Remote activation of synapses with channelrhodopsin-2 in *Drosophila* larvae

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Goals

1) Observe the behavioral effects of remotely activating motor neurons that express the blue light sensitive ion channel, Channelrhodopsin-2.
2) To record spontaneous miniature synaptic potentials (i.e. 'minis') at the neuromuscular junction (NMJ) of Drosophila larvae
3) To evoke excitatory junctional potentials (EJPs) at the fly NMJ by activating Channelrhodopsin-2 in motor neurons with blue light pulses
4) To perform quantal analysis and calculate the quantal content of light evoked EJPs

Part I: The life of the fly

The fruit fly, *Drosophila melanogaster,* has become a widely used model organism in the biological sciences. It has been particularly useful as a system for studying the genetic basis of neural function and animal behavior. One of the big advantages of working with flies is that their life cycle is short. The *Drosophila* life cycle can be broken into four main phases (Figure 1):

1) Embryo (22 hours @ 25^{o}C):
About ½ mm long, oval and white.

2) Larva: (4 days @ 25^{o}C).
Larvae burrow through food when young and go through two molts. The largest of the larvae are known as '3rd instars' and can often be found crawling on the walls of the vial directly before they begin pupation. Because of their large size, we will be using 3rd instar animals in our experiments.

3) Pupa (6 days @ 25^{o}C)
Once they have grown to full size, 3rd instars will crawl away from food and look for a high spot in which to pupate. Once they find a spot, they will stop moving and their body will darken and harden. During pupation, the animals will reorganize their entire body plan.

4) Adult (several weeks at @25^{o}C)
After pupation, adult flies emerge and immediately begin mating and laying eggs.

