The Alcohol Pharmacology Education Partnership

Drinks Like a Fish: Can alcohol damage developing neurons?

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Relevant APEP Module(s): Modules 3 and 5

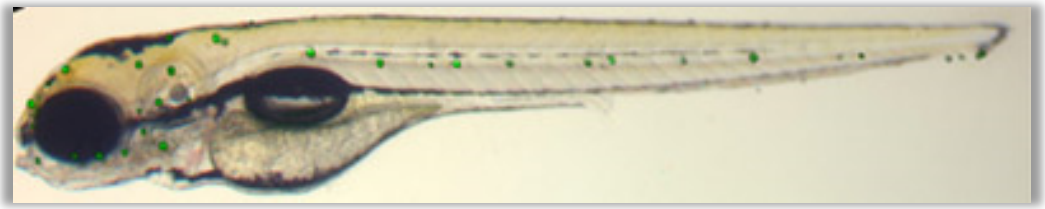
Target Group: A.P. Biology or a research course in Biology (would be a great collaboration with Chem too!)

Pre-requisite Knowledge: General understanding of organismal biology and development, chemical reactions (oxidation and oxygen radicals)

PROJECT SUMMARY

Background

Alcohol causes damage to many types of cells, including neurons in the developing fetus and young adult. The zebrafish is a useful model to study damage to developing neurons by drugs and toxins such as alcohol. The model involves measuring the acoustic startle response--a behavioral response consisting of rapid navigation that provides protection from environmental stimuli. All vertebrates have a startle response so that they can adapt to changes in their environment. In the zebrafish, the startle response is controlled by a group of neurons called the neuromast. The neuromast is an organ that controls balance (similar to the human vestibular organ) that is located along the fish's lateral line and helps the fish to orient in the water (Murakami, et al., 2003). In the picture of a 5 day old zebrafish below, the neuromasts have been stained with a green dye.



Thus, measuring how the alcohol affects the zebrafish acoustic startle response can be an indirect measure of actual damage to the developing neuromasts. One mechanism for alcohol-induced cellular damage is the generation of reactive oxygen species (as discussed in Module 5). The acoustic startle response of the zebrafish can be used to study whether reactive oxygen species are involved in alcohol's toxicity to the developing neuromast. This experiment is a model system to assess fetal brain development as well as postnatal brain development (i.e. neurogenesis in the young adult, as discussed in Module 3).

Objectives

The overall goal of this activity is to determine if alcohol (in concentrations relevant to human blood alcohol levels when consumed) can cause damage to developing neurons (i.e., the neuromasts) in the 4 day old zebrafish.

As a second experiment, students can use antioxidants to determine if reactive oxygen species are involved in the mechanism of alcohol toxicity.

At the conclusion of this lab activity, students should be able to:

- Discuss how to measure toxicity of alcohol on developing neurons
- Discuss how to determine if the production of oxygen radicals (or other reactive oxygen species) are involved in the mechanism of alcohol toxicity

National Science Education Standards
Science as Inquiry – CA1, CA23, CA25
Life Science – CC15, CC60, CC61, CC63
Science in Personal and Social Perspectives – CF13
Teaching Standards – TB1-4, TD1-4

MATERIALS

Breeding Materials (optional)

- ✓ Adult zebrafish
  - Your local pet store should carry adult zebrafish, or you can order a zebrafish breeding kit from Carolina Biological Supply http://www.carolina.com/
- ✓ Fishtanks (For maintenance of the fish during breeding and collection of embryos)

*For tips on zebrafish breeding and husbandry, see https://wiki.zfin.org/display/prot/ZFIN+Protocol+Wiki

Embryo Collection Materials

- ✓ Stock salt solution (to make egg water)
  - Dissolve 40 g Instant Ocean in 1 L distilled water (Instant Ocean can be purchased from your local petstore, or online at Carolina Biological Supply #671440)
- ✓ Egg water (60 µg/mL final concentration)
  - Add 1.5 mL stock salt solution to 1 L distilled water
- ✓ Sterile plastic pipettes with graduations
- ✓ Sterile petri dishes (10 cm)
- ✓ Fine mesh fish net to transfer eggs
- ✓ Incubator or oven set at 27º to 28º C (optional: see Useful tips below)

Drug Preparation Materials

- ✓ Alcohol (ethanol) – any concentration to start (e.g., 100%, 95%, or 70% ethanol
  *** Use caution when using ethanol ***
  Please check the safety standards in your school system
- ✓ 15 mL conical tubes (3 per student/group)
- ✓ Pipettes and tips to measure small volumes
- ✓ Surgical gloves (for handling solutions)
- ✓ Egg water
- ✓ Beakers
- ✓ 6-well plates with net well-inserts (24 mm membrane, 74 µm mesh) (1/student)
  - Net well-inserts are used to transfer fish during alcohol treatments 6 well plates & net well-inserts can be purchased at Fisher Scientific (6 well plates: 08-772-49; inserts: 07-200-213)
- ✓ Parafilm or plastic wrap to wrap the 6-well plates to prevent evaporation
- ✓ Optional: Tin foil to cover the 6-well plates (if using antioxidants, many are light sensitive)
- ✓ Tricaine for euthanasia of the fish

- Embryos should be euthanized in a solution of 250 mg/L Tricaine. Tricaine is available through Fisher (AC11800-0050).

