University of Minnesota

Nsci 5100: Introductory Neurobiology Laboratory at Itasca

Retinal Neurophysiology

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Developed by the Robert F. Miller Laboratory

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INTRODUCTION

To the artist or philosopher, the eyes may be the window of the soul, but to neuroscientists the retina is a window of opportunity for advanced studies of the **central nervous system** (CNS). The retina is the part of the brain that has the specialized task of transforming light energy into neuronal signals; the process known as **transduction**. More importantly, the retina begins the complex process of *analyzing* the spatiotemporal patterns of incoming photons to provide a coherent "view" of the objects in the world that reflect and emit those photons. This is to say that the retina does not act like a passive photographic film, but rather, is an active computational element in the visual nervous system.

In using light, which is the natural or **adequate stimulus**, the neuroscientist knows that the responses evoked for study are physiologically relevant, a special benefit of studying this tissue. Questions can be asked that are specific to the process of vision, and how the brain solves problems of seeing, much in the same way one could study a machine designed to perform a visual task and determine what the function of each component is. Keep in mind that the questions that can be asked (and the answers obtained) are *not* limited to the realm of sight. Many of the problems that must be solved by the retina are directly pertinent to other areas of the brain. Simple logic also would suggest that since the retina develops from CNS neural tissue, many of the cellular processes and "biological strategies" in retinal function are likely to be found elsewhere in the CNS.

As you will see, retinal anatomy is very well defined. Furthermore, all of the cell types in the retina have been subject to single cell electrophysiological study, providing a good basis for understanding many fundamental principles of retinal function. This structure may be complex, however, its processes can be interrupted and studied at different points and you will conduct experiments designed to accomplish this task.

We will be assaying retinal function by three **electrophysiological techniques**, the **electroretinogram** (ERG), the **extracellular recording** of ganglion cell (output neurons) spike activity and the **intracellular recording** of the graded voltage signals of retinal neurons. To accomplish this, in addition to electronic and surgical instruments, we will also be using pharmacological tools to alter the **retinal network**. The drugs chosen for study have well documented effects in many species.

During this week we will teach you a number of techniques used to study the retina (as well as the brain). While this will provide a useful and, we hope, exciting learning experience, it is only the secondary goal of our module. The primary goal for this week is to develop your ability to think critically and analytically. We encourage you to develop and test hypotheses. The planned experiments have specific tasks and goals, however we emphasize that we want your creative input. The week begins with well defined plans for the daily exercises, but as we progress, you will be given more and more freedom in setting the goals for your group. The faculty is here to teach you and one of the best ways to do this is to also ***have fun***. We strongly encourage you to try experiments of your own, because this will lead you to ***ask questions*** and that is when you will learn the most. It is our intention to be flexible during each day, so that you may pursue something out of the ordinary should you choose to. This course is more than an opportunity to learn about the retina and the techniques used for study. It is also a chance to learn how to do science. Let yourself take full advantage of the environment and enjoy the spirit of discovery!

Goals for the Week

1. **Learn critical thinking skills associated with the scientific method.**

2. **Learn general anatomy of the eye and especially the neural retina.**

3. **Learn how to perform field potential, extracellular single unit and intracellular recording techniques in a superfused retina-eyecup preparation.**

4. **Explore several principles of sensory information processing: Stimulus transduction, stimulus encoding and processing.**

5. **Record the electroretinogram (ERG) and identify the source of this response.**

6. **Record intracellular responses from retinal neurons: Identify cell type, identify the general rules for excitatory synaptic transmission in the retina.**

7. **Record extracellular action potentials and identify the functional differences between these signals and the ERG or intracellular voltage responses.**

8. **Apply a variety of pharmacological agents to the retina and determine the role of various neurotransmitter candidates in retinal information processing.**

To help achieve the goals:

a) Please look over the lab manual the night before each day's experiment. You do not have to read in depth, but looking things over will help prepare you for the day's goals. You can "ignore" the technical details, but try and focus on the scientific questions for the day.

b) Be prepared to read the lab manual instructions when there are detailed descriptions of the procedures or experiments BEFORE ploughing ahead or asking an instructor for assistance.

c) Try to generate your questions in the form of experiments you would like to conduct. We will be happy to describe the results of such experiments (if known).

COURSE SYLLABUS

I. MONDAY

A. Warmup/Introduction - *Gottesman*
   1. Overview of the module goals
B. Description of the day's tasks
   1. Morning - *Faculty*
      a. View dissection demonstration
      b. Practice eyecup dissection
      c. Solutions and perfusion system practice setup
   2. Afternoon
      a. General retinal anatomy & techniques for study - *Fagerson, Purple & Zahs*
         (1) Introductory remarks
         (2) Labeling cells
         (3) Microscopy
         (4) Basic retinal anatomy
         (5) Types of preparations used for retinal research
         (6) Demonstration of dissociated cells and retinal slices
      b. Visual Psychophysics - *Gottesman*
         (1) Technical information
            (a) Visual Stimulation Electronics
         (2) Testing some basic properties of vision
            (a) Measure "frequency of seeing" curve
            (b) Measure Temporal Summation curve
         (3) Discussion

II. TUESDAY

A. Identification of ERG components - *Purple*
B. Description of day's task
   1. Morning - *Purple*
      a. Response properties of ERG
         (1) Amplitude-Intensity Relations
         (2) Temporal Integration
         (3) Dark adaptation
   2. Afternoon - *Purple*
      a. Light adaptation (increment threshold)
      b. Isolation of Photoreceptor-only ERG

C. Discussion - *Purple*
   1. Properties of the electroretinogram
      a. Compare full ERG to Photoreceptor-only ERG
   2. Related psychophysical phenomena

III. WEDNESDAY

A. Intracellular recording - *Gottesman*
B. Description of day's task
   1. Morning
      a. Brief introduction
      b. Turtle versus frog retinae
      c. Overview of intracellular recording procedure
      d. Develop strategy for identifying retinal cell types being recorded
C. Afternoon
   1. Presentation of student data

IV. THURSDAY

A. Retinal signal processing - *Gottesman & Zahs*
   1. Single cells versus field potentials
      a. Information coded by spike trains
B. Description of day's task
   1. Morning - *Gottesman & Zahs*
      a. Comparison of ERG and spiking cell discharge
         (1) Amplitude-Intensity relations
         (2) Temporal Integration
      b. Before Lunch Discussion
         (1) Qualitative comparison of responses
         (2) Comparison of criteria for thresholds in graded vs. spike train responses
   2. Afternoon - *Gottesman*
      a. Introduction to pharmacological techniques
         (1) 2-amino-4-phosphonobutyrate (APB)
         (2) kynurenic acid (KYN)
C. Discussion - *Gottesman*
   1. Discussion of student's pharmacological data
   2. Outer vs. inner retinal neurons
      a. Outer retina
         (1) Transduction, "dark current", photoreceptor transmitter release
         (2) Graded neural responses
         (3) ON and OFF "channels"
            (a) Origins of b- and d-wave of the ERG

b. Inner retina
(1) Transformation of graded responses of outer retina to spike discharge.
(2) Neural information sent to rest of CNS is encoded temporally rather than by signal magnitude.

V. FRIDAY

A. Basic concepts of pharmacology - *Gottesman*
1. Agonists
2. Antagonists
a. Competitive
b. Non-competitive
3. Classes of transmitters
a. Excitatory
b. Inhibitory
c. Modulatory

B. Description of day's task
1. Morning
a. Begin study of glutamate receptor subtypes using agonists
(1) Kainate
(2) Quisqualate/AMPA
(3) *N*-methyl-D-aspartate
b. Use of antagonists to assay receptor subtypes and role of subtypes in light-evoked activity
(1) amino-phosphonovaleric acid (AP5) & amino-phosphonoheptanoic acid (AP7)
(2) Kynurenic acid (KYN)
(3) Quinoxalinediones - DNQX, CNQX, NBQX
2. Afternoon
a. Continue glutamate receptor subtype experiments

C. Discussion - ***Class presentation to faculty***
1. Functional organization of excitatory synapses in the retina
a. What glutamate receptors exist at which synapses?
(1) What does the binding of glutamate at each site do:
(a) to channels
(b) to the resting potential

PREPARATION OF SOLUTIONS

In order to study the retina we must maintain its viability after removal from the host organism. This is done by bathing it in a salt solution which mimics the fluid normally surrounding the cells. This salt, or electrolyte solution, was first described by **Lord Harvey Ringer** around the turn of the century and is often referred to as **Ringer's solution**. It basically consists of 4 different salts: **sodium, potassium, calcium, and magnesium**.

Other compounds must be added to our basic solution. To maintain the normal **metabolism** of the retina, **glucose** is included. Finally, we bubble the Ringer's with a mixture of oxygen (95%) and carbon dioxide (5%). The retina has the highest oxygen demand of any tissue in the body and this bubbling saturates the solution to carry the O_2 to the tissue. The CO_2 reacts with the bicarbonate level in the Ringer's to maintain the **pH** of solution at **7.4**, which is normal for the frogs we will use. It is therefore important to be certain that your solutions are ***always*** being bubbled. If the gas tanks go empty, or your bubbling lines or valves are incorrectly set, the retina will die due to lack of oxygen and pH poisoning.

The amount or concentration of a compound in a solution is usually reported as **molarity**. This is done because it literally tells us how many molecules are in the solution. One mole of any material is equal to the molecular weight of that material in units of grams. A 1 molar solution (abbreviated as 1 M) has 1 mole of a substance (or solute) in 1 liter of solvent. Solutions used for biological purposes are usually much less than 1 molar and are usually reported as millimoles (mM; milli = 0.001).

The formula for our basic salt solution for *frogs* is:

SALT	mM	Molec. Wt.	gm/L	mOsm
NaCl	86.0	58.44	4.988	172.0
KCl	2.0	74.56	0.149	4.0
$CaCl_2$	1.8	147.00	0.265	5.4
$MgCl_2$	1.0	203.30	0.203	3.0
$NaHCO_3$	25.0	84.00	2.100	50.0
GLUCOSE	5.0	180.2	0.901	5.0
			Total	**239.4**

We will save time by having our Ringer's solutions almost completely prepared in advance of our experiments. The Ringer's will have all the required salts, but will not include glucose. This is because glucose will support bacterial growth even when we refrigerate the solutions. Since we will make enough to last the entire week, we would have serious "infection" problems if glucose were present. This means that you will need to add glucose to your Ringer's salts ***every morning*** on Tuesday through Friday.

If you prepare 500 mL of Ringer's, how many grams of glucose will you need to add to your 500 mL of salts? You will not need the same accuracy in weighing out your glucose that you would require for the other ionic components (**why is that?**). This means that you should not obsess about the exact weight to 2 decimal places. This is not a license to be wildly inaccurate, but you can err by ± 50 mgs without seriously compromising the preparation.

There are two other things to consider regarding the Ringer's. We will use a low concentration of phenol red as an indicator to monitor the pH of our solutions. If the color of your solutions are not pink, but change to orange or yellow, discard the solutions and get fresh stock. Also, calcium bicarbonate has limited solubility and will precipitate if there is not enough water available. If your Ringer's appears at all ***cloudy***, this will indicate a loss of calcium and bicarbonate in the solution and you should discard it and get new Ringer's. We will use a slightly different Ringer's for experiments with the turtle on Wednesday.

EQUIPMENT

Electronics:

1) **Digital oscilloscope** and associated **oscilloscope printer**.
2) **Grass S48** stimulator for driving LED light flash stimuli
3) **Dagan S900** stimulator
4) **Light sources** to stimulate the retina
   a) Light Emitting Diode or **LED** - driven by **S48** stimulator
   b) **Fiber Optic** illuminator with **neutral density filters** and a filter holder
5) **Headstage amplifier** made at U of M for the transretinal ERG and intracellular recording
6) **Grass P-15** amplifier used for the intraretinal ERG and spike recording. This an **A.C.** or **Alternating Current amplifier**, measures transient changes in voltage
7) **Dagan Dual Channel Amplifier** for intracellular recording and cell staining
8) **Audio monitor**. Allows experimenter to monitor neuronal spike activity without constantly watching oscilloscope
9) **Utility amplifier**. Allows adjustment of neuronal signals to make them easier to display on output devices
10) **Window discriminator/rate meter**. Sums or integrates neuronal action potentials over fixed time periods. Turns brief action potentials into slow signals that are easier to monitor
11) **Chart recorder**. Provides continuous hard copy of electrical potentials
12) **Computer**. You will use the computer for creating graphs of your data

Some of this equipment you have used before, while others are being used for the first time.

Superfusion/Pharmacology:

In addition to electronic tools we will be using neuroactive agents which act as pharmacological tools. These drugs were chosen because they modify normal retinal function in network selective fashions.

Drugs will be delivered to the retina through a gravity driven superfusion system. Normal Ringer's or perfusate will be stored in a 500 ml **siphon bottle**. Test solutions will be stored in 12 ml **graduated syringe reservoirs**. Each container has its own valve and is connected to a common **manifold**. The output of the manifold is brought to the retina by a long, thin piece of Teflon tubing (**input tube**). The superfusate will be drawn off via a wick system to a 400 ml beaker. *Note that the source bottle holds **500 ml** but the output reservoir holds **only 400 ml!*** You must be sure to periodically check the catch beaker to ensure that you do not have a "flood"!

Preparation Chamber

The preparation chambers that you use are sturdy, but they do contain critical parts that you must take care not to damage. **Please be aware that the equipment you are using is designed to last *years* and requires appropriate care**. The chamber is made up of two parts, a base and a "top piece" or dome cover. Both parts have electrical leads attached to them and you need to take care never to pull on these wires or you can break the electrical contact with the electrodes embedded in the chamber components. Likewise, ***never*** attempt to unscrew the black dome piece located in the base! If you look under the dissecting scope, you will see that there is a hole in the black rubber dome of the base and that there is a grey cylindrical object in that hole. A similar cylinder is present in the top piece, if you look inside the edge of the hole. These are silver chloride pellet electrodes and are critical for our recordings. The pellets are very susceptible to mechanical damage, so avoid rough contact with them. The pellets and both chamber components do require a daily cleaning to ensure that there is not bacterial growth or contamination of the pellets. The top and base should be rinsed with distilled water and carefully wiped with a Kimwipe at the end of every day's experiments.

Surgical Tools

Your experimental success will depend greatly on the quality of your preparation. Your surgical tools will be critical in determining your ability to have a good prep. It goes without saying that each group has several hundred dollars in precision tools that are used, not only for this course, but in other courses later this summer and in future years. ***Nothing will upset the faculty more than seeing a disregard for your equipment***. Treat the tools as if you would have to purchase new ones if you damage yours. Be sure to carefully clean and dry your tools at the end of your surgery ***and do that when you finish your surgery, NOT at the end of the day***.

DISSECTION

This week's laboratory exercises require the sacrifice of live frogs and turtles. If you find this task unpleasant you should not hesitate to fully examine your feelings on this topic; either individually, or with classmates or faculty members. ***Please do not ask a classmate to sacrifice an animal for you unless you are willing to do it for them the next time. We do not expect you to like to do this, but you need to be responsible and not pass the buck just because the task is unpleasant. If you cannot perform the protocol because of an ethical problem, a faculty member can do this for you***. We are not taking time in the lab to directly address the question of the use of animals in research, however it is a serious and important issue that should not be dismissed. Any individual considering a career in neuroscience needs to carefully formulate their personal position on the ethical, responsible use of animals in research and, in this learning environment, the faculty are quite willing to discuss their own views. We will also *listen* to you and try to facilitate your own decision-making process if this can be of assistance.

Before beginning the surgery, you need to prepare/examine the **preparation chamber**. This is made of a **base piece with a rubber dome** over which the retina will be *everted* and a **dome cover**. The dome cover has a set screw which fits into a slot in the front of the dome support. The fit of this screw to the slot should be such that the dome cover can not rotate laterally, but the cover can be slid down over the dome. The screw should be tightened and then loosened *just enough to be able to slide the dome cover off*. This will allow you to tighten the screw into a locked position after the retina is in place with minimal rotation. If the cover still can "wiggle", check with an instructor about getting a different cover.

Step 1: Sacrifice the frog by decapitation. Using a strong pair of scissors or guillotine, make the cut about 3/4" behind the eyes (see Fig 1-1). It may be easier to put one blade of the scissors in the frogs mouth. Step 2: Pith **both** the head and the body of the frog. Step 3: Split the head between the eyes (**mid-sagittal cut**). It would be preferable that two groups share the eyes; however if one eye is not to be immediately used, wrap it in a wet paper towel and store it in the refrigerator.

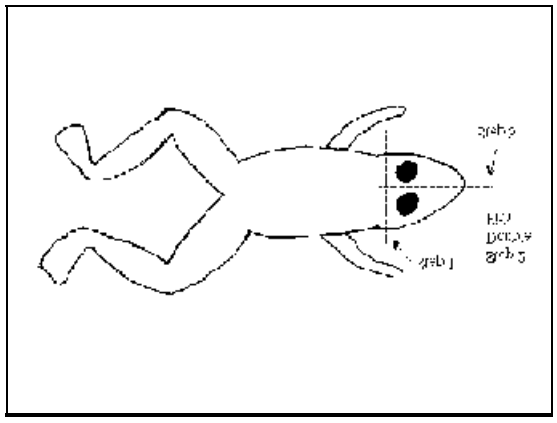


Figure 1-1

Unlike the rest of the central nervous system, the retina is not encased in bone. It is supported within a fluid filled globe, whose outside covering is flexible though surprisingly strong. The trick is to maintain the support of the **sclera** (tough tissue which makes up the outside of the eye) while removing everything that is in front of the retina. The first task after sacrificing the animal is to remove the eye from the orbit (called **enucleation**). If you have never dissected a frog before, take time to observe as many anatomical features as you can. On the first day, speed is *not* as important as learning the technique and you can take

advantage of this to discover some comparative anatomy. Use the second practice run to try and remove the eye as quickly, but safely, as possible.

