Video Article

Electrophysiological Recording of The Central Nervous System Activity of Third-Instar *Drosophila Melanogaster*

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Abstract

The majority of the currently available insecticides target the nervous system and genetic mutations of invertebrate neural proteins oftentimes yield deleterious consequences, yet the current methods for recording nervous system activity of an individual animal is costly and laborious. This suction electrode preparation of the third-instar larval central nervous system of *Drosophila melanogaster,* is a tractable system for testing the physiological effects of neuroactive agents, determining the physiological role of various neural pathways to CNS activity, as well as the influence of genetic mutations to neural function. This *ex vivo* preparation requires only moderate dissecting skill and electrophysiological expertise to generate reproducible recordings of insect neuronal activity. A wide variety of chemical modulators, including peptides, can then be applied directly to the nervous system in solution with the physiological saline to measure the influence on the CNS activity. Further, genetic technologies, such as the GAL4/UAS system, can be applied independently or in tandem with pharmacological agents to determine the role of specific ion channels, transporters, or receptors to arthropod CNS function. In this context, the assays described herein are of significant interest to insecticide toxicologists, insect physiologists, and developmental biologists for which *D. melanogaster* is an established model organism. The goal of this protocol is to describe an electrophysiological method to enable the measurement of electrogenesis of the central nervous system in the model insect, *Drosophila melanogaster*, which is useful for testing a diversity of scientific hypotheses.

Video Link

The video component of this article can be found at https://www.jove.com/video/58375/

Introduction

The overall goal of this approach is to enable researchers to quickly measure the electrogenesis of the central nervous system (CNS) in the model insect, *Drosophila melanogaster*. This method is reliable, quick, and cost-efficient to perform physiological and toxicological experimentation. The CNS is essential for life and therefore, the physiological pathways critical for proper neural function have been explored extensively in an effort to understand or modify neural function. Characterization of the signaling pathways within the arthropod CNS has enabled the discovery of several chemical classes of insecticides that disrupt invertebrate neural function to induce mortality while limiting off-target consequences. Thus, the ability to measure the neural activity of insects is of significant interest to the field of insect toxicology and physiology since the nervous system is the target tissue of the majority of deployed insecticides[1]. Yet, continued growth of fundamental and applied knowledge regarding the insect nervous system requires advanced neurophysiological techniques that are limited in feasibility, since current techniques are labor intensive and require a high expense, insect neural cell lines are limited, and/or there is limited access to the central synapses of most arthropods. Currently, characterization of most insect neural proteins requires the target to be cloned and heterologously expressed for subsequent drug discovery and electrophysiological recordings, as was described for insect inward rectifier potassium channels[2], insect ryanodine receptor[3], mosquito voltage-sensitive K^+ channels[4], and others. To mitigate the requirement for heterologous expression and the potential for low functional expression, Bloomquist and colleagues aimed to induce a neuronal phenotype in cultured *Spodoptera frugiperda* (*Sf*21) cells as a novel method for insecticide discovery[5,6]. These methods provide a valid approach for the development of new chemistry, yet they oftentimes create an insurmountable bottleneck for the characterization of pharmacological agents, identifying mechanisms of insecticide resistance, and characterization of fundamental physiological principles. Here, we describe an *ex vivo* method that enables the recording of electrical activity from a model insect that has malleable genetics[7,8,9] and known expression patterns of neural complexes[10,11,12] to enable the characterization of resistance mechanisms at the level of the nerve, the mode of action of newly developed drugs, and other toxicological studies.

The fruit fly, *D. melanogaster,* is a common model organism for defining insect neural systems or insecticide mechanism of action and has been established as a well-suited model organism for the study of toxicological[13], pharmacological[14,15], neurophysiological[16], and pathophysiological[17,18,19,20] processes of vertebrates. *D.melanogaster* is a holometabolous insect that performs complete metamorphosis, including a larval and pupal stage before reaching the reproductive adult stage. Throughout the developmental process, the nervous system

undergoes significant remodeling at different life stages, but the larval CNS will be the focus of this methodology. The fully developed larval CNS is anatomically simple with thoracic and abdominal segments that are fused and form the ventral ganglion, which represents an array of repeated and almost identical neuromeric units[21,22]. Descending motor nerves originate from the caudal end of the subesophageal ganglia and descend to innervate body wall muscles and visceral organs of the larvae. **Figure 1** describes the gross anatomy of the larval *Drosophila* CNS.

