner and pliers are used to maintain worms. **B**. Picture of a worm pick which is used to transfer worms. **C**. Higher magnification of spatula-like end of worm pick. **D**. Picture of worms through microscope**.** Arrow points to an L4 worm with a distinctive bright crescent on the ventral (left) side. Small, round eggs are also present (some labeled by astericks). Smaller worms are L1-L3 and larger worms are young adults or gravids. **E.** NGM plate seeded with OP50 in center. **F.** NGM worm plate with a "chunk" from a starved plate.

Growing C. elegans in the lab

C. elegans have a life cycle that lasts ~3 days and includes four larval stages (Brenner, 1974; Riddle, 1997; Wood, 1998). The fourth larval stage (L4) is easily identified under a dissecting microscope by the presence of a crescent-shaped structure on the ventral surface. This crescent (which will become the vulva) is unique to an L4 hermaphrodite worm (Figure 1D) and can be used to age synchronize worms. Typically, 2-3 L4 worms are placed on an OP50 seeded, small (35mm) NGM plate to maintain a strain (Figure 1E). This is done by transferring the worms under a dissecting microscope using a "worm pick" (Figure 1B,C). After 3-4 days at room temperature, a new population of *C. elegans* will be present on the plate. At this time, another 2-3 L4 worms can be transferred to a new plate to avoid starvation (e.g., exhaustion of OP50). To slow the rate of growth, worms can be stored at 15^0C and maintained once a week, rather than once every 3-4 days. This process of picking and growing worms is referred to as "maintaining worms."

The method of picking 2-3 L4 worms to a new OP50 seeded NGM plate is a reliable method to maintain worm strains. However, to quickly grow large numbers of nematodes for a fairly large number of students, this instructor chooses to "chunk" worms from starved worm plates ~1-2 days before students' first worm lab. *C. elegans* left on an NGM plate over time will eventually exhaust their food supply and starve. In the absence of food, worms enter a dauer state, also known as the "enduring" larval stage (Brenner, 1974; Riddle, 1997; Wood, 1998). This dauer state can last months. If dauer worms are exposed to food, they will transition to the fourth larval stage (L4) and continue through the life cycle. An investigator can "chunk" a plate by using a sterile spatula (stored in 70% ethanol and quickly passed through a flame) to cut a small "chunk" of agar (approximately 4x4mm cube) from a starved plate and place the chunk (worm side down) on a new OP50 seeded NGM plate (Figure 1F). Numerous worms reliably crawl out from the chunk at room temperature within 16-48 hours and this serves as a great starter plate to give to students. "Chunking" takes less than 1 minute and this allows for rapid preparation of worms. This method is reliable, but the first generation of worms that grow out from a chunk should not be used for behavioral assays as the recent starvation state may influence behavior.

Another advantage of these nematodes includes the ability to store *C. elegans* indefinitely at -80^0C and thaw when needed (Wood, 1998). This allows the investigator to keep the strains and eliminates the need to repeatedly order the same strains from the CGC.

STUDENT PREPARATION FOR INDEPENDENT PROJECT

Technique labs

Students complete 3 technique labs prior to beginning their independent projects. These technique labs are designed to empower students with the research skills needed to design and conduct subsequent experiments of their own design (Switzer and Shriner, 2000; Lemons, 2012). These 3 technique labs train students to: 1) maintain *C. elegans* strains, 2) stage worms, 3) execute an aldicarb assay and 4) plot and analyze data from aldicarb assay. Concurrent with these lab activities, students are immersed in the field of *C. elegans* in lecture via analyzing primary science articles that employ *C. elegans*. Connections between lecture and lab are abundant and heavily emphasized.

Technique #1 lab

In technique lab #1, students are introduced to *C. elegans*. Most students do not have any previous experience with this model organism. In this lab, students: 1) practice using a dissecting microscope to observe *C. elegans,* 2) become familiar with *C. elegans* nomenclature, maintenance and life cycle, 3) make a worm pick and 4) practice picking (e.g., transferring) worms from one NGM plate to another.

To achieve these goals, each student is given an OP50 seeded NGM plate (Figure 1E) with wildtype N2 worms, a dissecting microscope, a pair of pliers and a Bunsen burner (Figure 1A). Students are directed to wipe their bench down with 70% ethanol before starting. Students are challenged to observe the worms (after removing the lid) under the microscope and take notes of their observations. Lids of agar plates are removed for visualization of worms under the microscope and lids are quickly replaced when dishes are not in use to minimize possible contamination.

At the discretion of the instructor, students are asked to list at least 3 questions based on their observations. Students can then share their observations with their lab partner and the instructor can lead a student-question based discussion while projecting a live image of *C. elegans* taken from a camera-mounted microscope (or still images) onto the projection screen. Based on this instructor's experience, its helpful to cover several points to prepare students for upcoming research. These points include the various sizes (and ages) of worms growing on a plate. It is important review the life cycle of *C. elegans* and mention the four larval stages of this nematode (Brenner, 1974; Riddle, 1997; Wood, 1998). Based on experience, it is also helpful to include a description of the NGM plates seeded with a lawn of mutated *E. coli*, OP50. Other points that often arise are the 2 sexes of these worms; hermaphrodites and males (Brenner, 1974; Riddle, 1997; Wood, 1998). The class discusses the advantages that these 2 sexes can provide for research.