Being careful to avoid the eye, pin the hemi-head in a dissection dish or on a "frog board". This allows better control with your forceps. Be careful not to puncture the globe of the eye with your scissors. To avoid this, always try to point the scissors at a tangent or away from the eye. The skin surrounding the eye (especially the upper eyelid) is both slippery and tough so use a **mouse toothed pair of forceps** and a **medium size scissors**. The back of the eye is attached to the skull by a fairly large set of **extra-ocular muscles**. You can leave a large amount of extra tissue on the eye as you remove it from the head.

Once the eye is free, place it on the holder provided (small cylinder that has a concave recess on the top face) and cut/strip away any remaining muscle or extraneous tissue from the back of the eye. Make sure the **optic nerve** stump is cut flush to the back of the eye; *again do not cut into the eye itself*. Making cuts with the scissors blades at a tangent to the scleral surface will be the most successful.

With the clear part of the eye (**cornea**) pointing to the side, pierce the eye just below the **gold colored tissue that forms the iris** using a **scalpel, your scissors or a razor blade**. As in Fig 1-3, use the micro scissors (**iris scissors**) to cut the front of the eye (**anterior chamber**) away. This is done by placing one blade of the micro scissors in the incision with the blades cutting parallel to the table top and continuing around the circumference of the eye. Do not stick the scissors deeply into the eyecup. Think of trying to cut through the skin of an orange without damaging the fruit inside.

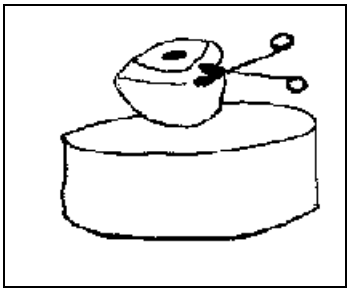


Figure 1-2

Gently lift one end of the anterior chamber and you will see the lens of the eye. The lens is usually imbedded in the jelly like substance found in the posterior chamber of the eye, the **vitreous humor**. As you lift the anterior chamber, use the iris scissors to cut the lens free of the vitreous as you go along. Be careful not to put the scissors too deeply into the eyecup, instead cut just below the clear lens as you remove it. This must be done in small steps because simply pulling the lens will detach the retina from the **pigment epithelia** and detachment will result in a damaged preparation.

With the anterior chamber removed, you can clearly see the optic nerve head and a few blood vessels running over the retina. A healthy eyecup will be black; a detached or damaged one will appear grey. Removing the vitreous will be necessary in pharmacology experiments, but it must be done gently so as not to detach the retina. As illustrated in Fig 1-3, make a series of triangle shaped wedges of Kimwipe. Place the small end of these wicks into the eyecup, just at the edge, and repeat the process until the eye is surrounded. When the fluid level is substantially reduced remove the wicks gently by pulling them out perpendicular to the edge of the eyecup. Do **not** pull the wicks up, rather out, parallel to the table top. If a wick sticks, add a drop of Ringer's to the eyecup. The surface of the vitreous can dry and form a membrane. The best way to see this is under a dissecting scope where you can gently grab just the surface of the vitreous and make a cut with your fine scissors. Then you can "tear" the remaining membrane toward the edges of the eyecup and continue draining with Kimwipes.

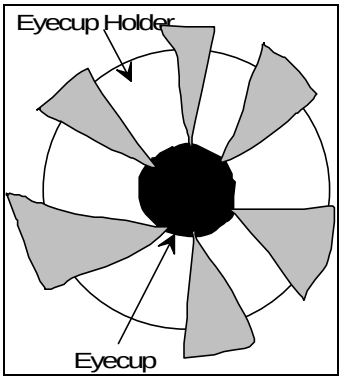


Figure 1-3

Once the vitreous is drained, place the eyecup on a hard flat surface and make four cuts into the eyecup as shown in Fig 1-4 (the figure shows a razor blade being used, but you can also use your fine scissors). Keep in mind that the drawing makes the prep look **flat.** The eyecup, even after trimming, is more like a hemisphere. These cuts allow the eyecup to spread open into a shape like a Maltese cross (as seen in the right panel of Fig 1-4) and the length of the cut will depend on the size of the eye you are

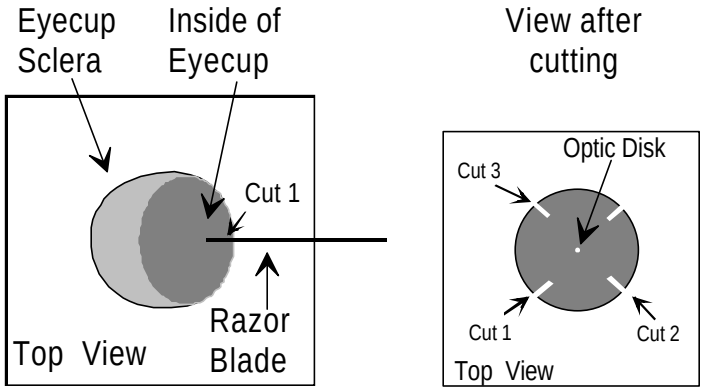


Figure 1-4

working with. Typically they will need to be 2-3 mm. Ideally, there should be an area about 1/4" in diameter in the middle of the eyecup (with the **optic disk**) that remains untouched.

The retina is now ready to be mounted in the recording chamber (Fig 1-6). 1) If you have a lot of eyecup (check with an instructor if you aren't sure), hold the eyecup by one edge and hold it on it's side on top of the black rubber dome. Then at the opposite end from where you are holding it, drive a fine dissecting pin (or 25 gauge hypodermic needle) through the edge of the eyecup into the dome. This should anchor it so that when the eyecup is spread, it's center will be at the apex of the dome. Take a set of forceps in each hand and gently spread the eyecup over the dome. 2) If you have a relatively flat, small piece of eyecup, place the piece on the top of the dome so that it is centered. The piece should balance in place by itself. You can then carefully put the dome cover on as described below.

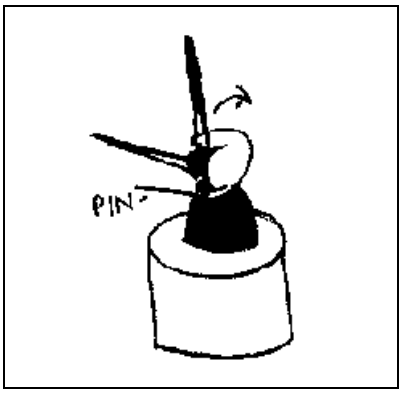


Figure 1-5

Remove the pin (you may need to gently hold the retina down with forceps so that the tissue does not stick to the needle) and gently lower the dome cover over the retina, as in Fig 1-7. ***DO NOT PRESS DOME COVER INTO RETINA!*** It should just sit on the retina. With caution, place a drop of bubbled Ringer's on the retina through the opening of the dome. If the liquid runs out, **GENTLY** reseat the opening of the dome on the retina by lightly pressing it down. When Ringer's stays on the retina tighten the set screw being careful not to wiggle the dome cover. After the first person has finished the procedure, remove the cover and discard the retina. Carefully clean the dome and dome cover. Each piece contains a silver/silver chloride pellet electrode, and these are somewhat fragile. Rinse off the chamber, but **do not** harshly scrub the pellets. The next partner can now practice the dissection. Save the last retina mounted and place the chamber on its stand in the experimental setup. We will practice connecting the superfusion system next.

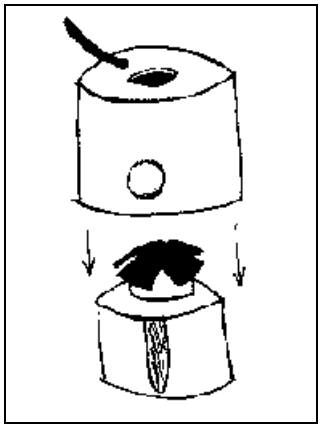


Figure 1-6

SUPERFUSION SYSTEM

We will keep the retina alive by providing it with the Ringer's solution described earlier. This solution will be provided through a gravity feed superfusion system that is designed to also permit the introduction of a variety of pharmacological agents. We will just set up the flow at the end of the morning to help you feel more familiar with the system for tomorrow's experiments.

A. Fill siphon bottle with Ringer's & place it in superfusion cage. Place the black rubber stopper with the air stone into the bottle and turn on the gas flow to the siphon bottle.
B. All the containers will empty into a common output called a *manifold*. This part is the brown plastic piece with 6 metal tubes pointing up mounted in the front left side of the perfusion cage. Connect the manifold to all containers; the siphon bottle should be the rearmost manifold input and the 5 syringe reservoirs should be connected in some order (*ie* left to right syringes connect to back to front manifold connections).
C. Open siphon bottle valve and then the first reservoir valve and allow perfusate from siphon bottle to fill manifold, tubing and as small an amount of perfusate as possible to remain in each syringe. Repeat for the other reservoirs. If air bubbles persist, cover the hole in the black rubber stopper in the siphon bottle with your finger. This will build up air pressure that will force fluid through the line & purge the system of remaining air.
D. Repeat the procedure for each of the other reservoirs. **Be sure to avoid air bubbles when filling the lines & reservoir or your prep will die due to stoppage of flow.**
E. Close all valves and be sure the end of the long piece of tubing that will go to the retina (**input line**) has been put in the 400 ml "catch" beaker. Fill the line with Ringer's by opening the manifold output valve and then the valve on the siphon bottle. If the line doesn't fill, put your finger over the hole in the stopper on the siphon bottle to build up gas pressure to "blow" fluid through the line. If this does not work, get a "suction" syringe from an instructor to get the flow going.
F. When we run drug experiments, the reservoirs will contain Ringer's & they must be bubbled also.
G. Place the end of the **input line** through the "tubing guide" glued to the dome cover. The **input line** should extend just near to the edge of the hole in the dome cover.
H. Take a precut piece of toweling that we are using as our **output wick** (on the front table). Move the 400 ml beaker just in front of the prep stand and place the flat end of the wick into the beaker.
I. Place the pointed end of the wick so only the tip contacts the fluid in the hole in the dome cover. Do not stick the wick deeply into the hole and do not let it touch the retina.
J. Keep the wick taut to prevent fluid from collecting at the bottom of the prep chamber instead of filling the catch beaker.
K. Put some Ringer's into one of the reservoirs and try switching the valves to change the flow to the reservoir.
L. See if you can estimate the flow rate of our system.
M. Turn off the flow and bubbling. Rinse the air stone.
N. Label your siphon bottle, seal it with parafilm and refrigerate.
O. Clean up the prep chamber.
P. Flush the superfusion system using a syringe and distilled water.
Q. Use the 95/5 gas to *dry* out the superfusion manifold and lines.

VISUAL PSYCHOPHYSICS

I. Introduction

Psychophysics is the study of the relationship between stimuli (physics) and perception (psychology). Not surprisingly then, visual psychophysics investigates the application of visual stimuli with the resulting sensory experiences. One way to characterize this field is to think of mapping one domain onto another. For example, one might vary the wavelength of stimulating lights and record the verbal responses of subjects as to the hues experienced for each stimulus. The resulting "input-output map" would relate the physical stimulus domain to a subjective or psychological domain of color. This field has two major areas: 1) The measurement of thresholds; for example, determining just detectable energy levels for sensory stimuli, and 2) Measurement of perceptual responses to highly detectable, or suprathreshold, events.

Neuroscience is interested in psychophysics since a fundamental assumption of the brain sciences is that the psychological responses measured in psychophysical experiments are derived from physiological processes interposed between the initial physical stimulus and the final behavior. This is true regardless of whether one is considering the input-output relations of sensory systems or, instead, the conversion of a pattern of brain activity preceding a desired action to a final set of muscle movements.

Measurement of thresholds in *both* human and animals have provided one of the primary means for understanding the relation between the physiology of nerve cells and behavior. The two experiments that we will conduct serve as examples of the basic methods and rationale of threshold measurement.

II. Goals

There are 3 goals to our exercises this afternoon:

First, you will become comfortable operating the visual stimulators we will be using this week. Second, you will develop an initial appreciation of problems facing any visual system. Finally, you will conduct experiments that will be repeated using purely physiological measures later in the module. The data you collect can be compared to the results that you obtain from frog retinae. This comparison may serve to clarify some of the fundamental properties of vision and also highlight the extent to which we understand the role of the underlying physiological mechanisms involved in how we see.

III. Visual Stimulators

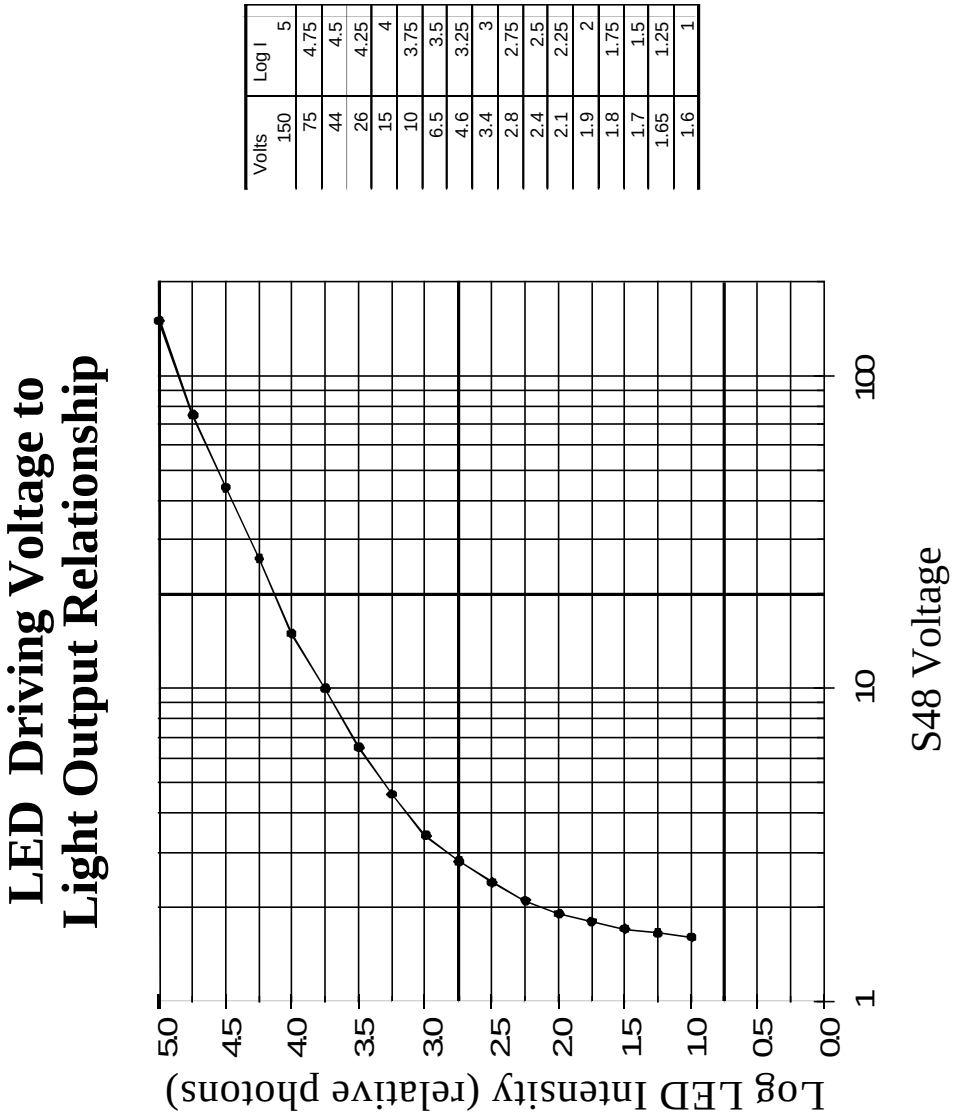
A. Fiber Optic Illuminator

1. Filter set
   a. We have calibrated pieces of filter material that we will use to reduce (or attenuate) the intensity of the fiber optic light for various experiments.
   b. *Log units of light intensity* - remember that a 1 log unit filter reduces light intensity by a factor of 10, a 2 log filter attenuates by a factor of 100

B. Light Emitting Diode (LED)

The main method of stimulating the retina will be via a light emitting diode. This electrical device emits photons when current flows through the device. LEDs provide a compact and economical visual stimulator at the expense of having any great control of spatial aspects of the stimulus.

1. We can position the LED via the three dimensional manipulator on which the LED holder is mounted. The system has been approximately positioned, however, you may need to make some adjustments to be certain the light is where it should be. This will be covered in more detail below. *Be aware that if the LED holder is moved intentionally or accidentally you will be unable to conduct your experiments. It would be equivalent to having your stimulating electrodes out of the prep chamber for the neuromuscular junction experiments. SO - if things don't work, be sure that a stimulus is getting to the retina (yours or the frogs).*
2. The LED receives its driving voltage from the Grass S48 stimulator. The LED output cable is already coming out of the recording cage and the double banana plug is connected to the voltage output of the S48. Be sure that the double banana ground is correctly connected. The second, small clear lead is optionally connected to the Grass output ground post. Leave this off for now, but you can attach the LED shield by loosening the lower post and then inserting the gold pin connector on the shield into the revealed hole and re-tightening the post.
3. Temporal control of the light comes from the timing of the Grass output.
   a. *STIM DURATION* controls flash duration or length
   b. *STIMULUS INTERVAL* controls interflash interval
      (1) Given in pulses per second which is the **inverse** of the interval, so smaller numbers mean slower rates of presentation.
4. Intensity control is provided by varying output voltage
   a. Voltage to light intensity relationship (***non-linear***)
      (1) See the figure (Fig 1-7) on the next page. You will be referring to this figure often, so you will want to take care of it over the course of the week.
   b. We use the **GRASS S48** because it provides
      (1) Highest light output from its large voltage range (150 Volts, max)
      (2) Fast setting of voltages possible (single turn pot)



Volts	Log I
150	5
75	4.75
44	4.5
26	4.25
15	4
10	3.75
6.5	3.5
4.6	3.25
3.4	3
2.8	2.75
2.4	2.5
2.1	2.25
1.9	2
1.8	1.75
1.7	1.5
1.65	1.25
1.6	1

Figure 1-7

IV. Visual Threshold Measurement

A. Introduction

There are various methods used to measure thresholds and we are going to use two different ones in our experiments this afternoon. The first technique, known as the method of constant stimuli, is often used in computerized collection of threshold data. You will implement a simplified version. The second technique is the method of limits and is a truly venerable procedure. It was used by Gustav Fechner in the late 1800's, when he coined the term psychophysics.