The *Drosophila* blood-brain barrier (BBB) develops at the end of embryogenesis and is formed by subperineurial glial cells (SPG)[21]. The SPG cells form numerous filopodia-like processes that spread out to establish a contiguous, very flat, endothelial-like sheet that covers the entire *Drosophila* CNS[23]. The *Drosophila* BBB has similarities to the vertebrate BBB, which includes preserving the homeostasis of the neural microenvironment by controlling the entry of nutrients and xenobiotics into the CNS[21]. This is a prerequisite for reliable neural transmission and function, yet the protection of the CNS by the BBB restricts the permeation of synthetic drugs, most peptides, and other xenobiotics[24,25], which introduces potential problems when characterizing potencies of small-molecule modulators. The method uses a simple transection to disrupt this barrier and provide ready pharmacological access to the central synapses.

The greatest strength of the described methodology is the simplicity, reproducibility, and relatively high-throughput capacity inherent to this system. The protocol is relatively easy to master, the setup requires little space, and only an initial financial input is necessary which is reduced to reagents and consumables. Further, the described method is completely amendable to record the central descending nerve activity of the house fly, *Musca domestica*[26].

Protocol

1. Equipment and Materials

1. Prepare the required components (listed in **Table of Materials**) of the electrophysiology rig to perform suction electrode recordings of the *Drosophila* CNS.
   NOTE: Prior to experimentation, it is necessary to construct chambers for dissection of the *Drosophila* CNS and to be used for bathing the ganglia in saline during recordings. A step-by-step outline of chamber construction is provided below.
2. Prepare the larval chamber.
   1. Melt the black wax using a hot plate.
   2. Pour 2 mL of melted wax into a chamber that has a maximum volume of 2 - 2.5 mL.
   3. Let the wax cool and harden for approximately 2 h.
   4. Use a razor blade to carve a hole in the hardened wax that will hold a volume of 500 µL, which has a surface area of approximately 0.5 cm^2 and a depth of approximately 0.25 cm.

2. Equipment and Software Configuration

NOTE: The setup of the extracellular recording is briefly described below.

1. Prepare equipment for dissection and recording.
   1. Position the tissue preparation, microscope, suction electrode, micromanipulators, light source inside the Faraday cage to reduce noise and eliminate extraneous electrical fields (**Figure 2**).
   2. Connect (preferably solder) ground and positive wires onto shielded alligator clips that will be connected to the bath and microelectrode holder, respectively.
      NOTE: Soldered and exposed wires can be covered with aluminum foil to reduce noise.
   3. Connect the ground and positive wires to input 1 of the AC/DC differential amplifier.
   4. Connect the *data acquisition system* to the AC/DC differential amplifier by connecting a Bayonet Neill-Concelman (BNC) cable from input 1 of the data acquisition system to the channel 1 output of the amplifier.
      NOTE: It is recommended to incorporate a 50/60 Hz noise eliminator between the data acquisition software and the amplifier. The use of a BNC T-connector is required.
   5. If desired, include an audio monitor into the setup by connecting input 1 of the audio monitor to input 1 of the data acquisition software.
   6. Fill the 10 mL syringe with saline and connect it to the pressure port of the electrode holder.
      NOTE: Ensure that no air bubbles exist between the syringe, the Ag/AgCl pellet, and the electrode opening.
2. Prepare the software for dissection and recording.
   NOTE: The outline of methods for software setup is based on the acquisition/analysis software listed in the **Table of Materials** which will digitize the raw electrical output and convert the data to spike frequency. However, other software can be used and multiple recording conversions can be used.
   1. Open the acquisition/analysis software.
   2. Click on "setup" from the main toolbar and select "channel settings," which will open a dialogue box.
   3. Reduce the number of total channels to three.
   4. For Channel 1, select a range of 100 mV.
   5. For Channel 2, click on the "calculation" tab that will open a drop-down menu. Select "cyclic measurements," which will open a second dialogue box.
   6. Select channel 1 as the source. Then on the drop down menu measurement, select unit spike at events. Finally, in the detection settings area, from the drop down menu select simple threshold.
   7. To convert the electrical activity into a rate plot expressed in hertz, the 3rd channel must be set in arithmetic mode: On the input window, type in "1000*smoothsec(ch2,2)". Then select as the units drop down menu hertz.
      NOTE: Successful recordings can be performed with multiple different recording setups, but "Unit spike at events and simple threshold" is likely the easiest, since it allows for adjustment of the threshold above the background activity for each individual recording.

