

## Video Article

# Electrophysiological Recordings from the Giant Fiber Pathway of *D. melanogaster*

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## Abstract

When startled adult *D. melanogaster* react by jumping into the air and flying away. In many invertebrate species, including *D. melanogaster*, the "escape" (or "startle") response during the adult stage is mediated by the multi-component neuronal circuit called the Giant Fiber System (GFS). The comparative large size of the neurons, their distinctive morphology and simple connectivity make the GFS an attractive model system for studying neuronal circuitry. The GFS pathway is composed of two bilaterally symmetrical Giant Fiber (GF) interneurons whose axons descend from the brain along the midline into the thoracic ganglion via the cervical connective. In the mesothoracic neuromere (T2) of the ventral ganglia the GFs form electro-chemical synapses with 1) the large medial dendrite of the ipsilateral motoneuron (TTMn) which drives the tergotrochanteral muscle (TTM), the main extensor for the mesothoracic femur/leg, and 2) the contralateral peripherally synapsing interneuron (PSI) which in turn forms chemical (cholinergic) synapses with the motoneurons (DLMns) of the dorsal longitudinal muscles (DLMs), the wing depressors. The neuronal pathway(s) to the dorsoventral muscles (DVMs), the wing elevators, has not yet been worked out (the DLMs and DVMs are known jointly as indirect flight muscles - they are not attached directly to the wings, but rather move the wings indirectly by distorting the nearby thoracic cuticle) (King and Wyman, 1980; Allen *et al.*, 2006). The di-synaptic activation of the DLMs (via PSI) causes a small but important delay in the timing of the contraction of these muscles relative to the monosynaptic activation of TTM (~0.5 ms) allowing the TTMs to first extend the femur and propel the fly off the ground. The TTMs simultaneously stretch-activate the DLMs which in turn mutually stretch-activate the DVMs for the duration of the flight. The GF pathway can be activated either indirectly by applying a sensory (e.g. "air-puff" or "lights-off") stimulus, or directly by a supra-threshold electrical stimulus to the brain (described here). In both cases, an action potential reaches the TTMs and DLMs solely via the GFs, PSIs, and TTM/DLM motoneurons, although the TTMns and DLMns do have other, as yet unidentified, sensory inputs. Measuring "latency response" (the time between the stimulation and muscle depolarization) and the "following to high frequency stimulation" (the number of successful responses to a certain number of high frequency stimuli) provides a way to reproducibly and quantitatively assess the functional status of the GFS components, including both central synapses (GF-TTMn, GF-PSI, PSI-DLMn) and the chemical (glutamatergic) neuromuscular junctions (TTMn-TTM and DLMn-DLM). It has been used to identify genes involved in central synapse formation and to assess CNS function.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/2412/>

## Protocol

### 1. Equipment and Materials

1. These experiments use a standard electrophysiology setup comprised of a stimulator, a stimulus isolation unit, two microelectrode amplifiers, a data acquisition system and a computer with collection software. Additional equipment includes a Faraday cage, a stereomicroscope on a boom stand, a vibration isolation table, a light source, and a recording platform.
2. Five micromanipulators are used. Two micromanipulators require fine controls for positioning the recording electrodes, while the other three micromanipulators only require gross controls to position the two stimulation electrodes and the ground electrode. The micromanipulator for the DLM recording electrode is placed at the tail end of the preparation (left of experimenter) and the micromanipulator for the TTM recording electrode is placed between the experimenter and the side of the preparation (slightly to the left of the experimenter). The two micromanipulators that will hold the stimulation electrodes are placed at the head of the preparation (right of experimenter). The micromanipulator for the ground electrode is placed at the far side of the preparation.
3. Pull glass recording microelectrodes with resistances of 40-60 M $\Omega$  and store flat in a dish supported by wax. For stimulation, two electrolytically sharpened tungsten electrodes are used. A tungsten wire, or a third electrolytically fabricated electrode is used as a ground. The stimulating and ground electrodes are prepared and attached to the micromanipulators before the start of the experimental session and need not be replaced for the duration of the session.

