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& BEHAVIORAL SCIENCES

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Dear Editor,

Attached is our revised manuscript (revised title: Methodological Description of the Laboratory Administration of Transcutaneous Auricular Vagus Nerve Stimulation (taVNS) in Humans: Technique, Targeting and Considerations).

We have included a tracked changes and clean version along with responses to all reviewer comments.

Sincerely,

Bashar W. Badran
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Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

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

OK, Completed

2. Please revise the title to reflect the method and its application.

Revised

3. Please provide an email address for each author.

Added to cover page

4. Keywords: Please provide at least 6 keywords or phrases.

Corrected.

5. Please spell out each abbreviation the first time it is used.

Corrected.

6. 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 and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Ten20, Matlab, CaviCide, etc.

Corrected. Thank you.

7. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution’s human research ethics committee.

Added.

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Corrected.

9. Please revise the protocol to contain only action items that direct the reader to do

something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Corrected.

10. 1.1-1.4: 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 materials and equipment information to the Materials Table.

Corrected

11. 2.1: What are the inclusion/exclusion criteria for recruiting the participants?

Added.

12. 2.3, 3.1, 3.5, 4.1, 4.2, 4.5, 5.3-5.6, 6.1, etc.: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a “Note.”

Corrected.

13. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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. See examples below:

3.2: What is used to apply the conductive Ten20 paste?

3.3: How to verify the polarity of electrodes?

5.2: Please specify the pulse generating GUI used in this step.

Added more descriptions to the procedures.

Note- We would like to make the GUI open access. How do we do this? Can you host it as supplemental material?

14. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Corrected.

15. Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file.

Corrected.

16. Figure 1: Please use capitalized letters A and B for panel labels.

Corrected.

17. Figure 2: Please note that the sham stimulation site is labeled as S, not B. Please revise to be consistent.

Corrected.

18. Figure 3: Please remove the picture containing commercial language (Ten20 in panel B).

Fixed.

19. Figure 6: Please include a space between numbers and their units (100 μ s, 200 μ s, 500 μ s). Please define SD in the figure legend.

Fixed.

20. Tables 1 and 3: Please include a space between numbers and their units (3 mA, 1.5 mA, 500 μ s, etc.). Should 0.825 in Table 2 be followed by mA?

Corrected.

21. Please number the tables in the sequence in which you refer to them in the manuscript text.

Corrected.

22. Please remove the embedded Table of Materials from the manuscript.

Removed.

23. References: Please do not abbreviate journal titles.

Corrected.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is a useful protocol that may extend the application of the tVNS, the following are my comments.

Major Concerns:

1. My main concern is the location of the stimulation. Based on a recent manuscript published in brain stimulation (2018) by Andreas M. Burger, there may be no vagus nerve distribution at tragus. Thus, before new evidence come up demonstrating that there is vagus nerve distribution at tragus, it may be not a good idea to put electrode at tragus as shown in Figure 2.

The reviewer is correct in addressing a current debate as to localization of stimulation. Our group, and others have chosen to stimulate the tragus as an active stimulation, whereas others stimulate the cymba conchae.

This was addressed in the introduction (lines 86-89)
The field is still determining optimal stimulation targets²¹, although the two most common placements are the anterior wall of the outer ear canal (tragus) and the cymba conchae. Sham stimulation may be conducted by stimulating the earlobe of the ear, an area believed to have minimal ABVN innervation (Figure 2).

We have added a second active site on Figure 2 to clarify this statement.

2. Individuals' s response to electrical stimulation varies significantly. I would suggest the authors do not use the term such as "stimulation current is determined as 200% of an individual perceptual threshold (PT)". There is no evidence suggest that 200% is better than 150% or 250%.

Great point. We agree. We have changed some of the language in the introduction and softened to language in methods.

3. It seems to the reviewer the threshold selection is too complicated to apply in clinical setting, I would suggest just increasing intensity gradually till the maximal intensity the subject can tolerate, then reduce intensity gradually till subjects feel comfortable.

The perceptual threshold finding algorithm is similar to the one used in rTMS that was developed and validated over the past 2 decades. This is considered the gold-standard for threshold finding. The authors believe this is an important

dosing parameter and respectfully will be maintaining it as a method for finding PT in the laboratory and clinical trial setting to maintain carefully controlled studies in the infancy of taVNS as a modality.

4. The Figure 4 is confusing, please just include the tVNS related part, delete all other materials that are unrelated to tVNS.

We have changed figure 4 to a more participant-focused image of a laboratory-based taVNS setup,

Reviewer #2:

Introduction

Paragraph 1: There is reference to '(C)' - unclear what this is referring to. Good mention of the financial cost of VNS, would also be worthwhile mentioning the safety profile of VNS, especially as safety and tolerability of taVNS is a key part of the paper.

Great catch. Typo deleted.

Added the safety profile of VNS to the introduction.

Paragraph 2: Mention could be made to the work by Colzato and colleagues- cognitive and social effects of taVNS.

We have added these. Thank you for the suggestion.

Paragraph 3: First sentence - should be 'is' instead of 'in'.

Fixed. Thanks.

Evidence needed for the tragus and cymba concha being the most popular areas of taVNS administration and for the earlobe having minimal ABVN innervation. Further explanation of why the tragus and cymba concha are innervated would be good.

