Responses to Sounds in the Central Auditory System of the Frog: An Advanced Electrophysiology Laboratory in Sensory Processing

Michael J. Ferragamo¹ and Janine M. Wotton²
Departments of ¹Biology and ²Psychology, Gustavus Adolphus College, St. Peter, MN 56082.

Animal brains must be able to extract and display information about their world in order to perform daily activities. Owing to the diversity of ecological habitats, a variety of systems have evolved receptors that convert specific forms of physical energy, like light or sound, into neural impulses. The nature of the spike code and how it is deciphered has been an intense area of investigation by neuroscientists for many decades (Rieke et al., 1997). A traditional approach to that question has involved recording the time-of-occurrence of action potentials evoked in response to stimuli in which a single parameter, for instance amplitude, has been manipulated systematically. In this paper we describe how students might apply this methodology towards understanding some of the basic principles of sensory processing in the auditory system of an awake frog.

Frogs rely upon vocal communication to advertise for potential mates, to defend territory and to alarm neighbors of danger. Cells in the auditory midbrain of an awake frog display tuning to the spectral energy present in calls based upon discharge rate and encode the temporal properties of calls in the timing of their discharges. This laboratory experiment is designed to allow students to explore the relationship between stimulus amplitude or frequency and response rate, and how the timing of responses can also be used to encode behaviorally relevant features of the stimulus. Action potentials in the midbrain auditory nucleus, the torus semicircularis, are evoked by delivery of free field sounds and recorded. Most cells are broadly tuned to frequency, yet some can be fairly precise in preserving periodic structure. The use of a comparative model of study should help students understand principles common among all sensory systems, and an appreciation that the architecture of each system is adaptively matched to the ethological task at hand.

Key words: Rana, sensory coding, comparative model, undergraduate neuroscience

Goals
Our neuroscience minor offers an entry-level course (Introduction to Neuroscience PSY260), in which students get some practice with basic electrophysiological
methodology and are exposed to neuroethological models to learn the history, basic principles, and language of neuroscience. After completion of this course students may take the upper-level neurophysiology laboratory-intensive course (Neurobiology BIO384). The experiment described here is a discovery-based module for BIO384.

Goals for this module are to provide hands-on experience when introducing students to the following: (1) Animal Preparation: performing surgeries on a small animal. (2) Stimulus Preparation: working with sounds as sensory stimuli. (3) Electrophysiological Recording: isolating units and presenting stimuli during the data collection procedure. (4) Data Analysis: assembly and manipulation of data to display fundamental concepts of sensory physiology.

| Lab Schedule |
|---------------|
| Week 1        | Discussion of Experimental Objectives and Design |
|               | Orientation to Instrumentation and Software |
|               | Electrode Fabrication |
| Week 2        | Brief Experimental Proposal Due |
|               | Construction of Acoustic Stimuli |
| Week 3        | Instructor Demonstration of Surgery and |
|               | Electrophysiological Recording |
| Week 4        | Student Recording |
| Week 5        | Student Recording (continued) |
| Week 6        | Data Analysis |
| Week 7-8      | Final Paper Due |

Table 1. Typical lab schedule. Each session is three hours, but students are informed that additional unscheduled time may be necessary to extend recordings, and to complete signal generation and data analysis.

MATERIALS AND METHODS

*Rana pipiens* (leopard frog) or *Rana catesbeiana* (bullfrog) can be purchased from most biological supply houses. We prefer to use leopard frogs weighing 40-80 gm. Animals can be housed in a 10-gallon aquarium the bottom of which is covered with about one to two inches of gravel or sphagnum. Frogs should be provided with a small pool of water (e.g., a plastic container) and fed regularly with live crickets that are lightly dusted with reptile vitamins.