## 2. Preparing the *D. melanogaster*

1. Once your equipment is set up, it's time to prepare the flies. Anesthetize the flies by cooling on them ice or by using CO<sub>2</sub>. If CO<sub>2</sub> is used, then allow sufficient time (about 20mins) for the effects of the gas to wear off prior to beginning the experiment.
2. Use forceps to transfer the flies gently by their legs to a dish containing a platform of soft wax sloped at an angle of approximately 45°. The next four steps are done under a dissecting microscope away from (but close to) the recording equipment.
3. The next step is to secure the fly in wax. Orient the fly ventral side down, with its anterior facing upwards on the slope. Using a pair of fine forceps, extend the legs outwards, in pairs, and push them into the wax.
4. Familiarize yourself with the location of the muscles to be recorded from: the dorsal longitudinal muscle, or DLM, and the tergotrochanteral muscle, or TTM. The subcuticular attachment sites of the DLMs correspond with the region between the thoracic midline and the anterior dorsal bristles (or setae). The TTM attachment sites are located dorsally of the posterior and anterior supra-alar bristles. Making sure that the wings will not obstruct access to the DLM or TTM fibers, hold the wings outward and 'glue' them to the wax.
5. Using a fine pair of forceps, pull the proboscis outward carefully, and secure it by immersing it into the wax. This is a critical step that requires some practice since the proboscis is soft and is easily detached from the rest of the head. If that happens, discard the fly and start over. Failure to secure the head in this way leads to problems when inserting the stimulating electrodes through the eyes.

## 3. Placing the Electrodes

1. Once the fly is anchored to the wax, transfer the dish with the attached fly underneath the stereomicroscope that is located inside the Faraday cage. Orient the fly sideways with the head of the fly to the right of the experimenter.
2. The next step is to insert the electrodes. Ground and stimulating electrodes can be inserted without looking through the microscope. Good recordings rely on precise impalement, so it is a good idea to practice handling the micromanipulators. Bring the electrodes close to the sites of insertion with the help of micromanipulators to facilitate their proper placing and subsequent recordings.
3. Lower the ground electrode into the posterior end of the abdomen using the adjustment wheels on the micromanipulator. To place the sharpened tungsten stimulating electrodes in the brain, use the micromanipulator to position the tip of one of the electrodes so it just touches one of fly's eyes. Do the same with the other so both electrodes are just touching the outside of each eye. Then push the electrodes, in turn, through each eye so the tips of the electrodes reach the brain situated at the back of the head capsule (about 2-3mm).
4. Correctly placed electrodes will activate the Giant Fiber System. To test that the stimulating electrodes are placed correctly, apply a short (0.03 ms) stimulus of 30-60 V across the stimulating electrodes, and look for movement of the wings and twitches of the flight/leg muscle.
5. The next step is to back-fill the glass microelectrodes with 3M KCl using a Hamilton or heat-pulled plastic syringe, and place them into the fine-control micromanipulators. Properly inserted microelectrodes can be used for several rounds of experiments.
6. The first recording electrode will be inserted into a DLM fiber. There are two bilaterally symmetric DLMs; each one is composed of six individual muscle fibers. The recordings can be done from any of the six fibers; however, the most commonly used are DLM fibers 45a and 45b due to their good accessibility through the dorsal side of the thoracic cuticle, and the fact that both fibers are innervated by the same motoneuron.
7. Using the micromanipulator on the side farthest from you, insert a recording electrode into DLM fiber 45a or b. The slope of the platform allows the DLM electrode to enter the dorsal cuticle at a ~60-90° angle, which aids penetration. Use the software in oscilloscope mode and look at the computer monitor while inserting recording electrodes into the thorax. When the electrode has entered a muscle the baseline will drop to near zero or a negative value. Test with a single stimulus to see if you can observe the muscle response.
8. Insert the other recording electrode into the TTM closest to you. This electrode is inserted laterally, in front of you, due to the location of the muscle attachment site. Again observe the monitor while doing this and test with a single stimulus once the trace indicates the electrode is in the muscle.

