Summary of changes to the manuscript

1. We include information on single cell juxtacellular labeling as an alternative procedure for labeling cells, as requested by Reviewers #1 and #2.
2. We add specific experimental details and commentary as requested by all reviewers.
3. We include higher magnification views of pipette tips in **Figure 5.**

Reply to Reviewer #1:

Manuscript Summary:

This is a very nice compilation of a complex protocol, which is otherwise difficult to obtain. In most cases the details are worked out by time consuming trials and errors thus the paper is very timely.

We thank the reviewer for his/her cafeful read of the manuscript and recommendation to report our protocol.

I have the following notes:

Please indicate that blunt ear bars should be used to protect the ear drum during the operation.

We include a statement warning of this potential problem in protocol step 2.3, and suggest the use of blunt ear bars.

Please indicate to what extent the method can be used for mice or what modifications are needed.

This is presently covered in the “Modifications” subsection of the discussion section, however it is admittedly vague. Therefore, we now further clarify which parts of the protocol are likely to be applicable to other species, and which are species specific. Additionally, we state specifically in the introduction that juxtacellular monitoring has been successfully applied in previous studies in rats, mice, and birds.

Please give more details about the postoperative care, common problems (inflammations, loose implants) and solutions

We apologize for this omission. Post-operative care, including analgesics, antibiotics, monitoring, and reporting, is heavily regulated by law and local guidelines. We note this but include recommendations for alleviating potential post-operative complications in protocol step 2.18.

Indicate the optimal resistance of the quartz capillary.

We agree. The resistance is now indicated in protocol step 3.2.

Please indicate that Neurobiotin can also be added to the pipette solution, to label individual neurons.

A similar request was made by reviewer #2. As such, we state in the introduction and discussion that single cell juxtacellular labeling is an alternative strategy. This procedure is then described in protocol steps 3.3 and 3.11.

Please indicate, to what extent there is individual variability among the rats to be accustomed to the head restrain apparatus. What is the success rate of the handling, as described?

We thank the reviewer for bringing up this important point which we neglected. In fact the rats' behavior was closely monitored during training. Each rat was given a subjective score between 1-5 by the trainer based on signs of stress in response to training. The scoring system is as follows:

1 - quiet, calm, responsive to training
3 - quiet, but struggling against restraint
5 - struggling against restraint, tooth chattering, vocalization
Dropout – rat struggles too much that it cannot be placed in the sock for training

Score	1	2	3	4	5	Dropout
Day 1 (handling)	39%	33%	27%	0%	1%	N/A
Day 6 (body restraint in tube)	55%	36%	6%	0%	1%	2%

Although selection for surgery is subjective by the experimenter, in practice we implanted rats with final scores of 1 or 2. This corresponds to a success rate of >90% for Long Evans rats from Charles River labs (N=122 rats). We now state that rats should be monitored for these signs of stress in protocol step 1.1. We quote this expected success rate of "well-behaved" rats in protocol step 1.3. We further note the strain, sex, weight, and vendor of our rats at the beginning of the procedure.

Please indicate if the rats needs to placed to the apparatus AFTER the head-mount is made, if yes how many times? If no, how many days should pass between the surgery and recording?

We agree that the procedure is vague in this regard. This information, along with a more detailed description of how to initially head-restrain the animal, is now included in protocol steps 3.4 – 3.6

Editorial comment:
[Please keep JoVE's protocol guidelines and length requirements in mind while addressing reviewer comments(use short steps, imperative tense, proper spacing, etc).]

Reply to Reviewer #2:
In their paper 'Juxtacellular monitoring and localization of single neurons within sub-cortical brain structures of alert, head-restrained rats' Moore et al. describe procedures to recording and identify neurons in awake head-fixed animals. As the authors acknowledge there is nothing ground-breakingly new in the ms, but the special compilation of techniques used to identify cells in awake behaving animals has not yet been published.

I agree with the authors that such a publication makes sense, because there is a need to identify neurons in behaving animals and hence a need for this very powerful combination of techniques.

The paper is fairly didactical and I think it will meet its goal, i.e. it will make neuron identification more accessible. Prior to publication a few improvements are indicated.

We thank the reviewer for his/her cafeful read of the manuscript and recommendation to report our protocol.

1. The micrographs of the electrodes should include higher magnification views.

We agree. We include higher magnification views in **Figure 5**.

2. I am not sure that screws are still the gold standard for securing implants in rodents. Very stable implants can be achieved with various gluing procedures. More often than not skull screws lead to inadvertent damage of the underlying brain tissue.

We agree that for mice, the implants can be secured with dental acrylic alone. For rats, who can apply substantially more torque to the restraint mechanism, we prefer the use of screws for several reasons: (1) arranging the screws in a circular fashion at an oblique angle to the skull, as shown in Figure 3, ensures that if the rat resists against restraint the resulting forces will be distributed across the entire skull; (2) the screw heads provide a substrate to anchor the head restraint plate and bolt with acrylic. To address concerns of damage, we include additional details on the type of screw and the surgical procedure in protocol step 2.5.

3. I am not sure if the suggested laser puller and the use of quartz pipettes really make the technique more accessible for outsider. Conventional borosilicate pipettes can also be introduced through the dura and will be much more easily available in most labs. Perhaps the author can comment on this issue from their own experience.

We agree that the use of borosilicate would make the procedure more accessible. Therefore in protocol step 3.1 we specify, based on our experience, the experimental

conditions in borosilicate pipettes can be used as an alternative, as well as those in which quartz is preferable.

4. More experimental detail on labeling procedures would be desirable. A few lines from Deschenes could make a big difference for the investigator that actually tries out these procedures.

A similar request was made by reviewer #1. As such, we state in the introduction and discussion that single cell juxtacellular labeling is an alternative strategy. This procedure is then described in protocol steps 3.3 and 3.11.

5. I did not exactly get the application of Chicago Sky Blue. Can it also be used for intracellular labeling?

No, and this is stated explicitly in the introduction. However, as discussed above we now include information on juxtacellular labeling with neurobiotin or biotinylated dextran amine as an alternative strategy, as well as the limitations of such an approach. The reason we prefer Chicago sky blue is because of its high success rate, which is further explained in response below to comment 6.

6. I was surprised to see that the authors suggest several labeling procedures. What about the disambiguation of multiple labeled neurons? Can Chicago Sky Blue help here? Again a few words from a labeling expert could be very illuminating here.

We agree that it is important the protocol include an explicit description of how to differentiate between different units labeled with Chicago sky blue. This is now included in protocol step 3.12. Importantly, note that assigning recording sites to labels requires that 100% of labels are recovered. Thus, the high success rate of labeling with Chicago sky blue is the basis for our recommending it in this protocol.

Reply to Reviewer #3:

Manuscript Summary:
This is a excellent description of the methods used to implant a headholding device on rats used for recording neuronal activity in awake rats.

Major Concerns:
No concerns

Minor Concerns:
There is an internal inconsistency on lines 151 and 156. The coordinates should be 3 mm posterior and 3 mm lateral to bregma, not 1 mm lateral to bregma.

We apologize for the confusion. The mark must be made near the craniotomy but not on the craniotomy, because the mark must remain on the skull after the bone is removed.

In this case a mark is made 2 mm medial to the center of the craniotomy. The text in protocol step 2.7 is revised to clarify this point.

Additional Comments to Authors:
N/A