Recordings under the setting of "Custom-Source Input" increase reproducibility of the data assuming the background activity is not different between recordings.

8. Close dialogue boxes to return to the main screen. The y-axis should be expressed in Hz and the x-axis in time.

3. Dissect and Prepare the Larval *Drosophila* CNS

NOTE: Methods for larval CNS dissection are clearly illustrated in Hafer and Schedl[27], but these previously published methods reduce the length of the descending neurons that are important for measuring spike frequency. Here, an additional method is outlined to excise the larval CNS that maintains long, intact descending neurons.

1. Saline preparation.
    1. Prepare the dissection and recording saline to the following in mM[28]: 157 NaCl, 3 KCl, 2 CaCl$_2$, 4 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES); titrate pH to 7.25.

2. Dissect the larval *Drosophila* CNS.
    1. Identify and extract a wandering third-instar *Drosophila melanogaster* from the culture vial and place into 200 µL of saline (**Figure 3A**).
    2. Grasp the mouth hooks with a pair of fine forceps and grasp the abdomen of the maggot with a second pair of forceps (**Figure 3B**).
    NOTE: Do not apply too much pressure to the abdomen as this can result in tearing of the thin cuticular layer of the maggot.
    3. Gently pull the mouth hooks and abdomen in different directions to separate the caudal end of the maggot from the head region and expose the viscera of the maggot (**Figure 3C**).
    NOTE: The CNS will be intertwined with the trachea and digestive tract. Do not cut the CNS away from any tissue to prevent cutting the descending peripheral nerves.
    4. Tease the CNS out of the digestive tract and trachea with forceps (**Figure 3D**).
    5. If necessary, disrupt the blood brain barrier by manually transecting the CNS posterior to the cerebral lobes with Vannas spring scissors.
    NOTE: This should be done based on the physiochemical properties of the chemical being used. The red line in **Figure 4A** is the suggested transection point and the transected CNS with intact descending peripheral nerve trunks shown in **Figure 4B**. The transected CNS is ready for experimentation after completion of step 3.2.5.

4. Extracellular Recording of *Drosophila* CNS.

1. Prepare the CNS for recording.
    1. Pull a glass pipette electrode from borosilicate glass capillaries to a resistance of 5-15 mΩ.
    2. Insert the transected CNS into the wax chamber that contains 200 µL of saline. Clamp an uncoated insect pin with the alligator clip soldered to the ground wire and insert the pin into the saline to complete the circuit.
    3. Using the micromanipulators, orient the electrode to the caudal end of the transected CNS. Eliminate recording of background noise by adjusting the threshold level in the acquisition/analysis software prior to contacting the peripheral nerve trunks.
    4. Draw any convenient peripheral nerves into the suction electrode by applying slight negative pressure on the syringe.
    NOTE: The baseline firing frequency is correlated to the number of neurons drawn into the electrode where more neurons oftentimes result in increased resistance of the electrode.

2. Begin extracellular recording of CNS descending nerve activity.
    1. Start the recording on the data acquisition software and monitor the baseline firing frequency. Allow the firing rate to equilibrate for 5 min prior to collecting baseline firing rate data.
    2. Discard the preparation and recording if the pattern of firing of control treatment is not a bursting pattern similar to that shown in **Figure 5A**.
    NOTE: Altered pattern of firing suggests abnormal or unstable activity of the central pattern generator, which can alter neural function.
    3. After 5 min, add 200 µL of saline + vehicle to bring the total volume of the chamber to 400 µL to begin recording control firing rates.
    4. After baseline has been established (3-5 min), withdraw 200 µL of saline and add 200 µL of the experimental agent solubilized in saline.
    NOTE: Dimethyl sulfoxide (DMSO) is the recommended solvent for lipophilic compounds and should not exceed 0.1% *v/v*.
    5. Apply drug concentrations in a serial manner (low to high concentrations) and record each concentration for a select period of time (determined by the investigator based on the chemical properties of the drug[29]). Label this time point of drug application in the acquisition/analysis software by including a comment that includes the drug and the final concentration.
    NOTE: Firing activity of the CNS will decline by approximately 10-20% after 30-50 min after the dissection and therefore, appropriate untreated controls should be performed, and drug treatments should not exceed this time period.

