We thank the reviewers for their time and comments regarding our manuscript submission. Our response to the reviewers' comments are in green below each individual comment.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

This has been completed

2. Please provide an email address for each author.

This has been completed

3. Please rephrase the Abstract to more clearly state the goal of the protocol.

This has been completed

4. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: World Precision Instruments (WPI), Item #M325, PowerLab and LabChart, ADInstruments, A-M Systems Inc. Model 1700 or Model 3000, PowerLab 4/35, AD Instruments, Narashige PC-100 or Sutter P-1000, Carolina Biological Supply, #974228, LabChart 8 (AD Instruments), etc.

This has been completed

5. Please revise the protocol to be a numbered list: step 1 followed by 1.1, followed by 1.1.1, etc.

This has been completed .

6. Lines 100-138: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.), or move the solutions, materials and equipment information to the Materials Table.

This has been completed

7. Lines 139-141, 158-167, 175-176, 201-206, 216-222, 281-283, 287-295, 301-305: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note."

This has been completed

8. Lines 212-215: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

   This has been completed and we have reworded the protocol and clearly indicated notes for each.

9. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

   This has been completed and we believe it follows a logical flow for recording.

10. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

    This has been completed

11. Please reference all data and figures in the manuscript. For instance, Figure 3C is not mentioned.

    Figure 3C was referenced in the text. We did not alter this.

12. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

   This has been completed by adding information regarding the dissection and the importance of the descending neurons in the first paragraph

b) Any modifications and troubleshooting of the technique

   We have referenced the potential modifications to protocol.

c) Any limitations of the technique

We have included the limitations of this technique in the Discussion section and have described how it is necessary to pair this method with other electrophysiological methods for full validation of the data

d) The significance with respect to existing methods

We believe the significance of this method was clearly discussed in the discussion section by showing relevance to high school classrooms, insecticide toxicology, insect physiology, mammalian disease research, and others.

e) Any future applications of the technique

We believe this has been described in the discussion section.

13. References: Please do not abbreviate journal titles.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The article reads very well and I feel a video of this technique would be a very valuable addition to the JoVE both for academic researchers and industry members with an interest in discovery and characterisation of novel insecticides, and investigation of resistance mechanisms. I agree that this is a neat approach based on its relatively simplicity, although there are a few potential hurdles that could be potentially quite time consuming for the inexperienced operator to get right. My minor concerns are therefore just suggestions of small details that could be added to help students avoid lost time.

Major Concerns:

None

Minor Concerns:

Relating to caveats/limitations addressed at L411-417: Some additional explanation may be warranted here to make it clear that the recorded CNS output will not necessarily be influenced equally by all subtypes of receptors that have a key role in spike propagation and synaptic transmission. Genetic manipulations which alter the CNS response to a drug may not always read through to the whole-organism activity. It is possible that a drug/toxin will target a receptor or ion-channel subtype that is not well-represented by the output of the CNS recorded in this way, but may nevertheless have a critical effect on nervous system function. An example would be a genetic manipulation that clearly alters an insecticide's effect on this CNS preparation whilst not having much effect on its overall toxicity. The technique is nevertheless useful in such situations because it can highlight the possibility of an alternative mode-of-action and inform additional experiments.

Thank you for this comment and we fully agree with the reviewers comments. However, we believe adding a section to describe potential differences between in vitro and in

vivo responses will convolute the paper and reduce readability. However, we have added a limitation to the method by describing how it is possible that the spike rate is not equally influenced by all receptors.

Relating to drug application method described L271-279: Manual pipette evacuation of 50% of bath volume could be expected to lead to considerable disturbance of the prep and introduction of noise artefacts into the recording, so it will be important to demonstrate clearly in the video how this is carried out. Is it necessary, for example, to suspend data-capture during this process? If the authors do not recommend continuous perfusion of the preparation for some reason it could be useful to explain why, because many students may instinctively try to adapt the method to continuous perfusion in order to minimise contact with the prep during recording and to allow for a simplified chamber construction step.

Thank you for this comment and we agree that this would be a positive addition to the manuscript. Therefore, we have included the use of an automated profusion system as a modification to the protocol that is described in the discussion.

L316-317: "…when the threshold is set just above the baseline noise" suggests a threshold crossing event-detection method. It would be helpful in the video to show the positioning of the count threshold relative to the baseline activity, as this will give a good idea of the signal to noise ratio that a student should be aiming to achieve.

We agree and this will be included in the video.

Relating to description of CPG activity L310-320: Firstly it would be good to refer the student to articles which explain central pattern generator (CPG) activity and its physiological significance, as this may inform or inspire the use of this method for the study/manipulation of genes that may function in these neural circuits. I would suggest the following citations:

Mulloney B, Smarandache C. Fifty Years of CPGs: Two Neuroethological Papers that Shaped the Course of Neuroscience. Frontiers in Behavioral Neuroscience. 2010;4:45. doi:10.3389/fnbeh.2010.00045.

