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Rat Model of Widespread Cerebral Cortical Demyelination Induced by Intracerebral Injection of Pro-Inflammatory Cytokines

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Author Comments:	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. In addition, please note that we have changed the publication option from the Standard Access to the Open Access during revision in the Editorial Manager System. We have replaced the first page of the Author Licence Agreement with the a new one to reflect the selection of Open Access Option, as well. We will be happy to transfer the missing amount of publication fees for Open Access upon receipt of the bill from JoVE. With many thanks for your efforts and best regards, Sonja Hochmeister M.D. Ph.D.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

Graz, January 17th, 2018

Dear Editor,

Please find enclosed our manuscript „ Rat Model of Widespread Cerebral Cortical Demyelination Induced by Intracerebral Injection of Pro-Inflammatory Cytokines “ by M. Ücal & M. Haindl et al.

In this manuscript we give a detailed protocol on our new rat model of cortical demyelination, which was published in August 2017. This manuscript is a totally revised version of a previous submission. The protocol will allow readers to successfully reproduce cortical demyelination highly reminiscent to the pathology seen in autopsy material of human progressive multiple sclerosis sufferers. We hope that this model will contribute to the advancement of knowledge regarding the late progressive phase of MS.

Many thanks for your efforts and best regards,

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TITLE:

Rat Model of Widespread Cerebral Cortical Demyelination Induced by an Intracerebral Injection of Pro-Inflammatory Cytokines

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KEYWORDS:

Cortical demyelination; multiple sclerosis; animal model; progressive multiple sclerosis; neuroinflammation; demyelinating disorders

SUMMARY:

The protocol presented here allows the reproduction of a widespread grey matter demyelination of both cortical hemispheres in adult male Dark Agouti rats. The method comprises of intracerebral implantation of a catheter, subclinical immunization against myelin oligodendrocyte glycoprotein, and intracerebral injection of a pro-inflammatory cytokine mixture through the implanted catheter.

ABSTRACT:

Multiple sclerosis (MS) is the most common immune-mediated disease of the central nervous system (CNS) and progressively leads to physical disability and death, caused by white matter

lesions in the spinal cord and cerebellum, as well as by demyelination in grey matter. Whilst conventional models of experimental allergic encephalomyelitis are suitable for the investigation of the cell-mediated inflammation in the spinal and cerebellar white matter, they fail to address grey matter pathologies. Here, we present the experimental protocol for a novel rat model of cortical demyelination allowing the investigation of the pathological and molecular mechanisms leading to cortical lesions. The demyelination is induced by an immunization with low-dose myelin oligodendrocyte glycoprotein (MOG) in an incomplete Freund's adjuvant followed by a catheter-mediated intracerebral delivery of pro-inflammatory cytokines. The catheter, moreover, enables multiple rounds of demyelination without causing injection-induced trauma, as well as the intracerebral delivery of potential therapeutic drugs undergoing a preclinical investigation. The method is also ethically favorable as animal suffering and disability is relatively modest. The expected timeframe for the implementation of the entire protocol is around 8 - 10 weeks.

INTRODUCTION:

MS is an immune-mediated, inflammatory disease of the CNS, which primarily damages myelin sheets, but eventually leads to the axonal loss and permanent neuronal damage. MS is the most common immune-mediated disease of the CNS with an estimated prevalence of about 2.3 million people worldwide, according to the National MS Society[1], and represents a major personal and socioeconomic burden. The average age of the disease onset is 30 years and leads to the loss of productive years by causing severe disability. MS is currently incurable, and the present treatment modalities aim to manage the symptoms during an acute relapse in relapsing-remitting MS and to modify the course of the disease to decrease the relapse frequency by immunomodulatory therapy[2,3]. No treatment option has yet proven efficacious for the progressive types[4], with the exception of a recent clinical trial of B-cell depleting therapy, which was shown to be efficacious in a subgroup of primary progressive MS (PPMS) patients with active inflammation[5]. Whilst several potential genetic[6] and environmental risk factors[7] have been identified, the etiology of MS, however, remains unknown.

