

ARTICLE

Using the Tools of Behavioral Neuroscience to Determine the Identity of Different Mouse Strains in a Laboratory Course

Janine M. Wotton & Michael J. Ferragamo

Neuroscience Program, Gustavus Adolphus College, 800 College Ave., St. Peter, MN 56082.

Understanding the neural mechanisms underlying behavior depends on our ability to define and to measure these behaviors in the model animal. We describe an upper-level course which provides students with hands-on experience in the methods of behavioral neuroscience. There are many well-established behavioral tests which are relatively easy for students to conduct that can be used to determine the performance of animals in such tasks as anxiety, motor performance and memory. Laboratory mice bred specifically to exhibit particular behavioral characteristics are readily available from vendors along with well documented behavioral profiles for these strains. We used two albino strains CD1 and BALBc as our model animals. Students were given the task of identifying the strains based on the results of a battery of behavioral tests but were not given information about the mice. These two

strains were chosen for their clear differences particularly in tests of anxiety. Students conducted elevated plus maze and zero maze tests, open field test, light-dark exploratory task, rotarod, balance beam test, spatial or novel object learning. Students were able to correctly identify the two strains by comparing their own data with the published literature in the field. The course structure encouraged students to work in teams to design protocols, and then to collect and explore data. Students were enthusiastic about the hands-on laboratory experience and were able to demonstrate an appreciation for and understanding of these methods in behavioral neuroscience.

Key words: inbred mice, outbred mice, anxiety, motor performance, memory, biobehavioral

The availability of mice that express a variety of behavioral phenotypes produced by either mutation or transgenic manipulation has accounted for many important advances in biomedical research (Wahlsten, 2011). Students bound for research and clinical careers in neuroscience should understand the importance of evaluating behavioral phenotypes before selection of a model for further neurobiological investigations. Scrupulous characterization of behavior requires compiling a broad assessment of results gathered across multiple tests, with each targeted to a specific behavior (Crawley, 2007; Wahlsten, 2011). Furthermore, different mice with a similar behavioral characteristic may respond differently to particular paradigms therefore each characteristic should be analyzed by more than one test (Crawley et al., 1997; Crawley, 2008).

Fortunately, there is a wealth of carefully controlled, replicable studies that used conventional methods to detail the behavioral traits of many mouse strains (e.g., Crawley et al., 1997; Crawley, 2008). That literature, which is very accessible to students at the undergraduate level, opens up an opportunity to introduce them to each method along with its potential applications, strengths and limitations, and also represents a valuable resource of established hypotheses for them to test. Here, we describe one approach to turning that opportunity into practice in an upper level behavioral neuroscience course. Rather than have students simply run each test to compare the level of a trait displayed between two identified mouse strains, we ask them to determine the identity of two mouse strains by comparing the outcomes of a battery of tests to the

standards described in the literature. This approach motivates students to treat the work of an entire semester as if it were a puzzle to solve. Moreover, it obliges them to read widely since they cannot make a correct identification by limiting their reading to studies involving only two strains. The key to making this work is to select two strains that show consistent differences with respect to whichever behavioral tests are available to the instructor. Our strains (CD1 and BALBc) were chosen in consultation with the vendor (Charles River) with the expectation that clear differences in anxiety (especially light/dark exploration) and motor performance would be evident.

Purpose of course:

In this course students conducted a battery of behavioral neuroscience tests over a full semester in an investigative project. Students learned theoretical content about the methods of behavioral neuroscience, applied this knowledge in the laboratory and communicated the results of their experiments. Students worked in groups to define their measurements, agreed on details of protocols and collaborated to collect and analyze data. The analyses were open-ended to allow students to actively explore data and to apply techniques from the literature. Individually students were required to present the results of the laboratory session as written lab reports in the style of journal articles.

Specifically, the course aimed to provide opportunities for

- 1) data analysis and presentation
- 2) written communication

- 3) group cohesion and collaboration
- 4) analysis of the literature to understand the purpose and advantages/disadvantages of different tests
- 5) hands-on laboratory skill development

MATERIALS AND METHODS

Behavioral Neuroscience (PSY 345) primarily served juniors and seniors enrolled in our neuroscience minor who by that point in the curriculum have at least completed our Introduction to Neuroscience course and laboratory. The class met for five hours each week over a fourteen-week semester, divided roughly equally between meeting in the classroom and in a behavioral neuroscience laboratory. During the first few weeks, students were introduced to the critical importance of behavioral measurements, the variety of methods available in general and on our campus, and the rationale behind selecting among them to best address a particular set of scientific questions. This component of the course was seminar style. Each student was responsible for leading a discussion of material published in current journal articles that exemplified the application of conventional methods and analyses. Students read peer-reviewed articles which utilized the methods that would later be used in the laboratory component of the course. Topics were generally organized into three categories: (1) anxiety, (2) motor and (3) learning and memory. The motivation for this grouping was based upon conventional practices in the field and because it set up a rotational schedule for a laboratory outfitted with a single piece of equipment for each test.