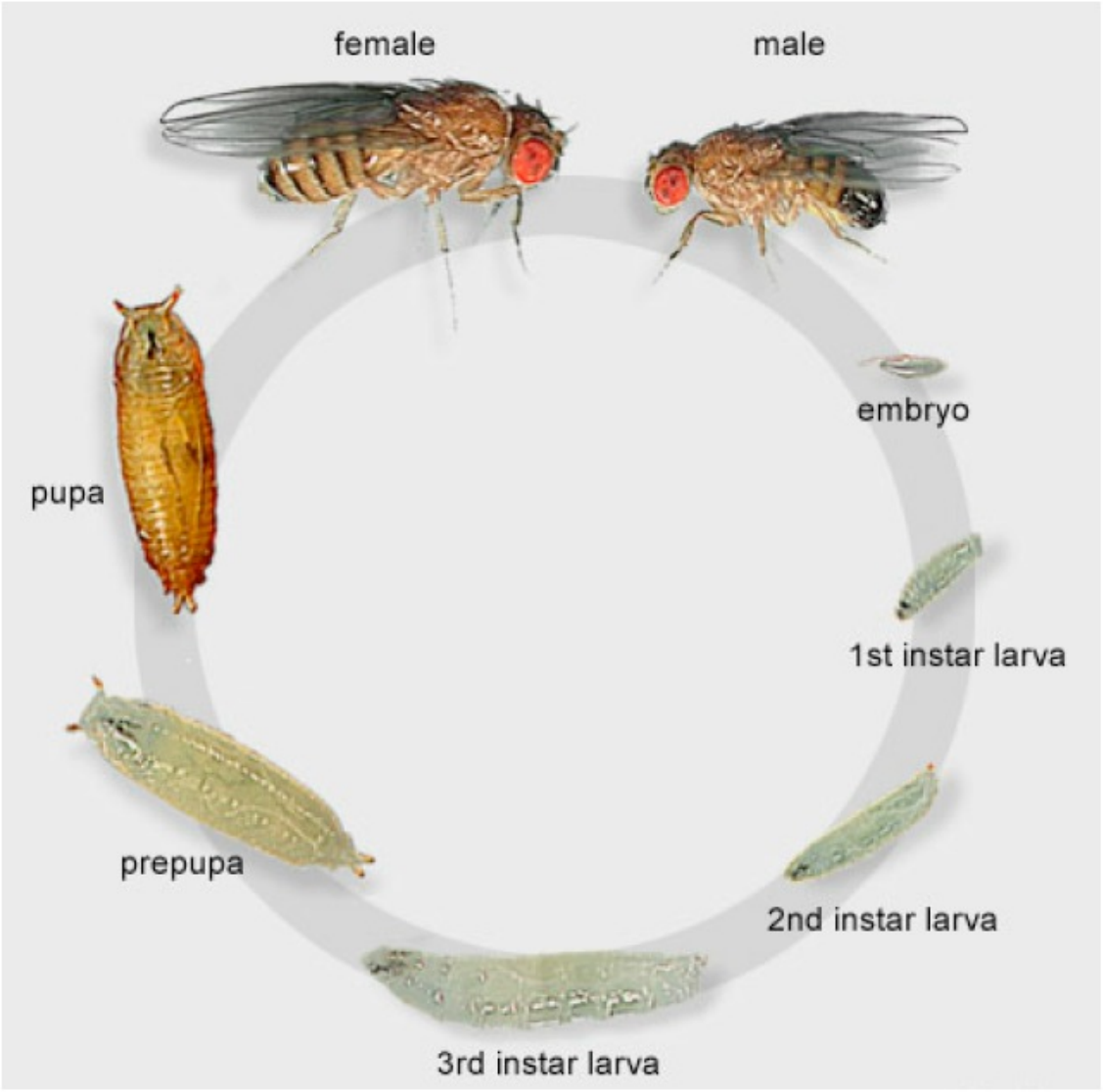

Figure 1: The life cycle of the fruit fly, *Drosophila melanogaster*

Part II: The larval neuromuscular junction

In the 1970's, *Drosophila* researchers began recording synaptic potentials from neuromuscular junctions (NMJs) in larvae. Since that time, this NMJ preparation has been used extensively to study the genetic basis of synaptic transmission. At this point, it is probably one of the most intensively studied synapses in all of neuroscience. In class, you will impale larval muscles with intracellular electrodes and record the activity of this synapse.

We will record from body wall muscles on the ventral side of the larvae. The cells themselves will appear as thin 'bricks' under a dissecting scope. The same muscle patterns are repeated over and over in each body segment. A diagram of the muscle organization is shown below in Figure 2. The cell bodies of motor neurons that innervate the body wall muscles are located within a structure called the ventral ganglion (looks like a carrot with two balls above it in Figure 2). Long, thin, nerves stretch from this structure to the each body wall segment.

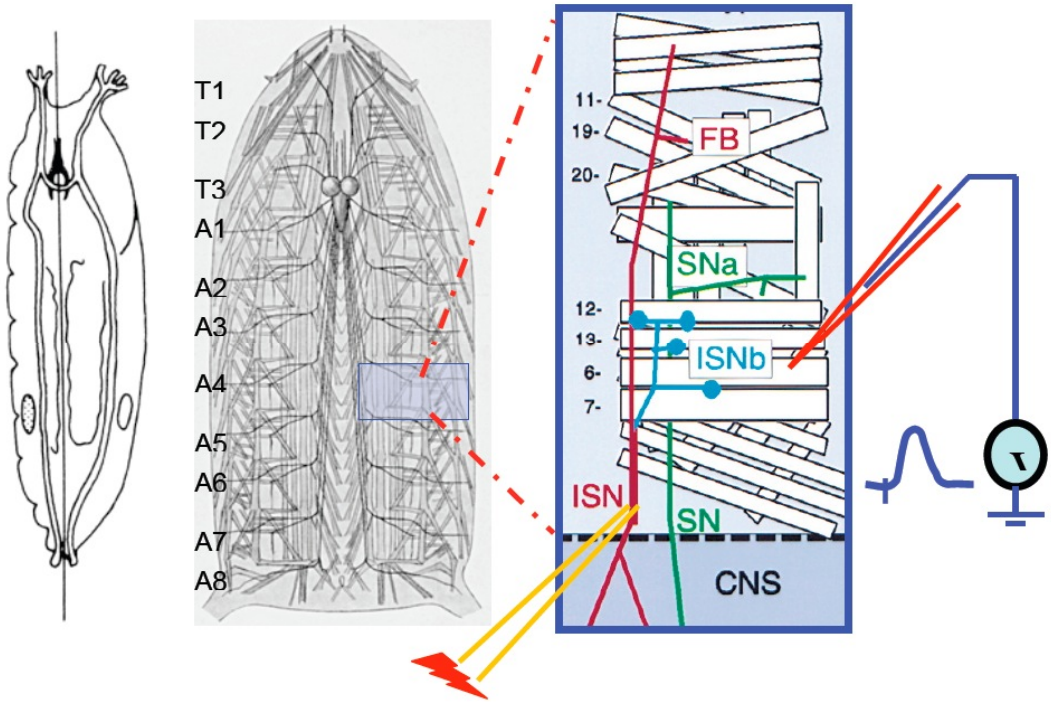

Figure 2: Schematic of the larval NMJ preparation. On left is an intact animal. The center shows what a larva looks like when it is dissected and filleted out. On right is a diagram of the area highlighted in the fillet (rotated 90^{o} counterclockwise). The muscle cells look like 'bricks'. Image courtesy of Bing Zhang, Oklahoma University.