Next, students are challenged to make and use a worm pick. This simple tool (a piece of wire anchored in a glass Pasteur pipette, Figure 1B,C) enables a researcher to pick and transfer worms. A worm pick is essential for all upcoming experiments. Students make a worm pick after watching their instructor demonstrate this task (see methods). Students are instructed to pass their worm pick through the flame of the Bunsen burner for 1-2 seconds to sterilize the pick. The wire cools quickly, but it can be helpful to pause for 1-2 seconds to ensure the wire cools

before continuing. Then, students are instructed to touch their worm pick to the OP50 in order to transfer some of the sticky bacteria to the bottom of the pick. Next, the end of the worm pick (with a dab of sticky OP50) is used to gently touch and pick up a worm. The pick (with the worm) transfers the worm to a new OP50 seeded plate by gently touching the pick to a new plate. After visual confirmation that the worm is on the new plate, it is important to flame the pick again. It is important to periodically "flame" one's pick (e.g., pass it through the Bunsen burner flame) to deter contamination or possible cross-contamination of various worm strains. Keeping lids on NGM plates whenever possible also helps to deter contamination.

Students use their worm picks to transfer worms from one NGM plate to a new NGM plate without nicking or breaking the surface of the NGM agar. This can be initially challenging for students for two main reasons. First, students must pick worms under the microscope. This task requires depth perception, which requires binocular vision. Students will quickly learn that looking through the microscope with one eye will make the task of picking worms exceptionally challenging. Second, it takes practice to move worms from one plate to another without breaking the surface of the agar. When first learning to pick or transfer worms, it is common to accidentally push the metal wire of the pick into the agar and nick the agar surface. This needs to be avoided because worms will dive into the nick and burrow deep into the agar, rather than growing on the surface of the agar. When worms burrow into the agar then it is difficult to get access to those worms. It is much easier to study and manipulate worms that are growing on the surface of the agar. For this reason, it is important to learn how to pick and transfer worms without nicking the agar upon which they grow. Despite these challenges, students quickly learn how to successfully pick worms during the first lab session. It has been the instructor's experience that the time it takes for students to learn this task is indirectly proportional to the empathy and encouragement given by the instructor.

Students are shown how to properly label the NGM agar plates on the bottom for proper identification. For example, using a sharpie, students write "N2" and the date on the bottom of the agar plate. "N2" refers to the strain (e.g., N2 Bristol wild-type strain.) Students are also instructed to include their initials on the plate and store all worm plates upside down (lid on bottom, agar on top) in a provided pencil box. Each group has their own pencil box.

As the final challenge, students are instructed to "maintain" their worm strain. This is done by picking 2-3 L4 hermaphrodites onto a new OP50 seeded NGM plate every 3-4 days when grown at room temperature. Students are charged to maintain their worm strains for the remainder of the semester. If lab accessibility (or time) is limited, students can choose to store their worms in a 15^0C incubator and maintain worms once a week. Worms grow slower at lower temperatures, including 15^0C. At our institution, it has proven helpful to limit the number of times students must come to lab outside of the scheduled lab time, and therefore our students keep their worm strains in the 15^oC incubator. The ability to pick L4 worms is important for future experiments in technique lab #3 (but not #2). About half of the students achieve this challenge by the end of the first lab, and all students achieve this challenge by the end of the second lab, which is timely for future experiments.

Technique #2 lab

The goals for this lab are: 1) enhance skills learned during technique lab #1, 2) practice staging worms, 3) learn steps to address possible contamination, 4) make and execute a plan to stage worms for technique #3 lab, and 5) learn how to "chunk" worms. Students obtain their worm plates from the previous lab and the instructor gives them two mutant strains (*unc-29 and unc-49*) that serve as controls for future experiments. Students begin the lab by maintaining their strains. They pick 2-3 L4 hermaphrodites to a new OP50 seeded NGM plate. This is done for each of the three strains.

Students learn how to age synchronize three worm strains (N2, *unc-29*, *unc-49*) for future experiments. The ability to age synchronize their worms (more commonly referred to as staging worms) empowers students to have a population of worms at a desired age for each strain, which is necessary for future experiments. Staging is done in addition to regular maintenance.

Students learn 2 methods (of several possible methods) to stage worms. One staging method includes picking 30-40 gravid hermaphrodites to a new OP50 seeded NGM plate. Gravid worms are adult hermaphrodites that have many eggs, which are easily seen through this transparent organism on the microscope. After 3-6 hours, students "flame" all gravid worms and leave eggs that were laid by gravids on the plate. Flaming worms refers to picking worms from a plate and then pass the worm pick (with worms on the pick) through the flame of a Bunsen burner, thus killing worms on the pick. All gravids are flamed (e.g., removed) and eggs that were laid by gravid worms remain on the plate. The eggs will hatch within a relatively close time window and will be age synchronized in a manner that will be appropriate for future experimentation.