Acoustic StartleTest Materials

- ✓ Timer or stopwatch
- ✓ Petri dish (10 cm) (either plastic or glass)
- ✓ Beaker
- ✓ Plastic transfer pipettes
- ✓ Egg water
- ✓ Something to tap the side of the dish to deliver an acoustic startle stimulus (a pencil or pen will work)
- ✓ Student data sheets with 60 lines for scoring (see student handout #2)

PROTOCOL

** If you plan to breed the zebrafish, the experiment will take 9 days to complete. There will be an initial investment of time to set up the breeding tanks.

If you can obtain the zebrafish eggs from another source (e.g., a university that is close by or a pet store), the experiment will take a student approximately 20-25 minutes to prepare the alcohol dilutions and approximately 30 minutes to administer treatments to the fish.

The acoustic startle test will require about 60 minutes for a 6-well plate containing 60 fish. Students should work together in groups of 3 or more. The time will vary depending on the number of fish to be tested.

Daily schedule for experiment		Time required
Day Before	Set-up breeding	30 minutes
Day 1	Collect eggs	30 minutes
Day 2 - 6	Observe eggs Remove dead eggs (white) Eggs hatch on day 3 Observe hatchlings	Time will vary with individual student interests
Day 7*	Prepare ethanol concentrations Treat fish (now 4 days old)	30 minutes prep; 30 minutes treat
Day 8	Acoustic startle test	60 minutes

*Note: you can also do this experiment with 5 day old zebrafish

Set-up

Preparation of Solutions

Note: these calculations are great exercises for your students to practice a little math. Have them plan out the solution dilutions ahead of time as a homework assignment!

The general equation to determine these dilutions is:

$Conc_1 \times Vol_1 = Conc_2 \times Vol_2$

Assume $Conc_1$ is what you have, set Vol_1 to 1 ml, $Conc_2$ is 10%, and solve for Vol_2 (total # of mls of egg water after dilution)

Make a stock solution of ethanol (10%)
Depending on the concentration of ethanol you have on hand (e.g., 100%, 95%, or 70%), make a 10% stock solution in egg water as follows:

If you have 100% ethanol:	use 1 ml plus 9 ml of egg water (total 10 mls)
If you have 95% ethanol:	use 1 ml plus 8.5 mls of egg water (total 9.5 mls)
If you have 70% ethanol:	use 1 ml plus 6 mls of egg water (total 7 mls)

Make working solutions (~100 ml) of ethanol from your stock solution
0.1% Ethanol
Use 1 ml of the stock solution plus 99 mls of egg water (total 100 mls)

0.2% Ethanol
Use 2 mls of the stock solution plus 98 mls of egg water (total 100 mls)

Set-up of treatment plates (6-well plates)

1. Take a clean 6-well plate, labeling the lid with code letters according to the chart below (A-F).
2. Fill each well with 2 ml of the corresponding solution prewarmed to 27º-28º C (see chart below).
3. Add net inserts into the wells
4. Add the remaining 7 mls of corresponding solution (this helps to avoid air bubbles)

Set up of 6-well plates

Well Label	Treatment (9 mls)
A	Control – Egg water alone
B	0.1% Ethanol
C	0.2% Ethanol
D	Control – Egg water alone (duplicate of Well A)
E	0.1% Ethanol (duplicate of Well B)
F	0.2% Ethanol (duplicate of Well C)

Neuromast Damage

1. Transfer 10 zebrafish into the net well-inserts containing 9 mls of prewarmed egg water (with or without alcohol) using a pipette (you can use less than 10 fish, but use at least 6)
2. Cover the plates with parafilm or plastic wrap.
3. Put plates in incubater/oven at 27º-28º C for 18-24 hours (you may need to do this experiment a few times before finding the best incubation time to produce the right amount of damage—you don't want too much or too little). If you incubate at room temperature, it will take longer to produce the neuromast damage, because development occurs more slowly at room temperature (see Useful tips).
4. The next day, fill 3 more 6-well plates with just fresh egg water at 27º-28º C (9 mls/well). Wash the fish with egg water for 5 minutes 3 times by transferring the net inserts (individually) to a new 6-well plate containing the fresh egg water. (This "wash" step removes any residual ethanol).