We will provide you with dissected preparations at first. Then if you have time, you can try the dissection yourself. The larval preparation is nice because huge parts of the prep can be damaged, but as long as there is one healthy segment, you can still get a good recording. Resting membrane potentials should be -30 to -60 mV. Impale these muscle cells just as you would a snail neuron or other cell.

The larval NMJ functions in many ways like a central synapse in our brains. Larval motor neurons generate action potentials and these travel down the axon and depolarize the nerve terminal. This depolarization opens voltage gated Ca^{++} channels, and Ca^{++} rushes into the nerve terminal. This, in turn causes release of synaptic vesicles containing neurotransmitter. Motor neurons in flies contain the

neurotransmitter glutamate. Released glutamate activates receptors on the muscle, causing depolarization (and in larvae, muscle contraction).

We will generate an action potential in the motor neuron, then record the resulting depolarization in a muscle. These are called ‘excitatory junctional potentials’ (EJPs); an example of one is shown in on the right side of Figure 3.

One of the interesting things about synapses is that they are actually very leaky things. Even in the absence of action potentials, synapses will continue to spontaneously release small packets (i.e vesicles) of neurotransmitter. Synapses are basically constantly ‘dribbling’ out neurotransmitter, even when nothing is happening in the neuron. These vesicles travel across the synaptic cleft and result in very small depolarizations. These are called miniature EJPs (or just ‘minis’). Minis are usually visible when recording from larval muscles. Examples are shown on the left side of Figure 3.

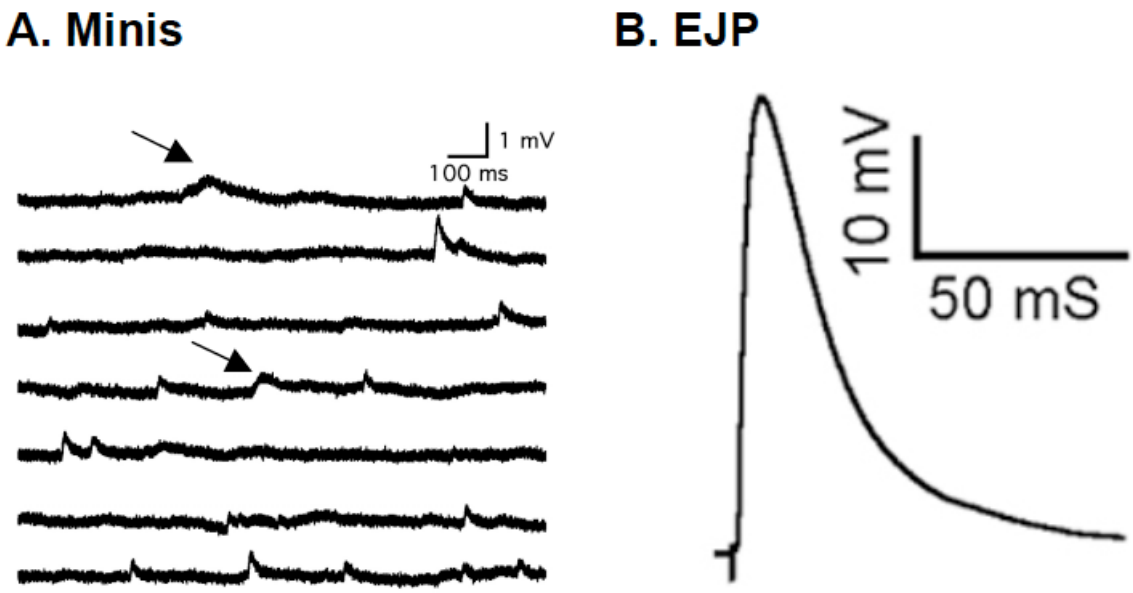

Figure 3: Mini EJPs and an evoked EJP from a larval muscle. Note that there are two types of minis. Some have sharp rise times, and some have slow rise times (arrows). The ones with slow rise times are actually from electrically coupled neighboring muscle cells. Image courtesy of Bing Zhang, University of Oklahoma.

Each mini represents the actions of single vesicle of transmitter and is the smallest building block of an evoked EJP. The amplitude of each mini is often called ‘quantal size’. The amplitude reflects the amount of transmitter release and how many receptors are present on the postsynaptic side.