Students were divided into lab groups of about 3-5 people (ideal maximum enrollment = 12-15) to make three groups. Each group was assigned 8-12 male mice, 4-6 from each strain (CD1 and BALBc). Animals were typically housed in groups of four mice of the same strain and the cages were labelled strain 1 or strain 2. This allowed students to collect data by strain and then compare across the groups. Individual mice in the cage were numbered and labelled by colored animal markers (Stoelting). Students were aware that they had to design tests to distinguish between two strains, one of which was inbred. The strains of the two groups of mice were not revealed and cannot be easily distinguished by physical features, like color, as both types are albino.

Each week a student group rotated through one of the methods in the category, thus enabling their animals to complete all of the experiments from a single category in 3 to 4 weeks, and across the three categories in 9-10 weeks. As mentioned, this schedule obviates the need for purchasing more than one piece of equipment for each method. An example of the schedule for one group:

Weeks 1-3 Anxiety Tests

plus maze, zero maze, light/dark box

Weeks 4-6 Motor Tests

rotarod, balance beam, open field

Weeks 7-10 Learning and Memory Tests

Y-maze, novel location, novel odor

The battery of these nine tests was sufficiently diverse to provide data for behavioral performance that indicated differences and similarities. Other tests could be substituted in this test battery depending on the equipment available, such as: Morris water maze, footprint analysis, gait analysis, startle or pre-pulse inhibition, strength grip tests, parallel bars or social tests. Many of these tests can be implemented with relatively simple and inexpensive equipment.

Extensive and detailed protocols for these tests are freely available, for example, International Mouse Phenotyping Resource of Standardised Screens (IMPreSS) (<https://www.mousephenotype.org/impress>), National Institutes of Health (Methods of Behavior Analysis in Neuroscience, 2nd edition *Frontiers in Neuroscience* Edited by Jerry J Buccafusco, 2009) and videos from JOVE (<https://www.jove.com>). In addition, there are excellent books such as Crawley (2007) and Wahlsten (2011) which describe the methods of behavioral neuroscience.

Method: Anxiety tests

The anxiety tests included the elevated plus maze, the elevated zero maze and the light/dark box, and all used a 5-minute test protocol. Prior to the test, students discussed and agreed on common behavior definitions and start locations. For example, in the elevated plus maze the mouse was always placed in the mid-section but facing a closed arm. Students agreed that a crossing from one zone to another required all four feet to cross a zone. Stop watches were used to time the duration in the open region and the duration in the closed region. Event measures such as crossing, rearing (standing on hind legs), stretch attends (stretching head and neck to explore without moving feet), dips (poking the head down the side of the open arms of elevated maze) and defecations were also clearly defined and recorded. Light levels were maintained at a fairly intense level (about 600-700 lux) to induce anxiety and ensure that animals would notice the difference between open and closed areas. It is recommended that the anxiety tests be performed first, as results on these tests can be influenced by other test experiences (Crawley, 2007, 2008).

Method: Motor tests

The open field test was used as a motor function test but can be used as a test of anxiety too. The measurements were recorded using an automated system of light beams (Kinder Scientific) but can also be done with stop watches. The measurements included the sum of all movements in the 5-minute test, the number of rearings, as well as the distance travelled, the time spent stationary, and the time spent moving within a center zone and a peripheral zone.

The rotarod is an automated system (Panlabs) which enables control of the speed and acceleration of a rotating rod. Students watched a video of how to conduct the test with particular emphasis on how to place the mice on the equipment (<https://www.jove.com/video/2609/measuring-motor-coordination-in-mice>). Mice were given two trials on the rotarod and the time on the moving rod was recorded

and the revolutions per minute (RPM) of the rod at the time the mouse fell off.

