October 16, 2009

Dear Dr. Nandita Singh,

Please find attached our revised manuscript “Murine Model for Parkinson's Disease: from 6-OH Dopamine Lesion to Behavioral Test” (JoVE 1376).

We are grateful to the reviewers for the very helpful comments and the opportunity to resubmit it.

Overall, our new version contains substantial changes that address all of the concerns noted in both reviews. With these new inclusions, we have also made this version more complete.

In addition to the revised manuscript, a point-by-point reply to all the reviews is also appended. In this response, portions of the reviews are restated, followed by our response in blue color.

Yours sincerely,

Stevens Rehen

Were animals used humanely and was the appropriate anesthesia or analgesia provided for potentially painful procedures?

No. This is a very poorly done surgical procedure and the video should not be approved for publication on JoVE.

We acknowledge the criticism and would like to thank members of the animal review board for pointing out these issues. The whole process of preparing and submitting this video protocol actually provided an excellent opportunity for us to discuss this issues deeply with representatives of our local committee and to reevaluate in detail approved protocols and methods of handling rodents in our research. A neurosurgeon recently joined our laboratory and is of great help for all related issues. Finally, our students attended a class on Biosafety and Ethics in the Use of Animal for Research, recently offered by our University.

**All recommendations and suggestions were considered in the revised manuscript and video, which were substantially modified** and now hopefully qualify for publication in JoVE, given the positive evaluation by the scientific reviewers.

In addition to the factors leading us to review some procedures, we also understand that, given a) the extended access to information available on the internet, b) the strong impact of visual scenes and, c) the wide use of this method in many research labs throughout the world, one should always demonstrate most recommendable procedures, keeping in mind that these might be adjusted to local conditions / specifics of the research project, animal strains, etc. Finally, we recognize that the experimenter being filmed experienced a certain level of stress due to the presence of the filming team. By affecting his ability to realize several procedures (e.g. stitching), which are normally performed smoothly in a quiet and less stressful environment, such artificial conditions indeed greatly impaired the quality of the demonstration.

Below are our specific answers to each addressed issue.

**SEE BELOW**

a) Absolutely not acceptable - for serious errors and deviations from the animal research standards.

There are numerous issues that need to be addressed, which render this video not worthy of publication, as follows:

1. Concern that the injection volume of the ketamine/xylazine via the IM route exceeded 0.1 ml (limit for IM injection volume in the mouse).

The actual dose for IM injection was below 0.1 ml (for a 30 g mouse, i.e. the heaviest animal used here, the total volume is 0.075 ml = 0.015 ml xylazine + 0.06 ml ketamine). However, since such volume is somehow close to the acceptable limit for IM way, and that IP injection might be performed with more safety and less pain, we now demonstrate application via IP route, as recommended.

Note that the dosage recommended by Bayer/Rompum that is compatible with a surgical tolerance of ca. 90 min in mice varies significantly according to the administration route: (IM: 16 mg/kg Rompum + 100 mg/kg Ketamine vs. IP: 10 mg/kg Rompum + 200 mg/kg Ketamine). The later route and dosage are now used and indicated in the manuscript. (http://www.rompun.com/index.php/fuseaction/download/lrn_file/dosage_mammals.pdf)

2. Dose of ketamine/xylazine is on the low end for this length of procedure. Generally ketamine is in the 90-120 mg/kg and xylazine is 10 mg/kg. The higher dose of ketamine is used for longer procedures. This procedure lasted at least 40+ minutes based on the comments made in the video.

The Xylazine dose was therefore adjusted to IP route. As 6-OHDA should be injected slowly into the parenchyma, this procedure does last about 40 minutes, but additional dose of ketamine should be applied if needed.

3. There is no need to pull the tongue with tweezers. This is an unnecessary trauma.

We find important do ensure airways are not obstructed as the animal gets sedated. This is done by gently pulling the tongue. Unpleasant frames were removed.

4. There wasn´t adequate skin preparation for the surgery. The fur should be shaved first. Then, the skin disinfected with a disinfectant (e.g., betadine) followed by alcohol and repeated 3X each. The area of hair removal in the video was insufficient and not properly done by shaving.

Recommended skin preparation is now demonstrated in both the text and the video.

5. Given the length of the anesthesia, one drop of saline in each eye would not prevent the corneas from drying out. Using an ophthalmic ointment product to prevent drying would have been required.

The application of one drop of warm saline on both eyes is repeated regularly throughout the procedure. This method prevents corneas from drying, as it also allows monitoring blink reflex as the animal recovers from anesthesia in his warmed post-op cage.

However, since small fragments of clipped hairs are more likely to hurt the eyes, ophthalmic ointment is needed after shaving, as now shown.

