

ARTICLE

Using *Drosophila* Motor Mutants to Teach Neurodevelopment in an Undergraduate Neurobiology Lab

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Most undergraduate neuroscience courses include a neurodevelopment component. Typically, the focus is on development of the mammalian central nervous system, including the concepts of neurulation, patterning of the neural tube, and differentiation of the various cells required to build a functional nervous system. However, it can be challenging to design an affordable undergraduate laboratory exercise to reinforce these concepts for students outside of lecture. Here we describe a laboratory exercise that takes advantage of the high level of conservation in neurodevelopmental pathways using *Drosophila* as a model organism to illuminate the connection between cell differentiation and nervous system function. Following a

lesson discussing spinal cord development, students use *Drosophila* larvae to assess the effects of mutations in highly conserved motor neuron differentiation genes on motor behaviors such as crawling. As outcomes of this laboratory, students are able to master important neurodevelopmental concepts, connect neurodevelopment to nervous system function, and gain experience with experimental design and data analysis.

Key words: Motor neuron differentiation, Neurodevelopment, Spinal cord development, Motor systems, Drosophila

Development of the mammalian nervous system is a standard topic of discussion in nearly all undergraduate neuroscience courses. Most courses focus on the embryological origins of the central nervous system (CNS), which arises from the ectoderm of the embryo during a process called neurulation. During neurulation, the neural ectoderm undergoes morphological changes that cause it to invaginate along the dorsal surface of the embryo, fold into a cylinder, and separate itself from the surrounding, non-neural ectoderm. Neurulation thus creates a cylinder of neural ectoderm that lies just below the dorsal body surface and extends from the anterior to the posterior of the embryonic body. This cylinder, the neural tube, is the precursor for the entire central nervous system. This initially simple structure undergoes profound changes to give rise to the complex cells and structures of the brain at its anterior end, and of the spinal cord throughout the trunk and abdomen of the body.

Development of the neural tube into the CNS is incredibly complex, requiring coordination of cell division, differentiation, and migration. The spinal cord is a good model for understanding these developmental processes. For example, extensive research has shown how diffusible signals produced by tissues proximal to the developing spinal cord, the notochord and the dorsal epidermal ectoderm, generate morphogen gradients that drive cell differentiation (Jessell, 2000; Lee and Pfaff, 2001; Lewis, 2006; Butler and Bronner, 2015; Gouti et al., 2015). A gradient of sonic hedgehog (Shh) arising from the notochord regulates differentiation of ventral cell types, while a gradient of bone morphogenetic protein (BMP) family ligands arising from the dorsal ectoderm regulates differentiation of dorsal cell types. Together, these opposing signaling gradients drive cell differentiation throughout the developing spinal

cord, yielding a variety of neurons and glial cells organized into distinct functional compartments along the dorsal-ventral axis.

These early developmental steps are critical for building a functional nervous system, but may be difficult for students to grasp for a variety of reasons. First, like all aspects of mammalian development, they occur while the embryo is sheltered inside the mother's uterus, and thus cannot be easily observed. Furthermore, events such as intercellular signaling and changes in gene expression are crucial developmental processes, but are essentially biochemical reactions that are invisible to the eye. Finally, the early development of the spinal cord, in which cells adopt distinct fates and developmental pathways, do not necessarily cause obvious external changes to the cell; for example, motor neurons are not immediately distinguishable from interneurons, and changes to cell morphology and tissue structure may not be apparent until much later in development. All of these challenges make it difficult for students to connect early developmental processes to the mature, functional nervous system with which they are more familiar.

We carried out a literature search to identify lab activities that would help students connect early development to adult nervous system function. Although it is easy to find innovative neuroscience labs aimed at undergraduate students (particularly those published in this journal), we discovered that few of these labs focus on neurodevelopment. Those we did find often utilized mammalian cell culture, a model that can be expensive and difficult to implement at institutions without proper equipment (such as cell culture hoods and incubators; e.g. Catlin et al., 2016 and Pemberton et al., 2018). We therefore designed lab that is inexpensive, easy to implement, and

helps students connect developmental processes like cell differentiation to the structure and function of the mature nervous system.

We focused our lab exercise on the development of spinal motor neurons as they are responsible for governing movement, a trait that can be readily grasped by students and directly assessed in a lab. Furthermore, motor neuron differentiation pathways are highly conserved, making it possible for us to use an affordable model organism and simple tools and techniques, appropriate for labs with any number of students and being carried out at any institution, regardless of available resources.

