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TITLE:

A Positioning Device for the Placement of Mice During Intranasal siRNA Delivery to the Central Nervous System

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KEYWORDS:

intranasal administration, brain delivery, siRNA, RVG9R peptide, mouse positioning device, nose-to-brain

SUMMARY:

Here, we present a protocol using a mouse positioning device that enables the appropriate placement of mice for the intranasal administration of a brain-targeting peptide-siRNA formulation enabling effective gene silencing in the central nervous system.

ABSTRACT:

Intranasal (IN) drug delivery to the brain has emerged as a promising method to bypass the blood-brain barrier (BBB) for the delivery of drugs into the central nervous system (CNS). Recent studies demonstrate the use of a peptide, RVG9R, incorporating the minimal receptor-binding domain of the Rabies virus glycoprotein, in eliciting the delivery of siRNA into neurons in the brain. In this protocol, the peptide-siRNA formulation is delivered intranasally with a pipette in the dominant hand, while the anesthetized mouse is restrained by the scruff with the nondominant hand in a “head down-and-forward position” to avoid drainage into the lung and stomach upon inhalation. This precise gripping of mice can be learned but is not easy and requires practice and skill to result in effective CNS uptake. Furthermore, the process is long-drawn, requiring about 45 min for the administration of a total volume of ~20–30 µL of solution in 1–2 µL droplet volumes per inhalation, with 3–4 min rest periods between each inhalation. The objective of this study is to disclose a mouse positioning device that enables the appropriate placement of mice for efficient IN administration of the peptide-siRNA formulation. Multiple features are incorporated into the

design of the device, such as four or eight positioning chairs with adjustable height and tilt to restrain anesthetized mice in the head down-and-forward position, enabling easy visualization of the mice's nares and a built-in heating pad to maintain the mice's body temperatures during the procedure. Importantly, the ability to treat four or eight mice simultaneously with RVG9R-siRNA complexes in this manner enables studies on a much quicker time scale, for the testing of an IN therapeutic siRNA approach. In conclusion, this device allows for appropriate and controlled mouse head positioning for IN application of RVG9R-siRNA and other therapeutic molecules, such as nanoparticles or antibodies, for CNS delivery.

INTRODUCTION:

The BBB prevents systemically administered molecules of >400–600 Da from entering the brain, posing a significant challenge to the delivery of therapeutic biomolecules for diseases affecting the CNS and brain¹. Direct drug delivery to the brain can be achieved by stereotactic injection; however, this requires surgical expertise and is highly restricted in delivery to areas proximal to the injection site, making it unsuitable for routine clinical use². IN delivery to the brain can also result in direct brain delivery by bypassing the BBB, allowing for the direct and rapid transfer of a variety of substances to the brain^{3,4}. This transfer is thought to occur by transport mechanisms via the olfactory and trigeminal nerves that connect the nasal passage to the brain, the cerebrospinal fluid, and the lymphatic systems⁵. As the direct nose-to-brain route does not involve peripheral organs and tissues, it substantially reduces systemic side effects and improves potency. IN administration is a promising noninvasive alternative to both local and systemic routes for brain delivery of therapeutic agents and may represent a powerful approach to combat neurological disorders, including Alzheimer's disease, Parkinson's disease, and brain cancer, and is being explored in several clinical trials^{6–8}.

Several experimental factors, such as volume and method of inoculation, as well as formulation pH, strongly influence drug delivery to the CNS via the nose-to-brain pathway⁹. In studies with mice, the success of IN drug delivery strongly depends on proper head positioning, which is critical for efficient brain deposition and to avoid drug drainage into the external environment or the airways. Notably, the majority of rodent studies employ a head-back position (supine) with a 70°–90° tilt for drug delivery to the olfactory epithelium, even though head positioning at 0° may favor drainage into the trachea⁹. IN delivery of drugs in mice that are awake results in reduced brain deposition compared to any application in the supine position, mostly due to scientists' inability to hold mice in the desired position for longer periods of time. Moreover, the upside-down position required by the skin grip method employed for awake mice results in drug deposition predominantly in the trigeminal nerve and the olfactory bulb, as well as peripheral organs, such as kidneys and lungs, within 30 min postinoculation¹⁰. The most appropriate body position for the delivery of therapeutics through the olfactory or trigeminal nerves in larger animals such as nonhuman primates in clinical studies appears to be the head down-and-forward position (i.e., the so-called "praying to Mecca position")¹¹. However, this position has not been well-studied in the mouse model, and the supine position is more widely used in rodent studies.

