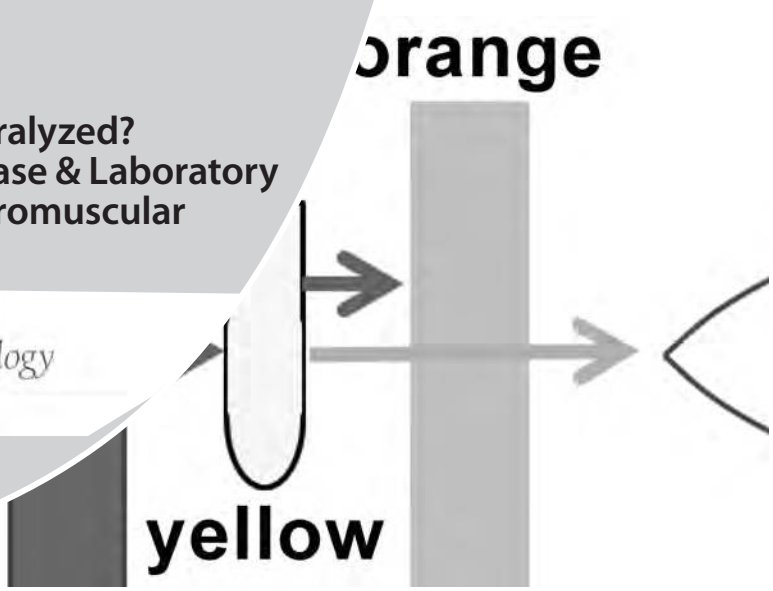
INQUIRY & INVESTIGATION

Why Is That Dog Paralyzed? A Problem-Based Case & Laboratory Exercise about Neuromuscular Transmission

RECOMMENDED FOR *AP Biology*

MARK MILANICK, KERRI GRAHAM, MELISSA WESSEL



Abstract

Students are provided with a mystery concerning dogs that are paralyzed. This motivates a laboratory exercise to measure parameters from the dog's "blood" to determine whether the paralysis is due to pesticide poisoning or an autoimmune attack on nerve myelin. Most of the materials are available from the grocery store. The real-world nature of the problem, and the mystery, engages the students in thinking about nerve, muscle, and immune system function. Alternative versions require less familiarity with physiology and can be used as engagement activities to encourage learning laboratory skills and experimental design or as motivation for learning nerve and muscle physiology.

Key Words: *Experimental design; nerve–muscle communication and function; problem-solving skills; autoimmunity; fluorescence measurement applications; laboratory skills.*

Students often don't see the immediate applicability of the subjects they study in biology. For example, as long as they are healthy, how nerves communicate with muscles may seem esoteric. Similarly, understanding enzyme function often seems disconnected from everyday life. Even the utility of commonly used laboratory techniques such as fluorescence detection (particularly using labeled antibodies) can baffle some students. However, if students can understand real-world applications, they become more engaged (Dinan, 2005).

Similarly, understanding enzyme function often seems disconnected from everyday life.

This laboratory exercise is a result of high school teachers and a scientist collaborating to develop a lab using readily available materials that will allow the students to connect these items to a potential real-life situation. This lab can be set up to run in four 50-minute class sessions, though we have done it in two 90-minute sessions. In the first 50-minute session, the students are provided with a real-life scenario in which they need to apply their knowledge about nerves, muscles, enzymes, and detection. In the second 50-minute session, the students design their experiments and further review the concepts of experimental design. In the third 50-minute session, the students actually do the experiment and record their results. In the fourth 50-minute session, the students discuss their results and return to think more about the real-life scenario. The problem can be made simpler, yet still engaging, so that it can be used in lower-level courses.

Students are asked to determine the most likely cause of paralysis in a dog when given two possibilities. The students review the key aspects of nerve and muscle function, as well as the neuromuscular junction. They also learn key aspects of enzyme activity as well as the use of fluorescent techniques to measure antibodies. Alternative versions of the lab are included that focus on learning laboratory skills, experimental design, or that can prepare students for a section on nerve–muscle physiology or the immune system.