As mentioned previously, Shh induces differentiation of ventral cell types within the developing spinal cord, including motor neurons. Intermediate levels of Shh activate expression of the transcription factors Pax6, Nkx6.1, Mnr2, and Olig2 within a ventral region of the spinal cord referred to as pMN, from which all spinal motor neurons will arise (Jessell, 2000; Price and Briscoe, 2004; Stifani, 2014; Zagorski et al., 2017). These proteins cooperatively activate a transcriptional program that drives motor neuron differentiation. An important step along that path is the activation of both Lhx3 and Islet1, which form a DNA-binding complex that directly activates genes specific to motor neuron function (Lee et al., 2012; Cho et al., 2014; Kania, 2014; Stifani, 2014). Once differentiated, motor neurons extend axons outward from the spinal cord toward the skeletal muscles of the body. Disruption of these developmental processes results in failed innervation of the skeletal muscles and thus impaired body movement.

In this lab, students used *Drosophila melanogaster* as a model to study the importance of motor neuron differentiation for movement and survival. We chose to use *Drosophila* as a model organism both because of its affordability and because it is easy to obtain mutants for most genes in its genome. Students worked with wild-type *Drosophila*, as well as *Drosophila* stocks carrying mutations in several genes that are critical for motor neuron differentiation in both mammals and *Drosophila*: *Olig2*, *Lim3* (the *Drosophila* homolog of *Lhx3*), and *Tailup* (*tup*, the *Drosophila* homolog of *Islet1*) (Thor and Thomas, 1997; Thor et al., 1999; Certel and Thor, 2004; de Navascues and Modolell, 2010; Oyallon et al., 2012). These genes were selected because they act at multiple levels of the genetic hierarchy involved in motor system development, providing students with an opportunity to compare and contrast their phenotypes. In addition, loss of function mutations in any one of these genes in *Drosophila* results in overt motor neuron differentiation defects and failed innervation of target muscles, reflecting conservation of gene function between *Drosophila* and vertebrates (Thor and Thomas, 1997; Thor et al., 1999; Oyallon et al., 2012). Students both qualitatively and quantitatively assessed motility in larvae of these various genotypes. Students then completed lab reports in which they presented and analyzed their data, made conclusions about the requirement of motor neuron differentiation genes for larval mobility, and discussed the importance of developmental processes such as motor neuron differentiation for adult success and survival.

MATERIALS AND METHODS

Materials

- Fly stocks (obtained from the Bloomington *Drosophila* Stock Center at Indiana University): Wild-type flies (Bloomington stock #5 or #3605; we used #3605), *Olig2* mutant flies (Bloomington stock #59233), *Tup* (*Islet* homolog) mutant flies (Bloomington stock #2207), *Lim3* mutant flies (Bloomington stock #3393)
- Fly culture vials and plugs (available from Carolina Biological Supply, Catalog #173078).
- Fly food (available from Carolina Biological Supply, Catalog #173210)
- Incubator at 25 °C (optional)
- Sharpies (one per group)
- 100 mm Petri dishes (empty; four per group)
- 20% sucrose solution (50 mLs per group)
- Paintbrushes (one per group)
- 100 mm Petri dishes with 2% agarose (eight per group)
- Graphing paper (0.2 cm², four sheets per group)
- Stereomicroscope (one per group)
- Timer (one per group)
- Plastic bags and freezer for disposal

Culture Preparation

Fly stocks were ordered in advance from the Bloomington *Drosophila* Stock Center at Indiana University (<https://bdsc.indiana.edu/>; allow one month for processing and delivery). Note that while the wild-type flies were homozygous, each of the mutant flies were delivered as heterozygous stocks due to homozygous lethality (Thor and Thomas, 1997; Thor et al., 1999; Oyallon et al., 2012). The adult flies for this lab had the following heterozygous genotypes: *Olig2/CyO*; *Tup/CyO*; and *Lim3/SM6a*. *CyO* and *SM6a* are balancer chromosomes, which are used in *Drosophila* fly husbandry to maintain heterozygous stocks. Balancer chromosomes help preserve gene mutations via two mechanisms: (1) balancer chromosomes contain inversions that impair genetic recombination, thereby preventing loss of the mutation due to genetic recombination; and (2) balancer chromosome themselves contain homozygous embryonic lethal mutations and thus can never become homozygous within the stock. As a result, a “balanced” stock will always include adult flies that are heterozygous for both the mutation of interest and the balancer chromosome. Readers can refer to the following website for further information if they are unfamiliar with fly genetics (https://bdsc.indiana.edu/stocks/balancers/balancer_intro.html).