Previously, we have shown that the RVG9R, a peptide designed based on the minimal receptor binding domain of the Rabies virus, displays tropism to cells expressing nicotinic acetylcholine

receptor subunits like neurons and macrophages and mediates the intracellular delivery of siRNA by a mechanism involving receptor engagement and temporary plasma membrane delocalization at the site of receptor aggregation^{12,13}. Importantly, systemic intravenous administration of RVG9R-siRNA complexes enables the transvascular delivery of siRNA into the CNS¹⁴. However, the systemic route dilutes the amount of siRNA delivered to the CNS, and recent data demonstrate that the IN administration of RVG9R:siRNA complexes to mice positioned in the head down-and-forward position elicits wide-spread target gene knockdown in multiple regions of the brain¹⁵. Importantly, this level of knockdown was achieved with as little as 13.5 µg of siRNA administered over a four-dose, 2 day regimen while the IV route requires a ~5 times higher dose per injection to achieve comparable knockdown. The only shortcoming of the IN approach is that it is an arduous procedure, requiring the use of both hands during the administration of the solution while continuously gripping the mice alternatively in the head down-and-forward and in relaxed positions between each inhalation for a considerable long treatment period (a 30–45 min procedure for the effective uptake of a ~20–30 µL volume per mouse). Using the mouse positioning device presented here enables the proper placement of mice with little physical duress to the animals and the personnel performing the protocol, as well as the treatment of multiple cohorts of mice within a reasonable period of time, enabling an in-depth study on using siRNAs as a therapeutic for West Nile encephalitis in mice at late stages of the disease¹⁵.

PROTOCOL:

All experiments were performed in compliance with guidelines and protocols approved by the Animal Care and Use Committee of the Hanyang University. In this protocol, 6-week-old Balb/c mice weighing 20–25 g were used ($n = 3$ per group). The animals were housed in a pathogen-free facility with 12 h light/dark cycles at a controlled temperature and humidity level with free access to water and food.

1. Device assembly

1.1. Assemble the individual parts of the positioning device as shown in **Figure 1A**.

NOTE: The device is supplied in individual parts that can be easily assembled and disassembled.

2. Material setup

NOTE: The IN administration of liquefied drugs requires the following pretreatment steps.

2.1. Prepare the required materials, including the positioning device, a 10 µL micropipette, 10 µL micropipette tips, a treatment solution (e.g., RVG9R-siRNA complex), a timer clock, and 70% ethanol (refer to **Figure 2A**).

2.2. Plug the power code to turn on the device heating system at least 15–20 min prior to the experiment. The optimal temperature for animal experiments is automatically maintained at ~37 °C.

2.3. Prepare the anesthesia ketamine:xylazine:phosphate-buffered saline (PBS) at a ratio of 1:0.5:8.5 and anesthetize each mouse with a single intraperitoneal (i.p.) injection in a final volume of 200 μ L per 20 g mouse.

NOTE: Please note that any optimized drug for ensuring the anesthesia condition for a period of 30–45 min can be used.

3. Mouse positioning

3.1. Assess the level of anesthesia by pedal reflex (firm toe pinch) to maintain the surgical plane.

3.2. Place the positioning device at an appropriate distance and height so as to provide for convenient access to all required reagents.

3.3. Once the mice are properly anesthetized, gently lift each mouse by the scruff of their neck with thumb and pointer finger and place it on the designated chair.

3.4. Proper positioning of the mouse on the chair is very important at this stage. Lay the mouse's back parallel to the back support of the chair, and at 90° to the chair seat. Lift the hands off the mouse and let the animal lie naturally in the head down-and-forward position, without pushing or pressing, as shown in **Figure 2B**. Make sure the mouse's forelimbs provide natural support while the animal is in this relaxed position, without any discomfort to the mouse.

3.5. Once the mice are properly positioned, strap the mice in with the chair belt, and immediately start the IN inoculation.

4. Intranasal delivery

NOTE: This protocol describes the IN delivery of RVG9R:siRNA complexes using the mouse positioning device. Using this protocol, a maximum of 20–30 μ L of solution can be easily administered to each mouse in 2 μ L drops. This volume is lower than that of the mouse's nasal cavity, which is 0.032 cm³, so the procedure does not result in lethal nostril obstruction or asphyxiation. This protocol allows for the simultaneous inoculation of at least four mice and a maximum of eight mice per device, resulting in time optimization and reduced variability.

4.1. Take the 10 μ L micropipette and adjust it to 2 μ L.

4.2. Load the micropipette with 2 μ L of RVG9R:siRNA complex solution (e.g., 5.2 μ g of siCy5 complexed with 104 μ g of RVG9R for the delivery experiment, and 13.5 μ g of siSOD1 complexed with 270 μ g of RVG9R for the silencing experiment, in a final volume of 25 μ L of PBS containing 5% glucose.

4.3. While holding the pipette in the dominant hand, adjust position by placing an elbow on the bench top. Support the hand holding the pipette with the other hand to avoid uncontrolled movements while administering.

4.4. Place a ~2 μ L drop very close to one nostril so that the mouse can directly inhale the droplet (refer to **Figure 2B**). If a tiny drop is not easily formed, then replace the pipette tip with a new one and repeat the operation. Use the stopwatch to verify the time interval between inoculations.

4.5. This device allows for the treatment of at least four mice at a time. If another group of mice is required to repeat the step 4.4, set another bar with four chairs above or below the first bar with four chairs to accommodate up to eight mice at a time on the device.

4.6. Repeat step 4.4 with the other nostril after 3–4 min from the first inoculation. This time interval is sufficient for the mouse to complete the inhalation of the first dose and restore normal breathing. If a drop of the inhaled solution is dislodged due to accidental snorting out from the mouse's naris, reinoculate an additional 2 μ L of solution.