B. Experiment 1 - Threshold and Frequency of Seeing Curve

1. Essence of Procedure
   a. Observer views stimulus target area
   b. Experimenter flashes light
      (1) Flash preceded by warning tone cue to aid observer
   c. Observer responds "YES" or "NO"; **no "MAYBE" responses allowed!!**
   d. This sequence is repeated many times at different intensities
   e. Resulting data are analyzed to determine threshold.
2. Initial Set Up
   a. Warning tone (from **Dagan S900**; triggered by **Grass S48**)
      (1) **Grass** *Delay Sync Out* to **S900** *Pulse Interval Trigger In*
         (a) Use double banana to BNC adaptor at **Grass**
      (2) S900 Settings
         (a) *Pulse Interval*= EXT*; Pulse Del*=Minimum; *Train Dur*=0.1sec; *Train Int* (sets pitch)= Values of 0.002-0.006 sec; *Pulse Width*= 0.0005 sec
         (b) **S900 *Output*** -> goes to Harvard Audio Monitor
            i) 1 Volt range; Pot in range of .5 to 1
   b. S48 *DURATION*= 100 msec; *VOLTS*= 15 V; *DELAY*= 600 msec; *STIM RATE*= 3 x 0.1 PPS*; STIM MODE*= REPEAT
   c. Position LED spot in the center of fixation marks on white card
   d. Illuminate the card with the fiber optic & allow observer to view white card for about 2 minutes then set the intensity so the flashing LED is ***just barely visible***
   e. *STIM MODE* = OFF
   f. Again, allow observer to view white card for about 2 minutes to adapt to the field.
   g. Experimenter should verify they know procedure from protocol sheet
3. Experimental Protocol
   a. **S48** *DURATION*= 100 msec
   b. Set interstimulus interval to 1 flash every 7.5 seconds
      (1) *STIM RATE* = 1.5 x 0.1 PPS
      (2) Slow this down if the experimenter needs more time
4. Practice Trials
   a. Run a few practice trials to get familiar with the task
5. Run the Experiment

a. Present stimulus intensities in order according to the protocol sheet
   (1) This specifies the sequence of the voltages at the S48
      (a) Remember the voltages are NOT light intensities
   (2) Work the top row left to right
   (3) Work the following row right to left
   (4) Continue to alternate direction with each row
b. Try and complete the entire protocol sheet without a break
   (1) Break if necessary
      (a) Observer **must** keep looking at card during break

Experiment 1 Protocol: Work across rows - alternating right to left then left to right. Put a **T** mark for "YES" responses and leave blank for "NO". Where there are asterisks do NOT present that stimulus (this means only 5 presentations each at 75 and 5 Volts). Use the computer to load Prism (see page 1.20 for instructions on using this data graphing package) and enter the data for your group in the appropriate column in Data Table 1-1.

S48 VOLTS	75	6.5	44	15	26	10	5
S							****
U	****						
B							****
J	****						
							****
R	****						
E							****
S	****						
P							****
	****						
# of Flashes Presented	0.00	10	10	10	10	10	5
# of Flashes Detected							
% Flashes Detected							
Log Intensity	4.75	3.50	4.50	4.0	4.25	3.75	3.30

Plotting Data Using GraphPad Prism

- To help you have more time to think about your data, we have created a project file in a scientific data graphing application, GraphPad's Prism. We hope that the program is sufficiently easy to use that you can use it to generate graphs of your data more quickly than doing it by hand.
- However, if you prefer to avoid the computer, we have preprinted Data Graphs that you can use; just ask an instructor for these.
- For those wanting to use Prism, here are some outlined instructions. Please appreciate that there is no hardcopy documentation available. The online help should be sufficient, but ask for help if something does not make sense.
- OVERVIEW
  - Run the program by double clicking on the Itasca97 Retina Icon in the GraphPad Software group.
  - The program is organized by 5 "file folders" which appear in yellow in the upper left of the screen. These folders are **Data, Results, Graphs, Layouts** & **Notes**.
    - All *raw data* will be entered into spreadsheets that will exist in the **Data** Folder. You will find that there are pre-existing data tables in that folder into which you can enter your raw data values from the data sheets in your lab manual.
    - If you need to analyze or transform your data (and this should generally ***not*** be the case) in any fashion (add a constant, multiply by a constant, find the mean & variance, etc....) You would click on the **Analyze** button just below the yellow file folder icons. The program will then prompt you to choose the operation you wish to perform. It will carry out the operation and the results will be placed into a newly created spreadsheet that will be located in the **Results** folder. You are free to perform further analyses on spreadsheets contained in the **Results** folder (such as fitting a regression line or even nonlinear curve fitting).
    - We have set up the project file with templates so that the program automatically creates a graph of data entered into the worksheets in the **Data** folder. This means that when you enter your values into the spreadsheet, you can click on the **Graphs** tab and you will see the graph of the data you just entered. While you can print from this page, you cannot control the size of the graph. This feature is applied in the next folder.
    - We have also created templates for graph *layouts* which are found in the **Layouts** folder. Each layout can be used to size graphs on the page, add text or spreadsheet data (if you select and "copy" columns in a **Data or Results** spreadsheet, you can then "paste" them into a layout). You can also print multiple graphs on a page if desired.
    - Finally, if you want, the **Notes** folder provides a simple word processor that allows you to add notes that are associated with your project.
- ***Be sure to <u>save your files</u> after entering your data.*** Prism does not have an autosave feature, so this will protect you from inadvertent disasters.

V. Temporal Summation

A. Introduction

Our second experiment will examine the extent to which the visual system integrates incoming photons and if it does, over what period of time.

B. Experiment 2

1. Essence of procedure
   a. Use classic Method of Limits to measure threshold for detection
   b. Again, Observers must respond **Yes** or **No** only
   c. Flashes begin clearly visible
   d. Subsequent flashes are reduced in intensity until the Observer responds **No**
   e. Threshold is *operationally defined* as the last intensity the Observer sees
   f. This procedure will be repeated two more times
   g. The average threshold will then be determined
2. Initial Setup
   a. Warning tone
      (1) Same settings as for Experiment 1 - ***EXCEPT:***
         (a) **S900** *TRAIN DUR* = 2.0 sec
   b. **Grass S48**
      (1) *DURATION*=1 msec; *VOLTS*=44; *STIM RATE*=3 X 0.1 PPS; *DELAY*= 600 msec
   c. Adjust fiber optic intensity so that the flashing light is ***just barely visible***
   d. Observer views card for a few minutes to establish constant conditions of adaptation
   e. Experimenter checks protocol sheets to be sure they understand experiment
3. Practice Trials
4. Run Experiment
   a. Follow protocol sheet on the next page
      (1) Run 3 descending series each at the following durations:
         (a) 2000, 50, 500, 1, 10, 1000 msec

Experiment 2 Protocol: Set **GRASS S48** *DURATION* to the first data column value (2000 msec). Set the *STIM RATE* to 1.0 x 0.1 PPS (go faster if you can do so comfortably; slower if needed). Set the *VOLTS* to 10. Present the first flash. Put a **T** in the box if the subject responds YES. Set the *VOLTS* to the next row value and continue until the Observer responds NO. Reset the *VOLTS* value to the start value and repeat until both columns are filled. Set *DURATION* to 100 msec and begin again. Note that some intensity values have already been filled in as "seen". This is based on our prior experience for responses at long durations, HOWEVER, if your Observer can not see the starting flashes, ignore our notation and start at higher intensity values.

Log Intensity	**S48** Volts	2000 msec		100 msec		50 msec		500 msec		1 msec		10 msec		1000 msec	
5.00	150	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**					**T**	**T**
4.75	75	**T**	**T**	**T**	**T**	**T**	**T**	**T**	**T**					**T**	**T**
4.50	44	**T**	**T**	**T**	**T**			**T**	**T**					**T**	**T**
4.25	26	**T**	**T**					**T**	**T**					**T**	**T**
4.00	15	**T**	**T**												
3.75	10														
3.50	6.5														
3.25	4.6														
3.00	3.4														
2.75	2.8														
2.50	2.4														
2.25	2.10														
2.00	1.90														
1.75	1.80														
1.50	1.70														
1.25	1.65														
1.00	1.60														

Calculate the threshold for each series by taking the intensity of the last YES (ie. Last yes =1.9 volts yields threshold= 2.00 log I). Take the average threshold for the 2 runs and put them into the spreadsheet for Data Table 1-2 in Prism.

General Retinal Anatomy

Goals/Questions for Day 1 Anatomy

1. Learn about staining techniques that can be used to study the structure of the retina. What types of microscopy are available and what are the advantages of each type?

2. Draw a simple diagram of the structure of the retina based on the data you have analyzed.

3. What different structures have you identified in the retina? How are these structures organized? What are some principles of retinal organization? In very general terms, what functions might these structures perform?

VI. Introduction

This morning you learned how to prepare an eyecup for physiological experiments to determine some of the functions of the retina. You saw the retina in the eyecup and saw that it appeared black. This was due to the pigmented epithelium behind the retina, which is black.

This afternoon, we want to learn something about the structure of the retina and how that may play a role in its function. The initial experiment needs no equipment. We want to find out two very basic functions of the retina. When you close your eyes, what do you see? What happens if the light in the room is turned off when you eyes are closed? Can you tell the difference? When your eyes are closed, can you imagine what you have just been seeing prior to closing your eyes? What are some basic functions of the retina that would be required for you to be able to answer the questions above the way you did? You will look at the results of some experiments in order to learn whether there are structures in the retina that might be involved in these functions. You should also keep your eyes open for other structures, to see if we can learn anything about some other possible functions of the retina.

You will look at some slides of fixed and fresh whole retinae under the light and fluorescence microscope, as well as radial sections through either whole eyes or retinae. You will see a variety of methods for staining specific elements of the retina and watch an experiment using fluorescent dyes in live retinae. We will also look at some 35mm slides showing cells dissociated from a retina as well as flat mount and radial views of retinae that were imaged with either fluorescence or confocal laser scanning microscopy.

The stains used include: 1) fluorescent dyes: fluorescein, sulforhodamine (a polar dye), Lucifer yellow, CY-3, tetramethylrhodamine, and BCECF, 2) horseradish peroxidase. Several of the dyes have been conjugated to antibodies which are used to identify the location of specific proteins.

There will be three slide projectors or viewers and four microscopes available so every one should be able to use either a slide viewer or microscope. The experiments will involve looking at 35mm slides or microscope slides of experimental preparations. You can do these in any order just be sure to look at each experimental preparation.

VII. General Experimental Questions

As you look at the results of the various experiments, try to keep in mind the following questions:

1. What type of stain was used and what are the significant characteristics of the stain? Also, is the method of application significant? How does the orientation of the retina on the slide (i.e. flat mount vs slice) affect what you can learn about its anatomy?

2. What are the two very basic functions of the retina hinted at in the Introduction to this section? What structures in the retina might be involved in these functions? Where are they located?

3. Are there other structures in the retina, besides those referred to in question 2, that might be involved in the function of the retina? If so, what other general conclusions can we draw about the function of the retina?

Experiment 1.

You will look at preparations of dissociated retinal cells and also a radial section through a retina.

35mm slides. Cells dissociated from the retinae of frog, salamander, and rabbit have been labeled with fluorescent dyes.

A. Dissociated cells from salamander labeled with BCECF, a fluorescent pH indicator.

B. Dissociated cells from frog, salamander and rabbit retinae labeled with the dye sulforhodamine. Images on the left are view with a light microscope using Differential Interference Contrast DIC). Those on the right are view with epiflouresence microscopy.

C. Retinal slices of a mudpuppy viewed with DIC.

Questions

What are some identifying features of these dissociated cells? Can you recognize cells in the radial slice that have these identifying features? What gross patterns do you see in the radial slice?

Experiment 2.

In this experiment, a substance called horseradish peroxidase (HRP) was injected into the optic nerve of salamanders. After several days, the animals were sacrificed and the retinae removed. The retinae were fixed and processed so that the location of the HRP could be identified by a brown (or black) color. The retinae were mounted onto a microscope slide with the inside surface up. You will look at these slides using light microscopy.

Light microscope slides.

A. Low power view of flat mount HRP labeled retina.

B. High power view of flat mount HRP labeled retina.

You will also look at a live retina which is labeled with a fluorescent dye. Approximately 20 hrs before you look at this retina, the eye of a mudpuppy was removed and crystals of tetramethylrhodamine, TMR a fluorescent dye, were placed on the stump of the optic nerve. You will view this retina on a

microscope slide (inside surface facing up) and examine it using an epifluoresence microscope. What you will be seeing are photons given off by the tetramethyl rhodamine molecules that have been excited by the stimulating light.

Questions

What structures do you see? What can you conclude about the optic nerve (where HRP was injected and the dye crystals were placed) and its relationship to the retina? Might any of these structures be involved in one of the basic functions of the retina?

Experiment 3.

35mm slides

A. Retinal slice from an eyecup into which the dye, Lucifer Yellow, was placed for 20-30 min. The image was viewed on a confocal microscope.

B. Retinal slice of a mudpuppy labeled with primary antibodies to neurotransmitters, and a secondary antibody conjugated to either horseradish peroxidase (HRP) or CY-3. On the left the neurotransmitter, glutamate, is viewed on a light microscope using DIC. The dark color shows the location of the primary and secondary antibodies. The tissue was treated with diaminobenzidine which reacted with the enzyme HRP on the secondary antibody to produce the dark precipitate. In the middle image a primary antibody to GABA and a secondary antibody conjugated to CY-3 is used and the tissue was visualized on the confocal microscope. In the right image a primary antibody to glycine is used and a secondary antibody conjugated to CY-3. The image was also viewed on a confocal microscope.

C. Mudpuppy retinal slice preparation labeled with a primary antibody to synaptobrevin, a synaptic vesicle protein, and Cy-3 conjugated to the secondary antibody viewed on a confocal microscope. The upper left image is a low power view of an optical section through the slice, showing nearly the full width of the slice. The other three images are high power views, focusing on the bright wide band, of three optical sections at different depths in the slice.

Questions

What structures can you see? In the 35mm slides, did you see some different structures? In each image are similar structures labeled? What differences can you see between the different methods? Do you think these structures are involved in one of the two basic functions of the retina or do you think they might perform other functions?

Experiment 4.

In between looking at 35mm and microscope slides, take a peek at an experiment which will be carried out during the afternoon in which a polar fluorescent dye, sulforhodamine, is applied to the eyecup for 15-20 minutes. This dye is known to be impermeable to cell membranes due to its charged elements. The retina will then be removed and radially sliced. The sections of the retina will be viewed on the fluorescence microscope again using the green light filter set. This experiment will be done on two eyes so that you can see how the dye is applied and also look at microscope slides of retinal sections that will have been prepared just prior to the afternoon session.

Experiment 4.

Microscope slide.

A. On the fluorescent microscope, look at the slide with retinal sections from one of the retinas prepared as described above.

35mm slides.

B. Retinal slice of a mudpuppy in which the eyecup was filled with sulforhodamine and kept in the dark for 20 min. The upper left image is viewed with DIC on a light microscope. The upper right image is viewed on a fluorescent microscope. The lower image is viewed on a confocal microscope.

C. Flat mount mudpuppy retina labeled with sulforhodamine as described above, viewed on a confocal microscope at a series of optical planes through the retina. The upper left image is at the inner surface of the retina and the lower left is near the outer surface of the retina. The depth in um is indicated on the lower right of each image.

D. Flat mount mudpuppy retina labeled with sulforhodamine. The left image is a 3-D composite of a series of confocal images taken through a portion of the retina approximately 20 um thick. The right image is a computer rendered view of the left image.

E. Flat mount mudpuppy retina labeled with both fluorescein and sulforhodamine. The fluorescein was applied as crystals of dye to the cut optic nerve for 24 hours similar to the TMR that was applied in experiment three above. The sulforhodamine was applied in solution to the eyecup for 20 min just prior to removal of the retina for viewing on a confocal microscope. The upper image is a fluorescent image of this retina using filters to excite and view the fluorescein. The middle image is a fluorescent image of the same location using filters to excite and view the sulforhodamine. The lower image is a computer rendered image combining the images of the two dyes.

Questions

How do you hypothesize the sulforhodamine dye became trapped where you see it? What kind of structures might be labeled? Think about where you saw the dye in the dissociated cells in Experiment 1B as well as in the microscope slide and the 35mm slides in this experiment. Are these structures different from ones you have seen previously? If so, in what way? Where are they located in relation to previously identified structures? Can you draw any conclusions about the functions of these fluorescent structures based on the methodology used in the preparation of the tissue and/or its location within the retina? Does the 35mm slide, E, appear to support your hypothesis about the function of these structures?

OUTLINE OF RETINAL STRUCTURE

I. Embryonic origins of the vertebrate retina

A. The retina is **CNS** tissue that migrates to the periphery during development

B. This means studies of retinal physiology and anatomy have relevance beyond the scope of sensory function.

II. History of the study of the retina

A. 1850 - first anatomical explorations of the retina - Heinrich Müller

B. 1850 - Max Schultze - pioneer of comparative anatomy and the implications for function that can be generated by careful observation of structures across species.