Approximately three weeks before the experiments were to take place, fly stocks were expanded by serially transferring adults to a new vial every two days for a period of ten days to obtain healthy and abundant populations of each genotype (these vials can be stored at either room temperature or in a 25 °C incubator if available; see Table 1 for timeline). Five to six days before the experiments were to take place, adult flies (10-15 males and 10-15

Timeline	Task
At least six weeks before Lab 1	Order fly stocks from the Bloomington <i>Drosophila</i> Stock Center
Three weeks before Lab 1	Transfer adult flies into fresh vials to expand stocks
19-13 days before Lab 1	Transfer adult flies into fresh vials every two days to expand stocks (keep previous vials)
Lab 1	Presentation and Pre-Lab assignment
Six days before Lab 2	Transfer adult flies into fresh vials to prepare larval cultures
Three days before Lab 2	Remove adult flies from vials; keep vials
Lab 2	Carry out motor behavior experiment

Table 1. Timeline of drosophila husbandry.

females) were transferred to fresh vials of food. Each student group should have one vial of each genotype. Vials were stored at either 25 °C or at room temperature and adults were allowed to lay eggs in the vials for two-three days, until there are obvious signs of larvae within the vials. Adults were then removed, and vials were stored in the incubator until needed for lab to allow larvae to develop.

Week 1: Introduction And Pre-Lab Assignment

The basic concepts addressed in this lab, including neurulation, early CNS development, and cell type differentiation in the developing spinal cord, were covered as part of lecture. However, the first week of this lab exercise included an additional presentation providing more detailed background (see Appendices). The presentation reminded students specifically of the function of motor neurons within the nervous system and the genetic pathways that regulate their differentiation in the embryonic spinal cord, with a particular focus on the genes that are mutated in the fly stocks utilized for this lab: *Olig2*, *Tup*, and *Lim3*. This presentation also included an introduction to *Drosophila* as a model organism as well as videos depicting the movement of wild-type larvae to illustrate normal motor function (Oyallon et al., 2012; Nichols et al., 2012). Supplemental readings were posted to assist students with the completion of these pre-lab reports, which were due at the beginning of the following week's lab (i.e., Price and Briscoe, 2004; Stifani, 2014; Gouti et al., 2015).

Students were then asked to complete a pre-lab assignment (see Appendices). The assignment asked students to summarize basic aspect of central nervous system development, such as neurulation and the roles of Shh and BMP in spinal cord patterning. These questions assessed student understanding of basic neurodevelopment. Additional questions asked students to explain the relationships amongst *Shh*, *Olig2*, *Tup*, and *Lim3* to assess student understanding of the genetic networks involved in motor neuron differentiation. A final set of

questions required students to apply that knowledge to larval behavior by predicting the effects of *Olig2*, *Tup*, or *Lim3* gene mutations in larvae. As part of this assignment, students participated in experimental design by choosing qualitative parameters to assess during their larval observations the following week.

Week 2: Evaluation Of Larval Mobility

Experiments were carried out during the second week of lab. For that lab meeting, vials of larvae and additional materials including sucrose, transfer pipets, Petri dishes, paintbrushes, agar plates, graph paper, stereomicroscopes, and timers were assembled (Figure 1). Each group received one vial of each genotype (four total), four empty Petri dishes, and eight agar plates. Students were instructed to label Petri dishes and agar plates, with one Petri dish and two agar plates for each genotype being analyzed.

