UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
Instituto de Fisiología Celular
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Dear Editor,

Please receive the revised manuscript entitled 'In vivo wireless optogenetic control of skilled motor behavior' by Diana L. Rodriguez-Munoz, Omar Jaidar, Marcela Palomero-Rivero, Mario A. Arias-Garcia, Gordon W. Arbuthnott and Violeta G. Lopez-Huerta.

In response to the accurate and appropriate comments from the reviewers, we have detailed several steps of the protocol and revised the figures for clarity. Also, we have edited the manuscript to address their concerns.

We really appreciate the reviewers' comments that encouraged us to improve the paper and we hope that after the revision the manuscript is now suitable for publication. In the following pages you can find a point by point response to each comment.

Sincerely

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Comments: We thank the editor and reviewers for their constructive suggestions and insightful comments that we believe have improved the clarity of our protocol. Corrections and changes have been highlighted in the text (blue text).

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. We have read the manuscript several times to spot and correct spelling issues and use grammar to the best of our knowledge.
2. Please revise the following lines to avoid previously published work: 185-199, 201-203, 204-206, 245-247. We have rephrased the text to avoid wording of previously published work. Please note that the line numbers have changed.
3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “The present protocol describes. …”. Here the word limit is exceeding. We have simplified our summary, lines 15-18.
4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). We have revised our manuscript to avoid the use of personal pronouns.
5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. 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 (including reagents, instruments, software, etc.). Please sort the Materials Table alphabetically by the name of the material. We have removed any commercial names and added more details in our Materials table.
6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. We have adjusted the numbering in our protocol steps.
7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. We have rewrite some of the steps in the protocol to offer more detailed information on how it is done.
8. Please add more details to your protocol steps:

Line 82: Please mention in detail what is meant by clean the surgery area? Please mention specifically what tasks are to be done. Done step 1.3 Line 96.
Line 95: Please write detail about the stereotaxic apparatus. Also, mention its relevance with the current study. Done in step 1.4 Line 99.
Line 104: Please specify the needle size. Done step 1.2 and 1.14 Line 92 and Line 134.
Line 109: Please mention in detail how the step is performed. Done step 1.16 Line 143.
Line 120: Please mention the composition of the mixture to be applied. Done step 1.21.
Line 125: Please mention the suture size. Modified step 1.24 Line177.
Line 173: Please mention how the euthanization was carried out. Modified steps 3.1 Line

259.
Line 177: Please mention how the processes will be carried out. In case you don't need to film this step, citations to published References should suffice. Modified steps 3.3-3.7 Lines 267-275.
9. Please include one-line space between each protocol step and then highlight in yellow up to 3 pages 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. Done.
10. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next and is in line with the Title of the manuscript. 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 the imperative tense.
11. Please note that the Figure legends are overlapping with the previously published works. Reword them or provide Reprint permission if they are being extracted from any previously published work. We will provide reprint permission from previously published work.
12. Please spell out the journal titles in the References. Done, we apologize for these errors it seems the citation software had some problems.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In, "In vivo wireless optogenetic control of skilled motor behavior," Rodriguez-Munoz et al. describe the use of miniature, wireless, head-mounted optogenetic control of skilled forelimb behavior. The single pellet reach to grasp task is a key tool for assessing skilled forelimb behavior in rodents and has been used to probe the roles of multiple motor circuits in skilled motor control. Forelimb movement during the task has been previously segmented into individual components by Ian Whishaw and the use of high-speed imaging would allow for a sensitive evaluation of deficits induced by injury or optogenetic control. Unfortunately, the extent of the analysis feasible with high-speed imaging is not touched upon in the manuscript. Movement errors are broken down into deficits in initiating reach, targeting the pellet, and grasping the pellet, while earlier work has segmented the reach into more discrete components. The most robust effect on success presented in the manuscript is the stance and initial positioning of the mouse, which does not depend on high-speed imaging.

We thank the reviewer for the insightful comments, indeed an important part of the current protocol is the high-speed imaging that we employed to perform kinematic analysis of the reaching movement. We apologize for not emphasizing its importance from the beginning but the analytical part is extensive, and we preferred not to focus the protocol on analysis. We observed that high-speed imaging allows to study even subtle effects over behavior that wouldn't be reflected by other forms of scoring. We added panel F in figure 3 to show how we used principal component analysis (PCA) to compare trajectories obtained with high-speed videography in different experimental conditions. Interestingly ipsilateral activation of D1SPNs does not change any performance parameters but it affects the

trajectory of the paw in hit and missed trials. We think that kinematic analysis can be useful in disease models where fine motor control is altered. We added a citation of the work by Becker et al, 2020 where they do a thorough analysis of the reaching movement kinematic.

