Graz, March 18th, 2018

Dear Dr. DSouza,

We would like to thank both you and the reviewers for the thorough and constructive review of our protocol manuscript “***Rat Model of Widespread Cerebral Cortical Demyelination Induced by Intracerebral Injection of Pro-Inflammatory Cytokines***” by Ücal & Haindl et al. We included the original editorial and reviewers’ comments and presented our responses to them below in red font; and amended the manuscript accordingly.

We hope the revised manuscript is now suitable for publication in JoVE.

With many thanks for your efforts and best regards,

Sonja Hochmeister M.D. Ph.D.

**Editorial comments:**

1. The manuscript has been modified to include line numbers and minor formatting changes. The updated manuscript 57879\_R0.docx is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink to download the .docx file. Please download the .docx file and use this updated version for future revisions. The file is also attached.

2. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has already been proofread by a native speaker that had been trained as a molecular biologist.

3. Please provide at least 6 keywords or phrases.

Two more keywords have now been added to make it total of six.

4. Please use SI units, e.g. please use “µL” instead of “µl”, “mL” instead of “ml”, “h” instead of “hour”, etc. Please leave a white space between the values and the units.

Usage of units has been standardized throughout the revised manuscript.

5. Please define all abbreviations before use.

All abbreviations have been defined in the first appearance in text.

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.

Revised manuscript is free of company names, trademark symbols and registered symbols.

7. Introduction: Please avoid sectioning of the Introduction.

Section headers have now been removed in the Introduction.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

All protocol section have now been proofread and revised for the usage of imperative tense.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. The Protocol steps should contain only 2-3 actions per step and a maximum of 4 sentences per step.

10. 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.

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

Protocol section has now been revised and personal pronouns have been removed.

12. Please avoid usage of phrases such as “could be”, “should be”, and “would be” throughout the Protocol.

Changes have now been implemented accordingly.

13. Please leave a blank line between all protocol steps as well as Notes.

Changes have now been implemented accordingly.

14. Protocol: 3.8: Please avoid using any personal pronouns in protocol steps.

Respective step has been revised and personal pronouns have been removed.

15. Protocol: 4.1: Please use the imperative tense for all actions in the protocol.

Respective step has been revised to use imperative tense, exclusively.

16. Protocol: 6.4: Please specify the pH of the PBS.

Detail added in the revised version.

17. Protocol: 6.7: Please use the imperative tense for all protocol steps.

Respective step has been revised to use imperative tense, exclusively.

18. Protocol: 8.4, 8.5: Please quantitatively specify the amount of the solution needed for washing the plates.

Missing information regarding the amount of solution has now been added.

19. Protocol: 8.6: Please specify the temperature.

Missing information regarding the incubation temperature has now been added.

20. Discussion: Please avoid sectioning of the Discussion.

Section headers have now been removed in the Discussion.

21. Figures 2, 4, and 5: Please add a scale bar to the images.

Scale bars have now been added to the images in respective figures.

22. If you are reusing figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [AUTHOR] et al.[REFERENCE]”.

Submitted version did not include any figures that were reused from a previous publication. However, in the revised manuscript such previously published images have been included. Re-print permission is uploaded along with the revised manuscript in the Editorial Manager site as a supplemental file. Reused images have now been cited appropriately in the respective figure legend.

23. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please list all the materials, equipment, instrument, and software used in your work.

Materials table have now been revised to include a missing material. Missing company names and catalog numbers have been included in red font in the revised .xlsx file. Some of the materials have not been given a catalog/product number by the producing company, thereby catalog/product numbers for them could, unfortunately, not be provided in the .xlsx file.

Company names and catalog numbers for some generic material, which experimenters/readers may obtain them from any suitable source, have not been provided (e.g. screw driver, exam gloves, surgical gown).

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**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
The paper describes a procedure to implant a catheter into the cortex of adult rat in order to induce an EAE type restricted cortical demyelination.  
  
Major Concerns:  
I have three major concerns:  
i) Even though I understand that novelty is NOT a requirement for publication in JoVE, The procedure is already quite detailed in their previous recently published paper (Uçal et al., 2017).

In the previous publication authors tried to keep the respective method section as detailed as possible. Nevertheless, authors believe that both the editor and the reviewer would agree that the extent of the details presented in this protocol manuscript is far beyond than what has been presented in the methods paragraph in Uçal et al., 2017.

When trying to establish a new animal model in a new setting/lab often successful reproduction is complicated or delayed by subtle details that the original working group perhaps does not even consider to be of special relevance. Protocol papers are specifically designed in a way to avoid such difficulties. Readers, in addition, would utilize a video for the implementation of the protocol, when the manuscript is accepted for publication in JoVE.

ii) The histological illustrations of what is described as a "widespread cortical demyelination" are far from being convincing (Fig. 6) and in large of mediocre quality (Fig. 7).