Prior to beginning the experiment, students were reminded of the genotypes expected in the larval populations. Because the parental stocks are heterozygous, their progeny are produced in a 1:2:1 ratio of balancer chromosome homozygotes to heterozygotes to mutant homozygotes. However, balancer chromosome homozygotes die as embryos and thus are absent from larval populations. Larval populations could therefore contain a mixture of heterozygous and homozygous mutant individuals depending on homozygous mutant phenotypes. In the case of *Tup*, homozygous mutants die as embryos,

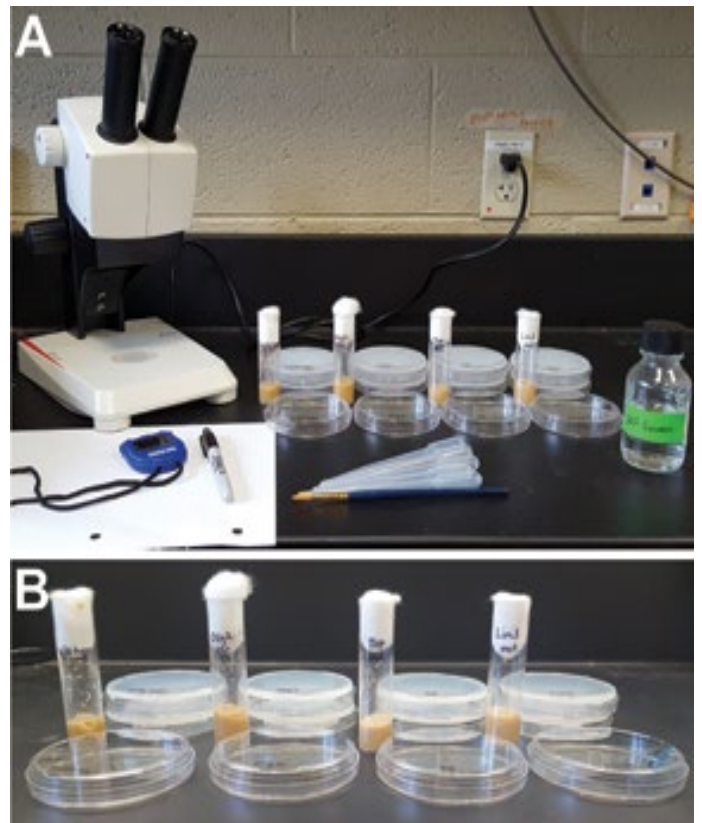


Figure 1. (A) A typical laboratory setup including all necessary reagents. (B) Vials containing larvae of various genotypes (wild-type, *Olig2*, *Tup*, and *Lim3*), along with correspondingly labeled petri dishes (one empty, two with 2% agarose).

and therefore all larvae are expected to be heterozygous *Tup/CyO* (Thor and Thomas, 1997). *Lim3* mutants die in late larval or pupal stages, and therefore larval populations within this stock are expected to contain a roughly 2:1 ratio of *Lim3/SM6a* heterozygotes to *Lim3/Lim3* homozygous mutants (Thor et al., 1999). *Olig2* mutants die as pupae or adults, and therefore larval populations within this stock are expected to contain a 2:1 ratio of *Olig2/CyO* heterozygotes to *Olig2/Olig2* homozygous mutants (Oyallon et al., 2012). Note that while the larval phenotypes of heterozygous *Tup*, *Lim3*, and *Olig2* mutants have not previously been reported, our unpublished observations suggest that heterozygotes of all three mutants may exhibit mild to moderate motor defects. However, we did not share this information with students, instead allowing them to reflect on the heterozygous phenotypes as part of their post-lab assignment.

To begin their experiments, students first retrieved larvae from their vials. Sucrose solution was added to each vial using transfer pipets, enough to cover food with about one inch of liquid. This step encourages larvae to crawl out of food and into the liquid sucrose solution. After five minutes, after multiple larvae were observed in the sucrose solution, transfer pipets were used to move larvae from the sucrose solution into the appropriately labeled empty petri dish. Students then carried out a qualitative analysis of larval movement. They first used a paintbrush to transfer one wild-type larva to an appropriately labeled agar plate and observed its movement (Note: students should be directed to use the largest larvae for these assays; larval size increases with age and students should attempt to use larvae that are similar in age for all experiments). Students were asked to describe the normal movement of the wild-type larva, paying particular attention to the qualitative parameters they had chosen to assess in their pre-lab assignments. Their observations were recorded on their lab assignments (see Appendices). This process was repeated until a minimum of ten wild-type larvae had been observed. Students then repeated this procedure with the mutant larvae, observing at least ten mutant larvae of each genotype to account for the presence of both heterozygous and homozygous mutants in some genotypes (see Oyallon et al., 2012; and Appendices for examples of mutant larval movement).