   Be sure the net inserts themselves are labeled and not just the 6-well plate so they don't get mixed up!

Acoustic Test

Zebrafish embryos have a startle response that allows them to react to vibrations in the water. This response is fully developed by 5 days post hatching, but there are reports that they can have an acoustic response as early as day 3.

A short video clip has been included so you can visualize the acoustic test.

<u>Before testing</u>

- Make groups of at least 3 students for observing and recording data.
- Provide each observer with a copy of the Student handout #2—the acoustic startle test. Instruct the observers to become familiar with the movement scale for rating fish.

- The observers should be "blind" to the drug treatment of the fish being tested. The teacher should reassign the well designations (L,M,N,O, P, Q) ahead of time in a random order to minimize the introduction of experimental bias during the scoring of fish movement (put a piece of tape over the old letters to hide them).

  The new code should be recorded on a separate paper and kept "secret". When the experiment is over, the teacher can "break the code"; tell the students which group corresponds to the new letters. Discuss with them why this is so important for **subjective** measurements.

- Assign one student in each group to be the person who gets a fish from the 6-well plate to put into the testing dish. This student will say out-loud the new letter code ("L"), but won't be blind to the treatment because he/she will know which well the fish came from. Make sure the student understands that he/she should not divulge the code to his/her group mates.

Performing the Acoustic Test

1. Keep a beaker of egg water in the incubator to keep it warm. Keep a second beaker of egg water (room temperature is OK) for "waste".
2. Prepare a 10 cm petri dish with 25 ml of egg water prewarmed to 27º-28ºC. Put the petri dish over a piece of white paper so you can visualize the fish easily.
3. Transfer one of the fish from a well to the 10 cm petri dish using a plastic transfer pipette. Wait approximately 30 seconds to 1 minute for the fish to acclimate to the new dish.
4. During the acclimation period, observe the fish to see that it's wiggling/swimming in the petri dish. Students should take notes on their observations.
5. Using a pen or pencil, Tap ONCE on the edge of the dish. This is the stimulus for the acoustic startle reflex. Make sure to tap with the same part of the pen each time you administer the stimulus. Very important to do it the same way every time!
6. Observe the fish immediately after the tap. The response to the tap may range from no movement (a 0 on the rating scale), to a tail wiggle (1 on the rating scale) to darting across the dish (3 on the rating scale). Ask each observer to score the movement on their Student Data Worksheet (which they will make themselves) using the movement scale below. Also record any other observations.

The movement scores are:
0 = no movement (it could be dead--if so, record that as well)
1 = wiggles
2 = swims 1 body length
3 = swims more than 1 body length

After recording the movement rating, move the fish with a plastic pipette into the waste beaker containing egg water

7. Repeat steps 3 – 6 for each fish.
   Replace warm egg water as needed.

8. Discard fish when done.

   Fish should be euthanized in a solution (>250 mg/L) of Tricaine (3-amino-benzoic acid ethyl ester) (powder should be stored in the freezer). This is an "overdose" of a potent anesthetic agent.

   Weigh out about 25 mg and put into 100 mls of water in a beaker. Prepare this solution just prior to the acoustic startle test and keep it in a beaker on the lab bench for easy access. After the experiments are over, using a plastic pipette, move the fish from the "waste" beaker and any other beakers or plates, into the Tricaine beaker. Once the fish have been euthanized, the remains can safely be disposed of in the sink. Euthanasia is complete in approximately 10 minutes.

Interpretation of Results

Damage to the neuromast should be reflected by rating scores lower than "3". There will be variability with a group of fish exposed to the same ethanol concentration. Ask students to reflect on why this variability exists. Reasons include genetics, health of the fish, experimenter variability in handling, etc.

Additional Experiment to Determine the Mechanism of Alcohol Toxicity

If you are interested, you can repeat this experiment to explore whether the damage from alcohol is due to the generation of reactive oxygen species. The way to "prove" this is to use antioxidants and determine if they can prevent the damage produced by alcohol. Choose a concentration that produced a moderate effect on the startle response from your first experiment—Do not choose a concentration of alcohol that was too toxic (i.e., killed a lot of fish) because it will be difficult to prevent the damage with an antioxidant.

