Neuroscience Laboratory 120L
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Adrenergic Control of Fish Melanophores

Introduction

The Beast
The Siamese Fighting Fish (Betta splendens) is a small tropical freshwater fish which is commonly found in pet shops as a “Betta.” This fish is native to parts of South East Asia (mainly Thailand, Cambodia) and Malaysia. The brilliant colors and long fins of male pet-shop specimens are the results of captive selective breeding. In the wild, Bettas are rather dull, grayish-green, short-finned fish.

Melanophores

Chromatophores are cells containing pigment granules whose dispersal or aggregation is under hormonal or nervous system control. In lower vertebrates, chromatophores are found in the dermis; in response to various stimuli, the pigment in these cells is transported to or from the center of the cell. Changes in the distribution of pigment permit the animal to display variations in color. These cells are divided into different categories, depending on the type of pigment they contain (e.g., yellow - xanthophores; red - erythrophores; black/dark brown – melanophores). Chromatophores are of
interest to neurobiologists because they are a specialized type of effector organ with an important role in animal behavior, and they respond to circulating hormones (e.g., epinephrine, melanophore stimulating hormone, melatonin) and neurotransmitters (e.g., norepinephrine) via identified cell-surface receptors. **Melanophores** (also called melanocytes) are one type of chromatophore that contain black or dark brown pigment granules (*melanosomes*), membrane bound organelles that contain *melanin*. The pigment granules are transported along microtubules at rates of about 1 μm/sec (about the same rate as fast axonal transport) either towards or away from the cell center. When pigment granules are aggregated in the cell center, most of the cell is unpigmented, and an animal bearing such cells would appear lightly colored. When pigment granules are dispersed throughout the cell, the cell is uniformly pigmented, and an animal bearing such cells would appear darkly colored. Movement of pigment granules in melanophores is easily visible by bright field microscopy.

**The isolated fish-scale preparation**

Many teleost (bony) fish have melanophores within a dermal layer on their scales. Release of the neurotransmitter *norepinephrine* by sympathetic nerve fibers in the scale dermis or blood-borne *epinephrine* both produce pigment aggregation and paling of the skin (“blanching reaction”). The isolated fish scale is a convenient preparation for examining these transmitter-receptor interactions and biochemical cascades mediating pigment aggregation and dispersion in melanophores. In this experiment, we will use isolated scales from Siamese Fighting fish.

**Signal Transduction**

The regulatory pathway controlling the direction of granule transport has been partially elucidated using live melanophores. By analyzing the effect of various agonists and antagonists on cell surface receptors as well as the effect of inhibitors or activators of signal transduction pathways, scientists have begun to identify components of the signaling pathways that ultimately control the motility of pigment granules. While there are several stimuli which can trigger a variety of intracellular signals affecting pigment granule motility, in this exercise we will concern ourselves with the pathways which include changes in the intracellular concentration of cAMP.

Pigment dispersion is activated by an increase in cAMP levels while aggregation occurs when cAMP levels are reduced. In fish, the hormone epinephrine binds to a cell-surface receptor, which interacts with a G-protein. G-proteins have GTP binding and GTP hydrolysis capabilities. Binding of epinephrine to its receptor causes the exchange of GTP for GDP; the G-protein is now considered
activated. In this case the G-protein is an inhibitory G-protein. When it is "activated", it inhibits the enzyme adenylate cyclase. Active adenylate cyclase converts ATP to cAMP. The cAMP activates cAMP dependent protein kinase (PKA) which can phosphorylate many targets. When epinephrine or norepinephrine is added to fish melanophores, adenylate cyclase is inhibited, cAMP levels drop, PKA is inhibited, and the pigment granules aggregate.

AGGREGATION & DISPERSION

Objective - Observe Pigment Granule Movement in Fish Melanophores

Melanophores can be induced to transport pigment via stimulation of cell surface receptors that result in a signal transduction cascade as outlined above. Our goal is to demonstrate that epinephrine- or norepinephrine-induced aggregation is mediated by interaction with alpha-adrenergic receptors. To induce aggregation and dispersion we will use epinephrine or norepinephrine and IBMX.

![Image of Pigment Granules](image)

Scale from *Betta splendens*, 200x magnification

Epinephrine and norepinephrine bind to adrenergic receptors, and, at the concentration we will be using, cause intracellular cAMP levels to drop. IBMX crosses cell membranes and inhibits the activity of phosphodiesterase, an enzyme that converts cAMP to AMP. In the presence of IBMX, cAMP levels remain high and dispersion is induced.
Methods

Motility assays
To answer the questions for the lab report, it is very important that you (1) take good notes describing what you observe during these experiments and (2) learn to use the QX3+ Computer Microscope before starting the experiment.

1. Prepare the following solutions and vortex each one immediately. Wear gloves, and be careful when handling stock solutions. The IBMX is dissolved in DMSO which readily penetrates skin:

- 1 ml of Ringers containing 100 µM epinephrine (10 µl of 10 mM stock into 1 ml of Ringers)
- 1 ml of Ringers containing 10 µM yohimbine (10 µl of 1 mM stock into 1 ml of Ringers)
- 1 ml of Ringers containing 100 µM epinephrine and 10 µM yohimbine
- 1 ml of Ringers containing 10 µM propranolol (10 µl of 1 mM stock into 1 ml of Ringers)
- 1 ml of Ringers containing 100 µM epinephrine and 10 µM propranolol
- 1 ml of Ringers containing 2.5 mM IBMX (10 µl of 0.25 M stock into 1 ml of Ringers)

The TA will do the following step (Step 2 only) for you:

2. Place a small blob of Vaseline on a slide and place the clear, non-tissue end of a scale into this dab (to immobilize the scale on the slide). Place a small drop of Ringers on the scale, then cover with a coverslip that has a small blob of modeling clay on each of its 4 corners. Place the coverslip over the scale with clay-side down so that the space between the slide and coverslip forms the perfusion chamber. Make sure this chamber is filled with Ringers.