**Surgical Preparation** Before allowing students to perform the surgery, we discuss proper handling of frogs, and students are reminded of the ethical considerations of conducting invasive procedures on live animals. A part of a session is devoted to students observing the instructor perform the surgery under a camera projected to a color monitor. Our general policy is to give students the option of having the instructor do the surgery without consequence to their grade. All experiments are done in accordance with the Gustavus Adolphus College Animal Care and Use Committee in compliance with Public Health Service Policy on Humane Care and Use of Laboratory Animals and USDA Code of Federal Regulations.

The surgery exposing the torus is simple and quick, and should be performed at least one day prior to recording in order to allow the animal to recover from the anesthetic and regain comfort. The frog is anesthetized by submerging it completely in a buffered (sodium bicarbonate, pH 7) solution of 0.2% 3-aminobenzoic acid ethyl ester (MS-222) for two to three minutes, after which the frog should be unresponsive to a firm squeeze of the foot.

![Figure 1. Location of torus semicircularis. A. The circle circumscribes the approximate size and location of the area on the surface of the skin overlying the TS, and the dashed lines indicate the shape of the cut used to reveal the underlying cartilage. The pair of tympani is shaded in gray. B. A photomicrograph of the left lobe of the TS. The most distinct structure in the TS is the principal nucleus, where large spherical cells are arranged in a lamellar structure resembling the layers of an onion. The bulk of the cell mass in the nucleus occupies the rostral portion and gradually diminishes towards the caudal border (Feng, 1983).](image)

The frog is placed dorsal side up and covered with moistened cheesecloth in a tray positioned under a dissection microscope. The skin is lifted with blunt forceps to make an H-shaped cut with scissors at the midline roughly from the eyes to the most rostral border of the tympanum (Figure 1A). In order to prevent the skin from quickly resealing, the flaps can be trimmed around the edges. The cartilage is now exposed over the optic tectum, and should be thinned to a pliable layer with a dentist drill or Dremel tool (Dremel engraving bits #106 or #108). Gentle pressure is applied in a circular pattern to remove thin, even layers throughout the circumscribed area (circle in Fig. 1A). Caution should be exercised to avoid the blood vessel in the muscle at the caudal border. If the surgery is performed carefully, there should be very little blood loss. Once the cartilage has been reduced and softened to where it can be deformed by a gentle push with a blunt forceps, it can be easily torn away with #7 forceps. To avoid penetrating the brain case with the engraving bit, it is best to test the thickness after each sweep. Before removing the cartilage it is advisable to have the instructor check that the region where the cartilage is reduced lies over one lobe of the tectum. Once the cartilage is removed, further use of the drill will risk damaging the brain. If exposing more of the tectum is necessary, then the diameter of the hole can be widened by shaving its edge with a scalpel. The optic tectum is covered with a semi-transparent membrane mottled with black pigment (Figure 1B) that, if left intact, will prevent the penetration of electrodes. A small region on the surface of the tectum is...
exposed by either making a small incision in the membrane with small scissors, or by gently tearing it with a fine probe. Although not difficult, this step must be done carefully to avoid touching the brain and it may be best for the instructor to help. After the surgery is completed, the hole should be filled with mineral oil to prevent drying, and the skin flaps will assume a natural position to cover the hole.

The frog can be placed in the tank without restraint, but should remain wrapped in the moistened cheesecloth until the animal resumes motor activity. Typically, the frog will appear alert and resume normal postures and behaviors within about two to three hours after being anesthetized. A single animal can be used in multiple recording sessions for several weeks, and if not, the surgical site will heal.

**Figure 2.** A cartoon of the sound delivery and data acquisition system. See text for details.

**Electrophysiological Recording** On the day of recording, the frog is immobilized with an injection of pancuronium bromide (0.007 mg/gm body weight) into the thigh muscle, wrapped in moist cheesecloth, placed on a Styrofoam board, and loaded onto a table-top vibration isolation table (Kinetic Systems). A Faraday cage lined with sound attenuating foam is desirable, but is not necessary as long as the background level of acoustic and electrical noise is low. A small pin connected to the indifferent input of the differential amplifier can be gently inserted into the thigh, and to reduce noise a wire in contact with the surface of the moist skin should be connected to the ground connection of the differential amplifier.