Materials

Use the same materials as in the first experiment, plus:

- ✓ Antioxidants
  - Nicotinamide, a form of Vitamin B3; available at Fisher Scientific (AC12827-1000)
  - OR Trolox (a water soluble form of Vitamin E) & Ascorbic Acid (Vitamin C), available at Fisher Scientific (AC21894-0010 & AC10502-1000)
  - OR let the students choose other antioxidants (e.g., curcumin, the active compound in the spice, tumeric). Make sure to check the solubility of other compounds. If they are not water soluble, use another solvent, such as dimethylsulfoxide or DMSO. Don't forget to use DMSO in the control well. Some other antioxidants are also toxic to marine life, so keep this in mind.
  - Antioxidant stock solutions may be kept in a refrigerator, but some are light-sensitive, so the beaker/tube should be wrapped with tin foil.

Preparation of Solutions

- Make a stock solution of the antioxidant. First determine the molecular weight of the compound, the proposed concentration for treatment, and whether the compound is soluble in an aqueous (i.e., water) or organic solvent (i.e., DMSO). [Calculations should be made well ahead of the experiment day.]

  Here is an example using 5 mM nicotinamide as the antioxidant:

  Make a stock solution of 500 mM nicotinamide

  Nicotinamide (MW = 122.12 g/mol and it is water soluble.)

  1. Weigh out 610 mg nicotinamide
  2. Dissolve in 10 mls egg water

Make 2 working solutions of 5 mM nicotinamide (~100 mls) from the stock solution: 1 in egg water and 1 in 0.2% ethanol (made in egg water). Label the 2 beakers appropriately.

Nic: Take 1 ml nicotinamide stock plus 99 mls egg water (100 mls total)

Nic + ethanol: Take 1 ml nicotinamide stock plus 99 mls 0.2% ethanol (made in egg water) (100 mls total) (note: the final concentration of ethanol is really now 0.199%)

Set up of 6 well plates as follows:

Well Label	Treatment (9 mls)
A	Control – Egg water alone
B	0.2 % Ethanol (made in egg water)
C	5 mM Nicotinamide in egg water
D	5 mM Nicotinamide in 0.2% ethanol
E	Same as B
F	Same as D

Notice that all solutions are made in egg water. Each well has the same amount of egg water, regardless of the drug present.

Proceed to do the experiment exactly as in the previous protocol by adding 9 mls of each solution to the appropriate wells.

Use the same kind of data collection and presentation in tables or graphs.

TEACHING TIPS

Breeding

- Fish breeding takes patience. Fish often produce the most eggs when they have been maintained for the least amount of time. If you want more information on male and female zebrafish, refer to the website (wiki.zfin.org under protocols). It is highly recommend that you find a source to get the eggs (from a nearby pet store or university/research lab that is willing to breed for you) instead of breeding on your own. It is much easier!
- Otherwise, use a calendar to coordinate your breeding to coincide with planned classroom activities.

- When possible, include students in the husbandry of zebrafish. This is particularly interesting for them and fosters a deeper engagement with this project.

Useful tips

- If you have access to an incubator or oven, keep all solutions warm and carry out the fish treatment at 27-28ºC. If you do not have an incubator, you can do the breeding and the actual experiment at room temperature. However, the developmental processes and the damage will proceed slower than normal so plan your schedule accordingly.

- Drug preparation, including calculations, may take a while. Students can participate in these calculations if you want to use it as a prelab activity. Students should look up the solubility and molecular weight of the reagents and determine how much to weigh out prior to arriving in the lab. In general, the teacher should prepare the stock solutions for the students (for safety reasons), but the students can help prepare the dilutions.

REFERENCES

Murakami, S.L., Cunningham, L.L., Werner, L.A., Bauer, E., Pujol, R., Raible, D.W., and Rubel, E.W. (2003) Developmental differences in susceptibility to neomycin-induced hair cell death in the lateral line neuromasts of zebrafish (Danio rerio). Hear. Res. 186(1-2): 47-56.

Parng C, Ton C, Lin YX, Roy NM, McGrath P. A zebrafish assay for identifying neuroprotectants *in vivo*. Neurotoxicol Teratol. 2006;28:509–516.

Ton C, Lin Y, Willett C. Zebrafish as a model for developmental neurotoxicity testing. Birth Defects Res A Clin Mol Teratol. 2006;76:553–567

http://www.scribd.com/doc/29532167/The-Developing-Utility-of-Zebrafish-in-Modeling-Neurobehavioral-Disorders

The Zebrafish Information Network
www.zfin.org or wiki.zfin.org for protocols

SUPPLEMENTARY MATERIAL

1. Student Handout #1 – Administering ethanol to zebrafish (4 days old)
2. Student Handout #2 – Acoustic startle test procedure
3. Student Handout #3 – Administering ethanol and antioxidants to zebrafish (4 days old)
4. Video Clip of Startle Response in Zebrafish