○ The Case

This fictitious case involves a veterinary lab testing company that has received 20 samples from dogs around the country. In all cases, the dogs have suddenly become paralyzed and the treating veterinarian has narrowed the possible causes to pesticide poisoning or an autoimmune paralysis. The case motivates the students to learn, or review, the basics of nerve conduction, the role of myelin in nerve conduction, and the role of acetylcholine in activating muscle cells, eventually leading to contraction. The students are given blood samples from one of the paralyzed dogs and from a healthy dog and then must determine whether the paralysis is due to pesticide poisoning, an autoimmune process, or both. Pesticide poisoning can be determined by measuring the rate of the enzyme acetylcholinesterase (frozen grocery store fish is a suitable source). An autoimmune response can be determined by measuring the presence of a fluorescent "antigen" (turmeric is used as the fluorescent agent). Appendix B (http://support.dalton.missouri.edu/milanickm/pub/AppB.pdf) has a simpler case where the pesticide has gotten into dog food and the students have to determine which dog food samples inhibit the enzyme. In Appendix C (http://support.dalton.missouri.edu/milanickm/pub/AppC.pdf), the case stresses the autoimmune process and measuring fluorescence.

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○ Nerve Conduction, Muscle Contraction, & Acetylcholinesterase

Next, the students are asked to discuss muscle weakness and the general mechanisms that can cause weakness. They should be encouraged to think about the general elements for signaling muscle contraction (summarized in Figure 1). The major steps include:

1. The motor neuron is activated.
2. The signal travels down the motor neuron axon.
3. Calcium increases in the nerve terminal, and acetylcholine is released.
4. Some acetylcholine diffuses to the muscle membrane and binds to the acetylcholine receptor.
5. Acetylcholine bound to its receptor leads to muscle depolarization.
6. Muscle depolarization leads to an increase in muscle cell calcium.
7. The increase in calcium allows actin to interact with myosin, and the crossbridge contract cycle occurs.
8. Acetylcholine is broken down by acetylcholinesterase and stops the signal.

One key learning objective of this exercise is that the neurotransmitter, acetylcholine, is released by the neuron and must be broken down by acetylcholinesterase in the synapse in order to allow the muscle to relax. Acetylcholine in this context is a neurotransmitter; that is, it transmits the signal from a nerve to another cell. The neuromuscular junction is the place where the nerve and muscle are in close proximity and where the acetylcholine-containing vesicles are located in the nerve terminal. On the muscle side of the junction, there is a high concentration of acetylcholine receptors and acetylcholinesterase.

The other key learning objective of the exercise is that the myelin coating on the nerve allows the nerve conduction to occur rapidly; if this is disrupted, the nerve conduction signal is decreased or prevented.

○ Background Information

Why Would Pesticides Cause Paralysis?

Most pesticides work by inhibiting the enzyme acetylcholinesterase (Weinbroum, 2004; Barthold & Schier, 2005). When this enzyme is inhibited, acetylcholine remains in the neuromuscular junction and continues to activate the muscle cell. The result is initial cramping and weakness that can progress to paralysis.

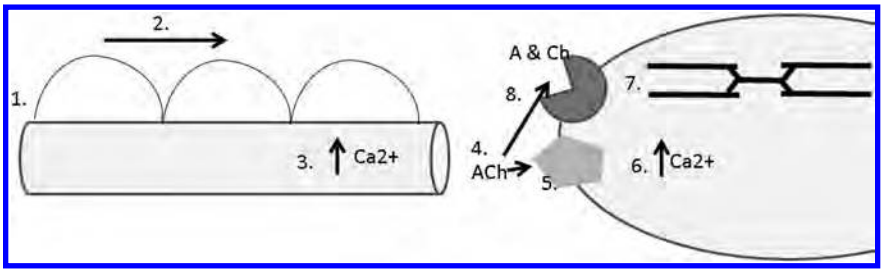

Figure 1. Some of the major steps during nerve–muscle activity. See text for individual steps.