Figure 2 is mentioned for sham stimulation, but not for stimulation on the tragus. Would help to make it clear in this paragraph that Figure 2 also shows where the tragus is. The text also mentions the cymba concha, but this is not illustrated on Figure 2. Another common target is the concha, but this is not mentioned - perhaps worth mentioning?

- R: A literature review reveals these two as the most common treatment positions for taVNS with the earlobe as the most common sham. Intervention is listed in citation 5.

Authors (and the field likely) concede that the field is operating under the auspices that the sole anatomical dissection study – Citation 5. We have made a statement to acknowledge this.

Peuker, E.T. & Filler, T.J. The nerve supply of the human auricle. *Clinical Anatomy*. **15** (1), 35-37 (2002).

We have fixed figure 2 to reflect two active sites and described in the figure legend.

May also be worth mentioning that sham can also consist of attaching electrodes to the same part of the ear which active stimulation is applied, but without passing current despite volunteers believing they are receiving active stimulation.

Interesting. Passive control added to the sham section.

Paragraph 4: Are there any reported side-effects of taVNS? - Left vs. right stimulation. Protocol

Well-detailed and clear. Can see this being useful for others. May be worth mentioning from the outset that this protocol seems to be specific for targeting the tragus. Would need to modify how the ear is targeted if administering taVNS to the concha and cymba concha.

Great point. Clarified in protocol and enhanced figure 2.

One concern is about the kit: does not look very portable or very user-friendly compared to others. Looks to be more used in experimental settings. Perhaps this is worth some consideration in the discussion.

Correct. This is a laboratory, non-portable setting that is used for parametric optimization and flexibility with opensource matlab script.

Electrode preparation and placement section:

Good detail provided regarding anode and cathode placement for the tragus. May be worthwhile being completely clear for anode and cathode placement for sham on the ear lobe.

Great point. Added.

Determination of perceptual threshold section is very good - provides a much more controlled way of determining PT.

May be worthwhile providing more detail about what to do if volunteers find taVNS too painful or uncomfortable.

Thank you. We have added a brief statement regarding this concern.

Delivering stimulation section:

Figure 5: 'd' could be more clearly labelled on the diagram.

Labeled d on the ON and OFF time. Duty cycle is hard to describe in a figure like this so we labelled d_1 and d_2 on the On/Off times.

If stimulation is typically administered at 200% of PT, reference is needed (5.3).

Fixed and noted.

After taVNS section:

Paragraph 1: References needed for first sentence to show that discomfort and side effects of taVNS are 'commonly' reported.

Added.

Representative results

It is interesting that for the same pulse width, a lower current is needed to sham site (earlobe) than tragus stimulation. From the values presented in Table 2, some of these active vs. sham differences may be statistically significant, would be worthwhile making this clear in this table.

Added. Thanks for pointing out this oversight.

Would also be of interest to see the pain rating for some of the other stimulation parameters for active and sham stimulation.

Pain ratings for other parameters can be found in the citation added to the manuscript.

Paragraph 1: There seems to be a slight mix-up with the numbering of the tables. More information about the participants would be helpful (e.g. number of males/females, age range, exclusion criteria).

Fixed.

Paragraph 2: NRS should be written out in full here (numeric rating scale). References are needed to support the first sentence. Check grammar of third sentence. More information about the participants would be helpful (e.g. number of males/females, age range, exclusion criteria).

Fixed.

Paragraph 3: Sentence 1 needs references.

Added.

Paragraph 4: Check grammar and references are needed.

Fixed typos as a result of merged edits. Thank you.

Discussion

Critical steps in the section:

Bullet point 1: What about mental health conditions? Why screen for cardiovascular disorders? Are there any particular cardiovascular disorders which may prevent participation in a taVNS study?

Inclusion/exclusion criteria included in the protocol. We had accidentally omitted this section in the initial submission.

Bullet point 2: Is there any evidence that their proposed skin preparation improves safety of taVNS? It is already a safe stimulus, so would be interesting to know how this further enhances safety.

Great question. Skin prep reduces resistance at the skin/electrode interface. This allows for less voltage needed to drive the required current through the ear.

Generally, we like to use low-current and low-voltage stimulators, to minimize the electricity needed to stimulate the nerve.

Bullet point 3: 'LOTES guidelines' should be expanded and further explained. Also, the group's published guidelines should be referenced.

Lines 47-0481 expand on LOTES and it is cited (#29).

Modifications and troubleshooting of the method section:

What about negative effects encountered by participants?

In our series of studies with over 150 individuals receiving stimulation over 4 studies, we have yet to have a negative effect encountered by participants.

Reviewer #3:**Manuscript Summary:**

The manuscript provides a relatively clear description of how to apply a new and promising technique that allows for a non-invasive stimulation of the vagus nerve

Minor Concerns:

1) It may be useful to extend the introduction to mention some of the recent studies that have successfully used taVNS to alter cognitive and social functioning in healthy participants.

We have added a brief bit in the introduction about this. Thank you for the suggestion.

2) the main differences between the authors' setup and the stimulation set-ups used by other groups should be pointed out

Noted. We have discussed different active placements with citation in the introduction and modified figure 2 to reflect other active position some groups use.

We also softened language regarding perceptual thresholds and current intensities as parameters are not yet fully established

3) It would be useful to share the script used to control the stimulator

We will be including the GUI as an open-source resource with this manuscript. Thank you for the suggestion.