1. Recognized the anatomical distinctions between rods and cones (see **Fig 1-8**)
2. Proposed the Duplicity Theory to ascribe a *functional* distinction between these two cell types based on his comparative data.
   a. Rods dominate the retinae of nocturnal animals
   b. Cones dominate the retinae of diurnal animals
   c. Therefore the function of these cells are specialized for operation in dim (rods) or bright (cones) illumination.

C. It required more than 100 years for science to develop the techniques required to demonstrate directly that the Duplicity Theory was correct and that the rods and cones are the biological elements that perform the essential process of **visual transduction**.

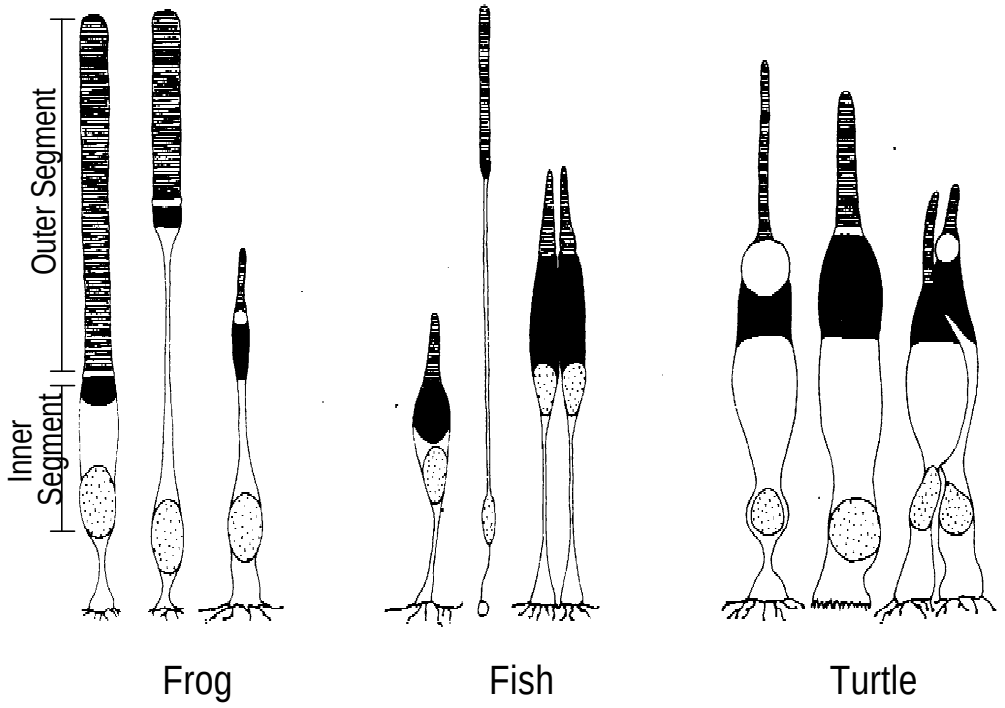


Figure 1-8 Representative Rod and Cone Photoreceptor Cells

III. General retinal anatomy

A. The eye (see schematic human eye: **Fig 1-9**)

1. The eye is a fluid filled globe with a hole at one end.
   a. The hole is filled with optical elements needed for image formation
      (1) Cornea and lens
2. Layers to the eye
   a. Sclera - tough outer coat
   b. Choroid - nutritive inner lining
   c. Retina - neural innermost layer

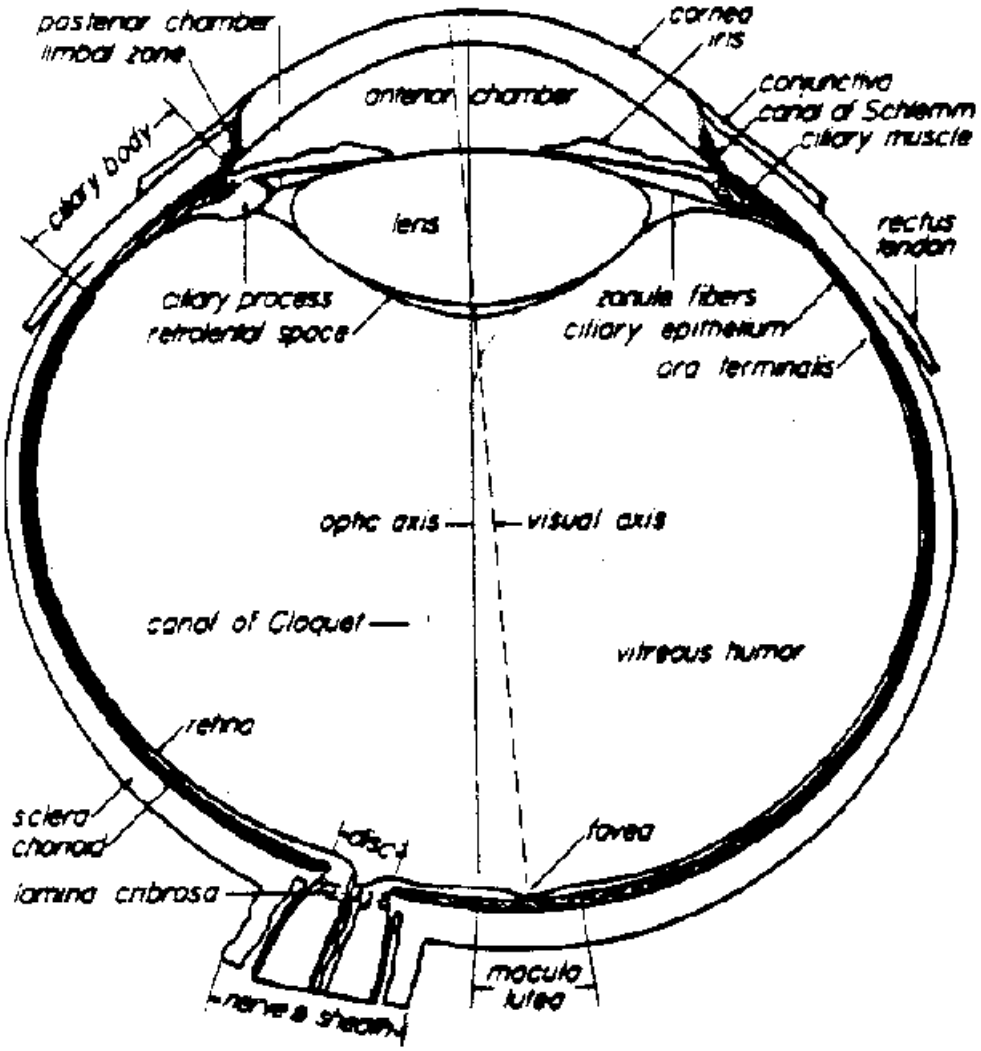


Figure 1-9 Cross section of the Human Eye

B. The retina
   1. Cell Types (see radial view of retina in **Fig 1-10**)
      a. Photoreceptors: Rods (R) and Cones (C)
      b. Bipolar cells (B)
      c. Horizontal cells (H)
      d. Amacrine cells (A)
      e. Interplexiform cells (I)
      f. Ganglion cells (G)
      g. Müller cells (M)
      h. Pigment epithelium cells (PE)

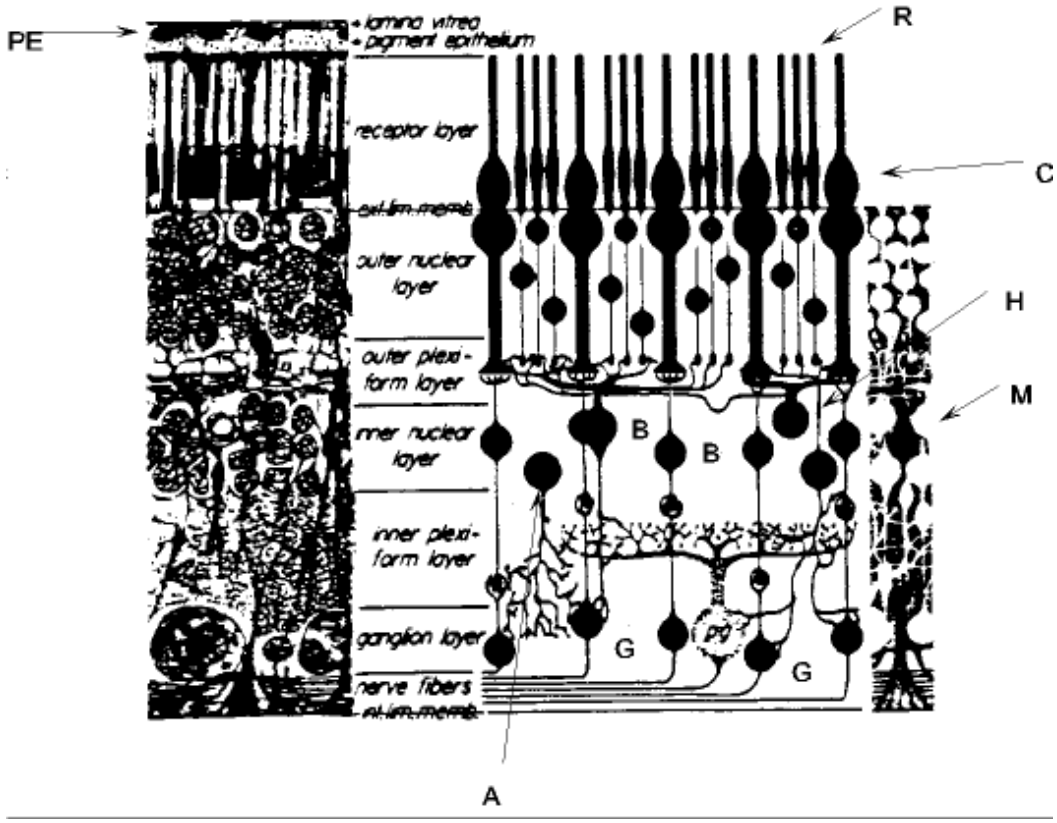


Figure 1-10 Radial view of the Human Retina (left side) and schematized view on the right

   2. General layered morphology - similar to brain layers
      a. Cell body layers
         (1) Receptor layer
         (2) Outer nuclear layer
         (3) Inner nuclear layer
         (4) Ganglion cell layer

b. Synaptic layers
   (1) Outer plexiform layer
   (2) Inner plexiform layer

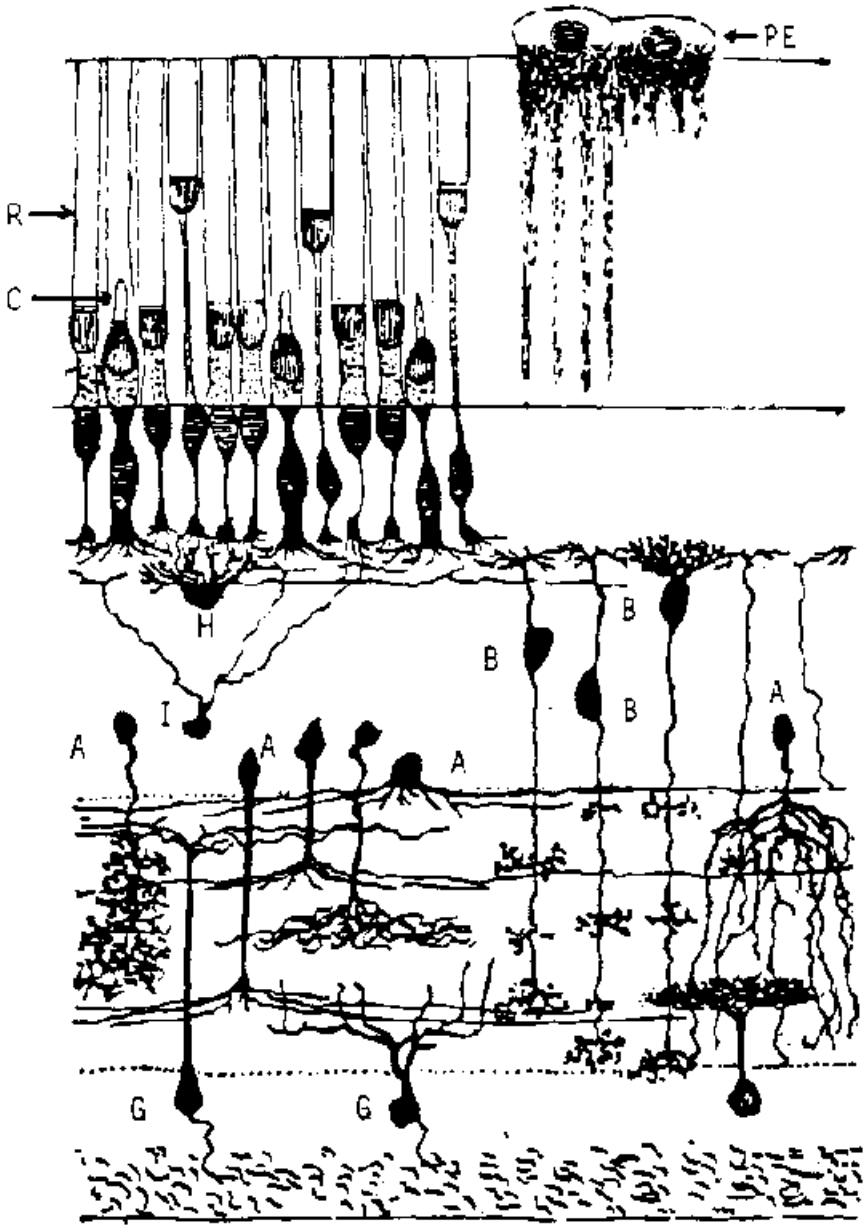


Figure 1-11 Radial view of the Frog retina

Synaptic Vesicle Cycling in a Presynaptic Terminal of an Axon

In this afternoon's exercise, you viewed sulforhodamine stained retinal cells. What possible mechanism for uptake of sulforhodamine in the retina does this figure suggest?

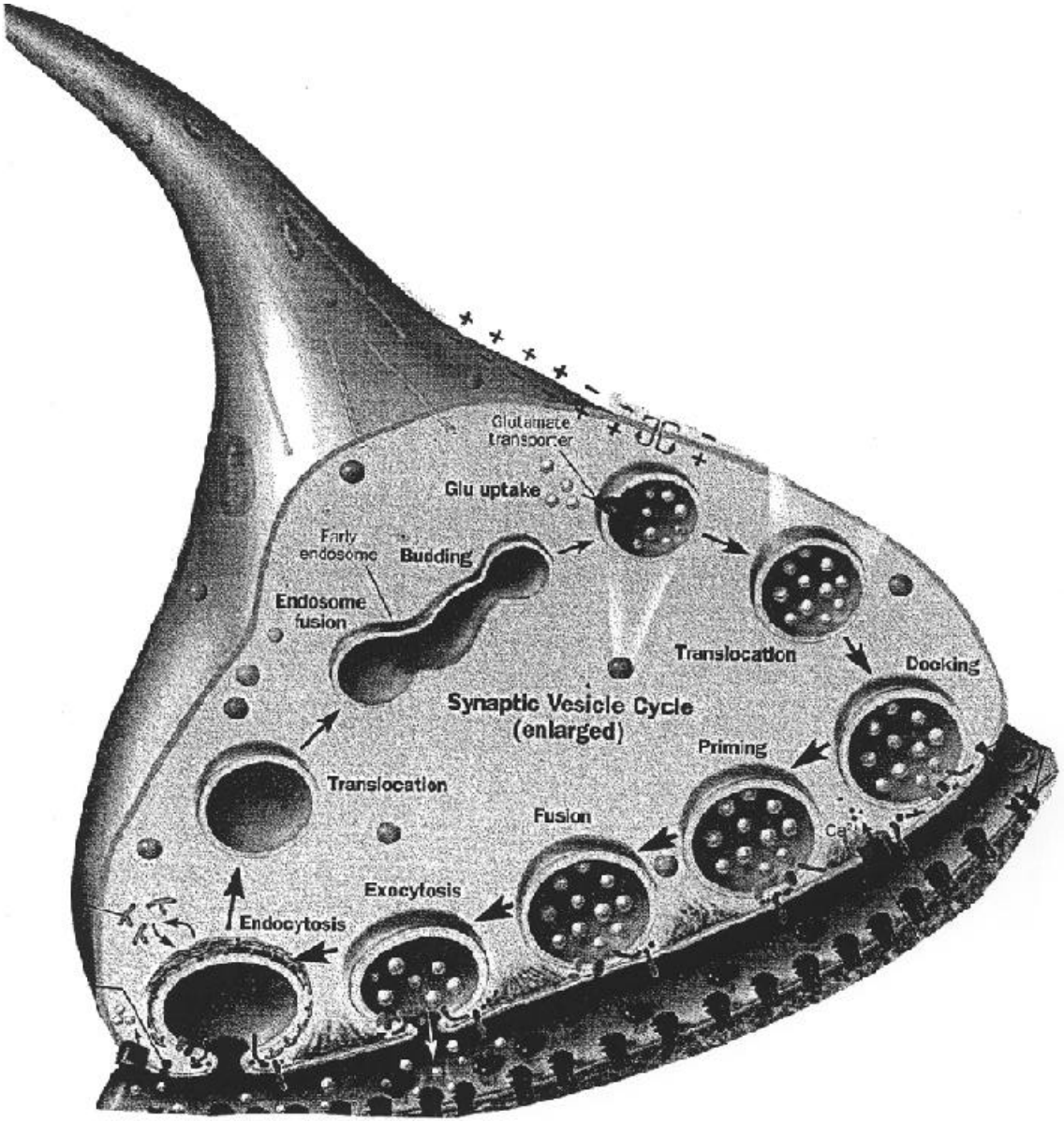
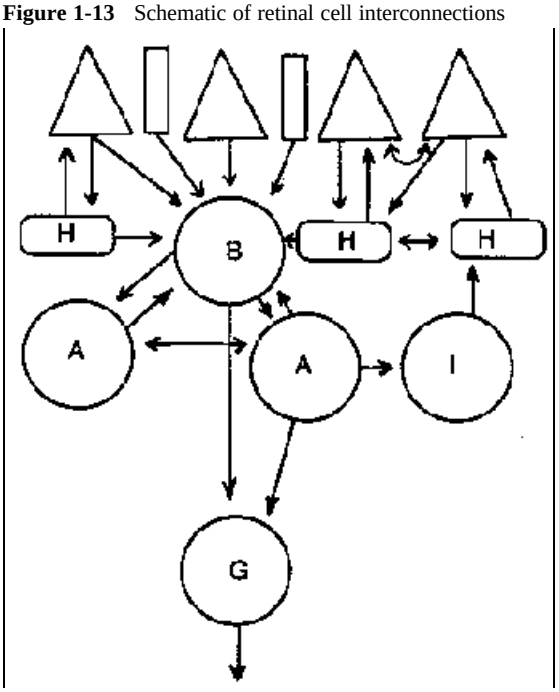