One common way to obtain information about the basics of how a synapse works is to figure out how many mini EJPs it takes to make an evoked EJP. This is called the 'quantal content' of a synapse. To do this, we divide the average amplitude of an EJP by the average amplitude of minis. This tells us how many vesicles (or 'quanta') are released in response to an action potential. This analysis can be a very powerful way to determine exactly how synaptic function is altered in mutant animals and even in human genetic disorders. In lab, you will measure the quantal content of EJPs at the larval NMJ.

Part III: Remote activation of genetically engineered neurons with blue light pulses

To measure the quantal content of a nerve terminal, we need to be able to evoke action potentials in larval motor neurons. One way to do this is to electrically stimulate the nerve with a suction electrode (like the ones for used for crayfish work). We're going to try something different. We will use a new genetic tool for remotely stimulating neurons with blue light.

In 2003, a researcher named Georg Nagel isolated and characterized a gene that codes for the blue light activated cation channel, Channelrhodopsin-2 (ChR2) (Nagel *et al.*, 2003). This channel serves as a blue light detecting mechanism the green algae, *Chlamydomonas reinhardtii*. Nagel showed that when ChR2 is expressed in neurons, blue light pulses could depolarize neurons and make them fire action potentials. This technology is now being exported to a wide variety of model organisms. This is opening up new possibilities for research aimed at understanding the neural basis of animal behavior. It is also being developed as a potential tool for restoring nervous system function in clinical settings.

In flies, we use the GAL4-UAS system (Brand & Perrimon, 1993) to express ChR2 in selected subsets of cells in the fly brain. This allows us to remotely activate specific neurons in freely behaving animals just by shining light on them. See the references below (Schroll *et al.*, 2006; Hwang *et al.*, 2007) for research articles using this technique in flies.

We have engineered flies with ChR2 expressed in larval motor neurons for this lab. We have also hooked up Blue LEDs to your electrophysiology rigs. You will be able to deliver short light pulses and, hopefully evoke EJPs in your larval preparations. You will also be able to activate neurons in intact animals; you will be able to examine the behavioral effects of stimulating motor neurons in freely behaving larvae.

Part IV: Lab exercises

Behavior:

1) Obtain two vials of flies (one has larvae with ChR2 in motor neurons, the other is a wild-type control)
2) Place larva of each type on separate Petri dishes
3) Observe their natural movement
4) Focus blue light on individual larva
5) Using the controller box, deliver short (~1 sec) blue light pulses to the larva. *Does the light-pulse affect either group in anyway? Does increasing the duration of the pulse affect either group in any way? Which group do you think has ChR2?*

NMJ Preparation:

1) Obtain pre-dissected larva from instructor
2) Rinse the preparation with fresh chilled saline (do this every 5-10 minutes)
2) Place preparation on rig and locate muscles
3) Load a 15-30 Mohm glass electrode with 3MKCl onto electrode holder
4) Adjust blue light so that it is focused on preparation. Do not stimulate the preparation constantly for long periods of time (i.e. over one minute).

Recording:

1) Insert electrode into cell (same as any other intracellular recording!)
2) Record minis and measure mini size
3) Use Rig software to deliver short (1-20 ms) pulses of blue light. Change the duration until you are able to see a large EJP. *Why would lower duration pulses not yield a response?*
4) Measure amplitude of light evoked EJPs at a constant duration (20 ms)
5) Calculate quantal content of the nerve terminal by dividing average amplitude of EJPs at 20 ms stimulation by average amplitude of minis
6) If you are unable to see minis in your recordings, try increasing the intensity of light pulses *Are there changes in the response as you increase duration?*
7) If you are unable to see minis in your recordings, try giving trains of high frequency light pulses (i.e. 10 events at >1Hz) *How do EJP parameters (amplitude, time course), etc change during stimulus trains?*

WORKS CITED

Brand, A.H. & Perrimon, N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, **118**, 401-415.

Hwang, R.Y., Zhong, L., Xu, Y., Johnson, T., Zhang, F., Deisseroth, K. & Tracey, W.D. (2007) Nociceptive neurons protect *Drosophila* larvae from parasitoid wasps. *Curr Biol*, **17**, 2105-2116.

Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P. & Bamberg, E. (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc Natl Acad Sci U S A*, **100**, 13940-13945.

Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Voller, T., Erbguth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E. & Fiala, A. (2006) Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in *Drosophila* larvae. *Curr Biol*, **16**, 1741-1747.