MS is characterized by large inflammatory demyelinating plaques in, and diffused injuries to, the white matter[8,9]. Focal lesions have been associated with an extensive T-cell mediated attack, oligodendrocyte destruction, reactive astrogliosis, and axonal degeneration, leading to a motor neuron decline. Grey matter demyelination and atrophy have gained recognition as additional histopathological features of the disease[9-11]. The latter has been suggested to contribute to the neurological dysfunction and cognitive decline in patients[12,13]. Three patterns of cortical demyelination have been distinguished, namely i) leukocortical, contiguous with the white matter lesions (34%), ii) small, perivascular (16%), and iii) subpial (50%). Unlike focal white matter lesions, these grey matter lesions have been reported to lack a T-cell mediated attack and are instead characterized by an enhanced microglial activation, apoptosis, and neuronal loss[12].

To date, it has not been possible to recapitulate human MS in a single animal model, largely due to the complexity of the disease. A variety of MS animal models, each simulating different aspects of disease pathogenesis and progression have, instead, been developed[14,15]. Current animal

models mimic three different disease processes: i) focal inflammatory lesions, ii) diffuse white matter injury, and iii) diffuse grey matter pathology.

Animal studies of MS white matter plaques have mostly been conducted in rodent encephalomyelitis (EAE) models. Test animals are actively immunized with an emulsion containing a myelin antigen [usually myelin oligodendrocyte glycoprotein (MOG)[16,17], myelin basic protein (MBP)[18], or proteolipid protein (PLP)[19]], together with a complete Freund's adjuvant (CFA)[20]. The disease can also be passively induced by an adoptive transfer of myelin-specific T-cells[21]. The disease course depends on the antigen/mouse strain combination that is used. MOG_{35-55} in C57BL/6, for instance, results in a monophasic chronic disease[22], whilst $PLP_{139-151}$ in Swiss Jim Lambert (SJL) mice leads to a relapsing-remitting disease course[23] in a gender-specific manner[24]. Rat MOG_{35-55}, further, induces an encephalitogenic T-cell response, whilst human MOG_{35-55} induces B-cell-dependent inflammation in C57BL/6 mice[25]. Various EAE models provide an excellent tool to study cell-mediated inflammation primarily in the spinal cord and cerebellum, but forebrain structures like the cortex, corpus callosum, and subcortical structures remain largely spared[26]. Neither diffuse white matter injury nor grey matter demyelination is, furthermore, adequately replicated in EAE models[26,27]. Cortical demyelination associated with active EAE induction has been reported in marmosets[28,29] and in certain Lewis rat sub strains, in the latter case attributed to the prevailing combinations of MHC class I and class II isotypes and alleles[30].

The cuprizone model[31] is a useful tool for studying diffuse white matter demyelination and grey matter pathology with an extensive demyelination of the cortical, sub-cortical[32], and hippocampal[33] regions, as well as the corpus callosum[34] and the caudate putamen[35]. Cuprizone intoxication, which, in principle, results in metabolic stress-induced apoptosis of the oligodendrocytes, mimics some characteristics of the cortical demyelinating lesions of MS brains such as a microglia activation, astrogliosis, and a relative lack of infiltrating peripheral immune cells. The lack of neuronal apoptosis and thalamic atrophy, as well as the complete resolution of demyelination with a robust remyelination observed upon the cessation of dietary cuprizone supplementation[32], however, limit the cuprizone intoxication's usage as a preclinical MS model. Toxic demyelination can also be induced by a focal injection of lysolecithin or ethidium bromide into the white matter tracts[36,37], but these methods are rarely used. Toxic demyelination models are especially suitable for the analysis of the complex mechanisms of remyelination, such as the requirement for oligodendrocyte progenitor cells and astrocytes[38,39]. Detailed information on EAE and intoxication models are provided in two recent reviews[15,40].

The cytokine-induced demyelination was initially developed to study spinal white matter lesions in EAE[41]. Later, it was modified to study cortical grey matter pathologies in MS. Dark Agouti (DA) or Lewis rats are first primed by a sub-clinical immunization with MOG_{1-125}[42,43] or MOG_{1-116}[44] in an incomplete Freund's adjuvant (IFA). Unlike classical EAE models, these primed animals do not exhibit clinical symptoms of focal inflammatory lesions in the spinal cord. Instead, an inflammatory response and demyelination in the brain are subsequently achieved by an intracerebral administration of a pro-inflammatory cytokine mixture [tumor necrosis factor alpha

(TNFα) and interferon gamma (IFNγ)] once the animals have attained a stable titer of anti-MOG antibodies in the blood.