A second method used to stage worms, as well as remove possible bacterial contamination, is "bleaching" worms. Students place ~30μl of worm bleach (see methods for recipe) onto a new OP50 seeded NGM worm plate. Worm bleach is placed on the NGM agar outside of the spot of OP50. Students pick gravid hermaphrodites from one strain and place them into a ~30μl drop of worm bleach on the new plate. Through the microscope, students can observe the hermaphrodites dissolve in the bleach and the eggs remain. The chitin-coated eggs are protected from the fatal effects of the bleach. The worm bleach will also remove possible bacterial contamination that may be present. At the end of lab, the plate is stored in a 24^OC incubator or at room temperature. Students must return in 16-48 hours to pick at least 12-15 worms that have hatched from the worm bleach and transfer to a new OP50 seeded NGM plate.

If students regularly wipe down the lab bench with ethanol at the start of experimentation and regularly flame their worm pick, contamination is rare in our experience.

This is somewhat surprising because three sections of microbiology run in the same space as this lab during the fall semester. While it is uncommon, there are times when bacterial or fungal contamination may occur. Worm bleaching will remove contamination. It is helpful to note that contamination can affect behavioral assays (described below) and therefore, worm bleaching can be used remove contamination or for staging, both of which are valuable for behavioral assays.

Students are charged to age synchronize three worm strains (N2, *unc-29*, *unc-49*). The goal is to have 12 "young adult" worms of each strain at the start of lab the following week (lab technique #3 lab). The term "young adult" refers to worms that are ~16-24 hours older than an L4. Thus, students must make a plan to stage their worms accordingly. Each student must stage three strains using a method that is distinct from their lab partner. For example, if one lab partner chooses to stage worms using the bleaching method, then the other lab partner must use the flaming gravid method. Between the two lab partners, they will often have enough staged worms to run a successful experiment for technique #3 lab, even though each student should technically have enough worms to run the experiment individually. It has been our experience that each pair of students collectively has an adequate number of young adult staged worms for each strain. Rarely does one student successfully get enough young adult worms for each strain on their first attempt with staging. This serves as a moment of reflection and discussion. Students learn which staging technique works best for them and possibly how to improve the technique. This is helpful because staging will be used repeatedly in the future. Moving forward, students can select a single staging technique that works best for them in future experiments.

Students must have their staging plan (which is recorded in their lab binder) approved by the instructor before leaving lab. Students are directed to refer to the known life cycle of the worm to determine when each step (of the bleaching or gravid technique) must be done to ensure that students will have staged young adult worms in one week. The instructor offers suggestions, if needed, and ultimately confirms a staging plan that is appropriate and likely to succeed. Regardless of the staging plan, students will need access to the lab outside of the regularly scheduled lab time to stage the worms for the following week. At our institution, this is possible by giving students access to the labs Monday-Friday 8AM-8PM. Students are allowed into the lab as long as another lab section is not running.

Lastly, students learn how to "chunk" worms. Chunking can be used to resurrect starved worms. It is also a helpful way to quickly grow a large number of worms. To chunk a plate, a sterilized small spatula is used to cut a "chunk" of NGM agar from a starved plate (see "Instructor Preparation" or Materials and Methods). The chunk is transferred to an OP50 seeded NGM plate, worm-side down (Figure 1F). Starved worms (e.g., in a dauer state) transition to an L4 in the presence of OP50. Worms that crawl out of the chunk can be transferred to another OP50 seeded NGM plate. Depending on the level of starvation, some worms crawl out the same day while others may crawl out of the chunk the following day. This is a very useful technique to quickly grow a large number of worms or to grow a strain of worms that had been previously starved. For experiments described here, it is not ideal to perform experiments on recently starved animals because this could impact their behavior.

Technique lab #3

The goals of this technique lab are for students to: 1) perform an aldicarb assay, 2) understand the biological basis of this assay, 3) interpret and analyze data from an aldicarb assay and 4) generate a figure using data gathered from the assay.

Students perform an aldicarb assay with young adult staged worms that they have prepared previously. Aldicarb assays are a powerful, yet simple method to quickly test if a mutation may impact synapse function. Aldicarb assays involve plating worms on NGM plates that contain the acetylcholinesterase inhibitor, aldicarb. Aldicarb passes through the cuticle of the worm and decreases native acetylcholinesterase activity, thus allowing acetylcholine to build up in the synapse, ultimately causing worm paralysis due to overstimulation of body wall muscle.

Prior to the start of the aldicarb assay, we discuss our expectations from this experiment. This can be done either in the previous lecture or at the start of lab. To promote an active discussion of the aldicarb assay, students are required to read a review paper that describes the biological basis for this technique (Mahoney et al., 2006) prior to the start of lab.