6. The animal was blinking after the application of the sterile saline into the eyes. Thus, there is concern that the animal was not fully anesthetized.

We now state clearly that surgical procedures can only be started as the anesthesia level as reached stage #3 (surgical).

7. The surgeon was not wearing sterile gloves, a mask, and his lab coat was open. This is not appropriate for survival surgery.

The new version includes surgeon wearing sterile gloves, a mask, and buttoned up lab coat.

8. The 6-hydroxydopamine and apomorphine were made from powder. There is no mention of the solutions being filter sterilized prior to injection. Thus, the solutions could have been contaminated.

It is now specified in the text that 6-OHDA and apomorphine are prepared in sterile conditions. We also added text to insist that both drugs should be manipulated and disposed according to institutional biosafety rules for environmental care and safety of animals used in research.

9. Silk suture should not be used in the skin. It is a braided suture that promotes bacterial growth and potential wicking into the surgical incision.

The use of appropriate nylon sutures is now specified.

10. There was poor tissue handling during the suturing due to excessive pulling of the skin.

Pulling of the skin was substantially reduced in this new version.

11. There is no discussion of using analgesics post-operatively.

We now specify that Ibuprofen suspension is added to drinking water for the first two days following the procedure, as recommended by institutional and international guidelines.

b) Improvement requires - for minor deviations, missing parts, etc....

1. Atropine isn´t absolutely necessary and could have been given subcutaneously versus injecting into the muscle. SQ injections are preferred when possible in the mouse due to the limited muscle mass.
Although not absolutely necessary, we found important to maintain this premedication to prevent cardio-respiratory depression, as also recommended by Bayer/Rompun (Cf. #1a1). The more appropriate subcutaneous way for Atropine administration is now clearly stated.

2. The neomycin ointment isn´t necessary if the surgery is done using aseptic technique, which clearly wasn´t the case in this video.

As fully recovered, operated mice are returned to their cages and housed in groups (of ca. 6 animals per cage) to ensure proper social interactions during the following weeks and months. We find thus useful to apply local antibiotics on the sutured wounds. Although best possible measures are taken, there is indeed no way that such procedures can be performed in absolutely aseptic conditions. In our hands, only 1 mouse from 60 operated displayed signs of infection - possibly promoted by the inappropriate use of silk sutures - and was immediately euthanized.

3. Fluids should be warmed prior to injection to help maintain body temperature.

This is now clearly mentioned.

4. There is no discussion of how the control animal was manipulated, if at all. If not, how does the author know if the lesioned animal was circling from the 6-OH dopamine or due to infection from using an unsterilized solution? This was mentioned, but it is now more clearly specified both in the text and video. Control animals underwent the same surgical procedure, but received injection of a corresponding volume of the vehicle solution (0.02% ascorbic acid in 0.9% NaCl in sterile water) instead of 6-OHDA. They were tested for apomorphine-induced rotational behavior under the same conditions as 6-OHDA animals. Since none of the control, vehicle-injected mice displayed body rotations after treatment with apomorphine. It is thus very unlikely that the motor impairment evidenced in lesioned animals was due to infection or side effects from the surgical procedure. Our data strongly suggest that they actually result from the genuine degeneration induced by 6-OHDA.This conclusion is also supported by micrographs inserted in the video, illustrating the blatant diminution of Tyrosine-hydroxylase immunolabelling one month after the lesion in the ipsilateral striatum .

5. There is no discussion of the time length between the 6-OH dopamine lesions and the behavioral tests. Specific discussion was added in the new text and stated in the video. A figure was added to illustrate the time between lesion and behavioral testing.

2009/5/26 Nandita Singh <nandita.singh@jove.com>

Dear Dr. Rehan,

I do have the reviews in and I am pleased to inform you that **your article has been accepted to be published with some revisions**. I, however, do not have the comments from our animal review board to ensure that the animals were treated humanely. I hope to hear from them within a week.

I am enclosing the reviews and the changes requested. I am also sending the template for sending us your revised text. Please send us your revised text as a Word file in this template.

Please do not send us the final version till I have sent the comments of the animal review board to you.

Sincerely,
Nandita Singh

Title: Murine Model for Parkinson's Disease: from 6-OH Dopamine Lesion to Behavioral Test
URL: http://www.jove.com/index/Details.stp?ID=1376

General Comments

The authors describe a protocol to produce lesions in the striatum using the compound 6-OH Dopamine as a model for Parkinsons disease in mice. The procedures are clear, well explained and the authors highlighted possible mistakes so other scientist are aware of potential pitfalls during the reproduction of this protocol. The incorporation of the behavioral test gives to the protocol an additional tool to study possible manipulations on this mouse model for PD.