Major Concerns:

What percentage of mice "never perform the task appropriately," (line 183) and what does that entail? Are these mice that continue to reach for the pellet with their tongues, or are they the over-shaped or nonlearner mice described by Chen, Gilmore, and Zuo (JoVE 2014)?

We apologize for not being clear before. We have included a paragraph (lines 232- 240) explaining in more detail the behavior of animals that “do not perform the task appropriately”. We have observed that all the animals are able to do a reaching movement but not all can successfully grasp the pellet either from the beginning of the training sessions or throughout them (those that have a ‘regression’ in performance), now we mention the percentages of non-learners 11.1% (6/54 mice) and over-shaped mice 18.51% (10/54 animals). As you correctly noted this has been discussed by Chen et al, 2014 and we have included this citation in the manuscript.

The camera position is confusing. I am assuming that the high-speed camera is meant to capture the forepaw movement; however, the statement that the high-speed camera is to "record from the rear of the cage," along with the illustration in Fig 1E don't indicate that the forepaw would be in the field of view. As the front camera is set at 30-60 fps, I am assuming that it is only for evaluating the position and stance. Is 100 fps for the high-speed camera sufficient to characterize reach when the entire movement is over in less than half of a second? Sathyamurthy et al recorded at 240 fps and Azim et al at 500 fps. Additionally, the figure legend indicates that two high speed cameras were used. The figure needs to be revised for clarity.

Thank you for noting this important point. We are sorry for the confusion and not explaining correctly, the camera is not placed at the rear of the cage but in the lateral side of the cage to have a rear view of the behavior. We have revised Figure 1 and added panel E to clarify this point with the exact measurements and position of the cameras. In our experience, you can track the trajectory with one high speed camera in 2 dimensions but if 3 dimensional analysis is needed a second high-speed camera should be added as in Azim et al, 2014. In our hands, 100-120 frames per second is enough to track the trajectory of the movement, we get around 60-70 points per reach in control conditions and usually that is increased with optogenetic manipulations. In a recent paper by Becker et al, 2020 they also employ 120 and obtain enough data for kinematic analysis.

Experimental details are sparse throughout. What is the strain and age of the mice used in these experiments? Strain plays a critical role in behavior and may affect outcomes on this task.

We have added lines 77-81 to give more details about our experimental model.

Please provide details on the optogenetics experiments. How was expression restricted to spiny projection neurons?

We now mentioned in line 76 that we used Drd1-Cre transgenic mice and we mention in line 147 that a cre-dependent AAV is used to restrict expression to spiny neurons.

How was the stimulus triggered? What were the stimulation parameters (mW, duration, frequency of stimuli, etc.)? What is the control? Is it stimulation with control vector or no light stimulus, and therefore, no additional heat?

The stimulus is triggered manually by the researcher with a remote controller, the stimulus was a continuous pulse 1.5-2 seconds and the intensity at the tip was 1 mW, we added details of the stimulation in lines 209-211. We have some control animals where we did not express the opsin (n=5). In these mice the light stimulation did not affect behavior.

It is unclear whether the analysis of initial distance and body angle (Fig 2D,F) represents effects of trials between animals or across trials regardless of animal.

In this analysis we pooled data from hit and missed trials of 28 mice.

Are trials defined as any reach beyond the opening of the box? Occasionally mice will reach out of the box in rapid succession; does this count as multiple trials? If the mice fail to make contact with the pellet, but rather mis-target, does that count as a trial?

We analyzed both multi-reach trials, where the mouse reached multiple times before retrieving the pellet, and single-trial events, where the mouse retrieved the pellet in a single reaching movement. The trial was finished when animals pushed the pellet away or reinitiate the trial by going to the rear of the cage.

As the higher error rates appear to be related to stance (distance to pellet, body angle), do these parameters change with the optogenetic stimulation?
We observe that the body angle changes with contralateral activation of dSPNs but it does not change when the ipsilateral hemisphere is activated. The end-point distance to the pellet also increases with some manipulations that affect the target accuracy, we did not show in the current manuscript but activation of the striatal indirect pathway neurons changes significantly this parameter.

Minor Concerns:
In describing the analysis of separate phases of single pellet reach behavior (lines 54-55), the authors should include mention of work by Ian Q. Whishaw (eg. Whishaw and Pellis 1990). Thank you for noting this important reference we were missing.

Are 20 trials sufficient to account for variability? Chen et al (JoVE 2014) performed 30 training reaches, Azim et al (Nature 2014) 20 successful reaches, and Sathyamurthy et al (Cell Reports 2020) 40 trials.
In our hands 20 trials are enough but we are aware that it depends on the goal of the study and animal model used.

The authors would be best served by employing the same scales for reaching trajectories throughout.

The trajectories come from a left view (right handed animal) and right view (left handed animal) and that's the reason why they are not the same.

References 6, 25, 27-29 are missing journal information.