3. Analyze the data.
    NOTE: Multiple analyses can be performed on the collected data, such as changes in action potential bursts[29], spike waveform amplitude and time course, and determining mean spike frequencies after drug treatment[26,30,31,32]. The most common is determining the influence a drug has to the mean spike frequencies over a specified period of time, which is described below.
    1. Tabulate mean spike frequencies by selecting the entire region of interest (*i.e.,* the drug treatment time period) and determine mean spike frequencies automatically through the "Datapad" located on the main toolbar of the acquisition/analysis software.
    2. Determine the mean spike frequency of the CNS after drug treatment to the mean spike frequency of the vehicle control (baseline). Calculate the percent change of firing rate after drug treatment by the formula: (Treated Frequency/Baseline Frequency) × 100.
    3. Use the mean spike frequencies or percent firing of control for each concentration to construct a concentration response curve with standard graphing software.

4. Perform statistical analysis (*e.g.,* unpaired *t*-test) to determine significance between time points, concentrations, or drug treatments.
   NOTE: There are two primary methods for generating and analyzing concentration-response curves. The first method is one concentration per individual preparation. Here, the spike rate of the single concentration is normalized to baseline spike rate for each preparation. The benefits are reduced run down of the preparation due to shortened recording time, while the pitfalls are increased dissection time because this method will consist of 5-7 individual CNS preparations per concentration, and larger error bars on the averaged data set. The second method is multiple concentrations per individual preparation. Here, the spike rate for each of the 3-5 concentrations are normalized to the same baseline spike rate. The benefits are less dissection time and less variability between replicates for drug concentrations, while the pitfalls are the requirement of a fast-acting drug and unknown impact of previous drug concentration treatments on subsequent chemical treatments.

Representative Results

Spontaneous activity of the descending peripheral nerves arising from the *Drosophila* central nervous system can be recorded using extracellular suction electrodes with consistent reproducibility. Spontaneous activity of the excised and transected *Drosophila* CNS produces a cyclical pattern of bursting with 1-2 s of firing with approximately 1 s of near quiescent activity. For example, the CNS is near quiescent (1-2 Hz) for 0.5-1 s, followed by a burst (100-400 Hz) for approximately 1 s, and then returns to a near quiescent state (1-2 Hz) for 0.5-1 s (**Figure 5A**). This firing pattern is repeated every 2-3 s. The average spike discharge frequency of *Drosophila* CNS ranges from approximately 20-50 Hz over a 3-5 min period, when the threshold is set just above the baseline noise. The baseline firing frequency is correlated to the number of peripheral neurons drawn into the electrode and the seal formed between the electrode orifice and the ventral ganglia. Importantly, application of the chemical solvent DMSO at a final concentration of 0.1% does not alter the spike discharge frequency of the *Drosophila* CNS (**Figure 5A**).

This electrophysiological preparation provides a method to characterize the excitatory or depressant properties of various small molecules on the spike frequency of a well-characterized insect neural system. Propoxur, a known inhibitor of insect acetylcholinesterase (AChE), is a neuroexcitant and increased the spike discharge frequency of the transected *Drosophila* CNS in a concentration-dependent manner (**Figure 5B**). On the contrary, gamma-aminobutyric acid (GABA), a known inhibitory neurotransmitter acting upon the insect GABA-mediated chloride channel, is a neurodepressant and reduced the spike discharge frequency in a concentration-dependent manner (**Figure 5C**). Mean spike discharge frequencies can be determined across the recorded time period for each concentration to enable the construction of a concentration response curve to determine the 50% effective concentration (EC_{50}) to elicit a response. Here, propoxur is shown to have an EC_{50} of 338 nM (95% confidence interval (CI): 241-474; hillslope: 1.8; r^2: 0.77) with a concentration of 1 μM producing maximal excitation of the *Drosophila* CNS at 300% activity of control (**Figure 5D**)[26]. GABA is shown to have an IC_{50} of 1.1 mM (95% CI: 0.7-1.5; hillslope: 1.5; r^2: 0.95) with maximal inhibition at 5 mM (**Figure 5E**).