Figure 1-12 Artists conception of synaptic vesicle recycling. Adapted from NIH Research

IV. Pathways of the retina - remember the *netzhaut* (see Fig 1-13)

Figure 1-13 Schematic of retinal cell interconnections



V. Neuroactive substances found in the retina
   A. Amino Acids
      1. L-aspartate
      2. (-aminobutryic acid (GABA)
      3. L-glutamate
      4. Glycine
   B. Amines
      1. Acetylcholine
      2. Dopamine
      3. Serotonin
      4. Melatonin
   C. Peptides
      1. Cholecystokinin
      2. Enkephalin
      3. Glucagon
      4. Neurotensin
      5. Neuropeptide Y
      6. Somatostatin
      7. Substance P

8. Vasoactive Intestinal Peptide

Goals/Questions for Day 2

1. **The ERG is a physiological *population* response of the retina measured as an extracellular field potential.**
2. **As you collect data, you should speculate about:**
   a. **The nature of individual cellular responses to light.**
   b. **Which cell types are most likely to contribute to the ERG.**
   c. **The dynamic range of individual cells.**
   d. **You measured threshold for your own eyes yesterday, is there an equivalent physiological measure for neural responses? How would you define it?**
   e. **Compare retinal versus behavioral temporal integration. Are they the same? If not, what are the implications of the differences?**
   f. **Some of you will measure the time course of dark adaptation for the retina/ERG. Why do you think this process takes time to occur? Where is this process taking place?**
   g. **Others of you will measure retinal responses in the dark and in the presence of various background lights. Would you expect a constant stimulus to elicit a constant physiological response (voltage) regardless of the presence or absence of a background light? If so, why? If not, why not?**
   h. **You will record the ERG before, during and after blocking synaptic transmission in the retina. What can you say about the "meaning" of the ERG under these conditions? What information about the derivation of the ERG will you obtain by making this measurement?**
   i. **Conceptually, pharmacological experiments are easy. You put the drug on and see if it has an effect; however in practice, it is not so simple. For example, if a drug you applied to the retina reduced the amplitude to all components of the ERG, how does one distinguish between the blockade of synaptic transmission and killing the retina? If there is no change in your measured neural responses can you safely conclude that the agent has no effect on the tissue?**

THE ELECTRORETINOGRAM

As we now begin our experiments on the retina you will find yourself concentrating on the procedures and equipment that you will be using for the first time. *However, please try and keep your mind free to **think** about the experiments that you are doing!* The measurements that you will be making have been chosen to help you recognize important principles of general physiological function as well as specific retinal or visual information processing. *Remember to ask questions and test your hypotheses.*

The electroretinogram (**ERG**) is an extracellular field potential generated by the cells in the retina. This response has seen (no pun intended) widespread use in the study of the visual system and continues to be used today. It is also a primary clinical tool in evaluating various disorders of the retina (many ophthalmology departments have an ERG clinic where patients with a variety of disorders have their retinal function assessed).

We are going to learn how to record the response, explore some basic properties of the response and try and demonstrate some aspects of the source of the response. The ERG provides a good entry into retinal physiology because it is fairly easy to record. Do not let this fool you. Interpreting the meaning of field potentials is <u>not</u> a simple procedure. With that caveat in mind however, we hope that you will gain some insight into retinal function and the nature of information processing in this neural structure through your experiments.

The ERG has several component waves that you will identify and measure. For a flash of moderate (why?) duration, you should see a waveform similar to that shown in Figure 2-1. The most obvious response is the positive going waves at light **ON** and **OFF**. These are the **b-** and **d-wave** respectively. The **a-wave** of the ERG is the small negative wave that precedes the b wave. The precise form of the ERG is dependent upon the specific stimulus conditions that evoke the response as the following experiments shall demonstrate.

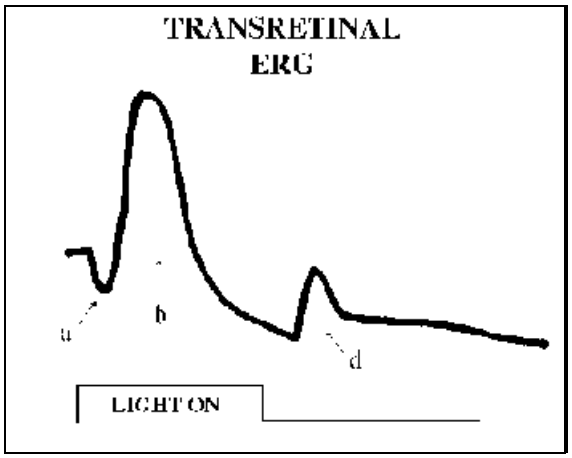


Figure 2-1 Idealized ERG.

METHODS:

1. **Divide labor**. One team member should attend to the dissection of the eye while the other prepares the setup.

2. **Prepare perfusion system**.
   a. Fill siphon bottle with Ringer's; begin bubbling with gas.
   b. Connect manifold lines to all syringe reservoirs.
   c. Sequentially, open reservoir valves and allow perfusate from siphon bottle to fill manifold and all reservoir tubing. If air bubbles persist, cover the hole in the black rubber stopper in the siphon bottle with your finger. This will build up air pressure that will force fluid through the line & purge the system of remaining air.
   d. Make sure perfusate can enter each of the 10 ml graduated cylinders; ***<u>however</u>*** <u>allow as small an amount of perfusate as possible to remain in the reservoir</u>.
   e. Close all reservoir valves, and fill the output line to the retina. This may require the air pressure method or, in extreme cases, applying suction to the end of the output tubing. Ask an instructor for a suction syringe for this purpose if your line does not fill.

3. Prepare electronics

a. Ready **Headstage Amplifier** (Fig 2-2); Be sure power supply to the rack is on.
b. Set at gain of 1K (times 1000); Set AC/DC switch to AC
c. Connect **CRO OUTPUT** BNC to Oscilloscope **CH1**.
d. Set Negative Capacitance, Current Inject and Resistance Balance controls fully counterclockwise (off)
e. Ready stimulus trigger; connect the **GRASS S48 DELAY SYNC OUT** to external trigger input of the Hameg scope and set the Hameg to trigger on that external input.

See Appendix at the end of the manual for information on gain and triggering.

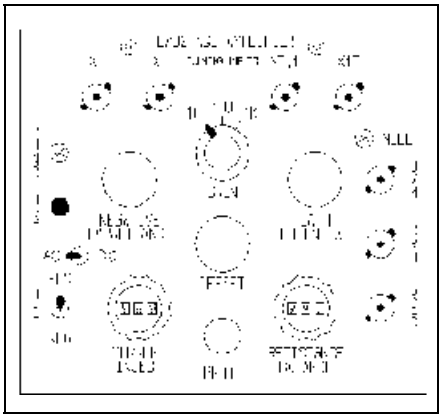


Figure 2-2 Headstage Amplifier Front Panel

4. Mount Preparation - Once preparation of frog eye is done and eyecup is mounted on the dome, insert recording chamber into stand, start perfusion. *Connect* **green ground headstage wire** to the **base** of recording chamber and the **black headstage input wire** to the **dome cover**.

5. Adjust Light Stimulus

a. Position one arm of the fiber optic illuminator so it will fall on the retina. Insert a 2.0 log unit filter in the holder mounted to the light source. [*Why?*]
b. **Set Grass S48 -** *STIM RATE*= 1 x 1 PPS; *DURATION*= 400 msec; *DELAY*= 200 msec; VOLTS= 44. Put *STIM MODE* in REPEAT and verify visually that the LED is repetitively flashing. If it isn't, be sure the *OUTPUT* of the S48 is ON and power is on. If LED still does not flash, check with an instructor.
c. Focus LED so that light falls on the hole in the cover of the prep chamber. If the prep stand has not been moved, the focus used from the psychophysics experiments should be nearly correct.
d. Stop flashes (*STIM MODE*= OFF) and close cage door.

6. Prepare oscilloscope. Adjust the time base of the scope to 0.2 sec/div and auto triggering (AT/Norm button out). Set channel 1 gain to 100 mV/div (these values may change). Remember, the total amplification (or amplifier **gain**) is 1000X so that the scope display is really 100 :V/Div. To insure that the scope trace will be visible, push in the GND button for CH1 to ground the input and position the trace two divisions below the middle of the screen (*do you know why you would want to do this?*). Push the CH1 GND button so it is out and be sure the AC/DC button is pushed in. You can use the Hameg printer to obtain records of your data.

Filtering of recordings (*Read this section when time permits, after the prep is up and going.*)

Whenever we have the ability to use prior knowledge regarding the signals of interest we can use the information to eliminate (filter) out signals that are "irrelevant" and would reduce the **signal-to-noise ratio** for data acquisition. The headstage amplifier can operate as either a DC (Direct coupled) or an AC (Alternating Current-coupled) amplifier. To understand these two modes of operation you can use your oscilloscope. Each channel has a switch that makes the scope operate in AC or DC mode, just like the headstage amp. If you take a 2 second, 1 Volt pulse out of the DAGAN stimulator and put it into the scope, in DC mode (time base set for .2 sec and gain at .5 V/div), you will see a trace like that at the top of Fig 2-3; a nice rectangular voltage step. Switching to AC mode displays a trace like that in the lower part of the figure. Now the display shows transient voltages that occur when the DAGAN input goes on and off. Remember that the DAGAN output has not changed! Only what is seen has changed. The AC coupled mode on the scope is only used when fast, transient events are the ones of interest. In AC-mode the input has a capacitor in its pathway that prevents any constant (non-time varying voltages) from being recorded. For our headstage amplifier this means that any constant offset voltages (like those produced by junction potentials) will be filtered from the recording. Where such potentials exist (or there is a lot of slow voltage drift), but are a contaminant to the signals being studied, AC coupling removes the unwanted signals. As long as the signals of interest are not also blocked by the AC coupling everything works well. AC coupling does not affect fast or high frequency signals while it attenuates (filters) slow (low frequency) signals. Another term for this mode of operation is called using a high pass filter. The complementary type of filter also exists, the low pass filter, which blocks high frequencies and passes low frequencies. DC coupling is inherently a lowpass amplifier setting. The DC coupling of the amplifier allows more slow signals (low frequencies) through than does the AC coupling of the scope. For the ERG, which contains slowly varying voltages, we selected a very low cutoff frequency so that we would not overly attenuate the ERG signal. Remember that the DC-coupled ERG is the true voltage signal generated by the retina so DC-coupled recording is the preferred mode. However, if you have a lot of baseline drift in the ERG signal (and many people do) you can perform all your experiments in AC-coupled mode. ***If you get time, you should compare the DC- versus AC-coupled ERG responses to the same stimulus. This will help you to appreciate the way the AC coupling modifies the waveform.***

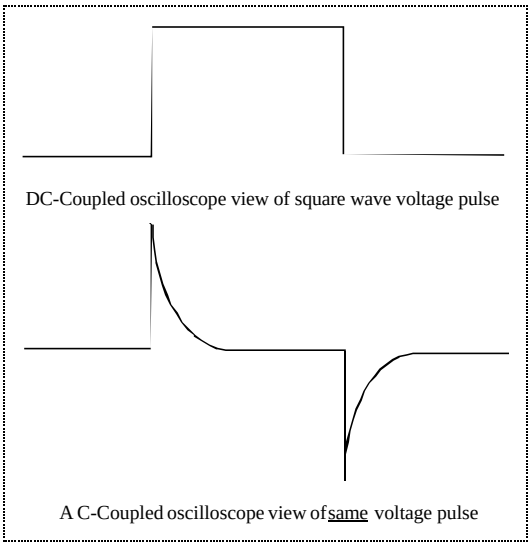


Figure 2-3 DC- versus AC-coupled signals

The ERG does not contain rapidly changing or high frequency signals, so we can filter the ERG record with a low pass filter. Since we are amplifying the retinal signal by one thousand there typically is a lot of unwanted high frequency noise in the voltage records. The headstage amplifier allows us to filter the CRO output by placing a lowpass filter capacitor across the two pin connectors located at the top of the front panel. If you want to determine the cutoff frequency here is the information you will need: the capacitor values we will use are 1 microfarad; the associated resistor in the filter circuit is 1000 ohms; Cutoff frequency = $1/(2\pi RC)$.

7. Find the ERG measured by the headstage amplifier.

a. With the scope in auto-trigger mode, wait until the trace is near the left edge of the display, and then flash the LED once by moving the *STIM MODE* switch down (SINGLE). You should see a deflection of the trace lasting 2-4 divisions. Congratulations!, you are recording the ERG! If you see no response, be sure there was a flash (verify Grass S48 **monitor** light comes on when a flash is supposed to occur and if that happens be sure LED still is illuminating the prep). If you still have problems, see an instructor.

b. Put the scope into Normal triggered mode (AT/Norm button in) triggering from the EXT. Input. Set Grass S48 *STIM RATE*= 2 x 0.1 PPS; *STIM MODE*= REPEAT. A sweep should occur every 5 seconds and there should be an ERG beginning about 1 div. after the sweep begins. If this happens, turn off the flashes (*STIM MODE*= OFF). You are now ready to begin the first experiment.

EXPERIMENT 2-1: Amplitude/Intensity Relations: 100 msec flashes

You will determine the nature of the relationship between light intensity and the response amplitude of the ERG. Evaluate the protocol of the experiments and make changes if you feel they can improve the data you collect. Do not be daunted by the lack of preprinted protocol sheets etc. Your lab notebook can accommodate your own creations as needed. If you don't make changes [which is perfectly okay] be sure that you have thought about the protocol and what might change if the data were collected differently.

1. You should begin your measurements with low intensity flashes. Set scope and chart recorder gain to resolve low amplitude responses. Be sure the trace will be on the screen, then set the scope to STORE and Ext Trigger. The scope timebase should be appropriate for the stimulus duration.
2. Set **Grass S48**: *STIM RATE*= 7.5 x 0.01 PPS; *DURATION*= 100 msec; *DELAY*= 100 msec; *VOLTS*= Level needed for 0.0 relative log intensity (see the protocol sheet next page). This intensity will most likely be too weak to elicit a response.
3. Present a single flash via the *STIM MODE*= SINGLE switch (think about stimulus rate and be aware of it). If a detectable response is evoked, carefully record the response amplitude of any ERG components seen (ie. **a**-, **b**- and/or **d**-waves). You can use the scope printer to make your measurements and enter them on the Experiment 2-1 protocol sheet.
4. Increase the flash intensity by 0.5 log unit steps. Set *STIM MODE*= REPEAT and adjust the flash intensity after each stimulus. Repeat until you reach the maximum light output for the LED. Note the need to adjust scope gain depending upon response amplitude.

EXPERIMENT 2-2: Amplitude/Intensity Relations: 4 second flashes

1. **GRASS S48** *STIM RATE*= 5.0 x 0.01 PPS; *DURATION*= 4000 msec; *DELAY*= 500 msec; *VOLTS*= Level needed for 0.0 log relative intensity. **Remember to adjust timebase of equipment if necessary.**
2. Repeat steps 3. and 4. from above and record on Experiment 2-2 protocol sheets.

Experiment 2-1 - Amplitude/Intensity Protocol (100 msec) (Red LED calibrated 8/90)

Log Intensity	Volts	a wave	b wave	d wave
0.0	1.46			
0.5	1.51			
1.0	1.60			
1.5	1.70			
2.0	1.90			
2.5	2.40			
3.0	3.40			
3.5	6.50			
4.0	15			
4.5	44			
5.0	150			

Experiment 2-2 - Amplitude/Intensity Protocol (4000 msec) (Red LED calibrated 8/90)

Log Intensity	Volts	a wave	b wave	d wave
0.0	1.46			
0.5	1.51			
1.0	1.60			
1.5	1.70			
2.0	1.90			
2.5	2.40			
3.0	3.40			
3.5	6.50			
4.0	15			
4.5	44			
5.0	150			

EXPERIMENT 2-3: Temporal Integration

The design and concept behind this experiment should be familiar to you from your own experiences on our first day. You will now collect data that will permit a fundamental comparison to be made; *perceptual responses versus physiological responses*. What hypothesis or set of postulates can you test regarding this comparison? In thinking of hypothetical results, what models of visual neurobiology might be in agreement with what kinds of outcomes?

1. From data collected in Experiment 2-2, determine the maximum b-wave amplitude (sometimes referred to as R_{max}). Divide this by 5 and then round up to the nearest 10 microvolts. You will use this as your **criterion amplitude** for the data collection and analysis.
2. Set **S48** *DURATION*= 10 msec; *STIM RATE*= 1 x 0.1 PPS (why this value?)
3. Adjust scope gain appropriately to allow you to easily and accurately determine when the ERG reaches the criterion response.
4. Adjust the LED voltage so as to evoke an ERG of the criterion amplitude. You may have some uncertainty given noise levels and variability of the response, but you should find that you can get the voltage set with fair accuracy reasonably quickly. Do not obsess over these measurements - collect the data quickly and accept some small inaccuracies.
5. Record the LED voltage on the Experiment 2-3 protocol sheet
6. Repeat steps D. & E. for *DURATIONS* of 1000, 50, 200, 1, 500, 100 and 2000 msec.