We have corrected this important issue.

What is the information on the high-speed camera used?

We included this information in the Materials table.

What is a Mototool?

It is the name we have in Mexico for the rotary tool (dental drill). We have changed the name in text to avoid confusion.

Reviewer #2:
Manuscript Summary:
Wireless optogenetics is an important technique. One great chanllenge in studying circuits involved in motor behaviors is to perturb the circuit activity with high spatial and temporal precision, but least interrupt the motor actions. Using the wireless technology to control optogenetic stimulation can be one of the solutions. Therefore, I appreciate authors' effort of putting together this protocol. But I think authors need to add lots of details. As one important goal of this paper, readers should be able to replicate the procedure based on this protocol. But I doubt this current version provides enough guidance.

We really appreciate the reviewer's accurate observations to make possible to replicate the protocol.

Major Concerns:
The quality of surgery matters to the success of experiments. To be able to perform the surgery in the right way, details are needed. However, I don't think the paper provided enough.
(1) In surgery procedures step 1, what is actually done to prepare the surgery area, whate PPE should be worn?

We added this information in steps 1.3 and 1.5 lines 96 and 102.

(2) 5% of isoflurane is pretty high. Do you keep the concentration at high throughout the procedure? Or only use high concentration to induce anesethia? In other words, what is the isoflurane concentration to induce anesthesia, what is the concentration to maintain anesthesia?

The reviewer is right, we apologize for the missing information. We have specified the concentration for induction and maintenance in step 1.8 line 111.
(3) In surgery procedures step 9, how many body axes need to be adjusted? only pitch?

We have added details in step 1.10 line 120.

(4) In surgery procedures step 12, what kind of pipette is used? glass pipette? how to prepare the pipette? What is the diameter and shape of its tip? What is the speed of viral injection? How much volume?

Thank you for pointing out these important missing information, we have included all the details in steps 1.2 line 92, 1.17 line 147 and 151.

(5) What is the purpose of meloxicam? Analgesic?

Yes, we have added it in step 1.26 line 184.

(6) In reach-to-grasp training, I don't fully understand how the food restriction is done. For example, appoximately how much food is provided a day, how long does the food restriction last? Every day throughout the training period? What signs indicate health issues resulting from food restriction? Is water also restricted or not?

We have modified our figure 2 panel A to clarify the time line of the experiment, there we specify the length of the food restriction period. We have added information about the amount of food during food restriction in step 2.1 line 194. We have included details about health evaluation of the mouse in lines 195-197.

(7) During habituation and pretraining (reach-to-grasp training steps 3-6), how much of pellets is given?

Since habituation until the last day of testing mice are given 20 chocolate pellets equivalent to 0.4 grams and 2.1-2.6 grams of standard pellets a day. We included this information in steps 2.2 line 199.

(8) In reach-to-grasp training step 7, how to train mice to run to the rear of the cage?

This can be achieved by waiting until the mouse is in the rear of the cage before placing a pellet in the indentation for each trial. We have added this information in lines 222-224.

(9) In reach-to-grasp training step 11, how the LED is turned on? Manually or controlled automatically by the video feedback?

Thank you for noting this information. The stimulus is triggered manually by the researcher with a remote controller, the stimulus was a continuous pulse 1.5-2 seconds and the intensity at the tip was 1 mW, we added details of the stimulation in lines 251-254. Although it would be ideal to have automatic video feedback we did not have the tools to develop such stimulation paradigm at the time.

(10) In reach-to-grasp training step 12, are those three cameras different types with different frame rate? Why is that? which one(s) are used to derive movement trajectory?

Thank you for noting this important point. We are sorry for the confusion and not explaining correctly. We have 2 high-speed cameras and one “normal” camera. In our experience, you can track the trajectory in 2 dimensions with one high speed camera but if 3 dimensional analysis is needed a second high-speed camera with the same characteristics of the first one should be added as in Azim et al, 2014. In our case we extracted trajectory data from only one camera (the high-speed camera in the side of the cage with a rear view of the behavior). The two high-speed cameras have a small field of view that does not allow to monitor the complete behavior. Then, we had a third camera (normal frame rate) to have a panoramic view of the behavior and a view of the mirror to collect body posture data. We have revised Figure 1 and added panel E to clarify this point with the exact measurements and position of the cameras.

(11) Is the cannula reusable? If yes, how to clean it, especially considering it is contaminated by PFA?

We have not re-used the LED cannula. But we would like to do in the future so we will start exploring ways to achieve this.

(12) What software or method is used to analyze movies to derive movement trajectory?
Minor Concerns:
Some part numbers and order information are needed.
(1) diameter and type of optical fiber glass optical fiber of 250 µm diameter line 85
(2) part number of eye spears We added the information in the Materials table
(3) size and part number of drill bit We added the information in the Materials table