Oftentimes, molecular probes of neural systems are not able to be used in physiological or toxicological assays due to their lack of proper physiochemical properties that enable penetration of the blood brain barrier. For instance, monomeric tacrine is a potent (*ca.* 200 nM) inhibitor of insect AChE[33], yet does not alter the spike frequency of the intact *Drosophila* CNS (**Figure 6A**). However, disruption of the neural lamella and the blood brain barrier through transection posterior to the cerebral lobes resulted in a near immediate increase in the spike frequency of the *Drosophila* CNS after exposure to 100 μM monomeric tacrine (**Figure 6B**). Similar data have been previously described[30].
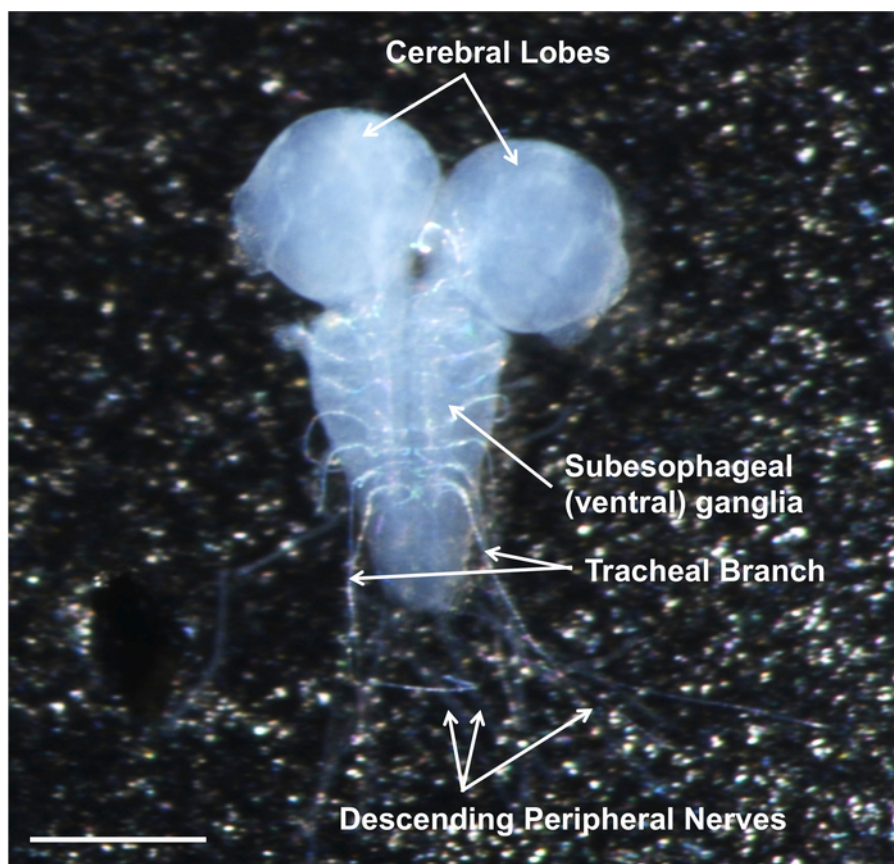
Figure 1: Excised CNS from third-instar *Drosophila melanogaster*. Arrows point to various anatomical structures of the CNS that correspond to the labels. The scale bar represents 250 µm. Please click here to view a larger version of this figure.
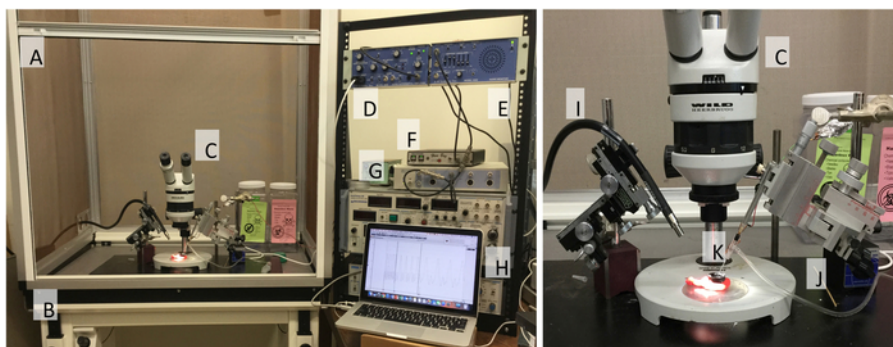