In the next part of the lab, students assessed larval movement in a more quantitative fashion. For this experiment, the remaining agar plates, one for each genotype, were placed on top of the graphing paper, and students prepared their timers in order to measure the rate of movement of each larval genotype (Figure 2). Students set the timer for one minute, transferred a wild-type larva to the appropriate agar plate, started the timer, and then counted how many grid lines the larva crossed before the timer indicated that a minute had passed. Larvae were counted as crossing grid lines when the anterior end of the larvae crossed into a new grid square. Students recorded their measurement on their lab assignments or notebooks, then repeated with nine additional larvae, for a total of ten

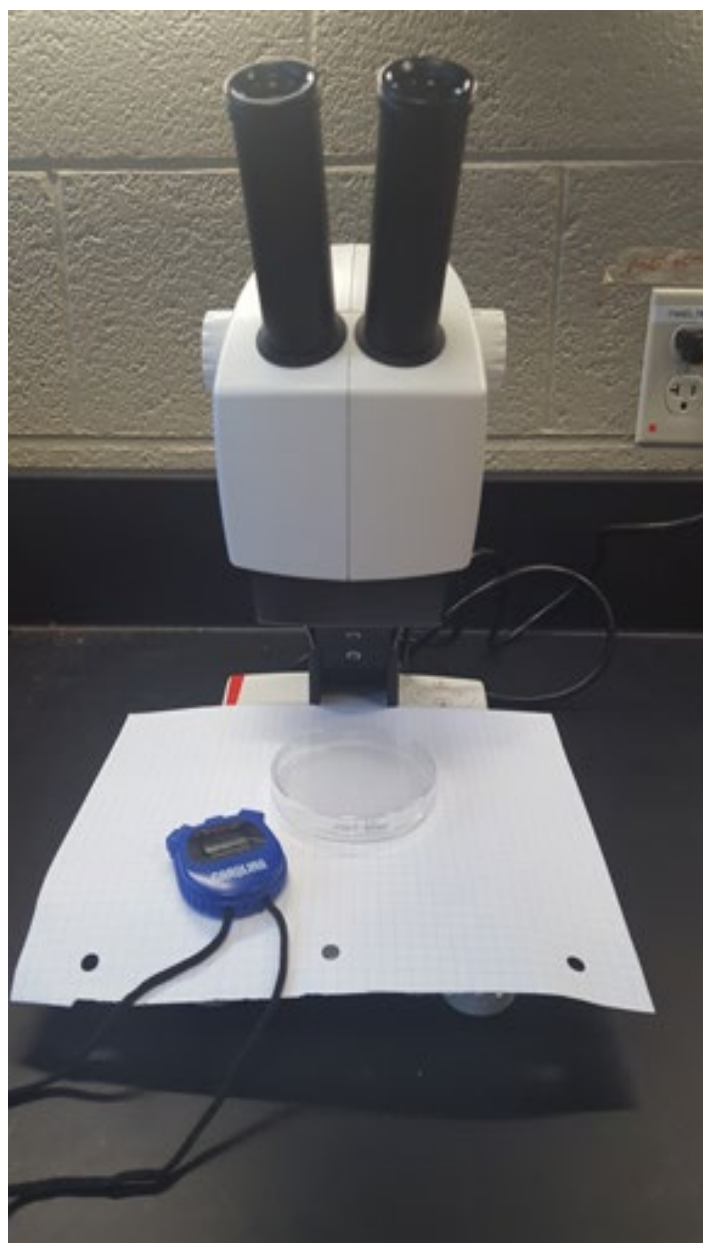


Figure 2: Experimental setup for quantitative assessment of larval movement. Agar plate should be placed over graphing paper and prepared for observation under stereomicroscope. Larva can then be transferred onto agar plate and its movement can be assessed for one minute (using timer to determine when a minute has elapsed).

measurements. Students then performed the same analysis with mutant larvae, again assessing ten larvae from each group.

After experiments had been completed, most experimental materials were discarded as non-hazardous waste in normal waste bins. Flies and larvae, and all materials that may have larvae on them, were placed in plastic bags, stored overnight in a $-20\text{ }^{\circ}\text{C}$ freezer to euthanize all flies, larvae, and eggs, and then discarded in a normal waste bin the following day.

Data Analysis And Evaluation

Following data collection, each student group calculated the average gridlines crossed per minute for each of the three mutant groups and the wild-type larval group. The calculated averages for the wild-type and mutant larvae from each student group (minimum of three student groups) were then compiled for the entire class, generating an overall average for the wild-type, *Tup*, *Olig2*, and *Lim3* mutants (see Figure 3 for an example of student data). Students were also asked to complete a series of post-lab questions to summarize their data and draw conclusions for each mutant. A final question asked students to consider whether their data matched their predictions or not, and to propose explanations for any discrepancies between their hypothesized and actual results.