In general this is a good demonstration of a procedure that is widely used in experiments related to Parkinsons disease.

**Revisions:**

1. **Comments on video**

1 Time: 7:11-7:13

Double voice in narration. Needs editing
Audio was modified as suggested.

2. **Changes in text**

1 Time: 2:30-2:34

Fur shaving should be done by a small animal electric razor or shaver, not scissors. Scissors are more likely to leave enough fur behind to cause infections during healing.

This concern was raised. Please address this in the text.

Modified as suggested, and now stated clearly both in the text and the video.

2 Time: 3:13-3:27

Earbars are too big for this size of animal. A mouse platform with smaller size earbars would be safer and less likely to induce tympanic membrane trauma.

We use a commercially available Stereotaxic platform (from Insight Ltda. Brazil) that allows handling both rats and mice. For mice, we employ mouthpiece and ear bars that were specially designed to cause minimum harm to the tympanic membrane, being both shorter and larger than rat ear-bars. This is now clearly stated this in both text and video.

Specifications about the equipment can be found at: http://insightltda.com.br.

Part numbers are #EFF 335 (for mice mouth piece), #EFF 400 (for mice ear-bars) and #EFF 332 for the stereotaxic platform we use.

Furthermore, experimenter did not test proper placement of ear bars by trying to move the animal´s head sideways and confirming that the skull is firm in place.

This procedure is now shown and explained as suggested.

3. Time: 4:14-4:17

Scraping away tissue from scull with the same blade that made the skin incision risks infection. Sterile blades are safer

We now specify and show that a sterile blade is used to gently scrape the periosteum.

4 Time: 6:09-6:15

6-0HDA is a toxin and its use in the US requires approval by the appropriate university Environmental Health and Safety Office and the IACUC Committee. It should be stated so in the narration and the protocol. Restrictions on 6-OHDA use should be described in detail and in accordance with IACUC and Environmental Health and Safety regulations.

Compliance to biosafety regulation is now stated.

5 Time: 6:10-6:16

Authors should mention the composition of the vehicle solution.

It is more clearly specified both in the text and the video. Control animals underwent the same surgical procedure, but received intrastriatal injection of a corresponding volume of the vehicle solution (0.02% ascorbic acid in 0.9% NaCl in sterile water) instead of 6-OHDA.

6 Time: 6:17

Authors should mention the composition of the vehicle solution

It is more clearly specified both in the text and the video. Control animals underwent the same surgical procedure, but received intrastriatal injection of a corresponding volume of the vehicle solution (0.02% ascorbic acid in 0.9% NaCl in sterile water) instead of 6-OHDA.

7 Time: 8:09-8:30

Inadequate post-op precautions. Systemic antibiotics are not mentioned or administered.

Post-operative analgesic and antibiotic treatments are now specified.

Other comments on the text:

1.- Abstract: Authors should define DBS :

Abbreviation was removed and replaced by “deep brain stimulation”

2.- Abstract: Define MPTP :

The full name was added.

3.- Abstract: 6-OHDA is missing the dash.

It was fixed as suggested.

4.- Abstract: If 6-OHDA is previously defined, use it in the sentence.

It was fixed as suggested.

5.- Abstract: Move the composition of the vehicle solution to the Anesthesia and Surgery section.

It was moved to the appropriate section.

6.- Anesthesia and Surgery: Gauge of Hamilton syringe must be mentioned.

It is now mentioned.

7.- Anesthesia and Surgery: ¡°6¡± after surface must be superscript.

It is now correct.

8.- Anesthesia and Surgery: Authors should mention the composition of the vehicle solution in this section. The actual one in the abstract section is not clear: ¡°10 ¥ìg in
NaCl with 0.02% ascorbic acid¡±. NaCl concentration?

Vehicle composition is now specified.

9.- How do authors control that the mouse´s head is even? Striatum is a big cerebral structure, and probably with correct AP, L and DV coordinates is easily targeted. However, to avoid variability among trials should be important to ensure that the head is always in the same position. Bregma and Lambda should be positioned at the same height (should be even) before the coordinates are zeroed. Authors should state how they achieve this issue, at least in the text of the article

Underlying principles of stereotaxy are now illustrated by new figures inserted in the video. The two-steps procedure for correct head placement is now explained in details in the text: 1) checking that the head does not rotate around any axis after the animal is placed in the

frame. 2) once skull landmarks are visible, checking that Bregma and Lambda are at the same height and spaced by 4.2 mm.

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11.- I would like to bring out some aspects regarding the aseptic conditions of the surgery. As a suggestion, scenes were the surgeon has the lab coat untied or the surgeon does not wear a face mask should be deleted.

Both text and video were significantly modified to specify and better demonstrate the experimental conditions.