PROTOCOL

1) Equipment and Materials

1.1) For all recordings a standard electrophysiology “rig” is used comprising a stimulator (e.g S48 Square Pulse Stimulator, Grass Instruments), a stimulus isolation unit (e.g. SIU5 RF Transformer Isolation Unit, Grass Instruments), two microelectrode amplifiers (e.g. Model 5A Microelectrode Amplifier, Getting Instruments), a data acquisition system (e.g. Digidata 1440A analogue-digital converter, Molecular Devices) and a computer with collection software (e.g. Axoscope 9.0, Axon Instruments, USA). Additional equipment includes a Faraday cage, a stereomicroscope (e.g. Wild M5) on a boom stand, a vibration isolation table (e.g. TMC), a light source (e.g. Fostec), a recording platform and 5 manual micromanipulators. Three micromanipulators only need gross controls (e.g. MM33, Sutter Instruments) to position the two stimulation electrodes and the ground electrode and two require finer controls for positioning the recording electrodes (e.g. MHW-3, Narishige)

1.2) Recording glass microelectrodes (resistance: 40-60 MΩ) are pulled using a Sutter P-95 puller. For stimulation, two electrolytically (NaOH) sharpened tungsten electrodes are used. A tungsten wire, or a third electrolytically fabricated electrode is used as a ground.

2) Recording Setup

2.1) Flies are anaesthetized by cooling on ice or by using CO_2 (allowing sufficient time for the effects of the gas to wear off).

2.2) Flies are then transferred gently by their legs to a dish containing a platform of soft wax sloped at an angle of approximately 45° . The fly is secured in the wax ventral side down, with the anterior facing upwards on the slope, by extending the legs outwards, in pairs, and pushing them into the wax. The wings are then held outwards and ‘glued’ to the wax as well. 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 (Allen and Godenschwege, 2010). It is important that the wings do not obstruct the access to the muscles since this can adversely impact the subsequent quality of the recordings.

2.3) Using a fine pair of forceps, the proboscis is pulled outward carefully and secured by immersing into the wax. This is a critical step that requires some practice since the proboscis is soft and gets easily detached from the rest of the head. If that happens, it is better to 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) Placement of Electrodes

3.1) Learning how to handle the electrode-holding micromanipulators deftly by practice is advised since good recordings rely on precise impalement. Bringing the electrodes close to the sites of insertion will facilitate their proper placing and subsequent recordings.

3.2) The ground electrode is lowered into the posterior end of the abdomen using the adjustment wheels on the micromanipulator.

3.3) The sharpened tungsten stimulating electrodes are then pushed through the fly's eyes. The tips of electrodes should reach the brain situated at the back of the head capsule. Correct placement of stimulating electrodes will result in GFS activation. If everything is done correctly, wing movements and flight/leg muscle twitches can be observed when a short (0.03 ms) stimulus of 30–60 V is applied across the stimulating electrodes.

3.4) The glass (recording) electrodes are back-filled with 3M KCl using a Hamilton or heat-pulled plastic syringe. These are then inserted into DLM fiber 45a or b using a fine-controlled micromanipulator. Each of the two bilaterally symmetrical DLMs is composed of six individual muscle fibers. Even though the recordings can be done from any of the fibers, the most commonly used are DLM fibers 45a and 45b (nomenclature according to Miller, 1965), due to their good accessibility through the dorsal side of the thoracic cuticle, and the fact that both fibers are innervated by the same motorneuron. It is best to place them into a DLM fiber furthest from the experimenter. The slope of the platform allows the DLM electrode to enter the dorsal cuticle at a ~60-90° angle, aiding penetration. Another filled recording electrode is placed into the contralateral (nearer the experimenter) TTM. The TTM electrode is inserted laterally, in front of the experimenter, due to location of the muscle attachment site.

4) Stimulation and Recording

4.1) The brain is stimulated as described in Step 3.3, setting the voltage 5-10 V above the response threshold. For response latency recordings, at least 5 single stimuli are given to each fly with a 5 s rest period between each stimulus.

4.2) The response latencies for DLM and TTM to a single stimulus can be monitored and compared. Expected values for control recordings indicate a healthy preparation and proper recording technique. Due to the small size of the muscle fibers, recordings from the TTM show more variability in terms of amplitude and shape of the postsynaptic potential compared to those from the large DLM fibers. This variability, however, does not affect the response latency values for the GF-TTM pathway.

4.3) The frequency of following is determined 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). A rest period of 2 s is given between each train.

4.3) The frequency of following at 100, 200, and 300 Hz can be compared by calculating the proportion of successful responses (out of 10) for both DLM and TTM pathways at each stimulation frequency.

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 and1.7 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).

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DISCLOSURES:

We have nothing to disclose.

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