In Figure 6, demyelination of the cortical grey matter at the ipsilateral and contralateral hemisphere has been represented by one field of view (FOV) for each side after PLP immunohistochemistry. Both ipsi- and contralateral cortical images at day 15 and 30 clearly demonstrate decreased PLP positivity in the subpial regions in contrast to the control [immunized + vehicle injected] brain (Figure 6A) with strong PLP staining at the respective regions. Nevertheless, authors agree with the reviewer that Figure 6 should better be revised as the widespread cortical demyelination could not be sufficiently represented with one FOV per hemisphere. Thereof, an overview image is now integrated to Figure 6 for that purpose (Figure 6G in the revised manuscript). Furthermore, images for day 3 (Fig. 6B in the original manuscript) and for day 15 (Fig. 6D and 6E in the original manuscript) have now been replaced with microscopy images with better quality (Fig. 6C, 6E, 6F in the revised version). A contralateral cortical image, in addition, for day 3 has now been included in the revised figure (Fig. 6D in the revised version). A quantification of PLP loss in both cortical hemispheres at all time points has now been included as Fig. 6J in the revised version. Finally, during the revision of the figure, authors took the chance to correct some mistakes in the scale bars of the images, as well.

Authors agree that in Figure 7 the demyelination is not easily distinguishable, largely due the lower magnification of the images. For that reason they are not part of the Figure 6 and they do not refer to the demyelination. Images in Figure 7, in fact, were intended rather to demonstrate the extent of “atrophy” in the brain upon a repeated cytokine injection through the catheter.

iii) Focusing the procedure on rat is of some interest, but the interest would be considerably increased if it was applicable to mice, due to the large array of transgenic that could be used by the scientific community.

Authors established the model first in rats on the grounds of their more extensive experience in working with rats than mice. In addition to that, establishment of the presented method in mice would have additional practical difficulties to be sorted out, some of which have been discussed in the submitted manuscript. The stability of the skull is an issue when implanting a permanent catheter and screws. Furthermore, catheter diameter also must be sufficiently large enough to avoid blockade by ingrown tissue whilst should not leave large zones of destruction after implantation. Nevertheless, authors agree with the reviewer on the remark that mouse models, indeed, provide a plethora of possibilities that could not be fulfilled with the rat models and implementation of the method in mice in future work would also be sought for by the authors.  
  
Minor Concerns:  
The references do not quote the initial papers:  
MOG-EAE  
Linington C, Bradl M, Lassmann H, Brunner C, Vass K. Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. Am J Pathol. 1988 Mar;130(3):443-54.  
and since it has been shown that M2 was identical to MOG:  
Lebar R, Lubetzki C, Vincent C, Lombrail P, Boutry JM. The M2 autoantigen of central nervous system myelin, a glycoprotein present in oligodendrocyte membrane. Clin Exp Immunol. 1986 Nov;66(2):423-34.  
Cuprizone:  
Ludwin, S.K. (1978). Central nervous system demyelination and remyelination in the mouse: an ultrastructural study of cuprizone toxicity. Lab Invest 39, 597-612.  
and even for the cytokine / MOG-EAE:  
Kerschensteiner M, Stadelmann C, Buddeberg BS, Merkler D, Bareyre FM, Anthony DC, Linington C, Brück W, Schwab ME. Targeting experimental autoimmune encephalomyelitis lesions to a predetermined axonal tract system allows for refined behavioral testing in an animal model of multiple sclerosis. Am J Pathol. 2004 Apr;164(4):1455-69.

Authors wish to thank Reviewer for the remark. These references are now added in the revised manuscript. In addition, initial papers for the MBP-EAE and PLP-EAE models are also added in the respective lines in the Introduction section.  
  
  
**Reviewer #2:**  
Manuscript Summary:  
The authors of this study present an adaptation of a model of repeated demyelination in the rat induced by subclinical immunization with MOG1-125 peptide and delivery of the pro-inflammatory cytokines TNF-α and IFN-γ via an implanted catheter. Overall this could be a useful model to test therapies specifically targeted at preventing brain atrophy in MS as it allows for repeated cycles of demyelination and remyelination  
  
Minor Concerns:  
-The authors comment on alternative anesthetic agents that can be used in the discussion. It would be helpful to mention these in the protocol description.

Alternative anesthetic agents have been discussed in the Discussion section to point out that the anesthetic combination used by the authors is not a prerequisite for the model. However, to keep the protocol steps clear and concise, authors avoided to mention them in the Protocol section. Nevertheless, the following note has now been added after Protocol Step 1.1. :

“Note: See Discussion section for alternative anesthetic preparations.”