Balance beam is one of the easiest tests to set up. It simply requires different diameter rods to be clamped in place and the mouse is given a time limit (two minutes) to complete traversing the rod (see JOVE video link above). If the mouse succeeded, then it was placed onto a rod with a smaller diameter, if it failed the task, then the maximum of 120 seconds was recorded for its time. The time taken to complete the rod was noted, the success rate and foot faults were also recorded. Video equipment can be used to improve the reliability of scoring, but with several students recording using stop watches it is not necessary.

Method: Memory tests

Light levels for the memory tests were conducted at low intensity (about 15-20 lux) to diminish the anxiety of the test. In different years we have tested 2-minute or 30-minute delays for these memory tests.

The Y-maze for spatial memory closes one of the arms during the initial 5-minute exploration and students recorded the duration of exploration of the available arm. A delay time was completed and then the mouse was given five minutes to explore the entire maze. Students recorded the amount of time in both the novel and familiar arms.

Tests of novel objects and novel locations can be done in any shape maze or in an open field box. In the novel object test, a mouse is presented with two identical objects during their first 5-minute exploration, followed by a delay, and then it is returned to the arena to explore one of the familiar objects and one novel object for five minutes. Students in this class have altered the shape (round or irregular) or smell of the objects (Q-tips sprayed with air-freshener odors). Tests of novel location are very similar, animals explore an arena with two objects, followed by a delay and then are returned to the arena, but one of the objects is now in a new location. Students measured the time in regions around the objects and events such as touching or sniffing the objects.

Method: All tests

Each mouse was tested separately, and between trials the equipment was cleaned with a non-alcohol based cleaning solution. It was important to emphasize to the students that mice be separated into appropriate cages to avoid inadvertently mixing the strains. Each test required less than fifteen minutes to run one animal and remove its scent by wiping the apparatus.

The results from the entire class were pooled at the completion of all experiments to provide robust sampling power for statistical analysis. Statistics were calculated in SPSS (v24) and plots were made using SigmaPlot (Version 13). This course has been taught three times (Fall 2013, Fall 2014 and Spring 2017) and the mouse data shown here is from the first two sessions

RESULTS

Mouse Behavioral Testing

Students collected multiple measures for each behavioral

Measure	BALBc Mean + SD (N)	CD1 Mean + SD (N)	Signif. (p)
Elevated plus			
dip	10.3 + 8.7 (24)	18.3 + 11.7 (27)	0.008 *
cross	7.4 + 5.8 (15)	13.1 + 9.7 (15)	0.062 #
rear	4.6 + 4.0 (24)	17.1 + 9.5 (27)	0.001 *
defecation	1.5 + 1.7 (24)	1.1 + 2.0 (27)	0.508
time open	49.5 + 52.3 (24)	54.2 + 45.0 (27)	0.731
Zero maze			
cross	5.0 + 4.0 (23)	12.1 + 6.75 (26)	0.001 *
rear	4.0 + 5.1 (23)	10.8 + 5.8 (26)	0.001 *
defecation	2.7 + 2.3 (23)	0.92 + 1.67 (26)	0.032 *
time open	56.7 + 52.4 (23)	58.3 + 32.9 (26)	0.894
Light/dark			
cross	4.8 + 4.6 (20)	24.1+6.9 (23)	0.001 *
rear	7.7 + 7.1 (15)	33.3+17.3 (15)	0.001 *
Open field			
movement	802.6 + 86.9 (15)	980.4 + 208 (15)	0.007 *
rear	49.35 + 26.2 (15)	76.0 + 25.6 (15)	0.009 *
distance	775.9 + 81.4 (15)	954.3 + 211 (15)	0.007 *
Rotarod			
time	33.8 + 13.3 (28)	62.7+23.5 (28)	0.002 *
RPM	8.2 + 1.7 (23)	11.3 + 4.5 (23)	0.004 *
Balance beam			
time	89.2 + 38.7 (15)	54.9 + 44.3 (10)	0.048 *
foot slips	3.2 + 2.45 (13)	1.5 + 1.5 (13)	0.039 *
Novel odor			
time novel	141.7+ 94.5 (15)	146.1 + 45.2 (15)	0.873
Novel object			
time novel	101.9 + 46.2 (15)	109.8 + 54.5 (15)	0.673
Novel Y-arm			
time novel	23.9 + 30 (11)	119.2+ 24.2 (13)	0.001 *

Table 1. The mean, standard deviation and significance of measures made during tests of the two mouse strains (difference between strains, significance * $p < 0.05$, # $p < 0.1$). Different measures were taken over the different years of data collection and therefore the number of mice vary for the measure shown.

test and only some of these are shown in Table 1. As some measures were clearly more helpful than others in determining the characteristics of the strains, students were forced to think about the value of specific measures.