The discussion of the aldicarb assay can begin with a series of questions. During the discussion, the instructor draws a model synapse on the white board and goes through various scenarios, similar to Figure 1 in Mahoney and colleagues (2006). Questions and answers are listed below to help students understand the biological basis underlying an aldicarb assay. Question: Knowing that aldicarb is an acetylcholinesterase inhibitor, what does one expect will happen to the motile behavior of an N2 worm on an aldicarb plate? Answer: The N2 animal will eventually paralyze. Why? Aldicarb will inhibit the breakdown of acetylcholine in the synapse, which causes an over-abundance of acetylcholine that leads to over-activation of cholinergic receptors and prolonged muscle contraction, resulting in paralysis. Question: What will happen to the *unc-29* and *unc-49* mutants on an aldicarb plate? Answer: The *unc-29* mutant will be resistant to the effects of aldicarb compared to wildtype, while the *unc-49* mutant will be hypersensitive compared to wildtype. Why? UNC-29 is an acetylcholine receptor subunit expressed in *C. elegans* muscle (Fleming et al., 1997). The *unc-29* mutant worms will not paralyze as fast as wildtype worms on aldicarb because there are fewer functional cholinergic receptors on postsynaptic muscle cells in the *unc-29* mutant. Thus, *unc-29* mutant worms will be resistant to the effects of aldicarb compared to wildtype worms. In contrast, UNC-49 is a $GABA_A$ receptor (Bamber et al., 1999) that is expressed in muscle. The *unc-49* mutant has a dysfunctional GABA

receptor subunit, thus decreasing GABAergic signaling at the synapse. The *unc-49* mutant will be hypersensitive to aldicarb due to decreased inhibitory GABAergic signaling. Without efficient inhibitory signaling at the neuromuscular junction, a worm will more rapidly paralyze on an aldicarb plate due to the build up of acetylcholine in the synapse, which will cause increased muscle contraction. When the inhibitory effects of GABA do not dampen the excitatory effects of acetylcholine, the excitatory effects overwhelm the system more quickly, causing a faster rate of paralysis of the *unc-49* mutant compared to wildtype. Contraction and relaxation of body wall muscle in *C. elegans* is regulated by acetylcholine and GABA, unlike skeletal muscle in vertebrate systems.

To begin an aldicarb assay, each student is given 3 aldicarb plates. Each aldicarb plate is designated for a distinct strain (e.g., N2, *unc-29*, *unc-49*). Students place 10 young adult N2 worms onto a single aldicarb plate. When all 10 worms are on a plate, this is recorded as time 0. Every 15 minutes, the student will score the number of paralyzed worms. Paralysis is defined by the inability of a worm to move after two touches with the worm pick near the pharynx. At each 15 minute interval, worms that are considered paralyzed are recorded and immediately flamed (e.g., removed from the plate). Students repeat this for two hours. During the first 15 minute interval, students start the aldicarb assay for the *unc-29* and *unc-49* mutants. When students stagger the start of the aldicarb assay for each strain, it is possible to score three (or more) strains of worms during the same 2 hour time block.

Students execute the aldicarb experiment and collect data. They are challenged to plot their aldicarb data from all three strains, as done previously (Petrash et al., 2013). Time in minutes is plotted on the x-axis, and % paralyzed worms is plotted on the y-axis. Students submit this graph as a figure, with a figure caption, to the instructor within 48 hours for assessment. This motivates students to practice graphing data and preparing data for documentation in the form figure, which will be useful for their final lab report and PowerPoint presentation at the end of this module. Constructive feedback from the instructor for this assignment can be used to generate proficient figures for the final report and presentation.

Preparation for students to answer the question: What experiments can I design that reveals if a "mystery" mutation impacts the function of the presynaptic or postsynaptic component?

Students are well equipped to develop informative independent projects after completing these three technique labs, in combination with reviewing primary science articles that employ *C. elegans*. Student groups are given a "mystery" mutant worm strain and are told that the mystery mutant has a defect in cholinergic signaling. Each group receives a distinct mutant. Students are charged with designing, executing and analyzing experiments that determine *how* the mutation may impact the presynaptic or postsynaptic side of the synapse. Students are instructed to imagine that they are the first to characterize this mutant and they will subsequently prepare a manuscript for publication (e.g., lab report) and execute an oral presentation based on their findings. Strains that could be used are listed in Table 1. Students remain blinded to the identity of their "mystery mutant."

What can an aldicarb assay reveal about synaptic function?

To initiate discussion and experimental planning, the instructor reviews the strengths and limitations of the aldicarb assay. The aldicarb assay can be used to determine if there are defects in synaptic transmission (Mahoney et al., 2006). It is possible that students will find their assigned "mystery" mutant is hypersensitive to aldicarb. Knowing that all assigned "mystery" worm strains have a mutation that impacts cholinergic signaling, what would this data suggest? This data would be consistent with a mutant that has: 1) enhanced release of acetylcholine, or 2) increased number or function of cholinergic receptors. Conversely, a student could find the mutant is resistant to aldicarb. This data could suggest: 1) the mutant is defective in acetylcholine release from the presynaptic compartment, or 2) the mutant has dysfunctional or decreased number of postsynaptic acetylcholine receptors (Mahoney et al., 2006) The Mahoney et al., paper can be used as a basis for this discussion.