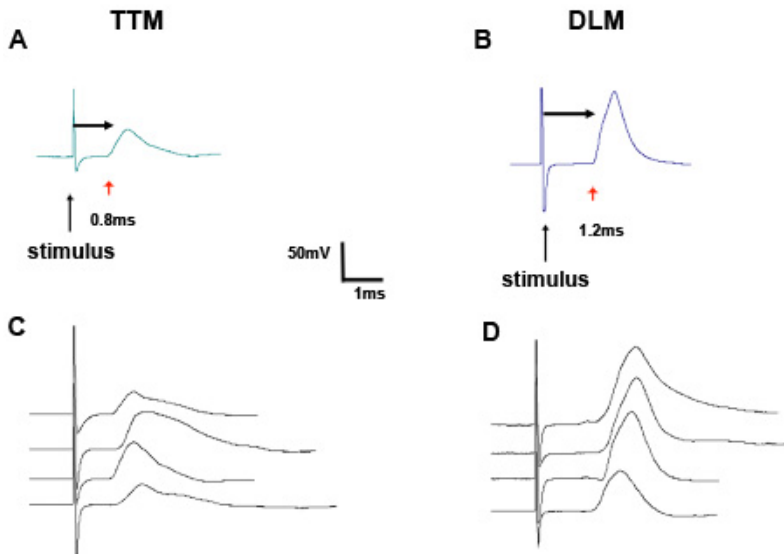
## 4. Stimulation and Recording

1. You are now ready to begin stimulating the brain and recording responses from the leg and flight muscles. Apply a short (0.03 ms) stimulus across the stimulating electrodes starting at 30 V and increasing to 60 V until you observe a response (i.e. a muscle twitch, and a muscle cell depolarization as observed on the computer monitor). For the remainder of the experiment, set the voltage 5-10 V above the response threshold.
2. To measure response latency, give at least 5 single stimuli with a 5 second rest period between each stimulus.
3. Determine the "frequency of following" by providing trains of stimuli at different rates. Typically 10 trains of 10 stimuli are given at 100Hz (10ms between each stimulus), 200Hz (5ms between each stimulus) and 300Hz (3ms between each stimulus). Allow a rest period of 2 seconds between each train of stimuli.

## 5. Results: Response Latencies and Frequency of Following in the Giant Fiber Pathway

1. The response latency is the time between stimulation of the brain and depolarization of the muscle. This figure compares the response latencies for DLM and TTM to a single stimulus. Latencies between 0.7 and 1.2 ms for the GF-TTM pathway and between 1.3 and 1.7 ms for the GF-DLM pathway indicate a healthy preparation and proper recording technique. The latencies can vary with genotype, genetic background, temperature and age.  
**Figure 1 (A and B).** Representative traces showing responses recorded from the TTMs and DLMs following a single stimulus applied to the brain.
2. As shown here, recordings from the TTM show more variability in terms of amplitude and shape of the postsynaptic potential (PSP) compared to those from the large DLM fibers; this increased variability is due to the small size of the TTM muscle fibers. This variability, however, does not affect the response latency values for the Giant Fiber-to-TTM pathway.  
**Figure 1 (C and D).** Further 'response latency' traces from 4 individual flies for both the TTM and DLM. Note TTM traces exhibit variability in PSP shape but response latency is unaffected. For DLM there is less variability in PSP shape.

### Response Latencies



3. Compare the "frequency of following" at 100 Hz, 200 Hz, and 300 Hz by calculating the proportion of successful responses (out of 10) for both DLM and TTM pathways at each stimulation frequency. At 100 Hz, both TTM and DLM follow the stimuli 1:1. At stimulation frequencies above 100 Hz, the DLM responses start to show failures because the intermediary chemical synapse between two interneurons does not have sufficient time to recover between stimuli. The TTM responses, however, remain 1:1 with stimuli even beyond 300Hz.
- Figure 2.** Representative traces showing the "frequency of following" recordings. At 100Hz, both TTMs and DLMs respond to all 10 stimuli (left). At 200Hz, the DLM responses start to fail (asterisk).