RESULTS AND DISCUSSION

Although neurodevelopment is an important topic in undergraduate neuroscience courses, it can be a challenge to design and implement an easy, affordable lab exercise that allows students to explore embryonic development of the nervous system and directly connect the events of embryogenesis to the functional, adult nervous system. Most published neuroscience lab exercises focus on aspects of adult nervous system function rather than development, and those that do feature development may be expensive or difficult to implement at schools with limited resources (such as Catlin et al., 2016; Pemberton et al., 2018). The exercise we have described here is based on one of the most basic concepts of neurodevelopment, differentiation of functional cell types within the embryonic central nervous system, and helps students understand how this process is required for adult processes such as movement. In addition, our lab uses a simple model organism and readily available reagents, making it easy to implement at any undergraduate institution.

The primary goal of this lab exercise was for students to meet important learning objectives for the course. For example, students should be able to describe basic neurodevelopmental processes such as neurulation and explain how various cell types form during development of the nervous system. This lab exercise also required students to apply those basic concepts by predicting how disruption of embryonic development affects the function of the adult nervous system. Finally, students completing this lab gained experience in experimental design, data collection and analysis, and critical evaluation of results. Student mastery of these learning objectives and skills was assessed in pre- and post-lab assignments.

Student Outcomes

The pre-lab assignment assessed student mastery of several important learning objectives: (1) Describe early development of the spinal cord; (2) Describe the genetic networks that control motor neuron differentiation; (3) Predict the effects of gene mutations on motor neuron development and larval nervous system function; (4) Design a qualitative experiment to assess larval nervous system function. Evaluation of the pre-lab assignments suggests that students mastered these learning objectives

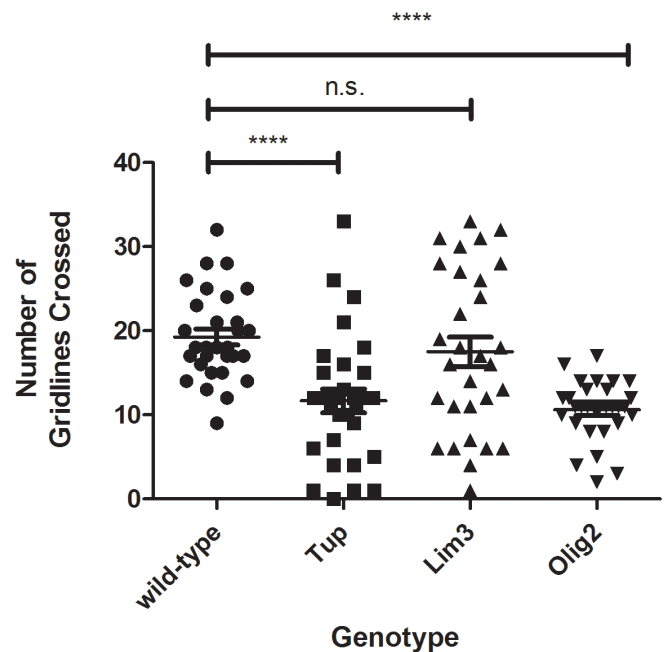


Figure 3. Results of quantitative analysis of motor behavior in motor mutant larvae. The average number of gridlines crossed per minute are shown for wild-type (WT), *Tup1*, *Olig2*, and *Lim3* motor mutant larvae. Averages were calculated by compiling data from three student lab groups; each group assessed ten larvae/genotype. Error bars represent standard error of the mean. Data were analyzed using t-tests; **** indicates $p < 0.0001$; n.s. indicates $p > 0.05$.

by the end of this lab exercise. Lab Assignment 1, Questions #1-2 assessed the first learning objective; 100% of students answered these questions correctly by describing the process of neurulation accurately and clearly explaining how Shh and BMP act as morphogens to pattern the dorsal-ventral axis of the spinal cord. Lab Assignment 1, Questions #3-4 assessed the second learning objective; 82% of students answered these questions correctly by describing the correct roles of *Shh*, *Olig2*, *Tup*, and *Lim3* in motor system development. Lab Assignment 1, Question #5 assessed the third learning objective; 100% of students answered this question correctly by predicting that loss of function mutations would result in failed motor neuron differentiation and defective larval movement. Importantly, 70% of students predicted that loss of *Olig2* would have more severe effects than loss of either *Tup* or *Lim3* because of its higher placement in the genetic hierarchy, further suggesting that students had mastered this learning objective. Lab Assignment 1, Question #6 assessed the fourth learning objective; 90% of students answered this question correctly by suggesting qualitative measures such as slow, uncoordinated movement and incomplete peristalsis. These responses suggest that students successfully mastered key neurodevelopmental concepts and were able to connect embryonic development to larval behavior.