How can a combination of aldicarb and levamisole assays further characterize synaptic function?

The inability to more clearly distinguish if a mutation impacts the presynaptic or postsynaptic compartment is a limitation of the aldicarb assay. However, this can be addressed by the additional use of the levamisole assay. Levamisole is a cholinergic receptor agonist that directly activates acetylcholine receptors (Brenner, 1974; Lewis et al., 1980a; Lewis et al., 1980b; Fleming et al., 1997). Similar to aldicarb, levamisole can pass through the worm's cuticle and diffuse into the synapse. Wildtype worms paralyze over time due to excessive excitation of the acetylcholine receptor. Worms with mutations that negatively impact presynaptic function paralyze at either a similar rate or a mildly faster rate on levamisole plates due to possible postsynaptic compensatory mechanisms such as increased cholinergic receptors or function. However, worms with mutations that decrease cholinergic postsynaptic function are usually resistant to levamisole. The levamisole assay is executed in the same manner as the aldicarb assay. Young adult worms are plated on levamisole plates and scored for paralysis every 15 minutes for two hours.

Levamisole assays, in combination with aldicarb assays, can further clarify how a gene may impact synaptic function than either assay alone. For example, levamisole assays can resolve if an aldicarb resistant strain has a dysfunction in the presynaptic compartment (e.g., decreased acetylcholine release) or in the postsynaptic compartment (e.g., decreased cholinergic receptor function). If a mutant is resistant to aldicarb and is also resistant to levamisole, this data would suggest that the mutation impacts the postsynaptic compartment, such as

decreased cholinergic receptor function. However, if a mutant was resistant to aldicarb but was *not* resistant to levamisole, this would suggest that the mutation impacts the presynaptic compartment, such as decreased acetylcholine release.

Interpreting data collected from aldicarb and levamisole assays is thought provoking and challenging. It allows students to consider what the data does and does not reveal about the mutant strain. Students are challenged to answer the question: What could be altered in the synapse that would be consistent with the data collected? This question requires students to think deeply about: 1) their understanding of the synapse, 2) how aldicarb and levamisole impact the synapse and subsequent worm behavior and 3) what mutation would be consistent with the data generated. Students must apply their understanding to interpret their data. This tends to be the most challenging part of the project for students and often initiates critical discussion about the data and what can be inferred from the data.

Students develop an experimental plan and timeline

Students are challenged to design, execute and analyze experiments using both aldicarb and levamisole assays. While students have not practiced the levamisole assay during the technique labs, this has never proven to be a problem. The levamisole assay execution is executed in the same manner as an aldicarb assay. Students are reminded to plan for appropriate controls, plan staging of worms, etc. This data for these student-designed experiments can be collected over 2 lab periods. However, it can be helpful to plan for time to repeat experiments if possible. In addition, if students have 3 weeks, instead of just 2 weeks for this independent project, they can repeat experiments and possibly use statistics to determine if their mutant data is significantly different from wildtype.

Students are given a handout that guides them through the process of developing hypotheses, designing experiments to test those hypotheses, selecting controls, and planning a detailed timeline of their experimental design/plan. During the next lecture, each group presents their hypotheses, experimental plan and possible outcomes and interpretations. This is done as a PowerPoint presentation and is assessed by the instructor. Students must refer to the primary literature and cite references. After each presentation, each group receives immediate feedback from the audience and from the instructor. The instructor also meets with each group separately for ~10 minutes to give detailed feedback and to ensure optimal planning.

Each group is also required to provide a detailed timeline of experimental details to the instructor. The timeline is written in an outline format. For example, groups are required to list which staging procedure will be performed, when the staging procedure will be executed and which investigator is doing each step. Controls must also be included in the plan. This timeline also serves as a contract between lab partners. The timeline makes each step of the experiment clear as well as who is responsible for each step. For example, one partner may be responsible for maintaining worm strains on Monday, while the other partner must stage the worms on Monday. The timeline serves as a contract and a point of reference for students to ensure that each experimental step is executed in a timely manner. The instructor emphasizes the importance of designating time to repeat experiments due to unforeseen problems. This timeline document is submitted to the instructor for assessment and is not included in the students' PowerPoint presentations.

INDEPENDENT PROJECT EXECUTION

Students are given 2-3 weeks to characterize their mystery mutant. The 2-week time frame provides sufficient time for students to execute experiments that utilize aldicarb and levamisole assays. A third week allows additional time to repeat experiments and/or to gather more data that could be used to complete a statistical analysis. The instructor chooses which time frame is best for their objectives.

Students create and follow an experimental plan and timeline that they design. Some of the plans may not require students to be present for the full three hours during the lab scheduled meeting time, however, all students must be present at the start of each regularly scheduled 3-hour lab session during the independent project phase. This allows for the instructor to provide general lab announcements and to address issues that may be of importance for the class. Also, each week in lab, the instructor meets with each group to gauge how experiments are progressing and to help guide students find solutions to possible unexpected challenges or problems.