-In the results describing figure 6 the authors mention slow behavior-please specify what this means as the rotarod test does not show significance. Is it specific testing or just observation?

The slow-down in the behavior was an observation by the experimenters and animal caretakers between 9-15 days after cytokine injection. We sought to check whether there was a significant weakening in the coordination and motor skills of animals, however, a rotarod test did not show any statistically significant difference between cytokine injected animals and controls.

-One advantage given for this model is that drug delivery to the cortex can be performed via the catheter. Please comment in the discussion on the time frame when therapeutics would be administered.

The following lines were added to the Discussion section:

“One advantage of the model is that the implanted catheter allows for testing of potential therapeutics administered to the cortex via the catheter to support remyelination at or after the peak of histologically detectable cortical demyelination (day 15 or later), whilst in a pretreatment setting this would be after immunization but before cytokine injection. The decision on the time frame when therapeutics would be administered, therefore, will be depending on the particular research question and the drug of interest.”

-The authors stain with PLP to indicate the timeframe of demyelination. Additional information on the timing or distribution of gliosis (astrocyte or microglial response timeframe) would be helpful.

-References have different formatting

The reference list was prepared with EndNote using JoVE style format. Only the first reference seems different since it citing a website, but not a journal article. Apart from that a difference in formatting did not catch our sight. If the reviewer provides more specific information, we will be happy to fix it.

**Reviewer #3:**  
Manuscript Summary:  
This protocol details a modified method to induce experimental allergic encephalomyelitis (EAE) in rats, a widely used animal model for studies of multiple sclerosis (MS). The protocol may surely be of interest to the readership of JoVE. However, there are some minor weaknesses that need to be addressed before the manuscript may be considered suitable for publication.  
  
Major Concerns:  
Authors should explain why male vs female rats have been chosen for the procedure. Compared with females, male Dark Agouti rats immunized for EAE exhibit lower incidence of the disease. Male rats possibly develop a more severe disease, but this needs to be clarified. Accordingly, authors' statement (pg.11) "....Whether such gender effects will occur for the protocol described here, is currently unknown." cannot be accepted, also considering the importance of the sex bias in both EAE and MS.

The reason for choice of males over females in the establishment of the model was based on a practical reason to avoid loss of a number of animals in the long experimental setting due to the known bile duct stenosis of Dark Agouti females. However, authors are aware of the fact that male DA rats usually exhibit a lower incidence for standard EAE, so a difference is to be expected. Authors, indeed, are currently conducting an experiment with female DA rats as well. So far the establishment of the titers after immunization appears similar to males, but they have so far no final histology as the experiment is still ongoing.

In the respective statement at page 11 pointed by the reviewer, authors’ in fact state that they have not specifically tested for validation of any expected gender differences. The respective lines have now been revised as follows to eliminate misunderstanding:

“Occurrence of such gender effects might well be expected for the protocol described here, yet remains to be validated in further experiments.”

Minor Concerns:  
-Throughout the manuscript the authors define MS as an autoimmune disease. This is not completely correct. Autoimmunity means that the immune system is reacting against normally-occurring antigens, as if these antigens were foreign. MS cannot be classified as an autoimmune disease at this time, since no specific antigen has yet been identified in MS and different structures and cells within the CNS are attacked and damaged by the immune system. "Immune-mediated" should be used instead of "autoimmune".

Authors wish to thank to the reviewer for this remark. The wording has now been changed throughout the text accordingly.

-Pg. 2/Background: "No treatment option has yet proven efficacious for the progressive types, with the exception of a recent clinical trial of B-cell depleting therapy". Authors should specify that B-cell depleting therapy was shown to be efficacious just in a subgroup of PPMS patients with active inflammation.

The respective lines have now been revised as follows:

“No treatment option has yet proven efficacious for the progressive types, with the exception of a recent clinical trial of B-cell depleting therapy, which was shown to be efficacious in a subgroup of PPMS patients with active inflammation.”

-English requires some revision: there are several typos, grammatical and verb tenses errors, as well as sentence structure errors.

The manuscript has been proofread by a native speaker that had been trained as a molecular biologist.  
  
**Reviewer #4:**  
Summary  
The authors propose a protocol for repetitive induction of cortical experimental autoimmune encephalomyelitis in rat by intracerebral catheter implantation. The advantage of this method is the non-invasiveness of interventions subsequent to the catheter implantation.  
Evaluation  
This seems like a useful method and the protocol is clear and easy to follow, even though video material will clearly help in understanding and implementing key steps. However, the drawbacks of the described approach should also be discussed.  
  