Figure 2: Electrophysiology setup that is used to perform extracellular recordings. (**A**) Faraday cage; (**B**) vibration table; (**C**) dissecting microscope; (**D**) AC/DC differential amplifier; (**E**) audio monitor; (**F**) noise eliminator; (**G**) data acquisition system; (**H**) computer running lab chart pro software; (**I**) fiberoptic cable with external illumination source; (**J**) micromanipulator; (**K**) microelectrode holder with pressure port with glass electrode and preparation wax dish. Please click here to view a larger version of this figure.
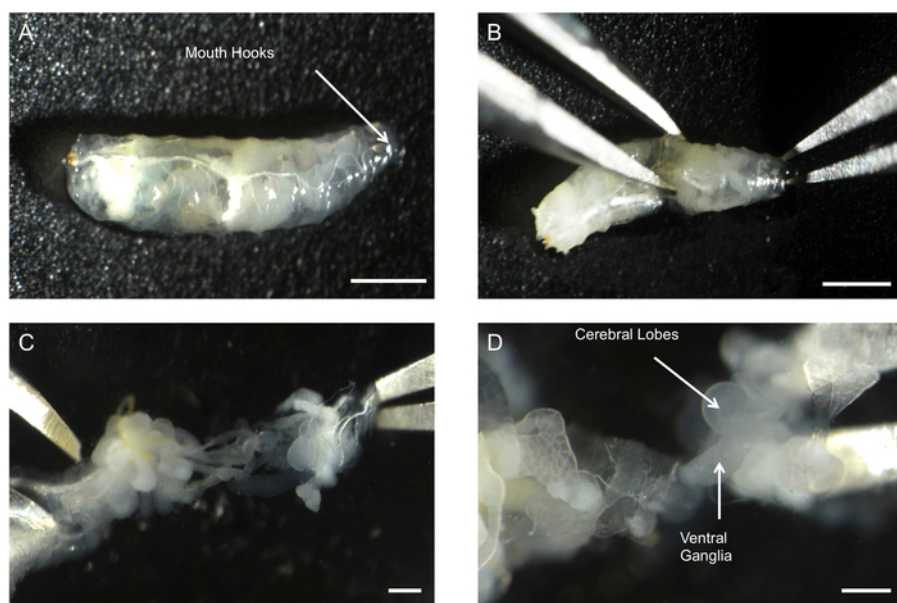
Figure 3: Method for excising the CNS from third-instar maggots. (A) Intact maggot submerged in 200 µL of saline. The arrow indicates the mouth hooks that are used for separation of the body wall. **(B)** Two pairs of forceps are placed at the middle of the maggot and on the mouth hooks to begin separation of the body wall. **(C)** Body wall is separated by applying slight and continuous pressure to expose the viscera. **(D)** CNS is clearly visible (white arrows) and is occasionally intertwined with the viscera. The scale bar represents 1000 µm, 750 µm, 500 µm, and 200 µm for panels A, B, C, and D, respectively. Please click here to view a larger version of this figure.
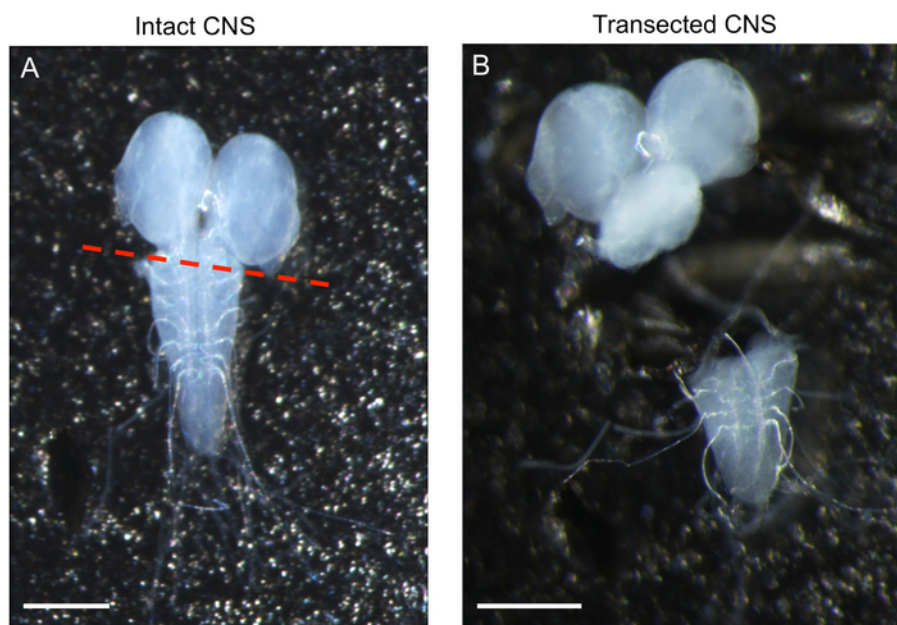