The fact that inhibition of an enzyme keeps the muscle contracted can be puzzling. To help them understand, the students are asked to consider analogies like a "double negative" or another from football: The neurotransmitter, acetylcholine, is like a halfback. The enzyme is the linebacker; it stops the halfback. If you block the linebacker, the halfback can keep running. Pesticides block the linebacker, allowing the signal to continue.

What Causes Demyelination?

Coon hound disease is thought to be an autoimmune response. Normally, the immune system responds to an external agent – in this case, a protein in raccoon saliva. The immune system identifies part of the raccoon salivary protein and makes an antibody to it. The sequence of the raccoon salivary protein where the antibody binds is called the *antigenic site*. In some cases, the antibodies against the raccoon salivary protein also recognize a related site on myelin. A short segment of a raccoon salivary protein and of a myelin protein look so similar, an antibody against one also reacts with the other (Ang et al., 2004).

Why Would Demyelination Cause Paralysis?

Myelin is like insulation around a nerve. The myelin has gaps along the axon; thus, the entire nerve is not insulated. Without insulation, the electrical signal moves down an axon, which would be analogous to taking steps where the heel of the stepping foot touches the toe of the stationary foot. With insulation, the electrical signal can jump over the insulation and get to the gaps faster; this is analogous to how much faster a person can travel down if they hop 2 yards at a time. When the myelin is destroyed, there are now more gaps in the "insulation" and the nerve impulse rate slows. The nerve loses its ability to properly signal the muscle to contract, resulting in paralysis. An alternative method of explaining the role of myelin is the passing game (Gathers, 2008). In this game, there is a line of students; for example, there are 3 males between every female. They pass an eraser down the line. An unmyelinated neuron passing its electrical signal down the axon is analogous to the eraser being handed to each student in turn. A myelinated neuron is analogous to having the male students covered up; the signal passes faster down the line just as the eraser passes faster down the line if only the female students have to handle it.

How to Test for Acetylcholinesterase

Acetylcholinesterase breaks down acetylcholine into acetate and choline, both of which are difficult to detect. Scientists have determined that acetylcholinesterase also breaks down acetylthiocholine; the products are acetate and thiocholine (Ellman et al., 1961; Schwartz et al., 2007). In thiocholine, the thiol group (a sulfur atom) is at an end of the molecule and is reactive. In acetylthiocholine, the thiol group is in the middle of the molecule and is not reactive. Conveniently, the compound dithionitrobenzene (DTNB) reacts with the end thiol and when it does, the reaction product is yellow, making thiocholine easy to detect (Ellman et al., 1961; Schwartz et al., 2007). Acetylcholinesterase, like all enzymes, merely speeds up the breakdown. Acetylthiocholine will break down even in the absence of enzyme, particularly if it is improperly

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stored. For this reason, we recommend including a control to be sure that most of the yellow color observed reflects enzyme-catalyzed breakdown and not just spontaneous breakdown.

How to Test for Antibody

In an autoimmune response, the antibody binds to a myelin protein; this causes the immune system to attack the myelin. One method to test for the presence of a specific antibody is to add a labeled antigen that also binds to the antibody. The label on the antigen is a fluorescent dye. A convenient way to determine the presence of a fluorescent dye is to put a drop of the sample onto filter paper. Fluorescent molecules absorb light of one color; in this experiment it is blue light. When the fluorescent molecule releases its energy and the electrons drop back to their resting shell, light of a different color is emitted – in this case, green. So the fluorescent dye can be detected by shining blue light onto the sample and viewing it through an orange filter. The blue light excites the fluorescent dye, but blue light will not go through the orange filter very well. The excited dye will give off greenish light, which can be viewed through the orange filter (see Figure 2).

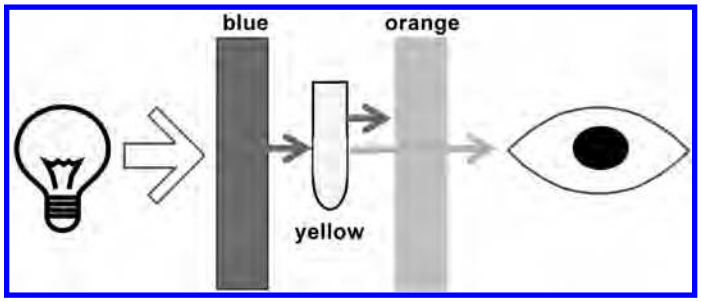

Figure 2. The tubes (yellow) are excited by blue light obtained by placing a filter between the light source and the sample. The fluorescent light is observed by having an orange filter between the sample and the observer.