EXPERIMENT 2-3: Temporal Integration Protocol

After collecting the voltages necessary for the criterion amplitude, you need to do one more thing before plotting your data. We leave it to you to do that.

Duration (msec)	Threshold Voltage 1 (Crit Resp =)	
10		
1000		
50		
200		
1		
500		
100		
2000		

Data Analysis

Experiment 2-1 & 2-2

1. Using the computer, run Prism (see page 1.20 if you need reminders) and enter the response amplitudes for the b- and d-waves from EXP 2-1& EXP 2-2 into Data Table 2-1 and Data Table 2-2 respectively. These data are automatically graphed in the combined Data Graph 2-1 & 2-2..

Experiment 2-3

1. Enter your measured stimuli required for obtaining the criterion response (Y-axis) vs. the flash duration on the Data Table 2-3 spreadsheet.

EXPERIMENT 2-4: Dark Adaptation (Groups 1, 3, 5 & 7)

(Groups 2, 4 & 6 turn to page 2.11)

You have all experienced the sensation of entering a dark room from a bright environment and finding yourself unable to see very much until you have been in the dark for several minutes. This phenomenon is called the process of **dark adaptation** and is the subject of our next experiment. The purpose of the experiment is to quantify the change in sensitivity of the retina following exposure to bright light (this allows us to determine if the retina is involved in this). To do this, we will measure the ERG response amplitude as a function of time in the dark following an adapting light.

1. Setup
   a. Be sure the LED is in position to stimulate the prep. Also be sure that one arm of the fiber optic illuminator is also in position to illuminate the retina.
   b. Make the prep as dark as possible by covering all light leaks into the cage and turning the room lights off. Once done, let the retina sit for 5 minutes.

2. Experiment
   a. Stimulate the retina with single **weak** stimuli (*DURATION*= 100 MSEC). The desired **criterion response** is approximately 5% of the maximum b-wave amplitude. Once you have set the intensity, begin repeating test flashes every 20 sec (*STIM RATE*= 5.0 x 0.01 PPS).
   b. Measure b-wave amplitude off the scope screen and continue stimulating until the response amplitude stabilizes.
   c. Remove all filters from the fiber optic filter holder and turn on the source on to its maximum setting (Position 3) for 1 minute. (Keep the LED flashing all the time)
   d. After 60 sec. turn off the fiber optic. This is TIME ZERO. Immediately measure the ERG response amplitude for the continuing flashes. You will want to collect data every 20 sec for the first 2-3 minutes, and every 1 minute thereafter (this requires manual presentation of the flashes). **Record the time after fiber optic offset at which the LED flashes occur**.
   e. Proceed with these measurements until the response has fully recovered. This can take an appreciable amount of time if you have a good prep.
   f. *If time permits (check with instructor),* increase the LED intensity until you have a near-maximal ERG (90% of maximum or so - do not use a super-intense flash).
   g. Repeat steps b to e.

3. Analysis
   a. Enter the ERG amplitudes and the corresponding times preceding (this means negative time values) and following the bright adapting light into Data Table 2-4a.
   b. Based on your data; what is the time course of the recovery of retinal sensitivity? How long does it take the eye to completely dark adapt?
   c. What might you expect the results of an experiment on visual perception to be?
   d. If you conduct the repeat experiment using a response that is 90% of R_{max}, enter those data in Data Table 2-4b. Data will be graphed on Data Graph 2-4.

EXPERIMENT 2-4: Dark Adaptation Protocol (Groups 1, 3, 5 & 7 *ONLY)*

Time in Dark (min)	b-wave amplitude (:Volts)	Time in Dark (min)	b-wave amplitude (:Volts)

EXPERIMENT 2-5: Light Adaptation (Groups 2, 4 & 6)

You will measure changes in the ERG when the retina is exposed to different levels of constant background illumination. This should be recognizable as a common requirement of vision (we can see in sunlight or in moonlight, for instance). While this adjustment may be needed frequently, the process is anything but "common". In fact, the visual system is able to adjust its operating range from low levels such as starlight up to bright sunlight. To put this in perspective, the levels of background illumination under these two extremes can be as much as 10 log units (10 billion to 1)! We will again measure the amplitude-intensity function repeating Experiment 2-1 from this morning. Then we will use the fiber optic to introduce steady background illumination (be sure that the fiber optic will illuminate the retina!) and investigate how the amplitude-intensity function may (or may not) be changed.

1. Experiment

a. Let the preparation sit in the dark with no flashes for 10 minutes.
b. Measure the b-wave amplitude-intensity function (*DURATION* = 100 MSEC) using the protocol sheet on the next page.
c. Determine the maximum b-wave response (R_{max}) from these data and set your intensity to approximately 5% of R_{max}. Once you have set the intensity, begin repeating test flashes every 20 sec (*STIM RATE* = 5 x 0.01 PPS).
d. Wait from 1 to no more than 5 minutes until you are certain that the response amplitude has stabilized. **Do not obsess about this**. If 3 consecutive flashes evoke responses whose amplitude is "unchanged" (within 5% of one another), you have reached stabilization.
e. Place 4 log units of N.D. filters in the filter holder and turn on the fiber optic.
f. Again, carefully wait for response stabilization and then measure the b-wave amplitude-intensity function. ***Stabilization may take different lengths of time on different backgrounds!***
g. Remove 1 log unit of neutral density filters from the filter holder. Repeat Step f.
h. If time (and your interest) permits, increase the adapting light by 1 log unit and repeat Step f.

2. Analysis

a. Enter b-wave amplitude for each background into Data Table 2-5a.
b. Calculate a value known as the **increment-threshold**. This is the intensity required to elicit a **criterion response** at each level of the background.
   i. First select a criterion response value of between 10-20% of the R_{max} ERG amplitude (in the dark).
   ii. Determine (via interpolation from Data Graph 2-5a) the LED log stimulus intensity needed to evoke this response.
   iii. Enter the log stimulus intensity required to elicit the criterion response versus the log of the background level into Data Table 2-5b and view the results on Data Graph 2-5b.

3. Questions

a. How does the amplitude-intensity relation change with the strength of the adapting light?
b. Does the sensitivity of the retina change when an adapting light is turned on?
c. What functional (ecological) role would such a sensitivity change have?
d. Does the maximal b-wave amplitude change with background?
   i. What are the implications of such a change?

e. What can you deduce about the perceptual correlates of light adaptation from your increment threshold plots? ["bonus" points for this one folks]

LIGHT ADAPTATION PROTOCOL

Adapting Light, Log intensity (**Remember to convert from attenuation with filters!**):_____.

Log LED Intensity	S48 Volts	b-wave Amplitude (∶V)
0.0	1.46	
0.5	1.51	
1.0	1.60	
1.5	1.70	
2.0	1.90	
2.5	2.40	
3.0	3.50	
3.5	6.50	
4.0	15	
4.5	44	
5.0	150	

Adapting Light, Log intensity:_____.

Log LED Intensity	S48 Volts	b-wave Amplitude (∶V)
0.0	1.46	
0.5	1.51	
1.0	1.60	
1.5	1.70	
2.0	1.90	
2.5	2.40	
3.0	3.50	
3.5	6.50	
4.0	15	
4.5	44	
5.0	150	

LIGHT ADAPTATION PROTOCOL

Adapting Light, Log intensity:_____.

Log LED Intensity	S48 Volts	b-wave Amplitude (∶V)
0.0	1.46	
0.5	1.51	
1.0	1.60	
1.5	1.70	
2.0	1.90	
2.5	2.40	
3.0	3.50	
3.5	6.50	
4.0	15	
4.5	44	
5.0	150	

Adapting Light, Log intensity:_____.

Log LED Intensity	S48 Volts	b-wave Amplitude (∶V)
0.0	1.46	
0.5	1.51	
1.0	1.60	
1.5	1.70	
2.0	1.90	
2.5	2.40	
3.0	3.50	
3.5	6.50	
4.0	15	
4.5	44	
5.0	150	

EXPERIMENT 2-6: Effect of blocking photoreceptor neurotransmission on the ERG.

Please be sure to begin this experiment no later than 3:00 !

We are now going to begin our experiments that "dissect" the living retina, by applying pharmacological agents that target specific functions. In this experiment we will be using SynaptoBlock™. The effect of this solution will be to block the neurotransmission from the retinal receptor cells to second order neurons. What cells will be generating the ERG that we will measure?

1. Setup

a. If not done previously, remove air from the tubing connecting one of the syringe reservoirs to the superfusion manifold.
b. Get a pre-made 5 ml of the SynaptoBlock™ solution.
c. *While wearing gloves,* place the 5 ml in the syringe receptacle and put in a bubbling line. Begin bubbling.
d. Set up S48: *DURATION*= 5 sec; *STIM RATE*= 7.5 x 0.01 PPS

2. Experiment

a. With the retina moderately well dark adapted, present an LED flash producing a near maximal ERG. Good a-, b- and d-waves should be present.
b. *If possible,* record the ERG in the DC-coupled mode. If that is too difficult, record in AC-coupled mode and simply try and record one or two DC-coupled ERG responses after the SynaptoBlock™ effects are stable.
c. Begin repetitive flashing and when stabilized print 1 or 2 traces on scope printer.
d. Switch to SynaptoBlock™ solution. ***BE SURE YOU FIRST TURN ON SynaptoBlock™ SOLUTION AND THEN TURN OFF CONTROL RINGER'S.*** This will avoid stoppage of flow and introduction of air bubbles into the system.
e. Note any changes in ERG over time. Record noteworthy examples.
   i. You can select the nature of the data to be recorded.
f. **Keep your eye on the reservoir**. Do not let it run empty.
g. When the ERG no longer changes (but do not run drug for more than 3 minutes, ***maximum***), switch back to control Ringer's.

3. Analysis

a. You are free to determine how you want to present the data you have recorded.
b. What changes are produced by blocking neurotransmission?
   i. What components of the ERG are most affected?
   ii. What does this suggest?
c. Are the changes you produced reversible?
d. Do you have any thoughts as to the time course of any changes that occur?

Goals/Questions for Day 3

1. **You will use intracellular electrodes to record light responses from cells of the retina.**
   a. **This technique will be classic "current clamp" recording of membrane potential (voltage) changes evoked by visual stimuli.**

2. **You will not impale individual cells under visual control and this poses several conceptual problems.** ***The class will address the following questions before beginning the experiments for the day, so be prepared with your answers:***
   a. **How can you determine from which cell type you are recording? See if you can generate 4 distinct approaches that actually could be used here at Itasca in answering this.**
   b. **What kinds of responses can you record with your electrode in response to light (is it equally likely to impale all cell types)? You should be able to think of 3 general, and easily discriminated types of responses.**
   c. **What kinds of physiological processes (at the biophysical level - think about mechanisms you studied last week) can account for the response types you just thought of? In some cases, you should have more than one way to generate a single kind of light evoked response.**
   d. **How can you distinguish between first order versus second order cells? (This employs one of the approaches you could use in a.)**
   e. **Assume that you have answered the prior question. If your analysis tells you that you only record second order cells, can you determine how a photoreceptor responds to light?**
      (i) What is the voltage response?
      (ii) What is the likely effect of this voltage response on transmitter release?
      (iii) What kind of transmitter (excitatory or inhibitory) is released?

INTRACELLULAR RECORDING IN THE VERTEBRATE RETINA

Yesterday we looked at the ERG. This method has the advantage of being easy to record and stable over long periods of time. Furthermore, by summing the activity of the entire retina, the ERG can be used as a measure of general retinal health. These reasons, along with being a non-invasive measurement, are why the ERG has been utilized for many years in clinical assessment of retinal function.

As you are aware, individual nerve cell's electrical responses to stimuli differ in different neurons (and even within regions inside a single cell!). Within the nervous system there are two classes of electrophysiological responses utilized for information processing. Cells that must transfer information over relatively large distances need amplification "stations" along these lengthy processes. This is accomplished by the energetically demanding use of regenerative mechanisms, the most well known of which is the **nerve action potential** or **nerve impulse**, which you will record tomorrow. The second class of cells, very often interneurons, do not require long distance signal propagation and these neurons carry out their role in information processing by altering their membrane potential without 'firing impulses'. Unfortunately, to record these **graded potential** changes we must place the tip of an electrode directly inside of the cell. We will employ a sharp, high impedance glass micropipette filled with a conducting solution to give us access to the intracellular voltages which underlie cellular communication.

In the morning presentation, the class will collaborate to design several experiments that will be used to answer the two fundamental questions for today's experiments: how can you determine what kind of cell you are recording from and what happens to the photoreceptor membrane potential when light is applied to the retina. Because these recordings are difficult to obtain, we will split the class into groups to permit some of the experiments you design to be done first by different groups. This will help obtain some data for all the possible questions. You will need to cooperate with your classmates to compare data and try and piece together enough information to answer the questions that are your goals for the day. Feel free to use your ingenuity to add experiments if you are not satisfied with the "answers" you are obtaining.

Today, you will be challenged in every regard as intracellular recording is not a trivial undertaking. To be successful one must have patience, perseverance, and black magic (see Dr. Gottesman for proper voodoo dance). Once you have penetrated a cell, you will typically only have a brief opportunity to observe this cell in action. You will try to identify the cell types that you record from using physiological, pharmacological and, if time permits, intracellular staining techniques. In the later case, to visualize the cell for post-experiment identification, your electrode will contain a dye called Lucifer Yellow. Steps to impale, record, and stain a cell are outlined below.

Methods

Setup:

1. Generate your experimental protocols *before* you record from cells! Intracellular recordings are notoriously short lived, so you need to be ready to make your measurements as quickly and efficiently as possible if you are to have the best chance of completing your experiment.

2. Prepare the perfusion system. Be sure to back fill the lines to the drug reservoirs as you did for the SynaptoBlock™ experiments. You will be applying drugs and you do not want air bubbles in the lines.

3. Connect the 10 V voltage output of the DAGAN amplifier (Figure 3-1) to channel 1 of the oscilloscope. You can set the gain of the scope based on the size of the voltage signals you expect to record

4. Mount an eyecup in the superfusion chamber and start superfusion.

5. Obtain an electrode holder. Plug the holder into the DAGAN amplifier headstage. Ground the chamber collar to the table. (We won't be using the electrode in the chamber bottom.)

6. Your instructors will make available micropipette electrodes. You will only have 2 or 3 high quality electrodes per group. ***BE CAREFUL*** *with these pipettes!!* They have been pulled on equipment in the Twin Cities that is unavailable here. We *will attempt* to manufacture more electrodes, but the pipettes required to record cells in the retina are quite different from those you used in the neuromuscular junction and we are not certain that we will succeed in modifying the electrode puller.
   a. The micropipettes need to be filled with an electrolyte solution. We will be using 3 M Potassium Acetate for the first experiments today. You should use this solution to backfill your electrodes for all your attempts at recording until you have been given permission by an instructor to go on to intracellular staining.
   b. If time permits you to try intracellular staining you will need to get another electrode. Backfill *just the tip* of the electrode using a syringe containing Lucifer Yellow and then backfill the rest of the electrode with the electrolyte solution containing Tris Buffer. Mount the electrode in the holder.

7. Be sure that the hydraulic manipulator is in the ***middle of its operating range*** so that you will have range to advance and retract. Verify that you know which direction to turn the dial to advance!

8. Position the electrode over the eyecup with the mechanical micro-manipulator while observing through the dissecting scope. Move the electrode until it ***just*** contacts the superfusate above

the retina. **Remember that the tip of the electrode is actually beyond the resolution of the dissecting scope, so you must be careful not to let the tip contact anything except the fluid.**

9. Display the voltage recorded at the electrode tip on the oscilloscope. Zero the potential by adjusting the **OFFSET** knob. This is the baseline potential for which all others will be compared. Turn the capacitance compensation (**CAP COMP**) knob fully counterclockwise.

10. The resistance of the electrode must be measured to gauge its sharpness. The concept is that the sharper the electrode, the smaller the opening and the higher the resistance to current flow through this opening. By rearranging Ohm's Law (R=V/I), we can determine the electrode resistance by passing current through the electrode and recording the voltage across the tip. Here we will pass +1.0 nA (via DAGAN **DC CURRENT** - Fig 3-1) so a 100 mV deflection computes to 100 MegOhms. Microelectrodes with resistance's less than 100 MOhms are probably too dull for our purpose. You may want to seek another electrode if this is the case.

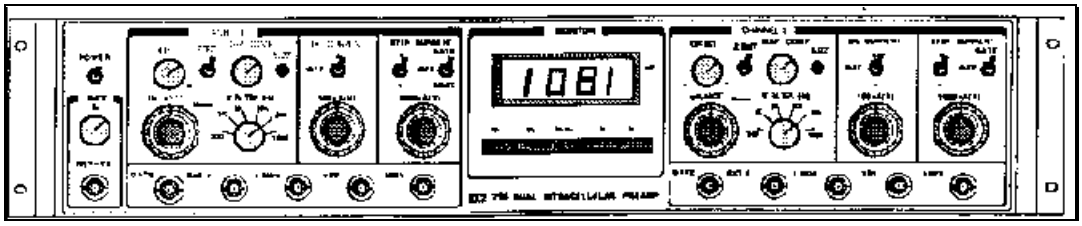


Figure 3-1 Front panel of DAGAN intracellular amplifier. Note left side controls related to text

Microelectrode Recording ("fishing for neurons"):

1. You are now ready to advance the electrode onto the retina. Begin by setting the oscilloscope gain very low (100 mV/div) and reduce the sweep speed to 0.5 sec/div. Temporarily close the superfusate stopcock to stop superfusate flow. After the chamber has drained, slowly advance the electrode until it just barely touches the surface of the retina. One of you can watch the oscilloscope for a rapid deflection indicating that the electrode has hit the surface. If you have the audio monitor hooked up to the electrode channel, there should be an audible signal of the change in voltage across the electrode that indicates contact with the retinal surface. Restart superfusion and be sure to verify that the flow is reestablished and that the chamber has refilled. You may still be some distance from the retina proper depending on the amount of vitreal material present (this can range from 0 to 1000 :ms).