4.7. Repeat the entire process with alternate nostrils until the 20–30 μ L dose is finished. It will take ~30–45 min to complete the entire inoculation procedure.

4.8. Put wet ointment on the mice's eyes before returning the mice back to their designated cages.

NOTE: Do not leave the mice unattended until they have regained sufficient consciousness to maintain sternal recumbency.

5. Data analysis

5.1. To confirm the brain deposition of Fluor-labeled RVG9R, anesthetize the mice (as described in step 2.3) and isolate the brain, as well as other peripheral organs, such as lung, liver, spleen, and kidney. Place each organ on the ImageStation and visualize fluorescence using ImageStation.

5.2. To visualize fluorescence due to the Cy5-label, prepare 20 μ m-thick cryosections, costained with Hoechst 33342, and perform full-brain scanning with a confocal microscope.

5.3. To assess gene silencing, extract RNA from various brain regions, such as the olfactory bulb, the cortex, the hippocampus, the thalamus, the hypothalamus, the cerebellum, and the brain stem, with an RNA purification kit, reverse-transcribed into cDNA using a cDNA synthesis kit, and perform qPCR with primer pairs as described previously¹⁵.

REPRESENTATIVE RESULTS:

Head position during IN administration is a major influence on the efficiency of drug delivery to the brain. Here we described the head down-and-forward position using a mouse positioning

device for the IN administration of a brain-targeting peptide-siRNA formulation for the delivery of the mixture to the mouse's CNS. To verify delivery through the IN route, we used the RVG9R peptide, previously shown to efficiently bind to neuronal cells both in vitro and in vivo^{14,16}.

We first tested nose-to-brain delivery of the RVG9R peptide labeled with Alexa Fluor 488 (RVG9R-A⁴⁸⁸) by using the mouse positioning device described here. At 48 h postinoculation, various organs, including the brain, the sinus, lungs, the liver, the spleen, and kidneys were excised to measure tissue-associated A⁴⁸⁸ fluorescence. A⁴⁸⁸ alone, or a control peptide (RVM9R-A⁴⁸⁸)¹⁴ that does not bind nAChR, were used as negative controls. As expected, neither A⁴⁸⁸ nor RVM9R-A⁴⁸⁸ were detected in any of the organs at 48 h postinoculation (**Figure 3A**, left). On the other hand, a strong fluorescent A⁴⁸⁸ signal was detected exclusively in the brains of the RVG9R-inoculated group. In addition, we compared this mouse placement position to the supine position method that has been used previously¹⁷, as well as to the awake method, for efficacy. We inoculated a fixed amount of RVG9R-A⁴⁸⁸ (100 µg) and assayed biodistribution at 48 h postinoculation. The results indicated that positioning the mice head down-and-forward improved the penetrance and deposition of RVG9R-A⁴⁸⁸ throughout the brain tissue (**Figure 3A**, right). In contrast, the animals inoculated in a supine position resulted in the delivery of RVG9R-A⁴⁸⁸ to the brain but not as strong as seen with the positioning device method. To further confirm siRNA delivery to the brain, we performed full-brain scanning after a single IN administration of RVG9R complexed to 400 pmol (5.2 µg) of Cy5-labeled siRNA. In contrast to PBS or RVM9R, complexing with RVG9R resulted in a strong accumulation of siRNA in major brain regions, including the olfactory bulb, the cortex, the hippocampus, the thalamus, the hypothalamus, the midbrain, and the cerebellum (**Figure 3B**).

Finally, we employed RT-qPCR analysis to verify the intracellular delivery of functional siRNA targeting the superoxide dismutase-1 (SOD1) gene. Mice were inoculated intranasally for 3 times on consecutive days with RVG9R:siSOD1 (13.2 µg of siRNA), and the SOD1 mRNA expression was analyzed 24 h after the last inoculation. RVG9R:siSOD1 administration resulted in a substantial reduction of the SOD1 expression in the olfactory bulb (63%), the cortex (47%), the hippocampus (61%), the thalamus (53%), the hypothalamus (55%), the midbrain (39%), and the cerebellum (30%) (**Figure 4**). In conclusion, the use of a positioning device enables an easy IN inoculation of RVG9R-complexed siRNA in mice, resulting in brain-specific siRNA delivery inducing functional silencing of the target gene.

FIGURE AND TABLE LEGENDS:

Figure 1: Photographic presentation of the device assembly. The positioning chairs are assembled on the stand at the appropriate height with screws. After the assembly, the device is connected to a power supply for heating to the physiological temperature during inoculation.

Figure 2: Photographic presentation of the IN inoculation procedure using the positioning device. (A) Experimental equipment required for the IN inoculation. (B) Animals in the head down-and-forward position seated on the device (left) and the deposition of a 2 µL droplet for IN inoculation (right).