○ Details for Teacher

Materials for Lab

All of these materials are needed for the complete laboratory exercise in Appendix A (http://support.dalton.missouri.edu/milanickm/pub/AppA.pdf). Parentheses indicate which supplies are needed for the exercise in either Appendix B or C.

- <1 ounce of frozen tilapia or salmon from grocery store (B)
- Ellman's reagent, also called 5,5'-dithiobis-2-nitrobenzoic acid or DTNB (B)
- Acetylthiocholine (B) (Important note: Acetylcholine will not work in this assay because it lacks a thiol group)
- Turmeric, from grocery store (C)
- Light source (C)
- Orange and blue filters (C)
- Test tubes (B, C)
- Test tube racks (B, C)
- Cheesecloth (B)
- Blender (B)
- Rubbing alcohol (C)
- Red and yellow food coloring (optional)

Teacher Setup

For Appendix B, skip the turmeric steps. For Appendix C, skip the acetylcholinesterase (fish) steps. Enzyme activity is detected by the breakdown of acetylthiocholine to acetate and thiocholine. The terminal thiol (sulfur) group is then able to react with DTNB, producing a yellow color. (Important note: Acetylcholine will not work in this assay because it lacks a thiol group.) A fluorescent antibody result is simulated in this experiment by using turmeric, which contains the fluorescent compound curcumin.

All the solutions should be made up in a buffer that has a pH between 7 and 8 (phosphate or tris buffers are fine). Two stock solutions must be made on the day of the experiment: a stock of 2 mM DTNB in buffer, and a stock of 2 mM acetylthiocholine in buffer. Dissolve 20 mg turmeric in 50 mL rubbing alcohol.

Partially thaw the fish, add 50 mL of buffer per gram of fish, then either blend it into fine pieces or homogenize it in the buffer. Strain through a cheesecloth or jelly cloth. Store on ice. Because the amount of active enzyme varies with different fish, test the amount of activity by diluting 1 mL, 1/3 mL, 1/10 mL, 1/30 mL of fish mixture into 4 mL of assay solution (1 mM DTNB and 1 mM acetylthiocholine). Choose the concentration of homogenate that gives a substantial yellow color in about 5 minutes.

Tubes:

- Sample from normal dog. For this sample, add fish homogenate so that the tube has enzyme activity but no "antibody."
- Sample from dog known to have been exposed to pesticide: This sample needs less enzyme activity, so either add no fish homogenate or add 1/10 the amount in the other tubes.
- Sample from dog known to have coon hound disease: For this sample, add fish homogenate and 10% turmeric, so the tube has both enzyme activity and "antibody" present.
- Sample from the sick dog. We made up different tubes for each group – there are four choices – each of the three above (no fish and no turmeric; fish and turmeric; fish and no turmeric) and one with no fish and turmeric, which corresponds to having pesticides and antibodies present. (Note: If it is too obvious that the tubes with control fish are cloudier than the tubes with little or no fish, soymilk can be added so that all the tubes look cloudy.)

Table 1 is an example of what the different tubes can contain.

To detect the fluorescence of turmeric, blue light is used to excite the turmeric. The

Table 1. Client tube contents.

	Normal Dog	Dog with Coon Hound Disease	Dog with Pesticide Poisoning	Dog with Both Coon Hound and Pesticide-Positive Results
Fish Homogenate	Yes	Yes	No	No
Turmeric	No	Yes	No	Yes

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Table 2. Expected results.