2. Set the oscilloscope to 5mv/div. Begin flashing a brief (50 ms), moderately bright light onto the retina (one log unit below saturation for the ERG is a good place to start). These flashes will help you determine when you have impaled a cell.

3. Advance the electrode ***3-5 microns (NOTE: THIS IS A VERY SMALL rotation of the control dial* - one full rotation equal 500 microns*)*** at a time using the remote hydraulic micromanipulator. Briefly press the DAGAN **BUZZ** button (Fig 3-1) and wait for a flash of light to verify whether the electrode has penetrated a cell or not. The oscilloscope should show a large transient when the buzz button is depressed. If it does not, then turn the capacitance compensation dial further clockwise until the transient is seen. This magic button is thought to cause the tip of the electrode to vibrate slightly thus causing a penetration if the electrode tip is very near the cell. Typically upon impalement, there is a hyperpolarizing shift from near zero volts to -40 to -80 mV. The baseline noise level may change and the voltage you are recording will change when the light stimulus is presented. Continue in this fashion of jumping a few microns and buzzing through the retina until you impale a cell. This is called "fishing for cells" for the simple fact that you typically spend a lot of time trying before you catch anything. Relax, they're there.

4. Think about the anatomy you've learned thus far. What cells are likely to be seen as the electrode touches the surface? How far should you advance the electrode? If you go too far, you are likely to find yourself in a pigment epithelial cell which usually has a large transmembrane potential (-90 mV). Going further will break the electrode and ultimately severely compromise your retina. If your recording becomes very stable and quiet, check your electrode resistance. If it has declined sharply from the beginning of the penetration, then you have broken the tip (you can't see this, even under a dissecting scope). If this happens, simply throw the manipulator into reverse, pull far back from the retina and go get an new electrode. If the resistance is still high, but you feel that you have passed through the retina, withdraw the electrode, ***pick a new spot*** and repeat the above steps starting with stopping the superfusate. If you observe any visible signs of injury at the locus of an electrode penetration you can move the position of the electrode to enter the retina at a different site.

Intracellular Staining:

1. Once you have impaled a cell and recorded from it you may be able to stain it.

2. Iontophoretically inject the dye using the **DC CURRENT** control (Fig 3-1). Be certain that the current output is initially set ***fully off***. Begin by turning the **DC CURRENT** switch to the on position and then slowly increasing negative current (over approx 15 sec) up to -1 nA. Apply current for 3 minutes then slowly turn off current.

3. Confirm cell's vitality by recording its response to a moderate light flash.

4. You may continue to record from this cell if it is healthy or pull out by quickly withdrawing the electrode (so the cell does not come out with the electrode). Please keep track of the location on the retina of cells you think you may have stained. (*Why?)*

5. Tell an instructor if you have a retina with potentially stained cells. The tissue will be processed and we will try to let you visualize your cells.

Final Discussion:

1. What cell types did we record from? What means did you use for distinguishing between different retinal cell types?

2. What is the nature of the signal that a photoreceptors transmits to the second order cells in response to light?

3. What are some of the difficulties in interpreting the intracellular voltage signals?

4. What are possible sources of noise in the system?

Goals/Questions for Day 4

1. **How would you characterize the difference between the amplitude-intensity relation for the ERG versus the cells that generate action potentials?**

2. **What are the implications of these differences in evaluating what the source of the ERG might be?**

3. **How do the properties of the spike generating cells match up with the psychophysical data you have collected? What are the implications of this comparison?**

4. **How can you determine what cell type(s) in the retina generate spikes?** ***[Think both operationally and theoretically]***

5. **We will be applying several drugs: 2-amino-4-phosphonobutyric acid, APB; kynurenic acid, KYN; and a mixture of Picrotoxin & Strychnine, P&S. Are all components of the ERG affected by the APB, KYN or P&S? Would changing the concentration have an effect?**

6. **What about the spike generating cell recordings? Are the effects of APB the same at light ON vs OFF? How about KYN and P&S?**

7. **What mechanisms might be mediating the effects of APB, KYN and P&S?**

8. **Do you have any insights into what our SynaptoBlock™ solution from Tuesday was?**

9. **How can you determine the locus (or loci) of action for applied drugs? Try and describe 2 different experiments that could prove that spike generating cells respond directly to applied drugs versus to being affected by presynaptic inputs.**

EXTRACELLULAR RECORDING FROM SINGLE RETINAL NEURONS.

I. Introduction

Today we will extend our measurements by simultaneously recording the ERG and the extracellular potentials of the cells in the retina. Doing two things at once isn't as hard as you may think, unless you are Gerald Ford. We will compare the ERG with recordings of cells that generate spike discharges. We will consider what information these spiking cells might be encoding. The electronics for today will be new and exciting, like an episode of Star Trek, almost.

II. Methods:

A. Prepare perfusion system and fill siphon bottle with Ringer's solution.

B. Get electronics ready (this will take a lot of "T" connectors). The headstage amplifier will be used to record the ERG again. The **GRASS P15** amp (inside the cage) will be used for the extracellular electrode. Set gain of P15 to 1000X and headstage amp to 1K. Set the **P15** filters: LO = 300 Hz; HI =3 kHz. Connect output of P15 to **negative input** of Utility Amplifier (on the equipment rack), turn off the positive input, and set to a gain of 1X. Take output of utility amp to both the Harvard Audio Monitor and the rate meter (see page 4.4). For now, connect the rate meter *input* to Channel 1 of the scope. Connect the output of the ERG amp (CRO Output BNC) to Channel 2 of the scope. GRASS S48 - *DURATION*=1 sec; *DELAY*=500 msec; *VOLTS*=44.

C. We will be using a flatbed chart recorder to help us observe the slow wash in and wash out of the drugs. The chart recorder has two channels, so we will simultaneously record the ERG and the spike generating cell records as seen in the rate meter output. To do this, use a BNC "T" connector to connect the ERG amp output to both CH2 of the scope and to one channel of the chart recorder. The same should be done for the signal being sent to CH1 of the scope (the rate meter output). The chart recorder input is a double banana connector, so you will need to use adaptors to make that connection. *Note: at the recorder, the input ground connection needs to be connected to the RED post.*

D. Determine the chart recorder gain that will match that of the scope (ie if the scope is set for 100 ΅V/Div, then the chart recorder can be set for 1Volt full scale (and thus 100 ΅V/Div, where the Div is one large division of the chart paper. To set this chart recorder gain, note that the gain settings refer to the full scale deflection of the pens. Thus, with the 10 mV button depressed AND the 100X button depressed, you have 1 Volt full scale & 100 mV per large division on the paper. With 1000X amplifier gain, this corresponds to 100 ΅V per division. The chart speed can be adjusted to allow you to see the ERG & rate meter waveforms, without overdoing it and using up paper at an alarming rate. Since you also have scope screen dump printers available, you can use either or both as you prefer.

E. Prepare frog eye for superfusion, mount recording chamber, start the perfusate flowing over the retina, and check the alignment of the optics.

F. Connect the black lead of the headstage amplifier to the dome cover electrode for the ERG. Connect P15 G2 (green lead) to the chamber base electrode with the green wire you used yesterday for the ERG.

G. Get a microelectrode from an instructor. ***Please be aware that there is a limited supply of these electrodes! These tools are fragile and need to be handled with care so that they can be REUSED by other students.*** Install the microelectrode in the holder. You will need to have a small piece of tape wrapped around the electrode so that it is held tightly between the plexiglas washer and the base of the electrode holder. Be sure that the electrode is well clamped to the holder. The actual tip of the electrode is much thinner than a human hair, so avoid touching it. Carefully connect the yellow lead with the red cap to the electrode and use a small piece of tape to secure the lead to the shaft of the electrode holder. This insures that the mechanical strain of the lead wire will not be transmitted to the electrode. Be sure that the electrode will not hit anything as you mount the electrode and holder in the clamp on the MO-1 hydraulic for advancing into the preparation chamber. Connect the end of the yellow lead to the yellow (G1) post on the P15 amplifier. The MO-1 and one axis of the 3D manipulator should be parallel to give you a fine and coarse position control for the electrode. Advance the electrode into the perfusate using the coarse advance. Temporarily stop the flow of Ringer's solution to allow the perfusate to drain from the retina. Viewing the prep through your dissecting scope, bring the electrode tip near retina. The actual tip of the electrode is dark and may be hard to see against the retina. You should aim the electrode toward an area of the retina that looks in good condition (as dark as possible). You should see both the image of the electrode and its reflection in the vitreous covering the retina. When the two images come together, the tip of the electrode will contact the fluid layer and you should see a small “dimpling” at the contact point. Once you see this, back the electrode away so that it is just off the fluid. Restart the flow of Ringer's solution and ***confirm*** that it is flowing. Close the doors of the cage and remember to turn off fiber optic. Let prep dark adapt for a few minutes.

H. Filtering of recordings (**Read this section when time permits, <u>after</u> the prep is up and going.**

1. Our ability to use prior knowledge regarding the signals of interest guides us in eliminating (filtering) out voltage signals that are "irrelevant" and would reduce the **signal-to-noise ratio** for data acquisition. The P15 is as an AC-coupled amplifier that operates like the headstage amplifier in AC mode, but it has the added ability to vary its filter settings. The LO frequency setting on the P15 sets the lower frequency bounds for signals appearing at the input. If we were recording the ERG, which contains slowly varying voltages, we would use a very low cutoff frequency so that we would not overly attenuate the ERG signal (for example the 0.1 Hz setting). As you all know, the action potential is a signal with a time course of 1 to 2 msec. This means that spikes have frequency information greater than 1 kHz, but little low frequency information. As a result, we use the specific filter settings for the P15 that will eliminate annoying baseline drift artifacts commonly associated with using metal microelectrodes (by filtering out signals slower than 300 Hz via LO=300) while not affecting spike amplitude or waveform. What else do we filter out of the spike recording when we set the LO filter to 300 Hz? We do not want to filter out the spikes themselves, so we set the HI filter setting to 3 kHz. This sets the cutoff frequency for the lowpass filter and blocks signals higher than 3000 Hz from “contaminating” the recordings.

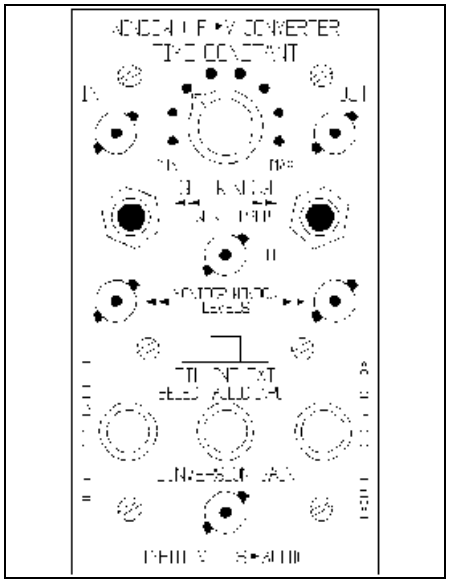
I. ERG check - If you have problems here just work with the spike generating cell recordings

1. The ERG display on the scope should not be a full screen deflection since the spike recording will be the focus of the morning exercises. You can set the scope gain somewhat low (ie 100 to 200 ∶V/div). Ground the ERG scope channel and position the trace 2 div from the bottom of the display. Return the channel to DC coupled mode.

2. Once you have an ERG, you can note the R_{max} value and then reduce the S48 intensity to a value that produces approximately a 25% R_{max} response.

J. Spike generating Cell Recording Setup

1. Initial Scope Setup
   a. Set the CH1 scope gain to 50 mV (what is the actual display gain?)
   b. Ground the scope and position the trace at the center of the display.
   c. Return scope to DC coupled mode and center trace using the offset control of the utility amp.
   d. You should see a horizontal trace which is about 25 microvolts in thickness.
      (1) If you have something else check with an instructor - you may have the dreaded *60 cycle noise* or some other problem.
2. Audio Monitor
   a. We use our ears as much as our eyes, especially when searching for cells in spike recordings. We use the audio monitor to listen for the spikes, which produce discrete pops/clicks when recorded.
   b. The P15 output or the output from the utility amp should be connected to the audio monitor inputs. Turn on the monitor about half volume. If you hear a *loud* hum you still have a 60 cycle problem. You should hear something that sounds like the hiss between stations on the radio.
3. Setting window discriminator/rate meter - Yet another new device!
   a. You will not be using the bottom 2 left hand dials (Threshold & Conversion Gain). Set the slider switch into the TTL Select position and turn the Audio Level fully counterclockwise (off).
   b. This device will help us measure the spike activity you record. It does this by converting incoming spikes to a step of voltage that decays with a time constant that we can set. If new events (spikes) occur before the voltage decay is complete, then the output voltage is increased. The conversion from spike waveform to voltage is determined by a window discriminator. This device simply sets a high and low voltage threshold (the window) and anytime the input signal crosses into that window without exceeding the upper threshold, a voltage pulse is generated. You must set these thresholds correctly relative to the input voltage to get the unit to operate correctly.
   c. You will need to switch BNC cables several times during this setup phase, but once done, you will not have to readjust the unit.
   d. Let the scope free run in single channel mode with CH1 on. *Do NOT change the gain or position of CH1*. The trace should be centered on the middle of the display. If it isn't, use the utility amp offset to center the trace. Disconnect the CH1 input cable at its source: the rate meter input (or utility amp



Window Discriminator/Rate Meter

output) and connect to the lefthand monitor window levels BNC. You now see a trace whose position on the screen can be changed via the lefthand set window control on the discriminator unit. Adjust the scope trace with this control so that the trace is one division above the center line of the display. ***Do not touch the CH1 Position knob at any time or you will have to readjust the system***. You can now reconnect the CH1 input to either the output of the discriminator or the output of the utility amp. Check to be sure that the right-hand set window control is turned fully clockwise.

e. What you have done is set the unit so that any time the voltage from the microelectrode vertically crosses the +1div point (from middle) on the screen - there will be an output from the window discriminator. You can hear this output by turning up the Audio Level control. Each time the discriminator detects an "event", it generates a TTL pulse that comes out of the audio on the discriminator (NOT the Harvard unit) as a click. You can create clicks by rapidly moving the CH1 baseline into and out of the window using the utility amplifiers offset. Obviously, it is critical, when you are collecting data, to have the baseline sitting just below the lower threshold of the window. If you don't, spikes will not cross into the window and the discriminator will not operate. Because we are using an AC coupled amplifier, there should be minimal baseline drift that would artificially trigger the discriminator and you can change scope gain as is appropriate ***as long as you use ONLY the utility amp offset control to position the trace.*** *Beware of recording an intraretinal ERG & confusing it for spikes!* Such a signal (the AC-coupled ERG) will cause a baseline "wiggle" and if you have too low a threshold (for too small spikes) the discriminator can't tell the difference between spike generating cell activity or filtered ERG activity.

f. The Time Constant knob determines how fast the output decays. We think that a setting that is one step less than maximum is the best, but when you are recording spike generating cell activity you should change this value and see the effect that it has on the chart record.

K. "Searching for Cells" - (finally!)

1. Start the stimulus light (previously set to elicit a 25 to 50% maximum ERG, but if you skipped the ERG set S48 to 4 Volts) flashing once every 5 sec. Let the scope free run at a timebase setting of .5 sec/div. ***Slowly*** advance the extracellular electrode with the hydraulic microdrive. Remember that the retina is only 150 - 200 microns thick and one full turn on the microdrive is 500 microns! You don't know how far away from the retinal surface you are so you don't want to be so conservative in advancing that it is the afternoon before you get to the cells, but you also don't want to drive the electrode through to the rubber dome. You can guide your advance by *listening carefully* to the audio monitor! When the electrode tip approaches the retina you should hear the a faint chattering sound at light ON and OFF. Moving in 2-5 micron increments advance the electrode until the sound is very distinct and crisp. If your electrode passes its optimal position, (noted by a decrease in sound level) simply reverse direction. Another way to confirm that you are past optimal position is to turn the LO frequency setting on the P15 to its minimum level, allowing the intraretinal ERG to be seen. If you then see a significant ERG coming from the electrode, you have probably gone past the spike generating cell layer. Whenever you suspect you have gone too far, back up and try again, to avoid breaking the electrode against the rubber dome. When you hear light evoked clicks and pops you should also be able to see spikes on the scope. Keep in mind that you are using

a **digital** scope. That means that the input voltage is discretely sampled. That is to say that there are a finite number of points per sweep. The slower the sweep speed the more crude is the temporal resolution of the scope. In other words, you filter the input, selectively reducing HIGH frequencies with slower sweep speeds. Since the spikes are fast events, if you really want to see the spikes you will need to go to fast sweeps speeds and trigger the sweeps not with the external trigger, but off of the internal CH1 signal.

a. You are quite likely to record multiunit activity. You can work happily with such recordings. If you want, once you are recording spikes, you can move the electrode slightly to try and "isolate" the activity of a single spike generating cell. If all the spikes on the oscilloscope have almost the same size and waveform, you have probably isolated a single cell. You also can use the Offset control of the utility amp to position the input to the discriminator so as to exclude small spikes, while "seeing" large ones. This is a sensitive adjustment, so ask for help if you need it.

2. If you never see spikes, open the cage and see (via dissecting scope) if the electrode has reached the retina. If not, close the cage and continue advancing. If it *has* entered the retina, pull the electrode into the fluid, and move to a new position. Take another "stab" at it.
3. **It almost always takes a few tries.**

Congratulations, you made it!

