GAP-FREE NEURAL CIRCUITS – CLASS #1: Introduction

OUTLINE:

- **Background & Motivation**
- **Neural signaling**
- **Experimental techniques**
    - Anatomy
    - Physiology
    - Manipulations

SELF-INTRODUCTION:

- **Skills and experience of Tots vs. Nikhil**

AUDIENCE POLL:

- **Who knows something about what neurons do?**
- **Who has actually done neurophysiology experiments?**
- **Who has little to no experience doing experiments in a biology lab?**

CLASS MECHANICS

- **Each day we'll discuss a specific neural circuit from an invertebrate as derived from experiments (8 total)**
- **Course website: http://mit.edu/nbhatla/gapfree**
    - **Syllabus with required and optional readings for each day**
    - **Class notes and slides will be posted soon after each class**
    - **Please read the required reading (1-2 papers) before each class**

[NIKHIL]

BACKGROUND ON NEURONS AND BEHAVIOR

Why do we have a nervous system?

- To survive, organisms must gather nutrients from the environment, in 2 basic niches:
    1. Species can diverse widely and randomly (plants and non-motile organisma)
    2. Species can sense nutrients at a distance and direct movement towards it (animals and motile organism, like bacteria) – these movements are what we call **"behavior"**

- DRAW BACTERIA CHEMOSENSING: Single-cell bacteria move towards nutrients by sensing the nutrient and modifying proteins which directly alter the flagella responsible for movement. How far does this signal need to travel? Even though bacteria are a few microns long, chemoreceptors localize just 100 nm from the flagella that they control. For a sense of scale,

the chemoreceptor proteins themselves are about 40 nm long. So bacteria can respond **on the order of milliseconds** to a chemoattractant **simply by diffusion**.

- TIMESCALES FOR SIGNAL MOVEMENT AT MILLIMETER SCALE:

Method		*Speed*	*Time to travel 1 mm*
1)	Diffusion		~1 hour
2)	Active transport (molecular motors)	~1 micron/sec	~15 minutes
3)	Electrical signaling (in neurons)	1-10 meters/sec	1 millisecond or less

- Neurons solve the speed problem of diffusion
    1. They function like **wires** that use electrical charge instead of diffusion to transmit signals, though they are not nearly as fast as metal wires
    2. They have drawn out morphologies that can span long distances and **specifically route** signals from one place to then next, instead of transmitting the signal in all directions like diffusion would.

So neurons evolved as multi-cellular organisms grew in size to enable fast signaling, in response to stimuli or just to generate undirected motion.

Neural circuits evolved to control the <u>movements</u>, or behavior, of the organism.

Behavioral output keeps us focused on a class of circuits with a <u>definitive</u> function.

There are 2 kinds of neural circuits:

Type	*Examples*	*Neural activity*	*Synaptic signaling*	*Non-synaptic signaling*
Acute (fast)	Vision, reflexes	Short window	Yes	No
Chronic (slow)	Hunger, mood	Much longer window	Likely yes	Likely yes

- <u>Acute, stimulus-dependent behaviors</u> (e.g. odor response, vision)
    - Causality is clear: stimulus can be given and behavioral response can be observed
    - Constrained time window of neural activity
    - Fast processing means that fast signaling must be used, ruling in anatomical synaptic connections and ruling out hormonal, non-synaptic connections.
    - Overall, hypotheses are more constrained so circuit will be identified more quickly.

- <u>Chronic, state-dependent behaviors</u> (e.g. hunger, mood)
    - Causality is more difficult: multiple stimuli may need to act together to elicit the behavior (e.g. food)
    - Less constrained time window of candidate neural activity

- Slow processing means that non-synaptic, "hormonal" connections might also be relevant as well as synaptic partners

This course will focus entirely on acute, stimulus-dependent behaviors.

PHILOSOPHY AND MOTIVATION

Why study invertebrates?