Units in the TS can be isolated by either glass microelectrodes (with impedances from 3-5 MΩ and filled with 3 M NaCl) or with tungsten electrodes. From our experience, glass delivers better isolation. The tips of glass electrodes can be dipped in India ink to aid visualization. A hand-driven micromanipulator with three-dimensional motion translation will work, but the ability to drive the electrode remotely is preferable. There are some relatively inexpensive hydraulic microdrives available (we use a Narishige MO-10). Positioning the electrode is a step that students find difficult initially but quickly learn after a few frustrating attempts that result in broken or bent tips. Occasionally some fluid will need to be cleared from the hole by absorption with small pieces of Kimwipe. The electrode is advanced slowly in a direction along the dorsal to ventral axis with the extent of the TS about 200 μm beginning about 1.5 mm below the surface of the tectum.

The set-up is shown in Figure 2. The signal from the electrode is amplified, filtered (Dagan EX-1; 0.3-3 kHz) and sent to a computer for digitization, and to an oscilloscope (40 MHz) and audio speaker to monitor the responses audiovisually. An inexpensive, battery-operated speaker is sufficient. Often stimulus-driven neural activity can be heard as a faint “hash” time-locked to the stimulus before units are first visualized. Students typically notice that they rely more on the audio rather than the visual feedback when searching for units.

Neural action potentials should be acquired using a sampling rate of at least 10 kHz. We use LabView to coordinate a fully integrated data acquisition and signal generation system with internal triggering. However, any of the data acquisition systems commonly used in student physiology teaching laboratories (e.g., 2-channel PowerLab) should be adequate. The stimulus can be sampled on a second channel and used to directly trigger a single sweep. If available, experiments can be stored on a tape recorder for off-line analysis.

**Figure 3.** Example stimuli. A. 500 Hz tone-burst; B. amplitude modulated tone; C. white noise; D. synthetic advertisement call from Rana pipiens.

**Stimuli** The behaviorally measured audiograms of commercially available species of Rana extend from tens of Hertz to several kiloHertz (Fay and Popper, 1999) and stimuli within this range should evoke responses. All of the equipment used to generate signals is inexpensive and available at consumer electronic outlets. Pure tone-bursts are all that are necessary to reveal how attributes such as frequency and amplitude shape the tuning or timing of discharges. Pure tones can easily be generated with an audio generator or from a digital sound-generating board, now standard equipment on most computers. A stimulus having a broader spectrum like a frog call, white noise or frequency-modulated sweep is effective when searching for auditory units. Each sound should be shaped with a brief rise/fall envelope to eliminate onset transients. Figure 3 displays examples of stimuli. We use MATLAB to synthesize WAV files. Included in the appendix is a list of internet sites that make available signal generation tools and field recorded frog calls stored in WAV format. Digital stimuli should be made prior to the start of the
experimental session and stored ready to play on the computer. A library of sounds including a range of pure tones and some broadband signals will give students the most flexibility in presentation. It is advisable to include a few pure tones between 100 and 500 Hz because these frogs typically have energy in their calls in this range. In order to increase the efficiency of this process the students will have developed an instructor-guided and approved proposal that includes a description of stimuli and how they will be used to address inquiry. The acoustic stimuli are amplified with a stereo amplifier and then presented through a transducer on the side contralateral to the recording site. The “volume” knob can be used to adjust the amplitude to different levels that can be quantified in units of dB SPL with a sound level meter (available at Radio Shack).

