**Reviewer comments:**

We thank the reviewer for their valuable comments and suggestions that have helped in a better presentation of the data in the manuscript. We hope to have addressed all the concerns with the changes in the manuscript and explanation below.

**Editorial comments:**   
Changes to be made by the author(s) regarding the manuscript:   
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.   
2. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”   
3. Abstract: Please do not include references here.   
4. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.  
5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).   
6. 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. Please move the discussion about the protocol to the Discussion.   
7. Please mention how animals are anesthetized.   
8. Please provide more details about full brain scanning and RT-qPCR. Alternatively, relevant references can also be cited.   
9. Please discuss all figures in the Representative Results. However for figures showing the experimental set-up, please reference them in the Protocol.   
10. Figure 2: Please use upper case “L” for volume and include a space between all numbers and their corresponding units (i.e., 10 µL).   
11. Figure 3B and Figure 4: Please explain each panel in the figure legend.   
12. Discussion: Please discuss critical steps within the protocol.   
13. References: If there are six or more authors, list the first author and then “et al.”  
14. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

We have revised the manuscript in accordance with the editorial comments.

**Reviewers' comments:**   
  
**Reviewer #1:**   
  
Manuscript Summary:   
In the present manuscript, Lee S. et al have presented a intranasal brain delivery set up for use in mice in a head down and forward position to be used under anesthesia.   
  
Major Concerns:   
One major problem with this manuscript is that no comparative data using other positioning methods have been presented to show that the present method is more effective than other currently used methods. The present method is more involved than the currently used methods. Superiority of this method over the currently used methods needs to be established to justify its usage.   
We had infact performed this experiment but not included previously as we didn't think it relevant to the manuscript or the protocol being detailed. We have now included data in revised manuscript (Figure 3A, right panel) comparing our positioning method with the more commonly used supine position and awake methods.

Minor Concerns:   
Need for anesthesia can interfere with the preclinical studies of brain diseases since anesthesia interferes with selected brain disease mechanisms.   
We agree with the reviewer’s point that anesthesia may interfere with some studies related to the CNS. However IN treatment of non-anesthetized awake mice results in very poor brain delivery of siRNA (Figure 3A, right panel) which is the major drawback associated with the protocol. Moreover, most procedures like brain surgery are performed under conditions of general anesthesia. We therefore believe that the protocol we detail will find use in a wide variety of applications.

**Reviewer #2:**   
  
Manuscript Summary:   
This article describes the use of a positioning device for the placement of mice during intranasal delivery of therapeutics to the CNS. Authors claim that use of this device for IN delivery can help reduce drainage of drugs or therapeutic peptides into the lung and stomach upon inhalation. Their protocol uses anesthetized mice in a 'head down-and-forward position instead of widely used supine position. The manuscript is well written and method section is sound   
  
Major Concerns:   
In the last two decades, there have been hundreds of publications where intranasal route has been used to deliver drugs, macromolecules and even cells. Most of these reports indicate that within 2 hours these compounds or cells are predominantly localize in the olfactory bulb. It is not clear why time dependent bio-distribution data were not collected or shown. In addition, it is important to compare and contrast bio-distribution data on the IN position in this device vs. supine position, at an optimal/fixed time interval for RVG-A488 (Fig. 3)   
Our previous data has indicated that non-targeted peptide/siRNA/ drug clear out from the brain and the CNS within 48 h of IN inoculation indicating that a neuron-targeted approach is required to retain drugs in the CNS after IN treatment (Ullah et al, Scientific report, 2018). We have incorporated this justification in the text of the manuscript in the *Discussion* section (line 316-319).

We also added data comparing biodistribution of peptide/siRNA using the supine, awake and positioning device protocols (Figure 3A, right panel).

Minor Concerns:   
Protocol: 4.6: What if there is an increase in volume due to nasal secretion/discharge? How that is going to be accounted for in recalculating the volume of lost drop(s) due to snorting out?   
If animal snort out, the volume snorted out (2 uL per drop) must be replaced with the same volume additionally. This point is been described in revised manuscript (protocol section 4.6, line 190-192).  
The discussion section should include few sentences comparing the anatomy of human and rodent nasal cavities, especially the olfactory region. Some investigators have used intranasal catheters for deep delivery of therapeutics. Some justification is needed to argue against the use of catheters.

We have now discussed the impact of anatomy on nasal delivery (line 300-306) and advantages of our approach over conventionally used catheter tubing in mice (line 278-286).