3. Open the QX3+ software on your laptop by double-clicking the desktop icon. Observe your fish-scale using (in this order) the 10X, 60X and 200X objectives on the QX3+ microscope. Can you make out the melanophores? Do they have aggregated or dispersed pigment? Take a still photo of the scale at 200X magnification by clicking on the “Snapshot” icon. Try lighting from above (“top” icon) and below (“bottom” icon) and adjusting the brightness and focus to get the best image. Label your photo right away (before you forget what it is) by clicking on the arrow in the lower left corner of the screen, then clicking on “paint” followed by the letter (“A”) icon. Use the second smallest font in black and place your label in a corner near the top or bottom of the photo where it will not cover the
melanophores. Example label: “Before epi – 3:10 pm.” Now go back to the “live” screen by clicking on the microscope icon in the lower right corner. Finally, you must EXPORT the image as a JPG file and give it a file-name that reminds you what it is (e.g., “before epi”). Export all your JPG images to a desktop folder labeled with your name.

4. Take still photos of the scale at 200X before and after perfusing 100 µM epinephrine solution between the coverslip and the slide, wicking with filter paper on the opposite side to enhance solution exchange. Observe the melanophores. Do the pigment granules move? In which direction? You may need to perfuse with several changes of the epinephrine solution in order to obtain maximal organelle movements. You can also make a time-lapse movie of the pigment granules moving in response to epinephrine. Get some melanophores in focus and get ready to perfuse the scales. To make the movie, click on the “Time Lapse” icon, then set it for 2-second intervals. When you’re ready to perfuse the epinephrine solution, click on the “Record Movie” icon – while keeping the “time lapse” window open. Stop the movie as soon as the melanophores seem to stop changing. You can save the movie as a compressed AVI file by going to the “collection” screen (arrow in lower left), selecting your movie, then exporting it as a video clip. Give the movie a name that indicates what it is (e.g., “epinephrine”).

5. Pretreat the scale with 1 µM yohimbine for 10 minutes, then perfuse with epinephrine + yohimbine. How does the response compare to that observed after epinephrine alone?

6. Flush scale with Ringers several times. Pretreat with 1 µM propranolol for 10 minutes, then perfuse with epinephrine + propranolol. How does the response compare to that observed after epinephrine alone?

7. Flush scale with Ringers several times. Perfuse with 100 µM epinephrine solution until you get strong organelle transport, then rinse again with Ringers several times. Next, perfuse with 2.5 mM IBMX. Do the pigment granules move? In which direction? Obtain still photos of before and after. We are saving the IBMX experiment for last because this agent is lipophilic and difficult to rinse out of the scale, once applied.
Data analysis

We will use ImageJ to analyze the size of melanophores in the images you collect with the QX3 microscope, before and after drug treatments. ImageJ is a public domain Java program that was created at the NIH for microscopic image analysis. It is available on-line at:

Measuring the average melanophore area (in square microns) in a selected region of your image...

1. Start the ImageJ program by clicking the desktop icon, then open your photograph file (“File/Open…”).
2. First, you must load the “analyze_particles” macro. A “macro” is a simple program that automates a series of commands. Click on “Plugins/Macros/Install…” and select “analyze_particles.txt”. Then click on “Plugins/Macros/analyze_particles.” This will execute a list of commands to modify your image (from Figure 1A to 1B, below) and create a square selection window (bordered in yellow on your screen). Using your mouse, drag and drop this selection window to the best spot on your image (a spot showing maximal change following drug superfusion).

Figure 1

3. Next, click on “Analyze/Analyze Particles.” You will see a pop-up menu like this… Change the settings so that they match those in this figure, then click on “OK.” Several new pop-up windows will appear, including one that displays numbered outlines of each “particle” (melanophore) in the selected area (see Figure 1C). The “Size Distribution” window displays the mean, std dev, min and max particle area in units of square microns (μm²).
4. To collect mean & std dev data, you can either write the numbers down in you notebook, or cut and paste the contents of the Size Distribution window into a Word document. Remember to note beside each set of numbers what image they were taken from (e.g., “before epi”, or “after epi”).

Questions
1. When you first observed your fish scale using the light microscope, were the pigment granules aggregated or dispersed? Write a short description of what you observed when you perfused the scale with epinephrine.

2. Do epinephrine or norepinephrine have the same aggregating effect when the scale is pretreated with yohimbine (an alpha-2 adrenergic antagonist)? What about when the scale is pretreated with propranolol (a beta adrenergic antagonist)?

3. What do the effects (or lack of effects) of yohimbine and propranolol suggest regarding the type of adrenergic receptors mediating catecholamine-induced pigment aggregation?

4. Why is it necessary to perfuse the scale with several changes of Ringers before adding IBMX?

5. Write a short description of what you observed when you perfused the scale with IBMX.

6. The IBMX is dissolved in DMSO which readily crosses the plasma membrane. Based on what you know about the action of IBMX, would IBMX dissolved in water have any affect on pigment granule movement? Why or why not?

References