	Correlated neurons	*Causal neurons*	*Gap-free circuit*
Vertebrates:	YES	Sometimes	NO – too many neurons, neurons vary, and connectivity varies or is unknown
Invertebrates:	YES	YES	YES – fewer neurons, neurons are identifiable and connectivity reliable and determinable

- How neuroscience is normally done in vertebrates:
  1. Identify a stimulus-dependent behavior of interest (e.g. new object attraction in mice)
  2. Drop an electrode into a part of the brain, present the object to the mouse, and see if neurons near the electrode respond to the stimulus.
  3. Occassionally, inactivate the neuron to show that the stimulus-dependent behavior goes away – shows that the neuron is in the neural circuit for the behavior.
  - If this is done in many parts of the brain, many neurons will be found
  - But there will be lots of GAPS between these neurons so the neural circuit will be fuzzy
  - Furthermore, same neuron is hard to identify across individuals, so may be studying different neurons with similar physiological response

- How we can do it differently with invertebrates
  1. Neurons are countable (302 in nematode *C. elegans,* 100,000+ in fruit fly *Drosophila*)
  2. Individual neurons can be named and identify from one animal to the next ("identifiable neurons")
     - Often a lineally-identical neuron can be observed across invert. individuals, and can be manipulated to show that they are in the neural circuit
     - These neurons have reproducible connectivity across individuals is or can be known, so the signal can be followed from one neuron to the next so that a GAP-FREE circuit can be determined
  3. **This course values precise circuit description of a simple behavior over vague description of a complex behavior**

Overall, invertebrates are much simpler, so why not start there. We know that genes and molecules are conserved from invertebrates to vertebrates, so perhaps neural circuit principles will be as well.

[END NIKHIL]

[TOTS]

HOW NEURONS SIGNAL

How does a neuron conduct its signal?

Neurons often have multiple protrusions, or processes, which are called either dendrites or axons. Dendrites are generally receive a signal from the environment or another neuron, while axons send a signal to another neuron or muscle.

A sensory neuron contain proteins which sense a specific stimulus, such as a photon of light or a molecule of odorant. Proteins are encoded by DNA and manufacture by basic processes of the cell. These proteins often reside in the cellular membrane. The stimulus generally causes a change in the shape of the sensory protein, which causes a molecular reaction which ultimately leads to a change in the neuron's membrane potential.

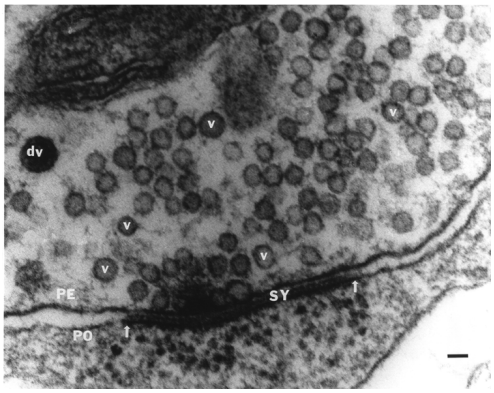
The intracellular space, or cytoplasm, of a neuron generally has a voltage of -70 mV relative to the extracellular space (**resting membrane potential**); the neuron is said to be polarized. This voltage, or difference in charge, is the result of differential ion distribution which is actively maintained by specific protein pumps. When a sensory protein responds to stimulation, it will cause nearby ion channels (a kind of protein) to open. This allows positive ions to flow into the cell, causing it to depolarize and bring the voltage of the neuron closer to zero. This will usually trigger nearby voltage-gated ion channels to open, effectively causing charge to flow down the dendrite toward the cell body. At the cell body or at the axon, special ion channels that specifically allow sodium ions into the cell (voltage-gated sodium channels) cause a spike, or action potential. This is a fast increase in voltage up to about 50 mV, which is rapidly hyperpolarized by ion channel inactivation as well as the opening of potassium channels that permit potassium ions to flow out of the neuron, repolarizing the neuron back to -70 mV within a few milliseconds. This spike travels down the axon and terminates at the synapse. The conduction speed can be as fast as 10 – 100 m/s (compare that to rates of diffusion or active transport of proteins). The action potential takes an essentially analog signal and converts it to a more discrete or digital signal. Why is this discretization important? Likely for the same reasons that much of our electrical communication is digital: digitization increases robustness to noise, which enables more reliable signaling over larger distances.

How does a neuron output a signal?

Chemical synapses

At the end of an axon, there are synapses that come very close (20 nm) to dendrites of other neurons. Synapses consist of synaptic vesicles that contain neurotransmitters. When an action potential reaches a synapse, voltage-gated calcium channels in the membrane of the presynaptic neuron open and allow calcium to flow into the cell. This **calcium** is required for fusion of vesicles to the membrane, which releases the neurotransmitter to diffuse across the 20 nm space (which happens on the order of microseconds). The dendrite contains proteins called receptors that bind the neurotransmitter and cause electrical changes in the post-synaptic neuron. Depending on the ion channel that is eventually opened, neurotransmitters can either excite the post-synaptic neuron as described above (i.e. depolarize it), or inhibit it (i.e. keep it from triggering an action potential).