Figure 4: Disruption of the blood brain barrier by transecting the CNS. (A) Intact CNS with descending nerves clearly visible at the caudal end of the ventral ganglia. The red line indicates the location of transecting the CNS to disrupt the BBB. **(B)** A transected CNS with the caudal end of the ventral ganglia still exposing long descending neurons. The ventral ganglia can be discarded. The scale bar represents 200 µm for both panels. Please click here to view a larger version of this figure.
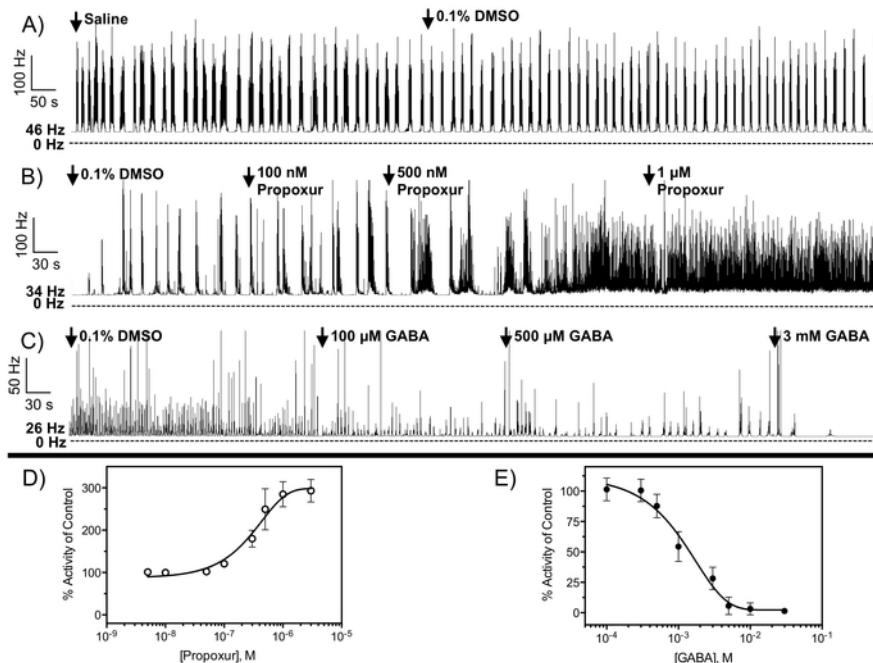
Figure 5: Neurophysiological recordings from the CNS of third-instar larvae of *D. melanogaster.* Representative nerve discharge traces before and after exposure to **(A)** DMSO, **(B)** propoxur, and **(C)** GABA. Initial firing frequencies in spikes/s (Hz) for each experiment are given to the left of each trace. Concentration-response curves for propoxur **(D)** and GABA **(E)** to CNS nerve discharge of *D. melanogaster* larvae from replicated recordings (n = 3-5 concentrations per curve, with each concentration replicated at least 5 times). Arrows represent point of drug application. Data points represent mean percent increase of baseline firing rate and error bars represent standard deviation. When error bars are absent, it is because they are smaller than the size of the symbol. Please click here to view a larger version of this figure.
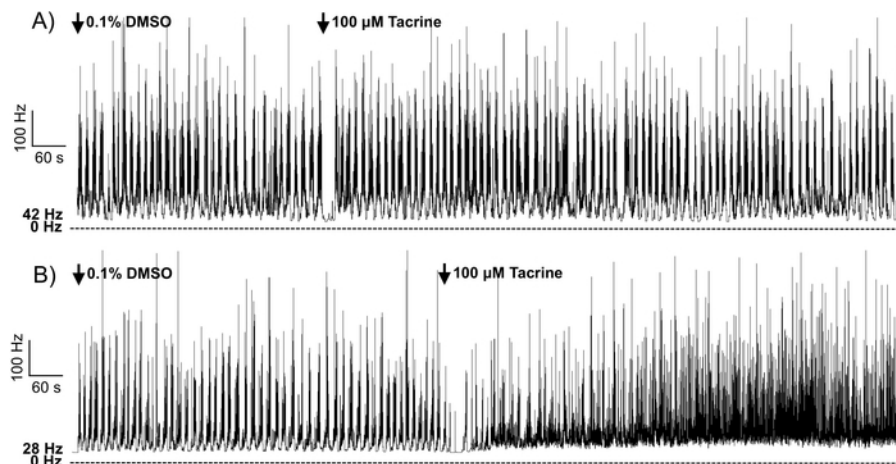

Figure 6: Increased penetration of tacrine into the nervous system after transection of the CNS. Representative recordings of **(A)** intact and **(B)** transected larval CNS exposed to monomeric tacrine, which was applied at the arrow. Initial firing frequencies in spikes/s (Hz) for each experiment are given to the left of each trace. Please click here to view a larger version of this figure.