III. Experiment 4-1 - Groups 1, 3, 5 & 7

A. Amplitude-Intensity Relations (100 msec flashes)

1. Okay, so now this is old hat to you. Refer to Tuesdays settings for the S48 if you need a reminder (Page 2.5). If you think that you need to change the protocol, do what you think is appropriate to make reasonable measurements.
2. Connect the output of the discriminator/rate meter and CH 2 of the scope to the chart recorder. Record your gain and timebase settings on the recorder. Collect amplitude intensity data as you have before using the protocol sheets. Use the b-wave for ERG amplitude. What are you going to use as the measure of spike generating cell discharge amplitude? How do you quantify it?

IV. Experiment 4-2 - Groups 1, 3, 5 & 7

A. Amplitude-Intensity Relations (4000 msec flashes)

1. See A.1. above
2. Simultaneously measure b- and d-waves and activity for the spike generating cells.

V. Experiment 4-3 - Groups 2, 4 & 6

A. Temporal Integration

1. Once more - repeat the protocol that you have employed for the ERG previously (Page 2.7) Except that you will run the protocol first for the spike generating cell responses and then, time permitting, for the ERG.

Experiment 4-1 - Amplitude/Intensity Protocol (100 msec) (Red LED calibrated 8/90)

Log Intensity	Volts	b-wave	Spiking Cell
0.0	1.46		
0.5	1.51		
1.0	1.60		
1.5	1.70		
2.0	1.90		
2.5	2.40		
3.0	3.40		
3.5	6.50		
4.0	15		
4.5	44		
5.0	150		

Experiment 4-2 - Amplitude/Intensity Protocol (4000 msec) (Red LED calibrated 8/90)

Log Intensity	Volts	b-wave	d-wave	Spiking Cell OFF	Spiking Cell ON
0.0	1.46				
0.5	1.51				
1.0	1.60				
1.5	1.70				
2.0	1.90				
2.5	2.40				
3.0	3.40				
3.5	6.50				
4.0	15				
4.5	44				
5.0	150				

EXPERIMENT 4-3 Temporal Integration Protocol

Duration (msec)	Spiking Cell Threshold Voltage (Crit Resp =)		ERG Threshold Voltage (Crit Resp =)	
10				
1000				
50				
200				
1				
500				
100				
2000				

Data Analysis

Experiment 4-1 - Groups 1, 3, 5 & 7

A. Enter the amplitude of the b-wave and the spiking cell response measures in Data Table 4-1.

Experiment 4-2 - Groups 1, 3, 5 & 7

B. Enter the response amplitudes for the ERG components and the spiking cell responses in Data Table 4-2.

Experiment 4-3 - Groups 2, 4 & 6

C. Enter the measured stimuli required for obtaining the criterion response (Y-axis) vs. the flash duration in Data Table 4-3.

These data will be plotted for you in Data Graphs 4-1 through 4-3. You need to take some time to consider the interpretation of the results in the context of the questions posed in the Goals for the Day.

VI. Experiment 4.4 - Introduction to selective pharmacology

Methods

A. You will apply 3 separate pharmacological agents. These have been premixed for your use and will be in labeled scintillation vials:
   1. *APB* (25 ΅M; also called AP4; **2-amino-4-phosphonobutyric acid**)
   2. *KYN* (1 mM; **kynurenic acid**)
   3. *P & S* (100 ΅M **Picrotoxin** + 10 ΅M **Strychnine**)

B. ***Remember to use gloves!*** Put no more than 5 mls of each drug into a reservoir (that is, put each drug in a separate reservoir) and begin bubbling. Remember, when switching between solutions always keep the flow running. Introduce the new drug while the previous solution is running, then turn off the prior drug. Never turn off the old solution before turning on the new one. This will result in all kinds of problems, from electrical artifacts to getting air in the lines.

C. Fill one reservoir with Ringers and use this to measure the flow rate of your system. If you have a flow rate less than .5 ml/min check with an instructor and remember *watch out for air bubbles!*

Experiment - ***Do not apply drugs for more than 3 minutes!!***

D. When applying drugs, one person should always watch to be certain that the level in the drug reservoir is going down, and therefore delivering drug to the preparation.
   1. Be careful not to leave 2 valves open all the time (this includes the main control Ringer's!)

E. You are to determine the nature of the data to be collected and your assignment is to address questions 4-8 on the Goals for the Day (page 4.1). We can provide a variety of graph paper for plotting and we have extra sheets of the Data Graphs used in prior experiments.
   1. If you are able to record both the ERG and spike activity you should make observations for both kinds of data.

Goals/Questions for Day 5

1. **You will observe the retina's sensitivity to more selective pharmacological agents designed to elucidate the excitatory pathways for visual information processing. These agents are selective drugs that act at differing subsets of excitatory post-synaptic receptors and you will be given both *agonists* and *antagonists* to test.**

2. **Based on your observations, do different types of excitatory post-synaptic receptors exist in the retina?**

3. **If the answer to the prior question is yes, can you perform experiments to test the hypothesis that these receptors are localized to different cell types or locations in the retinal network?**

4. **We will tell you which drugs are *agonists* and which ones are *antagonists*, but you must design experiments to allow you to determine which antagonists function at which post-synaptic receptor subtype (which antagonists will interfere with which agonists).**

5. **Your ultimate goal for the day is to combine the results obtained by all the lab groups and be able to draw a schematic of the vertical neural pathways in the retina and specify which post-synaptic receptor subtypes exist at each synapse in the pathway**

RETINAL/CNS PHARMACOLOGY

I. Introduction

A. Implications of neurotransmitters
   1. Synapses
      a. Term coined by Sherrington in early 1900's
      b. Concept championed by early anatomists

B. History of neuroscience
   1. Neuron Doctrine versus Neural Plexus
      a. Ramon ý Cajal versus Golgi (shared 6th Nobel prize!)
      b. Neurons as independent, separate units accepted in late 1800's
   2. "Animal Electricity" known since mid 1800's
      a. The initial thinking regarding cellular communication was that it did not involve chemicals, but electrical signal transmission instead.
      b. Gap junctions provide for the proposed "electrical synapse"
      c. Chemical transmission fully accepted only by about 1930-1940.
   3. Recognition of transmitter candidates was still flawed.
      a. Glutamate was *not* recognized/accepted for 20 years
         (1) Felt to be too ubiquitous to possibly be a transmitter

C. Steps in Synaptic Transmission
   1. Axon terminal depolarization
      a. Arrival of either action potentials or graded potentials
   2. Voltage-gated channels permeable to Ca^{2+} open
      a. Calcium influx triggers Ca^{2+}-dependent fusion of synaptic vesicles with the presynaptic membrane
         (1) Proof of calcium hypothesis
            (a) Prevent Ca^{2+} increase via a Ca^{2+} chelator like EGTA or BAPTA
               i) Depolarization does not cause release of transmitter
            (b) Prevent depolarization via voltage-clamp recording
               i) Increase Ca^{2+} via release from "caged" Ca^{2+} does result in transmitter release.
   3. Vesicle contents are emptied to the synaptic cleft via exocytosis
   4. Transmitter interacts with postsynaptic cell to yield excitation or inhibition

D. Postsynaptic Responses to Transmitters
   1. Transmitter interaction now often termed "binding"
   2. The term implies chemical interaction with something at the postsynaptic cell
      a. Is this intra- or extracellular?
      b. How do you test this?

3. Concept of "lock & key" is an old one
    a. The transmitter is the key
    b. An element on the postsynaptic membrane called the *receptor* is the lock
    c. Transmitter specificity requires such an idea
    d. If structure of the transmitter is critical for its effects, then biochemical "skeleton keys" should be possible
4. Synthetic compounds yield 2 benefits
    a. Easier to obtain than extracting native transmitters
    b. Their structure may shed light on nature of binding site located on the postsynaptic receptor
5. Early biochemical analogs of glutamate did not really help in understanding the binding site characteristics, but they *did* result in a very important discovery
    a. There were pharmacologically distinct varieties of glutamate receptor!
        (1) Glutamate is the native (or endogenous) transmitter, or ***agonist***
            (a) Glutamate can bind, and activate, **all** GLU receptors
        (2) Synthetic analogs of GLU show selectivity such that some mimic GLU only at specific synapses
            (a) In cases where one synthetic agent is ineffective, a different compound will mimic GLU
6. The different types of GLU receptors have been named for the specific synthetic **agonist** that activates that receptor type.
    a. You will be using several of these specific agonists in your experiments today.

II. General overview of today's experiments

Up to now your experiments had a common theme of "how variable X effects the response of the retina" and many of the answers obtained were specific to the retina. You now know more about the signaling in the retina, but do our results have relevance for someone working on the hippocampus? You will now apply drugs to the retina **which are known** to act similarly to the CNS neurotransmitter, glutamate (**agonists**), and to challenge their actions with blocking agents (**antagonists**). The class assignment is to determine the excitatory neuropharmacology of the retina. Specifically, you will coordinate your individual experiments so that at day's end you can indicate what the nature of synaptic transmission is at both the outer and inner retinal plexiform layers. The preparation is the same as yesterday's experiment, with both ERG and ganglion cell recording.

The agonists have effects that may (or should?) mimic that of GLU. You have used APB several times already. Are its effects consistent with the action of an agonist? You should use your data from today and yesterday to account for the effects of APB. You need to ***think carefully*** about what experimental results are consistent with applying an agonist whose receptor is present in the tissue being studied.

Suggestions:

1) Perform your pharmacology experiments carefully. That is, be certain that the drugs are being delivered to the retina, that you have good flow through the prep chamber and that the perfusion system is set up correctly. Be sure you know how to verify these items!

2) You will be collecting a lot of data, but do not become so focused on the data collection that you forget to **think about the *meaning* of the data**. You should have some predictions about possible results and see if your data support or reject them. Pharmacological experiments are **never** perfect. Compare your results with other groups to see if there is a consensus. The drug effects you observe (when a receptor is present) will be clear cut. Do **not** obsess about subtle effects or individual light responses. ***Ask the instructors if you can not explain your results!***

III. Hypotheses/Questions

A. Do different GLU receptor subtypes exist in the retina?
B. If multiple receptors exist, what role do they play in retinal function? Can you perform experiments that can determine if different receptors are located at different locations in the retinal network (ie. Outer vs Inner retina)?
C. Which antagonists work at which glutamate receptor subtypes?

IV. Agonists

Remember that each of these compounds is a good selective agonist for a specific subtype of glutamate receptor. For example, KA will, for all practical purposes, only bind to a kainate receptor and not to an NMDA receptor. You therefore have tools to test for the presence of 4 different types of GLU receptors.

A. Kainate (KA)
B. Quisqualate (QA)
C. N-methyl-D-aspartate (NMDA)
D. APB (or AP4)

V. Antagonists

These agents have been identified as antagonists to GLU, some of which are specific for certain GLU receptors and not others.

A. Di-nitro-quinoxaline (DNQX)
B. KYN
C. 2-amino-5-phosphonopentanoic acid (AP5) or
   2-amino-7-phosphonoheptanoic acid (AP7)

VI. Experiments

A. Explore **Hypothesis A.** above, via application of agonists. Start with 25 ΜM KA (Groups 1 & 4), 300 ΜM NMDA (Groups 2, 5 & 7), and 10 ΜM QA (Groups 3 & 6). Keep in mind that you have data on APB from yesterday's experiments that you must incorporate into your final "report".

B. **Be certain to limit your drug exposure time to no more than *3 to 4 minutes***. Long exposures with these drugs can cause toxic effects to the retina.

C. Explore **Hypothesis B.** by testing the effects of antagonists on light-evoked responses. Start with 20 :M DNQX (Groups 2, 5 & 7) and 300 :M AP5 or AP7 (Groups 1, 3, 4 & 6). Remember that you have data from yesterday's experiments for the antagonist, KYN, that you can compare to DNQX & AP5 or AP7. **Again, use *brief* drug delivery times!**

D. To answer **Question C.** each group will make a series of mixtures with one of the agonists and the antagonist. For example, Groups 2, 5 & 7 will apply mixtures of DNQX & KA, DNQX & QA, DNQX & NMDA and DNQX & APB. Groups 1& 4 will do the analogous experiments using KYN & Groups 3 & 6 will use AP5 or AP7.
   1. What should happen if an antagonist is mixed with an agonist for which there is not any competition? What if there is competition?
   2. How does one find out if the retina is dead or not?
   3. IMPORTANT!! In between the applications of the mixtures, perfuse the retina with the antagonist alone to be sure that the receptor of interest remains blocked. [*Can you think why you need to do this?*]

E. - Be careful not to let your reservoirs run dry!!

F. *You will obviously be required to coordinate with other groups to allow the class as a whole to "solve" the assignment for today.*

Appendix: Gain & Trigger - Technical Terms Defined

I. Gain

A. General definition

1. When you hear or read the term **gain** employed in the lab, you are hearing a term whose roots are in the realm of engineering. **Gain** refers to the amount of amplification that is applied to a signal of interest.

B. Oscilloscope Example

1. If you send some signal into an oscilloscope you can measure the size (amplitude) of that signal.
2. When you change the oscilloscope display in terms of its Volts/Division, you are changing only the gain of the display, the original signal is, of course, unchanged. In this case, it is "easy" to determine the amplitude of the original signal, by simply measuring the number of divisions and multiplying by the Volts/Division.
3. As the next example demonstrates, things become a little more complicated when the original signal is signal processed by some electronic device before it is sent into the oscilloscope.

C. Amplifier Example

1. The electrical signals generated by neurons are sufficiently small that we must amplify them before we can study them. This is true for field potentials (the ERG or extracellular spike recording) or intracellular recording.
2. Many amplifiers (including the Grass P15, the Headstage amplifier and the Dagan) have controls that allow the experimenter to set how much the original input signal will be amplified. For example the Headstage amplifier used on Day 2 has 3 fixed positions for 10, 100 and 1000 times amplification. These values of amplification are also referred to as the **gain settings** of the amplifier. So you might hear an instructor ask, "What **gain** did you record the ERG at?" In this case, you would look to see what the amplification switch was set to.
3. When amplifiers are used prior to the oscilloscope, the measurement of the original signal amplitude requires that you know and use the amplifier **gain** in calculating the correct amplitude. For example, if you have a **gain** of 1000 at the amplifier and an oscilloscope display setting of 100 mV/div, then a measured deflection of 3 divisions would represent an original signal of 300 microvolts (:V). This is obtained by dividing the display gain (100 mV/div) by the amplifier gain (1000X). The larger the amplifier gain, the *smaller* the display gain which makes sense since if you have to amplify a signal more it means the original signal was small.

II. Triggering

A. General Definition

1. Many devices have the capacity to have their operation controlled by events that occur externally to the device itself. The capacity is known as being **externally triggered**.
2. In our lab exercises we want to have the oscilloscope sweep be controlled by an external source and the following examples describe the various "types" of trigger settings that are commonly used.
3. Think of triggering as a decision box that looks at some signal and has rules on whether to cause a conditional event to occur or not.
   a. For oscilloscopes the conditional event is whether the oscilloscope sweep occurs or not.
   b. The decision criteria that can be selected are:
      (1) Source of the trigger signal - see below: internal or external trigger
      (2) Polarity of trigger signal - positive or negative going events can be selected
      (3) Coupling of the trigger signal
         (a) The trigger signal can be either AC- or DC-coupled (see lab manual page 2.3 for a description of coupling).
      (4) Delay - some 'scopes allow you to define a set amount of time that must pass after trigger conditions are met, before the sweep occurs. This is useful for offsetting the sweep start from an event that might occur later in time. As you will see below, devices like waveform generators or pulse generators often have a trigger output that can be set to occur before the waveform or pulse output is generated. If such signals are available, the delay setting on the oscilloscope is unnecessary.
      (5) Threshold - The experimenter can set a voltage level that the trigger input signal must exceed to meet the criterion of "go". If the trigger polarity is positive then the threshold must be crossed by having voltages greater than the threshold. If the polarity is negative, then the voltage must be less.

B. "Internal Triggering"

1. Oscilloscopes have the unfortunate history of calling one mode of triggering **internal triggering**. This refers to any time that the sweep of the oscilloscope is controlled by voltage signals that are located on one of the 'scope inputs that is also driving the Y axis of the display.
   a. Advantages
      (1) You do not need an extra cable for the trigger input - you already have one that brings the input in to one of the oscilloscope channels
      (2) You can **see the trigger signal** on the 'scope. This allows you to verify when the signal changes and adjust your trigger conditions with greater ease to get the timing of triggering as desired.
2. The Hameg 'scope has the ability to display 2 input channels, and it can be set so that the sweep will be triggered by either CH1 or CH2, but not both.

a. This is accomplished by setting the triggering for INT mode and a separate switch selects internal triggering off of CH1 or CH2 (I/II)

C. External Triggering
1. For this mode of operation a separate input cable is required to provide the voltage signal that the oscilloscope will use to determine trigger conditions.
2. The source of this signal is often a pulse generator (like our Grass S48 or Dagan 900 stimulators.
   a. Such devices often have separate trigger outputs that are designed to be inexpensive, but work well in providing external oscilloscope trigger inputs.
      (1) For example, the S48 SYNC outputs are designed to trigger 'scopes. The signals out of these channels are 30 Volt 100 :sec pulses! But big and fast is all an oscilloscope needs for its external trigger inputs.
      (2) Selecting the right trigger output helps to determine your view of the data on the oscilloscope. By selecting the Delay Sync Out from the S48, you can have a sweep begin at the Hameg a user-defined time prior to the S48 output to the LED. In this way, the light-evoked voltage response can be viewed in the middle of the 'scope display rather than being "crammed" to the left side of the display. It also provides the experimenter a chance to see what the voltage signals were *prior* to the stimulus, and help to be aware of potential artifacts like baseline drift or faster non-event related signals.

If you still have questions about these concepts (not uncommon if you have never used this equipment before) please don't hesitate to ask an instructor to clarify!