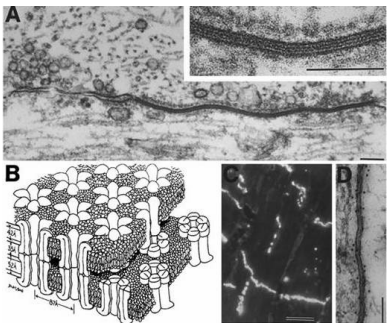
A neuron could also form a synapse with a muscle to generate behavior, and this synapse is called a neuromuscular junction (NMJ).
The canonical neurotransmitters are glutamate (an amino acid), GABA (a derivative of glutamate), glycine, and acetylcholine. Other common neurotransmitters include serotonin and dopamine.



http://web.as.uky.edu/Biology/faculty/cooper/CooperRes2.htm

[DRAW a chemical synapse]

Electrical synapses
The other form of signal output is through an electrical synapse, or gap junction. An electrical synapse consists of proteins called connexins that form a bridge between the membranes of 2 neurons. This 2 nm pore allows ions and other small molecules to move between the neurons. In this way, a voltage change can be transmitted quickly without any chemical intermediary.



http://electroneubio.secyt.gov.ar/Bennett-neurons'electrical_synchronization.htm

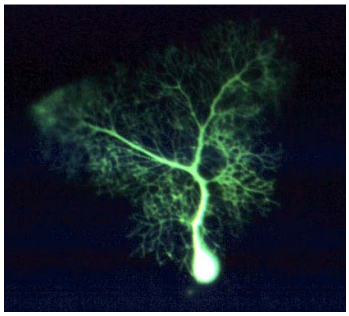
[NIKHIL]

TECHNIQUES FOR STUDYING NEURAL SIGNALING

Anatomy: the structure and connectivity of the nervous system

1) Electron microscopy: The "gold standard" for determining synaptic connectivity
    - EM is the best method for identifying synapses between neurons (as shown above).
    - If serial sectioning is done, you can trace a neuron's processes to ascertain the neuron's shape as well as all the synapses it makes.
    - In this way, the complete connectome of the *C. elegans* nervous system was reconstructed in the 1970s and 1980s.
    - The spatial resolution of EM is on the order of nanometers (10^{-9} m) or lower
    - But there is no ability to see changes over time as the sample must be immobilized and permeablized using fixation
    - Synapses, can be seen with basic EM

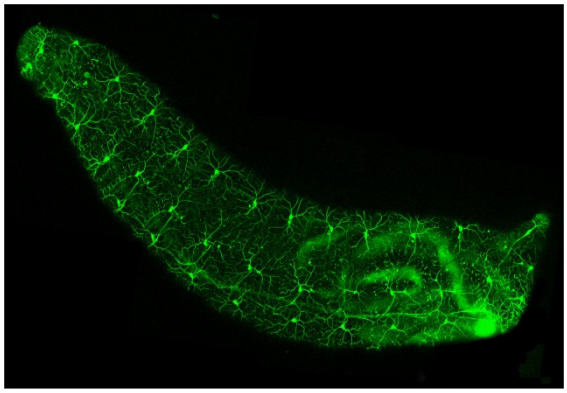
2) Light microscopy:
    - Usually much easier than EM
    - The morphology of a neuron can be visualized by filling the neuron with a dye.
    - Traditionally, this was done by inserting a small pipette to a neuron and allowing the dye to diffuse into the neuron, as shown below. This is commonly done to identify the cell type after intracellular or patch clam recording.



Purkinje cell from the cerebellum filled with Lucifer Yellow.
http://education.med.nyu.edu/courses/brainandbehavior/courseware/neurohist/html/0101.html

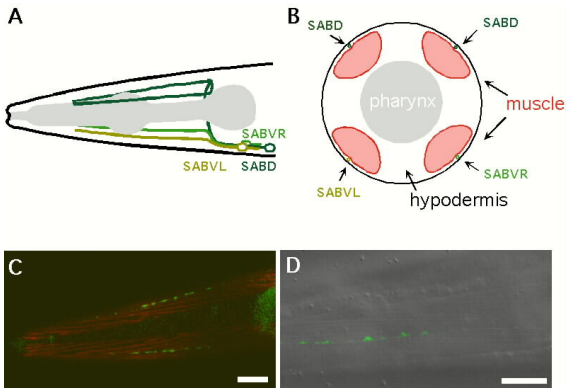
- TRANSGENES: Today, we can generate and incorporate DNA called transgenes into an organism
    - A promoter that expresses in a specific neuron or set of neuron can be fused with a coding gene (e.g. GFP or green fluorescent protein)