Figure 3: Distribution of fluorescently labeled peptide and siRNA after IN administration. (A) Biodistribution of intranasally inoculated RVG9R peptide at 48 h after a single IN inoculation. The brain and other peripheral organs were evaluated for the presence of fluorescence in mice inoculated with saline (PBS), A⁴⁸⁸, RVM-A⁴⁸⁸, and RVG-A⁴⁸⁸ (left). Imaging of the mice treated as mentioned above while they are awake, in the supine position, and in the mouse positioning device in the head down-and-forward position (right). (B) Biodistribution of the RVG9R:siRNA complex in the brain (*n* = 3 per group). The distribution of Cy5-labeled siRNA was visualized in full brain cryosections with a confocal laser microscope in animals inoculated with saline (PBS), siRNA complexed with control peptide (RVM9R-siCy5), or the brain-targeting peptide RVG9R (RVG9R-siCy5). The scale bar represents 1 mm. Abbreviations: o.b = olfactory bulb; ctx = cortex; hippo = hippocampus; hypo = hypothalamus; tha = thalamus; m.b = midbrain; cer = cerebellum.

Figure 4: IN application of RVG9R:siSOD1 induces target gene silencing in multiple regions of the brain. qPCR analysis for murine SOD1 mRNA in indicated brain regions 24 h after the last IN inoculation of saline (PBS), RVG9R:siCD4 (siCD4), RVM9R:siSOD1 (RVM9R), and RVG9R:siSOD1 (RVG9R). The relative SOD1 silencing in the olfactory bulb, the cortex, the hippocampus (upper), the thalamus, the hypothalamus, the cerebellum (middle), and the midbrain (lower) is shown. Data represent the mean \pm SD relative to GAPDH after normalizing with the corresponding data of saline-treated mice (*n* = 3 per group). The cartoon depicting the percentage of SOD1 mRNA in the indicated brain region after the IN inoculation is shown (lower right). ***P* < 0.01, ****P* < 0.001. Abbreviations: o.b = olfactory bulb; ctx = cortex; hippo = hippocampus; hypo = hypothalamus; tha = thalamus; m.b = midbrain; cer = cerebellum.

DISCUSSION:

We have developed a mouse positioning device for optimally positioning mice for the nose-to-brain delivery of therapeutics. The device is equipped with different functionalities, ensuring the easy simultaneous handling of animals. It is also equipped with heating pads for the maintenance of the animals' physiological body temperatures during experimentation. The anesthetized mice can be maintained in the head down-and-forward position by means of specifically designed chairs, with minimal discomfort to the animals. The height of the positioning chairs can be adjusted in such a way as is best to visualize animal nostrils while administering drugs.

IN brain delivery has several intrinsic limitations, including the small surface area of nostrils (allowing maximum volumes of 20–30 μ L per administration), nasal irritation, epithelium damage, and limited absorption across the nasal epithelium¹⁸. The IN administration of drugs to mice placed in the supine position has been used for brain delivery by dropping liquid drugs into the animals' alternate nares via pipette or polyurethane tubing (24 G x 19 mm) connected to microliter syringes^{19,20}. Although the use of tubing systems to release drugs near the olfactory epithelium is a potentially suitable approach, it causes irritation or nasal inflammation upon repetitive administration. Moreover, the small size of the nasal cavity limits this approach, especially in mice that may be overcome by repeating, as well as by drug formulations that persist in the nasal mucosa. Another option is the IN administration of therapeutics to awake mice¹⁰. However, this approach requires skilled procedures for animal handling during IN inoculation.

307 Additionally, it causes animal stress and, therefore, is not ideal for disease models, especially
308 infectious disease models. Furthermore, inconsistent dosing may easily result from drug drainage
309 into the lungs or the stomach due to imperfect animal gripping. The approach presented here
310 allows scientists to overcome these technical barriers. The head down-and-forward position
311 reduces the possibility of drug leakage from the nose to the lungs while inhaling, favoring direct
312 and selective siRNA delivery to the brain. IN delivery using the mouse positioning device does not
313 require any specialized technique for handling or gripping the animals during inoculation. Four
314 animals at a time can be treated for a period of at least 30–45 min. The procedure can be scaled
315 up by including an additional four-chair bar, allowing for the easy management of up to eight
316 mice in the same experimental session. Therefore, following this method, a single operator may
317 induce drug delivery to the brains of large groups of animals over extended periods of time.

318
319 The anatomical structure of the nasal cavity strongly influences the nose-to-brain delivery (as
320 reviewed by Merkus et al.¹¹ and Ruigrok and de Lange²¹). The relative surface area of the nasal
321 cavity in mice is 15 times bigger than that of humans and the relative surface area of the olfactory
322 epithelium is 6 times bigger. Although there are significant differences in the anatomy of the
323 nasal cavity in humans to rodents, there are approximately 45 clinical trials underway using the
324 IN approach to treat several brain disorders (www.clinicaltrials.gov). The study presented here
325 indicates that when using IN delivery of therapeutics, scientists should consider several factors,
326 such as head position, sleeping, and proper delivery agents.