Previous reading and discussions prepared the students for the likelihood that the same type of measure might differ between tests. For example, in Table 1 it is clear that the number of fecal boli counted was not a consistent indicator of anxiety for these strains, but that measures such as rearing and crossing were reliable.

The differences in behaviors between the two strains was particularly striking and consistent across all tests of anxiety. Figure 1 shows that BALBc mice display significantly fewer exploratory behaviors through rearing and zone crossing compared to CD1 mice. The BALBc were less active and travelled a shorter distance than CD1 mice (Figure 2). BALBc mice demonstrated impairments in performing motor tasks, as evidenced by falling off the rotarod sooner than CD1 mice and experiencing more foot slips on the balance beam, and taking longer to traverse

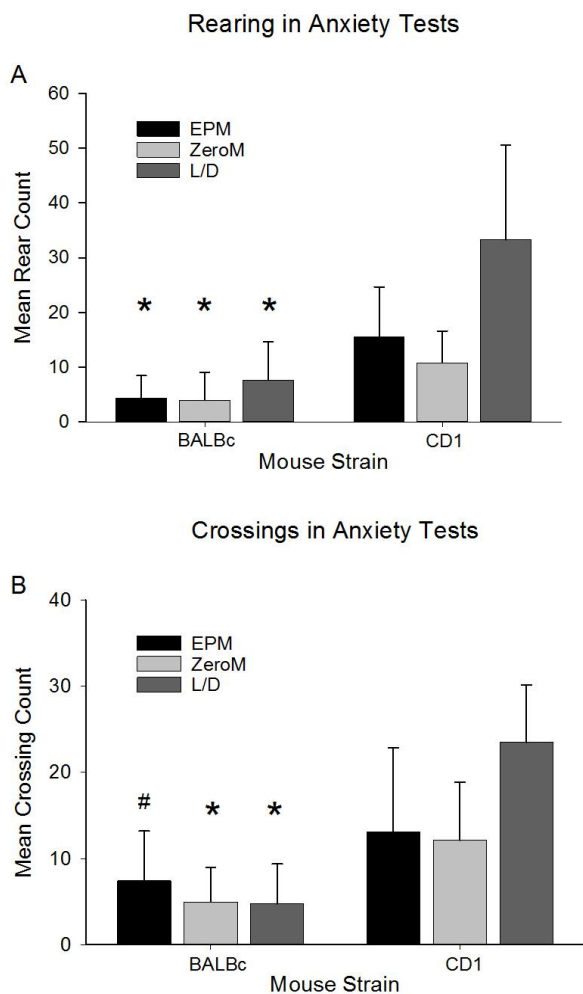


Figure 1. Behavioral measures of exploration during tests of anxiety. Decreased rearing (A) and zone crossing (B) by BALBc mice as compared to CD1 mice indicates greater anxiety in Elevated Plus Maze (EPM), Zero Maze (zeroM) and Light/Dark (L/D) tests (difference between strains, significance * $p < 0.05$, # $p < 0.1$).

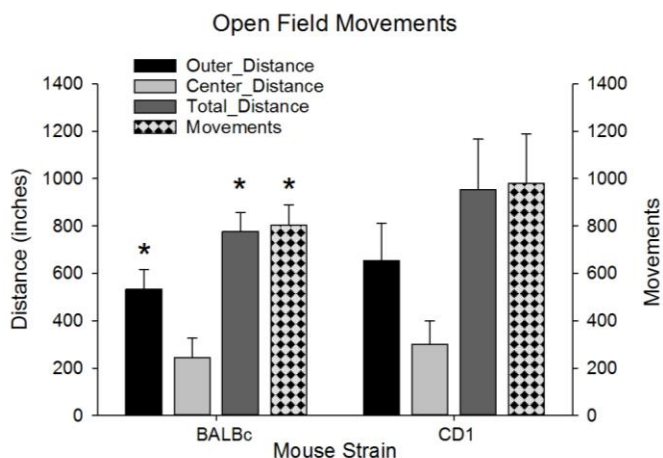


Figure 2. The 5-minute sum of inches travelled (calculated from beam breaks indicating movement from one grid to another in 1-inch grids): in the two-inch outer perimeter (of an arena 7 X 15 inches), within a center zone (measuring 3 X 11 inches), the total distance travelled and the total number of movements including postural shifts for the two mouse strains in the open field tests (difference between strains, significance * $p < 0.05$).

the beam (Table 1). In the spatial memory task (Y-maze), BALBc mice performed more poorly than the CD1 mice but there were no differences observed for object memory tests (Table 1).