Test	Coon Hound and Pesticide Poisoning Results	Normal Dog Results	Coon Hound Results	Pesticide Dog Results
Acetylcholinesterase (The more yellow the sample, the more enzyme activity. If pesticide is present, there should be little yellow color because of low enzyme activity.)	Not yellow	Yellow	Yellow	Not yellow
Autoimmune response (A fluorescent sample indicates that the antibody is present, which is consistent with coon hound disease.)	Fluorescent	Not fluorescent	Fluorescent	Not fluorescent

fluorescence is viewed through an orange filter, which does not allow the exciting blue light to pass through (see Figure 2).

If this amount of turmeric does not provide detectable fluorescence with the light source and filter combination, increase the turmeric. The tubes will then be slightly yellow in color. This is not a problem for the enzyme reaction because a bit more fish can be added so that the DTNB yellow soon overwhelms the turmeric yellow. To keep the students from guessing the result because of the turmeric yellow, add a bit of yellow food coloring to the non-turmeric tubes so that they all look about the same color of faint yellow. If desired, a bit of red food coloring can be added so that the tubes look like they contain blood; then the students are looking for an orange color as a result of the enzyme reaction, not yellow. Table 2 lists the expected results.

○ Results, Discussion, & Alternatives for Students with Less Prior Knowledge

We found that students enjoyed the challenge of thinking about the problem their dog had. The exercise allowed us to review many of the topics from earlier in the year. The students' excitement carried over into the laboratory session; it seemed to us they enjoyed designing the lab in part because of the introductory scenario.

This exercise can be modified to appeal to students at several different levels. As we used it (as set out in Appendix A), the lab works well for students who are familiar with experimental design, nerve conduction, neural transmission, and muscle contraction. It also helps if they know the basics about immune system function and enzyme activity. In this case, this exercise allows the students to apply their knowledge, develop additional problem-solving skills, and design real-life relevant experiments. We used this approach for a high school AP Biology class.

An alternative approach is to use this exercise as an initial engagement activity rather than a review activity. In this case, students need much less prior knowledge and the exercise is used to motivate them to learn new material. We have used this approach with college students in the summer before their freshman year as well as freshman and sophomore college students during their fall semester. These students were interested in working in research laboratories; this exercise allowed them to practice their lab skills and learn more about experimental design. Appendix B presents a scenario that only requires the measurement of acetylcholinesterase activity to introduce experimental design and the function of enzymes. Appendix C presents a scenario that only requires the measurement of fluorescence and introduces immunity and autoimmunity.

We have found that most students get excited about the real-world nature of the problem and are intrigued to work hard to solve the mystery.

References

Ang, C.W., Jacobs, B.C. & Laman, J.D. (2004). The Guillain-Barré syndrome: a true case of molecular mimicry. *Trends in Immunology*, *25*, 61–66.

Barthold, C.L. & Schier, J.G. (2005). Organic phosphorus compounds – nerve agents. *Critical Care Clinics*, *21*, 673–689.

Boydell, P. (2010). Coonhound paralysis in South Yorkshire? *Veterinary Record*, *167*, 351.

Dinan, F.J. (2005). Laboratory-based case studies: closer to the real world. *Journal of College Science Teaching*, *35*, 27–29.

Ellman, G.L., Courtney, K.D., Andres, V., Jr. & Feather-Stone, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, *7*, 88–95.

Gathers, A. (2008). Using a passing game to teach nerve conduction. *American Biology Teacher*, *70*, 11.

Schwartz, P.J., Blundon, J.A. & Adler, E.M. (2007). A biochemical assay for acetylcholinesterase activity in PC12 cells. *Science's Signal Transduction Knowledge Environment*, *394*, tr2.

Weinbroum, A.A. (2004). Pathophysiological and clinical aspects of combat anticholinesterase poisoning. *British Medical Bulletin*, *72*, 119–133.

MARK MILANICK (milanickm@missouri.edu) is Professor of Medical Pharmacology and Physiology and Investigator, Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO 65211. KERRI GRAHAM (kgraham@columbia.k12.mo.us) is a Biology Teacher and MELISSA WESSEL (mwessel@columbia.k12.mo.us) is a Biology Teacher and Science Chair at Rockbridge High School, Columbia, MO 65203.

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