327
328 We have shown the efficient and specific deposition of fluorescently labeled siRNA to the mouse
329 brain. Furthermore, the significant decrease in SOD1 gene expression observed after IN siRNA
330 delivery confirmed functional effects. We previously showed consistently that the IN
331 administration of RVG9R-siRNA complexes targeting the West Nile virus (WNV) RNA exerts a
332 strong therapeutic effect on WNV encephalitis¹⁵. Notably, nose-to-brain siRNA delivery required
333 a cell-targeting ligand (RVG) and a positively charged molecule (9R) to complex siRNA. In the
334 absence of these elements, molecules were cleared through the systemic circuit and lymphatic
335 vessels 48 h posttreatment (**Figure 3A**)¹⁵. Therefore, in the experimental setup described here,
336 we have examined peptide/siRNA localization 48 h after inoculation to image only the levels
337 retained specifically in the brain. This approach could be easily implemented for the delivery of
338 other molecules, such as proteins, peptides, and nanoparticles, or other therapeutics, for the
339 treatment of a number of brain-related disorders.

340 341 **ACKNOWLEDGMENTS:**

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343 344 **DISCLOSURES:**

345 P.K. and S.K.L. are cofounders of Signet Biotech. P.K. is coinventor of patent PCT/US07/12,152,
346 which partly includes claims relating to RVG9R. I.U., K.C., P.K., and S.K.L. are listed as coinventors
347 of a submitted patent (PCT/KR2016/014220), which includes claims relating to a mouse
348 positioning device.

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397 pharmacokinetic-pharmacodynamic studies: towards prediction of nose-to-brain transport in
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Assembly