Pulver SR, Bayley TG, Taylor AL, Berni J, Bate M, Hedwig B. Imaging fictive locomotor patterns in larval Drosophila. Neurophysiol. 2015 Nov;114(5):2564-77. doi: 10.1152/jn.00731.2015.

Possibly also this article, although the technical content is focussed on CPG activity that would not be recorded by the author's method: Sebastian Hückesfeld, Andreas Schoofs, Philipp Schlegel, Anton Miroschnikow, Michael J. Pankratz Localization of Motor Neurons and Central Pattern Generators for Motor Patterns Underlying Feeding Behavior in Drosophila Larvae PLOS One Published: August 7, 2015 https://doi.org/10.1371/journal.pone.0135011

We agree with the reviewer that inclusion of CPG neurons and the applicability of this assay to study the function of CPG gene manipulation to neural function. However, we do not think that discussing a list of neural proteins that this assay could be relevant do is pertinent to this methods paper. Therefore, we have not included a discussion of CPG activity from the perspective of using this assay to study the fundamental role in neural function.

Observed CPG firing patterns can be more irregular or erratic than those described in the manuscript, and this may be indicative of nerve damage or a poor transection of the brain. Rhythm greatly depends on sensory input and can become variable when sensory inputs are absent or intermittently failing (Reviewed Marder et al 2005 Curr Biol.). Some guidelines as to how to recognise abnormal or unstable CPG activity, as a guide to which preps should be rejected, would be therefore be a very useful inclusion in the video.

We have added a section (Step 4.2.2) noting the expecting pattern of CNS firing. This section reads, “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. Altered pattern of firing suggests abnormal or unstable activity of the central pattern generator, which can alter neural function.”

If the authors know of dissection/ablation procedures or particular measures that should

be undertaken in order to either preserve or eliminate these sensory inputs, or even to eliminate the CPG activity altogether, these would be very useful to include in the video. Generating statistically robust data for nicotinic receptor agents, for example, can be problematic by this method, because a combination of neuroexcitatory activity and receptor desensitisation leading to synaptic block will have contrary effects on the measured spike frequency -especially where the CPG circuit begins to fail. Eliminating the CPG activity before testing nicotinic agents would allow for more consistent and measurable output. [Eve Marder, Dirk Bucher, David J. Schulz, Adam L. Taylor Invertebrate Central Pattern Generation Moves along Current Biology , Volume 15 , Issue 17 , R685 - R699]

We agree with this reviewer that ablation of CPG activity would increase the stability and reproducibility of the recording. Unfortunately, we are unaware of a method to consistently reduce or eliminate CPG activity and believe that this step is outside the scope of this relatively simple method outline.

328-334: Description of analysis methods: Students may be unfamiliar with PowerLab 4/35 and Labchart software so a short statement on the way spike events are recognised (e.g. threshold crossing or template matching) would be informative.

This is noted in Step 2.2.7.

There are many options for processing the data, ranging from selecting the amplitude range of events to be counted, through to selection of the time ranges over which event counts are collected and compared. In the accompanying video some detail on the steps taken from handling of the raw data to generation of the C/R curves for a drug-response would therefore be helpful to the beginner. For example, do the authors recommend that dose response curves are constructed by generating multiple-dose response data from individual preparations and then averaging this data across different recordings, or that the data for each dose be obtained from individual larvae and normalised in each case to the pretreatment spike counts? My own experience is that C/R curves from this type of data tend to be noisy and reducing error bars can

involve a lot of replicate experiments.

Thank you for this suggestion. We have added in two different potential methods for CRC generation

Typos:

L50: of several chemical classes (of) insecticides

L261: where more neurons oftentimes reults (results)

L374-375: third instar larvae of D.melanogasgter. (melanogaster)

Thank you for pointing out our oversight. These have been corrected

Reviewer #2:

Manuscript Summary:

Swale et al. report a protocol for recording activity and spike discharge frequency of the central nervous system of the model organism Drosophila melanogaster.

This protocol is of great interest to researchers in the field of insect toxicology and its availability may help in elucidating mode of action of new insecticidal compounds.

Methods are described accurately and figures are very clear. Manuscript is also well written, so I suggest to accept this manuscript for publication in JoVe.

Major Concerns:

None

Minor Concerns:

line 395: "modest financial investment and minimal preparation time". "modest financial investment" I think the cost is a bit underestimated by the authors, as e.g. a data acquisition system and dissecting microscope are quite expensive; "minimal preparation time": could the authors give an idea about the time needed from preparation of CNS to first recording?

Thank you for this oversight. We have added two sentences into the discussion to describe the initial and continuous financial burden of this assay.