**RESULTS**

**Basic Recording**

An example recording made by a student using a glass electrode in the TS is displayed at the top of Figure 4A. This would be considered excellent isolation of a single unit that clearly discharged on multiple occasions during one trial of a 500 ms pure tone-burst. If more than one cell is isolated, action potentials can be sorted by eye based upon waveform characteristics. The duration of a recording from a single cell or a small number of cells can vary from seconds up to an hour depending upon the stability of the preparation. However, a reasonable representation of the temporal response properties will usually emerge in as few as 10-20 stimulus presentations. If the tissue is healthy and students are well prepared, two three-hour sessions should be adequate to collect sufficient data for subsequent analysis.

**Responses to Stimuli**

A number of potential manipulations of the stimulus can be used to explore various principles of sensory processing. The demands of on-line decision-making common to most single-unit experiments although exciting to the experienced investigator can be daunting to the novice. When a unit or group of units is isolated, it is helpful for the instructor to be on-hand to advise the group which stimulus to present based upon the unit’s response history. Of course, even the experienced investigator may later discover gaps in the data collection process, thus students should anticipate this and consider it a rite of passage.

The only information required for all of the analysis described here is the time-of-occurrence of each action potential. Analysis can be performed and displayed automatically (we use MATLAB), or by visual inspection of the record and entry into a spreadsheet.

The following are suggested foundations for experimental modules:

**Stimulus Specificity**

One of the first principles that students learn about sensory systems is that the receptors of sensory systems convert a specific type of energy into a bioelectrical potential. Students can easily demonstrate this because cells in the TS will typically respond to auditory stimuli but not to other modalities. The simplest experiment is to present a frog call and compare it with visual stimuli. If you adjust the amplitude of the call, you should see more spikes occurring at greater amplitudes.

**Adaptation**

Cells in the TS frequently show adaptation. If students record from more than one cell, then they will see different adaptation profiles. The middle of Figure 4A represents the time of occurrence of each spike with a dot. Each line represents the responses to one trial of a 500 ms pure tone-burst; the raw neural recording at the top of the figure is from the first trial. In this case, the cell fires a burst of action potentials beginning at about 15 ms after the onset of the stimulus that adapt well before the end of the activity.
stimulus. The temporal pattern of the response is more clearly visualized by accumulating the responses over all trials into time bins (Figure 4A, bottom). The resulting post-stimulus time histogram (PSTH) reveals a precisely timed onset response followed by an abrupt pattern of adaptation.

Receptive Fields (Frequency Tuning)
Frequency tuning is a receptive field property that originates in the auditory periphery and is preserved along the central pathways. The inset of Figure 4A displays a frequency-tuning curve plotted here as the normalized number of discharges to a pure tone-burst repeated over 20 trials. Each frequency was presented at the same relative amplitude measured at the tympanum. If the amplitude is too high, then the tuning curves become extremely broad. Before collecting data on the computer, students can quickly approximate the tuning range by listening to the unit activity as they repeatedly sweep through a range of frequencies by hand with an audio generator. From that initial scan they can choose a set of tone-bursts from their digital library that evenly span that range. At the minimum, the goal would be to delineate the high and low frequency edges and the most sensitive area, the best frequency, of the tuning curve. Again, this would increase the chance of completing a tuning curve if the unit is lost quickly. If the recording remains stable they can fill in more details by presenting more frequencies later. In the inset of Figure 4A, a notion of the region of best frequency and the edges are apparent despite the small number of data points.

Periodicity
Some cells in the TS can respond at fairly regular intervals to a stimulus that repeats at low frequencies. The ability to respond synchronously to the envelope of a tone modulated in amplitude (see Figure 3B) is displayed in the PSTH from another cell (Figure 4B). From the peaks spaced roughly every 50 ms it is clear that the cell was responding to the 20 Hz modulation rate.

Practical Considerations
An instructor with a background in neurophysiology or sensory biology can readily adapt this laboratory to meet needs and skills. It might be useful to acquire some passing familiarity with the acoustic signals but no real expertise is required because many auditory software packages are quite user friendly. It is advisable to set up and try out the equipment over the summer in preparation for use in a course.