- Once the neuron is transfected with the transgene it will glow and its structure can be visualized under a microscope.
- ADVANTAGE over dye technique: A whole strain is created so you have an infinite number of labeled animals to experiment with



Sensory neurons labeled in a Drosophila larva. A repetitive pattern can be seen.
http://www.nig.ac.jp/section/emoto/emoto-e.html

- Transgenic technique can be extended so that specific proteins can be marked with GFP, not just whole cells – this way you can see where the proteins are inside of a cell
- If a presynaptic protein is tagged with GFP, can see synapses!
- CAVEAT: But you don't know who the synapse is connected to



Neuromuscular junctions labeled by tagging a synaptic protein with GFP in a worm.
http://thalamus.wustl.edu/nonetlab/ResearchF/elegans.html

- But anatomy and the connectome don't tell us function: they allow us to <u>generate hypotheses</u> for circuit function
    1. It is important to recognize that just because 2 neurons synapse does not mean that they must use that capacity for a specific function.

2. Conversely, just because 2 neurons are not anatomically connected does not mean that they are not functionally connected, as they may use a non-synaptic slower signal to communicate in a slow circuit (we won't talk much about these)
    - Anatomy is just a starting point and does not in fact prove anything about the functional components of a neural circuit.
- People often wonder why the *C. elegans* nervous system wasn't solved when the connectome was detemined in the 70s and 80s – this is why. We have a map, and we're using this to help constrain the possible functional routes. But the functional routes still need to be determined!
- Now that we've identified neural structure, Tots will talk about how to study neural signaling

[END NIKHIL]

[TOTS] ***Physiology: molecular signals that underly circuit function***

1) Electrophysiology: Generally speaking, there are two ways of recording electrical activities in neural circuits. An electrode could be placed outside a neuron (extracellular recording), or inside a neuron (intracellular recording).
    - intracellular recording: One could record the membrane potential of a neuron by inserting a fine glass electrode inside the cell. This could be done with a sharp intracellular electrode or using a patch-clamp recording configuration. EXPLAIN DIFFERENCE with DRAWING. This could be done under visual guidance, or blindly. Intracellular recordings can not only record spikes but also subthreshold activities and synaptic currents. In fact, not all neurons signal via action potentials (e.g. neurons in the retina, non-spiking interneurons in invertebrates), and in these cases, it is necessary to record their membrane potentials directly. Intracellular recordings also allow the experimenter to stain the recorded cell by dye injection. Even though this method is very powerful, it is very difficult to maintain a stable recording over long time especially if the animal is behaving.
    - paired recordings: Electrophysiological methods could also be used to measure the connection within a neural circuit. Ideally, one would insert electrodes into both the presynaptic neuron and its postsynaptic neuron simultaneously. Then, one could inject a depolarizing current to activate the presynaptic neuron and examine its effect on the postsynaptic neuron to determine the whether the synapse is excitatory or inhibitory, and the strength of the synapse. If two neuron are connected via an excitatory synapse, one could observe an **exciatatory post-synaptic potential (EPSP)** from the postsynaptic neuron. Conversely, if neurons are connected via an inhibitory synapse, one could observe an **inhibitory post-synaptic potential (IPSP).** However, paired intracellular recordings are very difficult in practice. To overcome this difficulty, one could replace presynaptic current injection with optogenetic activation of the presynaptic neuron (e.g. channelrhodopsin-2-assisted circuit mapping).

It will be ideal if these electrophysiological measurements are done from freely moving animals. However, the rapid movement of the animal does not allow stable recording especially for intracellular recordings. Oftentimes, the nerve fibers that connect to the muscles are often cut to prevent the animal from moving. The behavior could still be read out by recording activities from remaining muscles or motor neurons. This is called "**fictive behavior**."

By combining transgenic labeling with electrophysiology, we can now reliably record from the same neuron across individuals.