The results of the tests conducted by students across all the sessions are summarized and compared to those published for similar experiments in Table 2. Students were able to distinguish the important behavioral characteristics and identify the two strains. An extensive repository of reference articles (see Table 2 for examples) was made available to students to assist them in matching the behavioral profiles of the mice to published data. Students were told that their mouse strains were represented in these papers and that they should use this literature to identify the mice. Strains in the literature which were not albino were obviously quickly eliminated as possible contenders.

Students were aware that one strain was inbred and one strain was outbred. Typically, BALBc are smaller and weigh less than CD1 mice and on many measures show less data variability (see Figures 1 and 2). Students generally correctly interpreted these pieces of information as indicators that this strain might be inbred. In all sessions, students correctly identified BALBc as the inbred strain and most students indicated CD1 as the most likely outbred strain, with a few students also considering strain OF1 as a possibility.

Student Outcomes:

Students were required to write individual laboratory reports to summarize their data and justify their choices of strain identification. These assignments helped all students to improve their approach to the analysis of data, their writing skills and their confidence in presenting information. At the end of the semester they were examined on their general knowledge of methods and

	Predicted behavior CD1	Predicted behavior BALBc	Observed BALBc	Test	Reference
Anxiety	Medium anxiety	Highly anxious	Highly anxious	Light-dark box	Crawley, 2008 Griebel, 2000 O'Leary, 2013
	Low/medium anxiety	Highly anxious	Highly anxious	Elevated plus maze	Crawley, 2008 Griebel, 2000 O'Leary, 2013 Sunyer, 2007 Arabo, 2014
Motor		Low motor activity Specific deficits	Lower activity	Open field	Crawley, 1997 Sunyer, 2007
		Possibly lower Variable Intermediate	Lower	Rotarod	Sunyer, 2007 Burket, 2016 McFadden, 2003
		Poor	Poor	Balance	Lepicard, 2000
Memory	Good spatial	Poor memory Poor spatial		Morris/8 arm maze	Crawley, 1997 Sunyer, 2007
	Good spatial	Poor spatial	Poor spatial	Novelty Y	Dellu, 2000

Table 2. The predicted behavioral profiles of CD1 and BALBc mice using published data and the corresponding observed data for BALBc mice. These types of comparisons enabled students to justify their identification of mouse strains.

techniques with a quiz. Students who had the hands-on experience of conducting the experiments attained an average score of 94% (SD =5.5) on this quiz (2013, 2014, 2017). Students in a purely seminar style of course on behavioral neuroscience in (2011, 2012) without the strong emphasis on the laboratory component attained an average score of 87% (SD =10.4) on a similar quiz. (Statistical comparison of these quiz results by course style using Mann-Whitney $p=0.052$).

Standardized assessment:

Students in every class at the college are routinely asked standardized questions for teaching and pedagogical assessment purposes. The measures are taken on a 5-point Likert scale with a score of 5 representing "very well" (or "a great deal") and score of 1 representing "poorly" (or "nothing"). The results of three of the most pertinent questions are given here as the mean and standard deviation of the three classes (2013, 2014, 2017: total 30 students).

Q1 "How much do you believe you have learned in this course" Mean 4.72 (0.37)

Q5 "How well did the evaluating techniques (exams, assignments etc.) seem to measure your mastery of the course material?" Mean 4.61 (0.5)

Q6 "How well do you think the instructor accomplished the objectives of the class?" Mean 4.83 (0.33)

Open-ended assessment:

At the end of the 2017 class, students were asked to specifically evaluate how well the course met the pedagogical aims outlined above. A selection of quotes has been included here, with representation from all members of the class, to indicate their opinions. The student evaluation clearly indicates that this style of course was very valuable and enjoyable.

1) Data analysis and presentation

"Often we, as students, are presented with lots of data while reading journals and have limited knowledge of how the analysis was carried out. This course helped bridge the gap by allowing us to explore the considerations of the analysis. I hadn't realized how many ways there were to formulate the data until I had the chance to work with it myself."

"This course strengthened my understanding of data analysis, manipulation and presentation. We addressed confounds, outliers and normalization through independent and small group setting discussions. I now feel competent in my abilities to know which test is most appropriate, why it is so, and how to accurately run the desired analysis."