Towards the end of the module, student groups generate 15-20 minute PowerPoint presentations that describe their questions, hypothesis, data and interpretations. Students are given a grading rubric for their oral presentations (Lemons, 2012) and referred to a book entitled, "A Student Handbook for Writing in Biology" (Knisely, 2013). This book was required for students to purchase in the mandatory pre-requisite courses and has a chapter devoted to preparing a PowerPoint presentation. In addition, the instructor gives a brief presentation that illustrates "what not to do" for an effective presentation as well as common strategies used for effective presentations. Devoting time to explain effective oral presentation skills has consistently proven beneficial for this instructor's students.

One week after student group presentations, each student (working individually) submits a written lab report describing their research findings. The delay between oral presentations and lab reports gives students time to receive feedback from the instructor and to use this feedback to further improve their lab report. The lab report is done individually (but could be assigned as a group assignment) to ensure that each student fully understands the experiments. This also helps prevent situations where one student is doing the majority of the work and the other is not. It also gives each student an opportunity to reveal their full understanding of the data. The figures from the oral presentations can be modified for the lab report. Each student receives a grading rubric for the lab report

(Lemons, 2012) and is referred to Knisely (Knisely, 2013).

Students are given the opportunity to assess their work and their lab partners' work. Students score each member of their group (including themselves) on a scale from 1-100. If each member fully contributed to the project, they would earn a 100. If they contributed less, then the student would provide a score that reflects the contribution. The student puts a check next to their name, so the instructor knows which student wrote each assessment. Only the instructor sees the assessments. Students are aware of this peer assessment at the start of the assignment. Therefore, students know they will be held accountable. If there is a discrepancy of scores or if there are scores that suggest that there was inequity within a group, then the instructor will have a meeting with each member of the group and determine the appropriate course of action. For example, in one instance a student contributed substantially less than planned. After an honest discussion, the student and instructor agreed that it would be appropriate for the student to lose points on the PowerPoint presentation due to their limited contributions.

MATERIALS AND METHODS

C. elegans strains

C. elegans are grown at either 15^0C or 24^0C on NGM plates seeded with OP50. All strains are derived from the N2 Bristol strain (Brenner, 1974). Mutant strains used as controls include: CB382: *unc-49(e382)III* and ZZ30*: unc-29(x30)I*. See Table 1 for a list of strains that could be used for experimentation. These worm strains are available from the CGC.

NGM plates

To make 100mls of NGM agar, add 0.3grams of NaCl, 0.25grams of Peptone, and 1.7grams of Bacto-Agar to a flask and bring up to 100mls with ddH_20. The solution is autoclaved and then allowed to cool to 55^0C in a water bath. The flask is transferred to a stir plate and maintained at 55^0C with gentle stirring while the following supplements are added: 100μl of 1M $MgS0_4$, 100μl of 1M$CaCl_2$, 2.5mls of 1M$KP0_4$ and 100μl of cholesterol (5mg/ml in ethanol). If the agar solution becomes too warm, the salts will precipitate out of solution. Approximately 4mls of solution is added to tiny plates (e.g., 35mm petri dishes). After agar is fully dried (usually 1-3 days, depending on humidity), the dishes are seeded with 30-50μl of OP50. The OP50 should be placed in the center of the plate and not allowed to extend to the edge of the plate. Allow 1-3 days to allow OP50 to adhere to agar. Plates (seeded or unseeded) are stored at 4^0C for months, lid-side down.

OP50 solution

100ml of LB broth is inoculated with a colony of OP50 and incubated overnight at 37^0C. Solution should become cloudy. Solution is stored at 4^0C for up to two weeks. During that time, the OP50 solution can be used to seed NGM plates.

Worm pick

A ~1 inch piece of platinum wire (0.2032 mm diameter, Alfa Aesar, catalog# 7440-06-4) is held with pliers and placed into the small diameter end of a glass Pasteur pipette. Approximately half of the wire should be inside the pipette and half out. The small diameter end of the Pasteur pipette (with the wire) is held in a Bunsen burner flame for ~20 seconds until the flame begins to melt the glass. The wire and Pasteur pipette are removed from the flame and allowed to cool. This process is repeated until the wire is held firmly in place. After the wire is well anchored in the Pasteur pipette, a pair of pliers is used to flatten the very end of the wire and to bend it in such a way to create a shape similar to a spatula (Figure 1B,C).