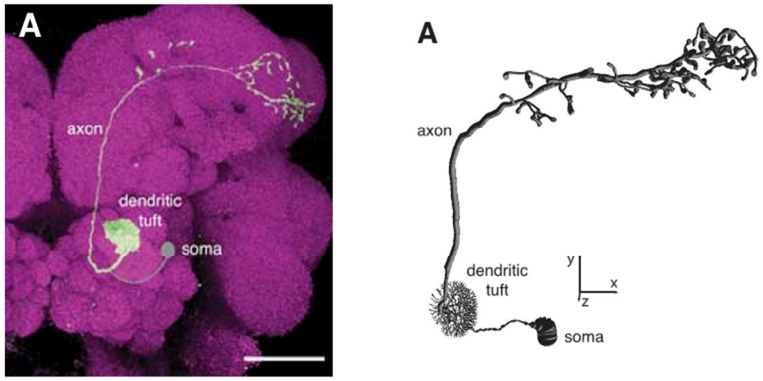


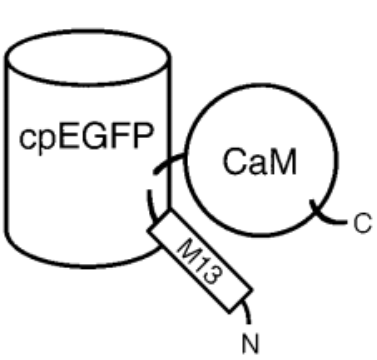
Figure 1: An example of a genetically-labeled invertebrate neuron. Projection neuron in the antenna lobe of Drosophila (Gouwens & Wilson, 2009).

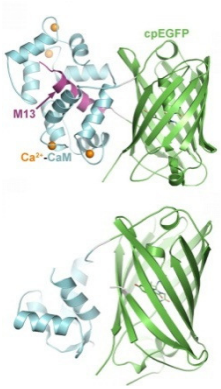
[END TOTS]

[NIKHIL]

2) <u>Imaging methods</u>:
   - Electrophys has excellent temporal resolution and the ability to measure currents of individual channels, it has some disadvantages:
     i. It can **only measure electrical signals**. If there is functionally relevant signaling that somehow bypasses current, it has no way of measuring it.
     ii. It **lacks spatial resolution**, and has some difficulty localizing current to sub-cellular compartments
     iii. It has **low throughput**, as it takes some time to setup the recording and it can only record from a small set of neurons at once
   - Imaging methods present an opportunity to solve these problems, though at a cost of temporal resolution.
   - Most common method uses a neural activity sensor called GCaMP
     i. A fluorescent protein like GFP is circularly permuted, and a binding domain is added on either side. When that binding domain binds its target, the conformation of the protein changes, reconstituting GFP and increasing its brightness. The most popular version of this approach is used in a calcium

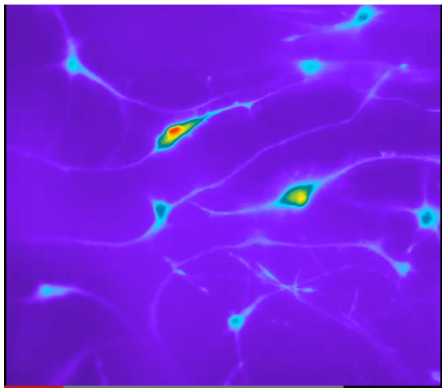
sensor called GCaMP, shown below. When calcium concentrations increase, GCaMP fluorescence increases, and vice versa.





http://www.nature.com/nbt/journal/v19/n2/pdf/nbt0201_137.pdf
http://en.wikipedia.org/wiki/GCaMP

- Another advantage is that with imaging you can monitor the calcium activity of **a whole set of neurons** all at once. This video also demonstrates the kind of subcellular resolution you can observe with imaging.



http://www.youtube.com/watch?v=SwuBk4l4y5E

- VOLTAGE SENSORS: In addition to molecular sensors, there are also voltage sensors which change fluorescence intensity in response to changes in voltage across the membrane. These are not yet widely used but I expect them to get better over time. They can have much lower latency than calcium imaging, but currently the signals are quite weak compared to calcium imaging.

- CONCLUSION: Like anatomical methods, physiological methods are also not definitive for identifying a functional neural circuit. They provide hints and hypotheses for what the circuit might be, but **just because a neuron responds to a stimulus does not mean that it is involved in the behavior-of-interest that that stimulus elicits**. It may be involved in a different response that you're not currently studying (examples of this will

be shown when we look at specific circuits), or perhaps not involved in any behavior at all.

- To definitively determine the components of a functional neural circuit, you can't simply observe but must also manipulate the candidate neurons to determine if they have a functional role.

- Neurons can be manipulated through either inactivation, which shows necessity, or activation, which shows sufficiency with regard to the measured output, which can be downstream neural activity or the behavioral response.