Chair belt

Screw

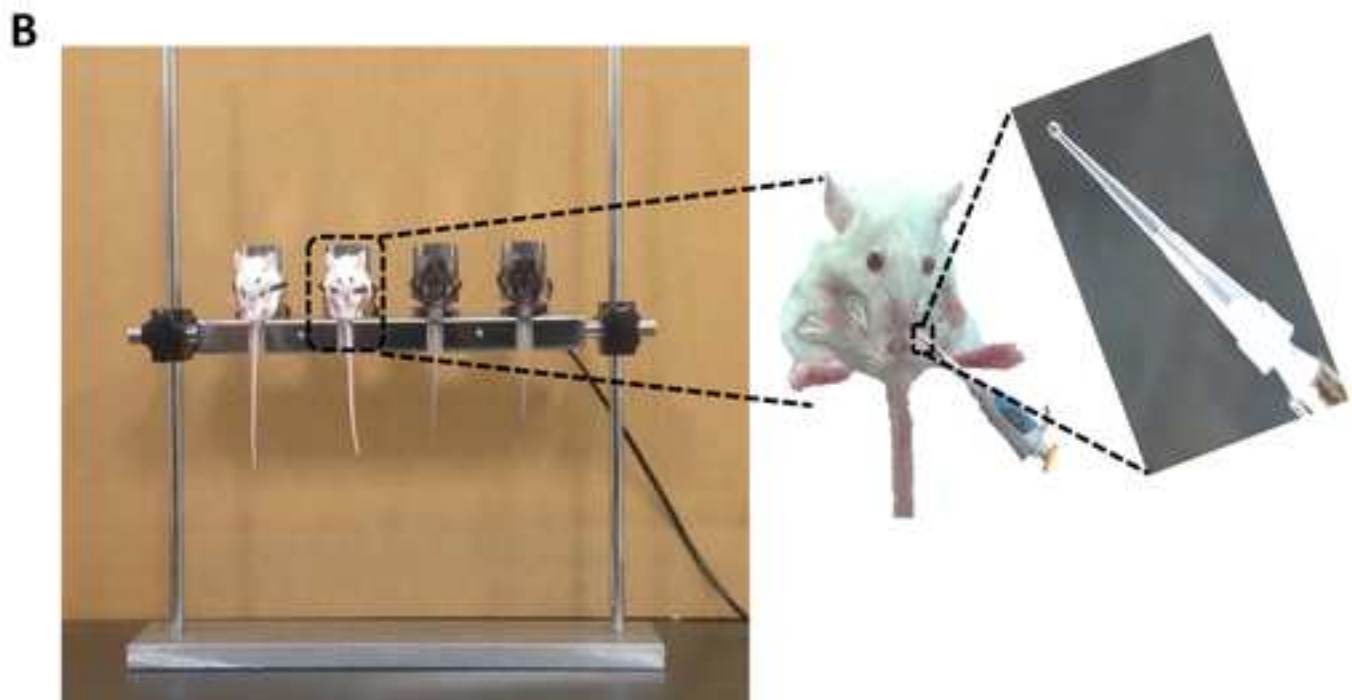
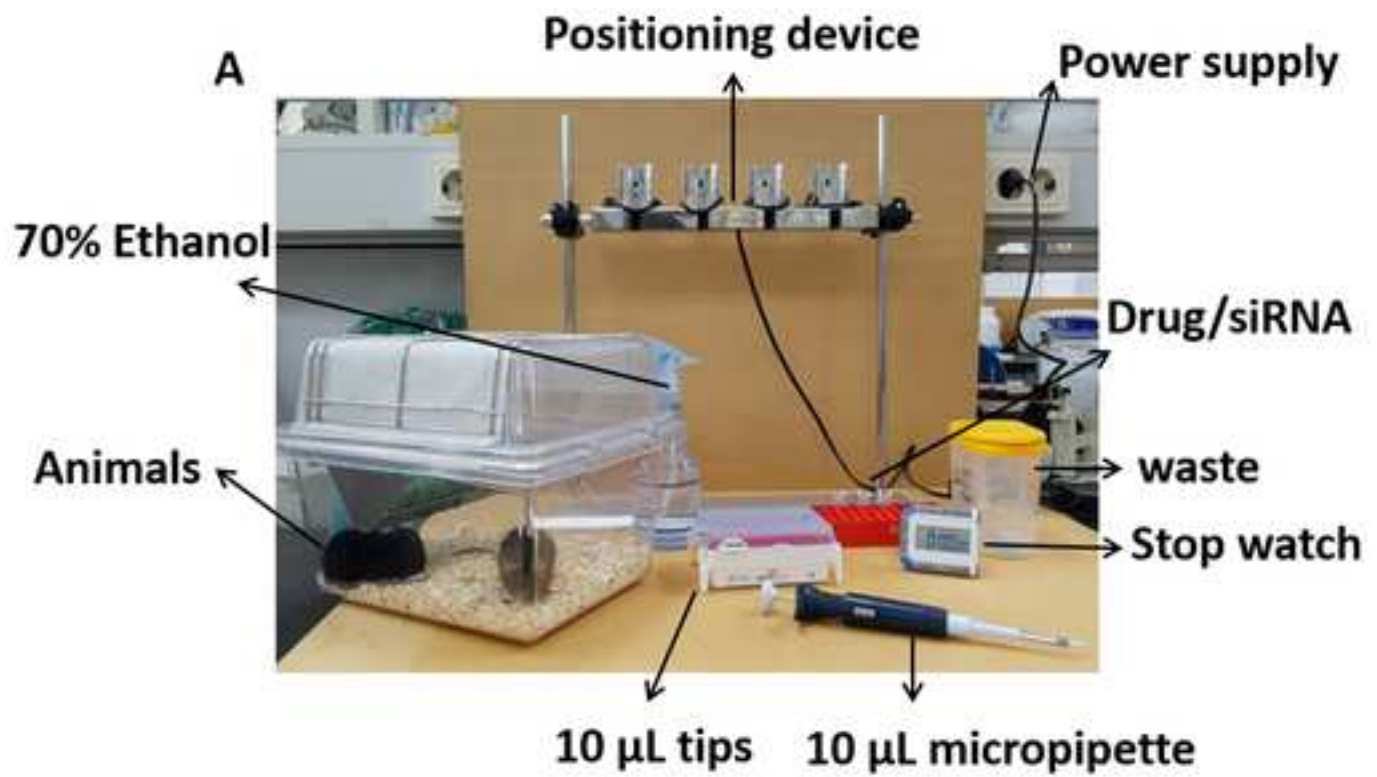
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