Aldicarb and Levamisole plates

For aldicarb and levamisole assays, the same procedure for NGM plates is followed with a few exceptions. First,

CGC strain	Gene name	Allele	Gene homolog	Reference	Response to aldicarb	Response to levamisole
ZZ37	*unc-63*	*x37*	α-nAChR subunit	(Culetto et al., 2004)	resistant	resistant
CB1072	*unc-29*	*e1072*	Non-α-nAChR subunit	(Fleming et al., 1997)	resistant	resistant
CB883	*unc-74*	*e883*	Accessory protein	(Boulin et al., 2008)	resistant	resistant
RM509	*ric-3*	*md158*	resistant to inhibitors of cholinesterase, endoplasmic reticulum protein	(Miller et al., 1996; Halevi et al., 2002; Boulin et al., 2008)	resistant	resistant
PR1152	*cha-1*	*p1152*	choline acetyltransferase	(Rand and Russell, 1984)	resistant	normal to mildly hypersensitive
CB993	*unc-17*	*e245*	vesicular acetylcholine transporter (vAChT)	(Miller et al., 1996)	resistant	normal to mildly hypersensitive
CB246	*unc-64*	*e246*	syntaxin	(Saifee et al., 1998)	resistant	normal to mildly hypersensitive

Table 1. Predicted responses of select worm strains on aldicarb and levamisole.

65mm petri dishes (instead of 35mm petri dishes) are used. Second, aldicarb (Sigma catalog #33386) or levamisole (Sigma catalog #L9756) is added to the 55^0C NGM agar immediately after the addition of the salts and cholesterol. 1ml of 100mM aldicarb (in 70% ethanol) or 1.25 mls of 100mM levamisole (in ddH_20) is added. This makes a final concentration of 1mM aldicarb and 250μM levamisole plates, respectively. Aldicarb is light sensitive and aldicarb plates are kept in the dark. Aldicarb and levamisole plates are not seeded with OP50 and are stored at 4^0C. These drug plates are used within two weeks because the drugs' efficacy decreases over time. The assay is performed on plates that had been allowed to warm to room temperature prior to the start of the experiment.

Worm Bleach

To make 5mls of worm bleach, combine the following: 2.75ml dH20, 1 ml sodium hypochlorite and 1.25 ml 1M NaOH. This solution is stored at room temperature for up to one month. Approximately 30μl of worm bleach is added onto an OP50 seeded NGM plate. Gravid hermaphrodites are placed into bleach puddle and worms are observed under a dissecting microscope. If worms do not disintegrate (as expected), additional bleach is added and worms are observed again.

Chunking

A small spatula, stored in 70% ethanol, is sterilized in a Bunsen burner flame and used to cut a small square or "chunk" of the agar of a starved worm plate. The chunk of agar is placed, worm-side down, onto a new OP50 seeded NGM plate. L4 worms crawl out from the chunk in 18-48 hours, depending on the severity of the starvation and the strain.

RESULTS

Each group has a different strain and results between groups are varied, as expected. Predicted results per strain are listed in Table 1. Students most often obtain trends that are similar to expectations. In a few instances, students may initially acquire unexpected results, but this is usually due to human error such as incorrectly labeling the aldicarb plate as a levamisole plate or vice versa. However, repeated experimentation (as previously suggested) often catches this error.

Students learn to have greater confidence in their results when data from controls (e.g., *unc-29* and *unc-49*) are consistent with expectations. These controls often reveal when a human error has occurred and students are surprisingly alert to this important point. The importance of running controls (e.g., N2, *unc-29*, *unc-49*) in parallel with the mystery mutant for each assay is made clear and quickly appreciated. The suggestion to include these controls is given by the instructor in the early planning phase. While students are initially discouraged to test so many strains in parallel, the value of these controls is fully understood within the first week of trials.

DISCUSSION

This lab exercise has been rewarding for students and the instructor. In an informal assessment, a student reported that this lab was "extremely challenging and forced me to think." Another student commented, "I thought synapses were simple structures, until I learned how complex they are." While another wrote, "I was surprised that we could learn about so much about neurobiology from a worm."

Modifications to suit instructor-specific goals

There are several ways this exercise could be modified to meet the diverse needs of instructors, various courses and student abilities. For example, this exercise could be modified such that students are provided with the identity of their mutant strain rather than referring to the strain as a "mystery mutant." This leads students to search primary research articles to learn more about the affected gene and its functions. In this instance, students can also determine if their results are consistent with their expectations, given what is known about their assigned worm strain. Another variation of this approach is to give students a "mystery mutant" strain, allow students to design experiments, and then announce that their "mystery mutation" has been recently identified during week 2 of the independent phase. In this case, students will have planned their experiments without bias and then use the primary literature to determine if their data is consistent with their expectations, given what is known about the gene. This latter option has been used twice before by this instructor and has been successful. Roughly half of the students reported that they found it more satisfying to "know" which strain mutation they were studying, while others reported they enjoyed speculating which gene was affected in their mystery mutant strain.

Modifications that further enrich student-driven projects

Time permitting, students can also propose and address additional questions based on their observations. For example, one group noticed their mutant strain tended to grow fewer progeny than wildtype. This group chose to investigate egg laying (a very well-defined and highly studied circuit) and design experiments to determine if the mutant hermaphrodites: 1) laid fewer eggs, 2) laid eggs that were less likely to hatch, or 3) produced fewer eggs compared to wildtype. Students used the literature, in combination with consultation with their instructor, to design, execute and analyze these experiments. Due to the worm's transparency, it is easy to count the number of eggs within a hermaphrodite. Students can count the number of eggs within a worm, the number of eggs laid by a given worm (by counting eggs external to the hermaphrodite on the plate) and the number of hatched worms within specific time frames. This data, which is easily collected, can be used to explore the initial observation of decreased progeny for the mutant strain. This work was done in addition to the aldicarb and levamisole experiments.