Discussion

The details provided in the associated video and text have provided key steps in order to record the activity and spike discharge frequency of the *Drosophila* CNS *ex vivo*. The dissection efficacy is the most critical aspect of the method because short or few descending neurons will reduce the baseline firing rate that will result in large variances between replicates. However, once the dissection technique has been mastered, the data collected with this assay are highly reproducible and amendable for a wide variety of disciplines. One modification to the described method is the inclusion of an automated profusion system that will prevent the need to manually pipette the saline and chemical solutions into the CNS chamber. Inclusion of the profusion system will reduce disturbances of the CNS during the recordings, which occasionally occur during the application of drugs with manual pipettes.

This electrophysiological method exploits the utility of the preparation for incorporation into drug/insecticide discovery research. Furthermore, this preparation is amenable to the classroom for demonstration of fundamental concepts in neurophysiology. The method requires relatively modest financial investment and minimal preparation time while providing a robust and stable recording that can be employed to illustrate the influence

of drugs to the function of fly CNS. For instance, the financial burden of the recording rig is an initial cost of approximately $10,000 USD with minor subsequent costs (*e.g.,* saline salts, fly colony maintenance, *etc.*). Further, the time needed from CNS preparation to first recording is approximately 10 min. Although *Drosophila* are easy to maintain in culture within a laboratory or classroom, it should be noted that this suction electrode assay can also be performed using the CNS of the housefly, *Musca domestica*, and probably other muscoid fly larvae, as well. The pest status of *Musca domestica* to livestock suggests this assay could be of significant utility to research programs aiming to characterize the neuronal sensitivity of flies from different populations or to determine the potency of newly developed neurotoxicants for eventual control of infestations.

In addition to characterizing the potency of drugs, this preparation can be used to characterize the influence of genetic mutations and manipulation on the activity of the *Drosophila* nervous system. Previous work has shown that CNS-specific knockdown of the gene encoding the inward rectifier potassium (Kir) channel increased the baseline CNS firing frequency by approximately 2-fold when compared to control flies[31]. These data were combined with pharmacological data to ultimately speculate the physiological role Kir channels serve to *Drosophila* nervous system function.

Although this technique represents a powerful assay to test diverse toxicological and physiological hypotheses, limitations to the assay do exist and must be considered. For instance, the data generated through suction electrode recordings rarely provide conclusive evidence for the exact mode of action of a drug or precise physiological role for an ion channel and must be studied in tandem with more cell-based measurements, such as patch clamp electrophysiology. An additional limitation to this method is that the various receptors and ion channel sub-types do not influence the CNS spike rate in an equal manner. Therefore, it is possible that a modulation of a specific receptor or ion-channel subtype may not significantly influence the spike rate of the excised CNS, but may indeed have a critical effect on total nervous system function and/or integration of signals.

Although limitations exist, the data collected through suction electrode recordings do provide quality proof-of-concept data and allow researchers to generate hypotheses regarding mechanisms of action that can be validated through voltage-clamp electrophysiology, biochemical analyses, or through additional pharmacological testing. For example, the latter was performed to test the hypothesis that the insect repellent, *N,N*-Diethyl-3-methylbenzamide (DEET), was inhibiting the octopaminergic system in insect CNS in an effort to describe the mechanism of toxicity to mosquitoes and flies[26]. DEET was shown to be a neuroexcitant of housefly CNS activity and also altered the evoked excitatory postsynaptic potential at the neuromuscular junction, which are cholinergic and glutamatergic systems, respectively[26]. These data suggested that DEET was not likely to induce toxicity through cholinergic inhibition, as was previously suggested[34]. Recording the discharge frequency of the fly CNS after exposure to phentolamine, a known octopamine antagonist, showed a complete inhibition of the DEET-mediated neuroexcitation, but not that of propoxur, which provided significant evidence that DEET was more likely affecting the octopaminergic system than AChE[26]. These published data sets highlight the variety of hypotheses and experimental conditions that can be investigated with this method.

Disclosures

The authors have nothing to disclose.

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