Students also gained valuable experience with skills such as data analysis and critical evaluation of results. Lab Assignment 2 assessed our final learning objective: (5)

Collect and evaluate data in order to either accept or reject your predictions about how gene mutations would affect motor neuron development and larval motor function. All students were able to successfully collect quantitative and qualitative data in Lab Assignment 2. Students were then asked to evaluate their results in Lab Assignment 2, Question #4. All students responded that they had observed altered motor behavior within all mutant larval populations, as expected. Examples of observed altered behavior included fewer gridlines crossed, frequent directional changes, difficulty initiating movement, and dragging of the abdomen. Furthermore, most students (73%) noted that the data were variable, the phenotypic effects were not as severe as they had predicted, and suggested that these findings may have been due to the presence of heterozygous larvae in all samples. Student performance on these post-lab questions suggested that they were able to successfully participate in data collection and critically evaluate the data they collected.

Suggestions for Implementation

The lab exercise as described here was successful in achieving our desired learning outcomes. We have several recommendations for implementation to ensure success. While instructors themselves can take advantage of videos of mutant larvae to help recognize abnormal larval movement, they should refrain from showing those videos to students to avoid influencing student observations. In addition, because of the mixed genotypes present in some of the larval populations, it is imperative that students assess at least ten larvae per genotype per group, and we recommend pooling class data to obtain a larger dataset. However, the presence of mixed genotypes with different phenotypes presents a valuable opportunity to have students connect genotype to phenotype; instructors that wish to emphasize this relationship can ask students to graph their data in a dot-plot format (as shown in Figure 3) to better enable the identification of distinct phenotypic subgroups within their dataset, possibly reflecting the distinct genotypes present.

Beyond the applications described here, this basic lab structure could easily be modified to address additional learning objectives if desired. For example, instructors who would like to incorporate more quantitative data analysis could have students assess more than ten larvae to increase the number of data points and carry out statistical analyses of their results. Instructors could also expand the lab by including additional *Drosophila* motor mutants and/or including additional motor assays (additional motor assay protocols described in Nichols et al., 2012). An instructor could choose to spend more time exploring evolution of the central nervous system as part of this lab exercise if desired. Although we did not focus on the differences between the *Drosophila* and human nervous systems in our course, one could readily do so. The instructor could assign additional readings describing the structure of the *Drosophila* central nervous system, along with the reviews we assigned describing the mammalian central nervous system, and ask students to compare and contrast the roles of *Olig2*, *Tup*, and *Lim3* in the development of these two species.

Finally, the approaches and methods used in this lab activity could be applied to other neuroscience topics such as diseases of the nervous system. Multiple neurological disorders affect movement, and some of those diseases have well-established *Drosophila* models, including spinobulbar muscular atrophy (SBMA), spinal muscular atrophy (SMA), and amyotrophic lateral sclerosis (ALS; Watson et al., 2008; Lloyd and Taylor., 2010; Grice et al., 2011; Casci and Pandey, 2015; Xu et al., 2015). The same experimental techniques could be used to help students investigate the phenotypes associated with these diseases. Therefore, this lab exercise not only effectively reinforces concepts in neurodevelopment but also provides a flexible template to be used for other undergraduate neuroscience labs.

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Group	# of Gridlines crossed in 1 minute	Description of crawling behavior

Post Lab Questions

1. Summarize your qualitative descriptions of WT and mutant larval crawling behavior.
2. Use your group's data to calculate the average number of gridlines crossed in 1 minute for each genotype.
3. Now use class data to calculate the average number of gridlines crossed in 1 minute for each genotype.
4. Based on your qualitative and quantitative data, did the mutants exhibit the types of motor defects you predicted in your pre-lab assignment? Propose explanations for your data and conclusions.