Students have been inspired by the freedom to pursue questions based on their own observations. A student

wrote, "I liked generating my own questions and then figuring out how to answer them through experiments." Another wrote, "I liked the freedom to follow what I thought was interesting in this lab." In the past, this instructor gave students an additional 1-2 weeks to work independently. This was sufficient for these experiments. These experiments were included in their proposed timeline and PowerPoint presentation mentioned above.

Another alteration includes providing more than one concentration of aldicarb or levamisole drug plates. For example, students could use 1mM, 500μM or 1.5mM aldicarb plates, rather than restricting the concentration to the 1mM concentration. Rate of paralysis is directly correlated with aldicarb or levamisole concentrations.

Modifications for courses without labs or for courses without sufficient lab time

This exercise can be modified and implemented in courses without a lab component or courses without enough lab time. Students can be challenged to understand the synapse and how drugs affect the synapse by working with data provided to them from the instructor. For example, students can be given aldicarb and levamisole data from wildtype, and from control strains *unc-29* and *unc-49*. Students are challenged to apply their understanding of the synapse to understand how the provided data is consistent with expectations for these strains. An instructor-student discussion similar to the one mentioned under the heading "*Preparation for students to answer the question: What experiments can I design that reveals if a "mystery" mutation impacts the function of the presynaptic or postsynaptic component?"* is used to guide students through the thought process of data interpretation.

After students gain experience with interpreting aldicarb and levamisole data for the wildtype and control strains, instructors can provide students with data from their assigned "mystery mutant" strain. Instructors can obtain data from the literature or they can request data from this instructor. Students must interpret provided data and determine if the mutation impacts the presynaptic or postsynaptic component. This "data provided by instructor" approach is highly valuable because students are challenged to apply their understanding of the synapse to interpret data. Instructors can choose to provide students with graphed data or they can provide raw data and have students generate graphs. Regardless, students will analyze and interpret data, which is often the most intellectually challenging component of this exercise. Thus, this option is a worthwhile exercise is worthwhile and beneficial.

Tips for success

It is important to keep aldicarb and levamisole plates stored at 4^0C and aldicarb plates also need to be stored in the dark. The efficiency of these drugs decreases overtime and the efficiency of aldicarb also decreases in the presence of light. These drug plates are best if used within two weeks of pouring. It is also important to allow the aldicarb and levamisole plates to warm up to room temperature immediately prior to starting the assays. Worms plated on cold plates will move differently and it will be difficult to score animals consistently on cold plates. Lastly, it is sometimes helpful to use a worm pick to move the worms towards the center of the plates during an aldicarb or levamisole assay. Worms tend to crawl towards the edges during the first 15 minutes of these assays and this can sometimes make it difficult to track the worms.

There can be variability in scoring paralyzed worms. Some variation stems from the individual researcher. Different researchers sometimes score paralysis in a distinct manner. It can be valuable for students to modify or more clearly define "paralysis" for their independent project execution. For example, some students chose to score a worm as paralyzed if the worm did not move its body within 3 seconds after a tap to the head. This same group chose to exclude any movement of the head. So, if the head moved and the body did not move within three seconds after a touch to the pharynx, this group would score that animal as being paralyzed. Other groups included head movement in their definition of paralysis. As long as each student researcher is consistent, the data will be reliable.

Optional class discussion regarding impact of drugs on synaptic function in humans

Time permitting, the instructor can guide a discussion about the effect of drugs on synaptic function in humans. One example that has strong connections to the student-driven experiments is a drug, pyridostigmine, which is an acetylcholinesterase inhibitor. Pyridostigmine is used as a treatment for Myasthenia Gravis (MG). MG is an autoimmune disease; the immune system attacks select components in the postsynaptic membrane including cholinergic receptors and this is associated with skeletal muscle weakness (Gilhus and Verschuuren, 2015). Patients with MG can benefit from pyridostigmine because the drug prolongs the time in which acetylcholine remains in the synaptic cleft, therefore allowing more time for ligand-receptor binding to occur with limited, functional cholinergic receptors.

These studies could also spawn discussion of other synaptic diseases, including mental depression, and their treatments. This discussion could include the role of neuromodulators in synaptic function and whether or not aldicarb and levamisole assays could be used to study neuromodulation.

SUMMARY

This lab exercise challenges students to: 1) design, execute and analyze experiments using *C. elegans* and 2) apply their understanding of the synapse to correctly interpret data. These experiments are reliable and are relatively inexpensive, thus making them ideal for an undergraduate setting. Furthermore, several modifications are described that would enable this module to be used in a variety of courses with or without lab component.

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