To make the best experience for all concerned, we strongly advise that an instructor not be too ambitious for a given session. We have presented several different possible modules but do not expect that an instructor would try to do all of them at once. If students are presented with too many choices, they will not gather data in a systematic fashion. Students need to have specific goals for a session, and for this we have found that it is useful to have a prepared protocol ready. For example, the simplest module would be to examine the specificity of the TS cells for auditory stimuli. The frequency tuning and adaptation module requires that students be a little more prepared by having a library of pure tones. More complex experiments involving time-based codes of AM signals should come only after students have successfully completed one of the easier modules. Students will almost certainly lose cells and have incomplete data sets but this is standard for any electrophysiology experiment. Each student may only successfully capture a couple of good cells but they do not need much data in order to see basic sensory properties. The first experiences of recording are tentative and full of mistakes but the hands-on opportunity allows students to grow in confidence and get the feel for it.

DISCUSSION
Male frogs begin to chorus at dusk to attract females; if a female approaches, the male will engage her in amplexus. In order to ensure its own mating success and to avoid the consequences of attracting heterospecifics, each species of frog, typically the male, has in its repertoire two calls, one to advertise to conspecific females and the other to claim territory in encounters with competing males (Gerhardt, 1988; Ryan, 2001). Advertisement and encounter calls have dominant frequencies. In some cases the spectral content may be similar between calls or to the vocalizations of heterospecifics; in that case, listening frogs must rely upon the temporal characteristics, such as duration or pulse rate, to identify and locate an individual (Ryan, 2001).

In the auditory system of frogs, the stimulated region of the two end organs, the amphibian and basilar papillae, determine the frequencies to which the neuron is tuned (Fay and Popper, 1999). Auditory nerve fibers that innervate the papillae have V-shaped tuning curves with the tip being the best frequency (Feng et al., 1990). The dominant frequencies of the call are over-represented in the best frequencies of the auditory nerve fibers. Tonotopy is maintained, albeit somewhat crudely in the midbrain. Frequency tuning curves in the TS are generally broader and more complex in shape than at the periphery. The breadth of tuning can be quantified by having students measure the width across the response at 50% of maximum. Some tones may produce inhibition. The emergence of receptive fields with more complex properties over what is observed in the periphery is not surprising in a nucleus that receives massive convergence of synaptic inputs from lower brainstem nuclei. The variety of response patterns observed in the TS can form the basis of a discussion on the role of the TS in identifying and locating other individuals, and in coordinating an appropriate motor action.

As mentioned above, calls with similar spectral structure can be distinguished based upon temporal attributes such as call rate or duration. A neuron’s response to a call can be quantified by discharge rate and by the timing of discharges. The auditory nerve can synchronize its discharges to the phase of a tone (Narins and Wagner, 1989) or to fluctuations in the envelope of a complex sound (Frishkopf et al., 1968; Feng et al., 1990). A relatively simple method for students to examine
synchrony is to plot an interspike interval histogram. Any stimulus-dependent regularity in the response should become apparent in a peak centered at approximately the period of the envelope. Occasionally a second peak is observed at a short interval (3-5 ms) that corresponds to an intrinsic bursting that is stimulus independent. While present in the TS (see Figure 4B), the precision of synchronization erodes as information is passed along the central pathway (Rose and Capranica, 1985). What emerges at the TS is the transformation from a time-based to rate-based code where some cells no longer synchronize to the envelope well, but selectively respond to a limited range of AM rates. Whether this is observed or not, students can be engaged in a discussion on the challenges in preserving the fidelity of timing as activity is conveyed across a number of synapses (Trussell, 1997).