Manipulating physiology to show causal function

1) Inactivation: To show that a neuron is functionally required (or necessary) in a neural circuit for behavior, you have to show that loss of the neuron results in partial or complete loss of the behavior. There are many ways to inactivate the function of individual neurons:
    a) Ablate the neuron: Historically this is one of the most popular techniques.
        - Ablation can be done by **laser** (e.g. in *C. elegans*), which is very precise.
        - A disadvantage of ablation is that it is usually done in younger animals while the behavior is measured in the adult, so the neuron may not be acutely required in the circuit but may just be required for the proper development of other neurons that are acutely required in the circuit.
        - It is also important to exclude the possibility that remaining neurites are not functional following soma ablation.
    b) Block the neuron's synapses: Popular in *Drosophila* and also used in *C. elegans*, synaptic silencing is a transgenic method where protein which blocks chemical synaptic transmission is expressed in a neuron of interest.
        - **Tetanus toxin** is the most commonly used.
        - Synapses can also be silenced by using genetic mutants that are defective in synaptic signaling.
        - SENSORY: This can be helpful in determining whether the input a neuron receives is dependent on synaptic signaling, or if the neuron itself receives input directly from the environment or via gap junctions.
    c) Hyperpolarize the neuron:
        - Traditionally, neurons were silenced through electrophysiological methods injecting a hyperpolarizing current via the electrode.
        - Recent advances in optogenetics allow for more convenient light-activated hyperpolarization.
        - Transgenic expression of a hyperpolarizer coupled with light activation can acutely block all synaptic output.
        - Gap junctioned neurons are presumably hyperpolarized as well.

- Removal of light restores function.
- Common hyperpolarizers include Halorhodopsin and Arch.

2) Activation: Once you've shown that neural inactivation disrupts the behavioral response, additional evidence for its function can be garnered through activation studies. In some cases, **inactivation will not show any effect because there is redundancy in the circuit**. Activation techniques can circumvent this problem.
    a) Restore genetic function in the neuron:
        - After showing that mutation in a specific gene disrupts the behavioral response, restoring that gene in a specific neuron shows that it likely functions there.
        - For example, a specific neurotransmitter may be released by a neuron in response to a stimulus.
        - Mutants lacking the gene for neurotransmitter synthesis will have disrupted behavior.
        - If specific restoration of the neurotransmitter synthesis gene in the neuron can restore the behavior, we say that the transgene "**rescues**" and conclude that the neurotransmitter functions in that neuron in normal animals.
    b) Depolarize the neuron:
        - Conventionally, electrodes were used to depolarize a neuron.
        - Additionally, changing the extracellular ion concentration (e.g. increasing potassium ions) also depolarizes the neuron (though not specifically).
        - Optogenetics now enables light-activated proteins which depolarize the neuron while light is on.
        - If the behavior can be elicited by activation of the neuron in the absence of the regular environmental stimulus, we're comfortable concluding that the neuron likely acts in the circuit for behavior.
        - A common depolarizer is channelrhodopsin.

If both inactivation and activation experiments result in the expected effect on the behavior, and a physiological response is also observed in the neuron, it's reasonable to conclude that the neuron acts in the neural circuit for the behavior.

[END NIKHIL]

[TOTS]

In addition to the basic manipulations discussed above, one could also manipulate neural activity to ask more specific questions about the neural circuit.

a) "reset" experiment: Let's say a neural circuit exhibit oscillatory behavior. How do you know whether a particular neuron is involved in the generation of that oscillation or simply following the oscillation generated by other neurons? A classical way to

disambiguate these two is to inject current into the neuron of interest, and observe whether that perturbs the phase of the ongoing oscillation. If it does, the neuron is part of a circuit that generates the oscillation. If the current injection has not effect, then the neuron is receiving oscillatory signals that are generated elsewhere (e.g. leech swimming, locust flight).

b) "substitution" experiment: Imagine a situation in which you have found a neuron that is activated when the reward is given to the animal. To show that this neuron is sufficient to act as a unconditioned stimulus (US), one could activate this neuron paired with conditioned stimulus (CS) and show that this is sufficient to induce conditioning.

c) cooling experiment: Cooling certain brain regions could be used to inactivate the neurons reversibly (e.g. differential time course in the involvement of different brain areas in honeybee conditioning). In addition, temperatures of a particular brain region could be manipulated in a more subtle way (within 10 degrees of normal temperature) to observe the effect of a neural dynamics within a specific brain region on behavior.

[END TOTS]