Major points:  
\* The catheter is a large-bore foreign body implanted into the cortex - this is bound to have biological effects; the authors should discuss these and potentially provide data regarding astrogliosis and microglial activation due to the catheter.

In the original publication of the model (PMID: 28457906), this question was specifically addressed with a full panel of histological slides depicting the astrocytic reaction around the catheter implantation site. Although there is some astrocytic reaction due to the catheter implantation, it appears to be minor and not reach the extent of an impenetrable glial scar. On the other hand, higher levels of astrocyte activation were observed around the catheter after cytokine injection (1-3 days), but decreased at later stages (15 and 30 days).

Microglia activation was also quantitatively assessed over the full course of the experimental timeline including the control groups in the original publication of the model. In the control animals without cytokine injection, microglial activation was observed to a much lesser extent compared to the animals with cytokine injection at different time points.

To address the suggestion by the reviewer, the respective sentences in the Discussion section has now been revised as follows together with references to previous publication (PMID: 28457906):

“The protocol uses a 26 gauge catheter, which is small enough to avoid extensive traumatic injury and large enough to avoid an increased rate of clogging of catheter tip over the long course of the experiment. Certainly, catheterization itself causes astrocytic and microglial activation, however this is minor when compared to the cytokine injected animals (REF).”

\* The authors nee to provide some quantifications of typical results so labs establishing this technique can confirm that they have successfully replicated the relevant effects.

A quantification of loss of PLP immunoreactivity in ipsi and contralateral cortex has now been integrated to Figure 6.

Minor points:  
\* Should animals not be controlled for hypothermia after the first surgery before brought back the cages?

Authors wish to thank to the reviewer for this remark. The protocol step 2.2 has now been revised to include following sentence:

“Place a homoeothermic blanket on the stereotactic frame before positioning the animal to avoid hypothermia throughout the surgery.”

In addition, Protocol step 5.1 has now been revised as follows:

“Return the animal to the modified cage and keep under observation for 1-3 h thereafter with application of infrared light to avoid hypothermia.”

\* The authors mention a greater depth spread of the demyelination (covering almost equally the entire layer 1-4 of the neocortex) using a single catheter mediated cytokine injection compared to previously described cortical EAE models. What could be the reason for such a difference?

Authors assume that the “catheter-mediated, atraumatic injection modus” at a “speed consistent with the physiological flow of the interstitial fluid” are keys to that observation.

The controlled delivery of cytokine mix via the catheter behind a restored, intact blood-brain barrier mainly allows for the transport of cytokines to the distant regions with the physiological flow of interstitial fluid. It should be noted that in earlier versions of the model (e.g. Merkler et al., 2006) pro-inflammatory cytokines were delivered by a single traumatic injection. Although injected slowly, this traumatic modus of delivery, most likely, leads to the traumatic opening of tissue capillaries and possible spilling (and thereby loss) of cytokine liquid into the blood stream, hindering the spread of demyelination both in depth and distance (i.e. contralateral). This largely explains why only a small rim of demyelination at the injection site could be produced in that method, which was then fully remyelinated within a few days.

Observation of a cone shaped pattern of spreading demyelination at the catheter site at day 1 in the current model supports this presumption as well. Furthermore, the first demyelinating areas at the contralateral site are the regions beneath the anchor screws. This is most probably due to the fact that a low CSF flow area is created in these regions due to the anchor screw, similar to the physiological low flow areas in the human brain such as the depths of sulci, consistent with a common theory on development of cortical demyelination in human progressive MS.

\* Concerning the Figures:  
- Comments on the diameter of the catheter and the lesion size that can be observed in (6A) should be made.

Catheter size was 26 Gauge; thereby the diameter was 404 µm. In Figures 6A, B, C, G, and H (in the revised manuscript), differences in the lesion diameter are observed, largely due to the 1) differences in slicing angle and 2) anteroposterior position of the coronal section with respect to the lesion center; as well as 3) dehydration of the brain after paraffin embedding, since the catheter was already removed during organ extraction.

It should also be noted that during the revision of the Figure 6, authors have taken the chance to fix some mistakes in the scaling information provided in the original submission.

- Does the depth/length of the catheter matter for the demyelination pattern?

Authors wish to thank to the reviewer for this important remark and wish to respond reviewer’s question in the following lines, which are also added to the Discussion section in the revised version:

“A similar depth of demyelination is, in fact, created in both ipsi- and contralateral regions with the presented method. This implies that the catheter depth/length might not play a major role for the pattern and extent of demyelination in the cortex. Therefore one might consider a modification in the catheter length in order to reduce the catheter-induced lesion size. Nevertheless, a significantly shorter catheter length might cause a slightly less pronounced cortical demyelination, whilst a conclusive answer would only be obtained by experiments specifically testing for the catheter length.”