Adaptation is a change in the firing rate during a maintained stimulus. In the vertebrate cochlea the biophysical mechanism underlying adaptation is a calcium-dependent process (Fain, 2003). Effectively, adaptation maintains high sensitivity to changes in the stimulus in a dynamic background. This makes sense for frogs competing to get their call heard over several meters in a loud, noisy environment generated by other chorusing animals and abiotic sources. Adaptation in the periphery is typically characterized by its speed. In the frog auditory nerve, the speed of adaptation is dependent upon the best frequency of the fiber (Megala and Capranica, 1982). Adaptation is often observed in the TS (Figure 4A). Students can quantify adaptation using a ratio (# spikes in first 50 bins / # spikes in last 50 bins) of the PSTH or by fitting a curve to its profile. Typically, the time constant of adaptation in the periphery can vary from less than ten milliseconds to tens of milliseconds (Eggermont, 1985). Although that range is similar in the TS, it is important to remind students that the temporal response patterns displayed by neurons in the TS have been shaped by processing in the brainstem. These observations can be used to provoke thought on how putative circuits sculpt sensory responses in the central auditory system.

CONCLUSIONS

When students are introduced to sensory systems, the text and the professor typically stress common fundamental principles. First, information is coded and conveyed to the brain about the nature of the signal. After transduction the information is represented by the rate of discharges or the temporal pattern of firing. Students can see both types of coding in this laboratory exercise. If the intensity or frequency of a signal is changed, then the firing rate will change systematically. When periodic signals are used, some cells will respond with a distinctive pattern of firing locked to the periodicity. Second, sensory systems discriminate change and do so reliably and rapidly. Over the course of numerous presentations (trials) of the same stimuli, students should recognize the fidelity of the coding and the relationship between the stimulus and the response. Third, a sensory cell is characterized by its frequency tuning curve and adaptation profile. The range of tuning of TS cells varies, but typically it is broad so overlap is likely to be seen if several cells are examined. Adaptation is easily seen in the response to relatively long duration signals and students should see considerable variability in the rates of adaptation in the TS.

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APPENDIX: Internet Sources of Stimuli

This site brings together many other websites about bioacoustics: http://zeeman.ehc.edu/envs/Hopp/sound.html.

Sound Analysis

There are a number of sites listed that provide free or inexpensive sound analysis software generally designed for birdsong but they work for frogs too. The analysis programs allow you to graph and analyze the signals used which are useful if you use real frog calls and would like to know the spectral and temporal structure of your signal.

1. Sound ruler (soundruler.sourceforge.net/) is a free shareware analysis program that has been positively reviewed in the journal Bioacoustics for use with frog calls. The demonstrations at the website include how to analyze a frog call.
(2) Avisoft has a light version of their analysis software and also some frog calls available for free download: www.avisoft-saslab.com/downloa_.htm

Natural Frog Calls
(1) The University of Michigan Museum of Zoology Diversity web:
animaldiversity.ummz.umich.edu/site/accounts/sounds/Anura.html
(2) allaboutfrogs.org/weird/general/songs.html

Signal Generation
There are many shareware and freeware programs for signal generation – these sites and availability change frequently. Some sites provide programs that can be downloaded and used for only a brief trial period.
(1) Virtins sound card signal generator works with standard PC sound cards – it can produce noise signals and pure tones as well as a variety of shaped signals and has the added advantage of a PC based virtual oscilloscope to view the signals. It is currently available for a free trial but is also relatively inexpensive (less than $50).
www.virtins.com/ScgenSetup.exe or also at
www.softpedia.com/progDownload/Virtins-Sound-Card-Signal-Generator-Download-18338.html
(2) Soundcheck by PassMark tests your PC sound card and includes a tone generator.
www.passmark.com/products/soundcheck.htm

Received January 19, 2005; revised March 26, 2006; accepted May 06, 2006.

Development of this exercise was supported by National Science Foundation grant 9952300 and the National Organization for Hearing Research. We are grateful to three anonymous reviewers for thoughtful comments and suggestions on the manuscript.

Address correspondence to: Dr. Michael Ferragamo, Department of Biology, Gustavus Adolphus College, 800 W. College Ave, St. Peter, MN 56082. E-mail: mferrag@gac.edu