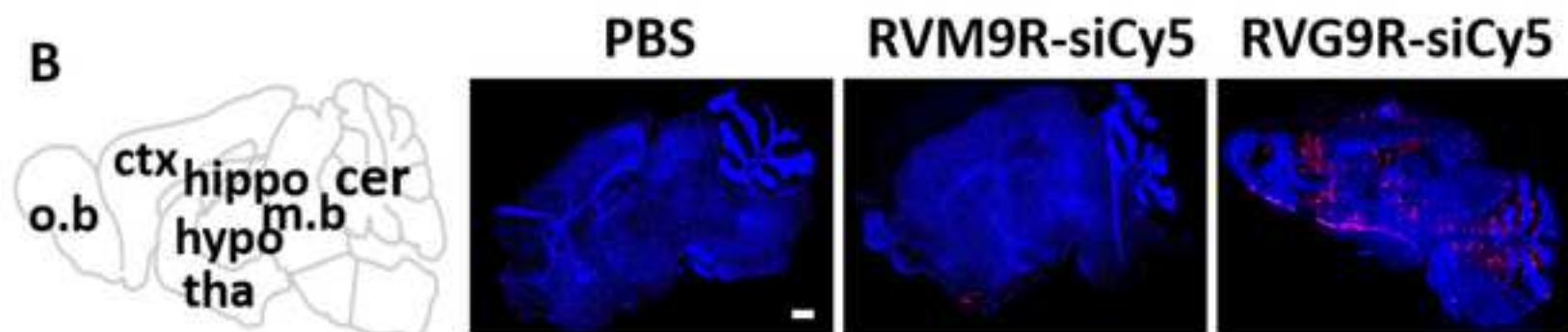
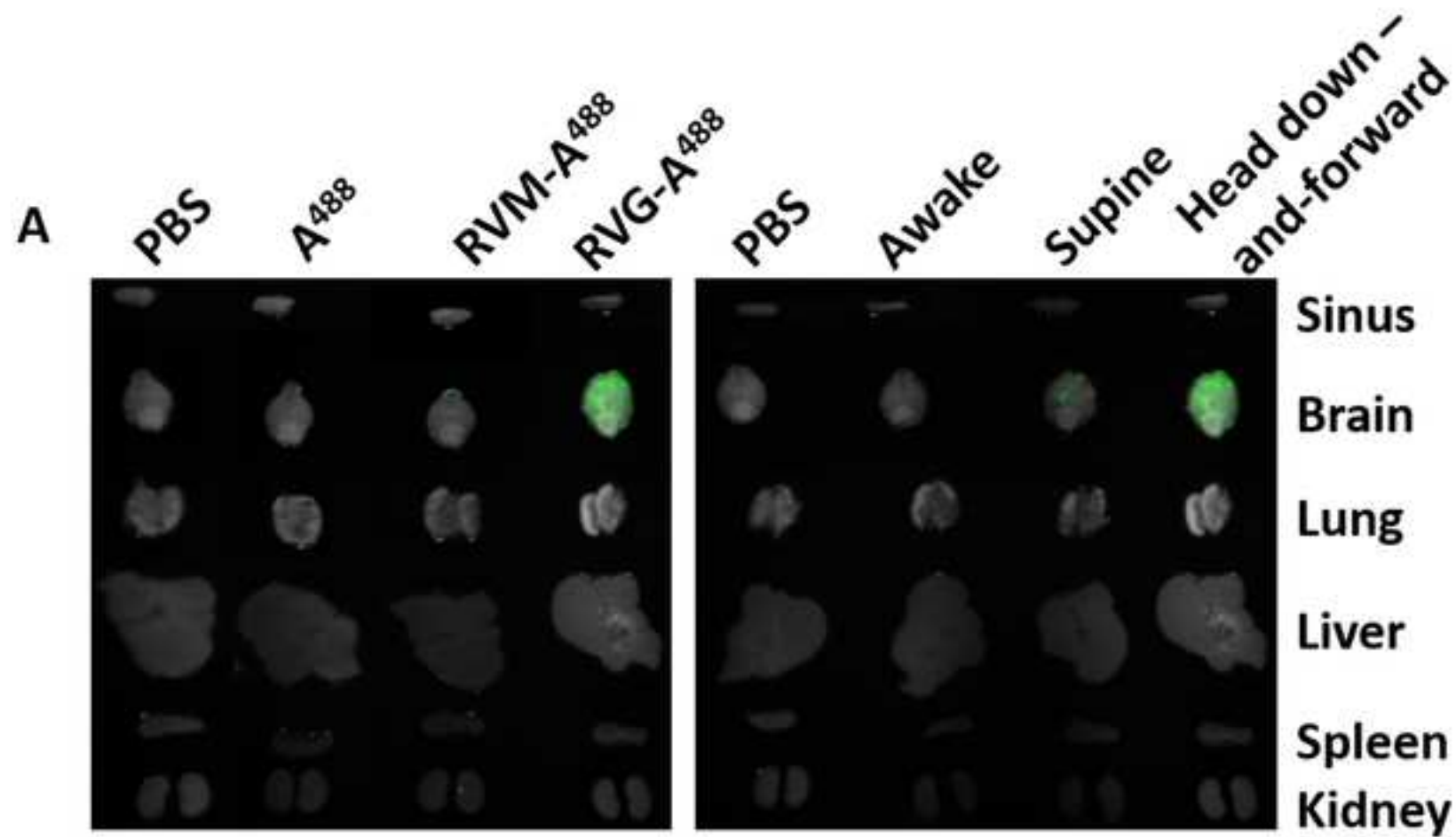
Positioning chairs