2) Written communication

"After each paradigm we wrote an extensive research paper mirroring a "typical" scientific paper. Each would include abstract, introduction, methods, results, and discussion sections. They would typically run about 10-15 pages in length and be accompanied by around 6-8 graphs. This not only strengthened my scientific writing skills, but also my scientific literacy as a whole. I feel much less intimidated when examining a primary literature article on an unknown topic as I can break it down and gather pertinent information."

3) Group cohesion and collaboration

"Planning protocols as a group facilitated collaboration among peers and fostered a give-and-take environment."

"This class really helped me learn to collaborate with a group in order to plan a well-designed experiment."

4) Analysis of the literature to understand the purpose and advantages/disadvantages of different tests

"The presentation part of the course allowed students to really engage with the literature and forced students to discuss the benefits and limitations of the study. It encouraged students to look beyond the basics of what was reported, and was helpful in understanding the limitations of different tests."

"The ability to critically evaluate protocols is essential to the field of science. This course challenged students to scrutinize and identify ways to improve methodology to avoid confounding variables."

"The constant examination of primary literature strengthened my understanding of when, why and how a particular behavioral assay is employed. We also discussed limitations (temporal effects, ambiguity, low sensitivity) and strengths (historical data, high sensitivity) of tests."

"This class was designed so that every test we ran in lab, we first learned about through literature we read or presented to the class. I found this especially beneficial in being able to analyze data"

5) Hands-on laboratory skill development

"A majority of this course was spent in lab, which was a great alternative to the traditional lecture learning. It allowed students to engage with relevant, hands-on experience that truly taught students the process of scientific data collection. It was a great alternative to the traditional classroom experience, and helped the students take control of their educational experience."

"Being able to interact with the paradigms was imperative to the course. The hands-on perspective made our classroom experience tangible. It brings it beyond reading about it in an article and allows us to practice real-world skills that extend beyond our experience at Gustavus."

DISCUSSION

The focus of this course can be varied to suit the instructor's needs and interests. Behavioral neuroscience techniques are used in literally thousands of published experiments which encompass a very wide range of topics (Wahlsten, 2011). We used the literature review to introduce the methods and discuss how to measure the relevant behaviors in an experiment. The literature varied each year as we looked for new and interesting papers which just happen to use the methods we wished to discuss. For example, in the 2017 motor module we discussed a murine model of Parkinson's disease (Filali and Lalonde, 2016). This paper introduced students to the SHIRPA assessment tool (SmithKlineBeecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment) as well as use of the balance beam, rotarod and open field tests. In 2014, we discussed a paper which used a model of Huntington's disease (Samadi et al., 2013). Discussion could also emphasize information about different mouse strains and genetics or could be thematic, such as the impact of alcohol or drugs on all motor,

memory and anxiety behaviors.

We selected the two mouse strains based on advice from the vendor and they have worked well for this class. However, there are many different mouse strains and knockout/knockin models available which could be used, and in the future we may try to add a new strain to the class. The inbred mice can be expensive and the strains need to be kept apart which adds to shipping costs. If budgetary constraints are tight then this may preclude getting two kinds of mice, but instead you can get a few more of only one type. The battery of tests can still be conducted on this one strain and compared to published data. Similarly, there is a lot of flexibility in the behavioral methods to be included. Instructors could focus on circadian, feeding, social, sexual or aggressive behaviors depending on their interests and the equipment available.

Conducting a battery of tests which includes multiple measures creates an open-ended opportunity for analysis and interpretation. If students do not know in advance which behavioral tests are likely to differentiate the two mouse strains, then they pay attention to all measured variables. Even if an instructor is limited to fewer pieces of equipment, it is still possible to do extensive open-ended analyses by examining multiple measures on each test. For example, in conducting the elevated plus maze test students might record time in open arms, time in closed arms, time in center, defecation, stretch attends, dips, rearing and grooming events. Each measure can be analyzed and compared and instructors can spend time discussing the reliability and variability of different measures on the same test.

We have used the course to teach students about measurement, data analysis and how to choose the appropriate behavioral tests for an experiment. The hands-on experience combined with the discussion of pertinent literature works well to reinforce the lessons and promote understanding of behavioral neuroscience methods.

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Address correspondence to: Dr. Janine Wotton, Neuroscience Program, Gustavus Adolphus College, 800 W College Ave, St. Peter, MN 56082. Email: jwotton2@gustavus.edu

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