### Frequency of Following



## 6. Representative Results

Wild type short-latency responses (stimulated electrodes are placed in the eyes, bypassing sensory receptors and triggering the GF circuit directly) depend on genotype, genetic background, temperature and age, and range between 0.7 and 1.2 ms for the GF-TTM pathway and 1.3 and 1.7 ms for the GF-DLM pathway (Tanouye and Wyman, 1980; Thomas and Wyman, 1984; Engel and Wu, 1992; Allen and Murphey, 2007; Phelan *et al.*, 2008; Augustin *et al.*, unpublished). This very short TTM latency is due to the robust GF-TTMn electrochemical synapse of the monosynaptic pathway and the longer DLM latency occurs because of the disynaptic nature of the pathway as well as the presence of a chemical synapse (PSI-DLMn). Intermediate- and long-latency responses (>3 ms) result from the activation of the GF afferents and are achieved either by using a lower intensity stimulation or providing a visual ("light-off") signal. At 100Hz both TTM and DLM should follow the stimuli 1:1. Above 100Hz DLM responses will start to show failures as the chemical synapse between PSI and the DLMns does not have sufficient time to recover between stimuli less than 10ms apart. TTM responses, however, will remain 1:1 with stimuli even beyond 300Hz (Tanouye and Wyman, 1980; Engel and Wu, 1992; Allen *et al.*, 2007; Martinez *et al.*, 2007). Mutations in the *shakB* gene, encoding a *Drosophila* gap junction channel ("innexin"), significantly increase the response latency of the GF-TTM pathway (~1.5 ms) while the GF-DLM branch is unresponsive (Allen and Murphey, 2007; Phelan *et al.*, 2008). The mutant response can be restored by stimulating thoracic ganglia directly, demonstrating that the delayed effect is not due to disrupted neuromuscular transmission. The ability to follow high frequency stimulation is also impaired in these mutants compared to wild type flies where the GF-DLM and GF-TTM pathways are usually able to follow 10 stimuli with 1:1 ratio up to 100 Hz

and 300 Hz, respectively. It is important to note that these frequencies are considerably above normal stimulation frequencies received by the contracting muscles during the sustained flight (3-10 Hz) (Hummon and Costello, 1989).

Another parameter used to describe the stability of the GFS outputs is the "refractory period", or the minimal time between twin stimulus pulses that still produces two responses from the muscle. The refractory time varies between 1-4 ms for TTMs and 7-15 ms for DLMs. The comparatively long refractory period for DLMs is due to relatively labile chemical synapses at the PSI-DLMn junction (Tanouye and Wyman, 1980; Gorczyca and Hall, 1984; Engel and Wu, 1992; Banerjee *et al.*, 2004; Allen and Godenschwege, 2010).

## Discussion

One of the most important things one has to pay attention to when trying to obtain high quality recordings is the proper orientation and health of the preparation. Ideally, the fly should still be alive at the end of the recording session and responsive to electrical stimuli. For the recording electrodes to most efficiently penetrate the thoracic exoskeleton, the fly should be glued to the surface in such a way as to form a right angle with the electrodes; if necessary, the insertion of electrodes can be facilitated by removing a portion of the dorsal thoracic cuticle with a tungsten scalpel thus exposing the DLM flight muscle (this step offers an additional advantage of making it harder for the tips of glass electrodes to break). Also, the care must be taken to avoid pushing the electrodes through the subcuticularly located DLMs and TTMs. The head of the fly should be well secured to allow for the stimulating electrodes to be properly inserted into the brain and to prevent them from being pulled out during the recording session.

Due to its size and well-described morphology, the GFS represents one of the most accessible neuronal pathways in *Drosophila*. The permeability of electrical synapses to small molecular weight tracer dyes allows for the visualisation of electrically coupled neurons, and several available GAL4 lines make it possible to manipulate gene expression levels in a subset of cells or cell groups (Jacobs *et al.*, 2000; Allen *et al.*, 2006) In addition to the above mentioned advantages, both afferent and thoracic components of the circuit display properties such as habituation, spontaneous recovery and dishabituation, making the *Drosophila* GFS a convenient model system for studying neuronal plasticity (Engel and Wu, 1996).

## Disclosures

No conflicts of interest declared.

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