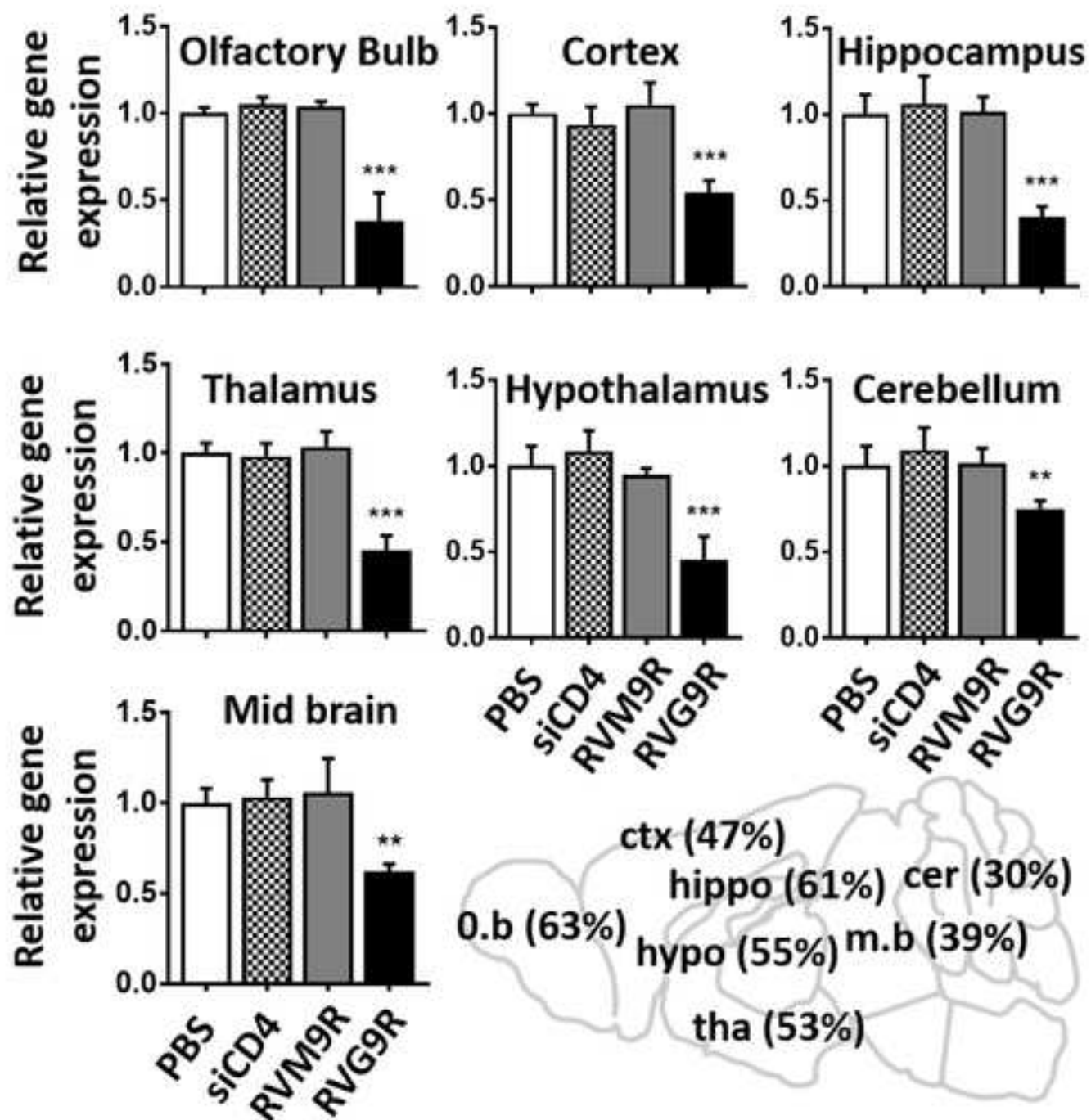
Heating system

Power supply

Positioning device







Name of Material/ Equipment	Company
Comercial assays	
iScript cDNA synthesis kit	BioRad
RNAiso plus	TaKaRa Bio
SYBR Premix ExTaq	TaKaRa Bio
Dyes	
Alexa fluor 488	ThermoFisher
Mouse strain	
Balb/c	Orient Bio
Oligonucleotides and primers	
Human CD4	ST Pharm
siSOD1	ST Pharm
GAPDH primers	ST Pharm
SOD1 primers	ST Pharm
Peptides	
RVG9R	Peptron
RVM9R	Peptron
Software, algorithms and devices	
FlowJo software 4.3	FlowJO, LLC
Mouse positioning device	Signet Biotech
Prism software	Graphpad
Prism software	Graphpad

Catalog Number

Cat# 1708891

Cat# 9108

Cat# RR420A

Cat# A30052

N/A

N/A

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N/A

N/A

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N/A

N/A

N/A

Comments/Description

6-8 week old, 20-30g

Sense: 5'-GAUCAAGAGACUCCUCAGU-3'

Sense: 5'-GGUGGAAAUGAAGAAAGUA-3'

F: 5'-AACTTTGGCATTGTGGAAGG-3' R: 5'-GGAGACAACCTGGTCCTCAG-3'

F: 5'-CCAGTGCAGGACCTCATTTT-3' R: 5'-CACCTTTGCCCAAGTCATCT-3'

YTIWMPENPRPGTPCDIFTNSRGKRASNGGGGRRRRRRRRR

MNLLRKIVKNRRDEDTQKSSPASAPLDGGGGRRRRRRRRR

<http://docs.flowjo.com/vx/>

<https://www.graphpad.com/scientificsoftware/prism/>

<https://www.graphpad.com/scientificsoftware/prism/>



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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 μ L).
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).

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please use standard SI unit symbols and prefixes such as μL , mL, L, g, m, etc.

[We have now used standard unit symbols and prefixes as suggested.](#)

3. Please use h, min, s for time units.

[We have now used suggested units in our revised manuscript.](#)

4. JoVE cannot publish manuscripts containing commercial language. This includes company names of 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. Examples of commercial language in your manuscript include Carestream, Leika, TaKaRa Bio, etc.

[Now we have corrected in revised manuscript as suggested.](#)

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

[The text in protocol has now been revised.](#)

6. Step 1.1: The Protocol should contain only action items that direct the reader to do something. Please write this step in the imperative tense.

[Now this has been corrected in revised manuscript \(line 118-120\).](#)

7. 2.3: Please ensure that all text is written in the imperative tense.

[Now this has been corrected in revised manuscript \(line 134-135\).](#)

8. 5.1: Please ensure that all text is written in the imperative tense. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Please split this step into two or more sub-steps to include all details.

[Now this has been corrected in revised manuscript \(line 202-211\).](#)

9. Step 5: Is the mouse euthanatized before fluorescence imaging? If yes, please specify that. Please include the details of euthanasia, organ isolation, staining and imaging.

[Now this has been corrected in revised manuscript \(line 202-211\).](#)

10. Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

[This has been corrected in revised manuscript \(line 196\).](#)

11. For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

We did not perform any survival strategies in our protocol.

12. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

This has been described now in revised manuscript (line 197-198).

13. Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

We did not perform any surgical procedures in our protocol.