Studies by Merkler *et al.*[42] and Gardner *et al.*[44] have proven the efficacy of a subpial cortical demyelination induction by a sub-clinical MOG immunization and an intracerebral or subarachnoidal cytokine injection. The reported duration of the demyelination was, however, too short—a complete remyelination occurred in 14 days or less, thereby limiting the window for any pharmacological intervention testing. Both models, moreover, utilize a traumatic injection modus, which could *per se* cause an injection trauma and a blood-brain barrier (BBB) breakdown and thus lead to an uncontrolled recruitment of inflammatory cells into the parenchyma. Both studies, furthermore, demonstrated demyelination that is restricted to a limited area, in the ipsilateral cortex, or in the close vicinity of the site of the cytokine injection.

To overcome these limitations, we implanted a catheter in the right parietal cortex of DA rats, with the catheter tip located just above the corpus callosum. To allow a full recovery of the BBB integrity, the animals were allowed a resting period of 2 weeks after the catheter implantation. Subsequently, the rats were sub-clinically immunized with 5 µg of recombinant MOG_{1-125} in IFA. Following the attainment of a stable anti-MOG antibody titer after around 4 weeks, 2 µL of cytokine mixture was injected *via* the catheter within 10 min using a programmable syringe pump. This procedure elicited a widespread cortical demyelination of both the ipsi- and the contralateral cerebral hemispheres in 15 days, with a partial remyelination around 30 days post-cytokine injection[43]. Multiple demyelination phases could, moreover, be induced by the repeated administration of pro-inflammatory cytokines through the catheter, and global brain atrophy, a common feature of progressive MS subtypes[45], could be induced as early as after the second demyelination phase[43]. Importantly, the implanted catheter could also be used to test pharmacological interventions.

The protocol described below provides a detailed explanation of the experimental steps for a reproducible generation of widespread cortical demyelination in both cerebral hemispheres of DA rats using an intracerebral catheter.

PROTOCOL:

All methods described here have been approved by local authorities (Bundesministerium für Wissenschaft und Forschung (Austrian Ministry of Science and Research); License Number: 66.010/0132-WF/V/3b/2014). The adult male DA rats (10 - 12 weeks of age) were housed in a 12/12 h light/dark cycle with free access to food and water.

1. Material Preparation

1.1. Prepare an anesthetic mixture: 0.02 mg/mL of fentanyl, 0.4 mg/mL of midazolam, and 0.2 mg/mL of medetomidine (final concentrations in the mixture).

Note: See the respective **Discussion** section for alternative anesthetics.

1.2. Prepare an antidote mixture: 0.07 mg/mL of flumazenil and 0.42 mg/mL of atipamezole (final concentrations in the mixture).

1.3. Assemble the catheter and the catheter cap with the inlet and the screw. Cut the catheter to a length of 2 mm with a scalpel (**Figure 1**).

Note: Do not use scissors for this, since they squeeze and distort the circular cross-sectional shape of the catheter tip.

2. Surgical Preparation

2.1. Anesthetize the rat by an intraperitoneal (i.p.) administration of the anesthetic mixture (1.5 mL/kg of body weight).

2.2. Shave the head of the rat between the ears using the electric shaver. Place a homeothermic blanket on the stereotactic frame before positioning the animal, to avoid hypothermia throughout the surgery.

2.3. Immobilize the rat's head in the stereotactic frame using the ear bars and bite plate, ensuring that the head is horizontal and stable. Check the stability by applying pressure to the skull with finger or forceps.

Note: A loose fixation and non-horizontal positioning within the stereotactic frame may cause a deviation from the intended coordinates.

2.4. Apply lubricating eye drops to prevent cornea dryness during the surgery. Cover the eyes with an opaque material to prevent any surgical light exposure.

2.5. Clean the shaven area by alternating the application of 70% ethanol and 10% povidone-iodine complex.

Note: Follow all precautionary measures during the surgery to avoid any infection.

3. Catheter Implantation

3.1. Make a longitudinal incision of about 2 cm of length in the middle of the head skin. Use bulldog clamps to hold the skin off to the sides. For an overview of these steps, see **Figure 2**.

3.2. Remove the blood using a cotton-tipped applicator.

3.3. Remove the skull periosteum. Clean the tissue with the cotton-tip applicator and expose the skull bone. Allow the skull to dry for about 1 min.

3.4. Identify the anatomical landmarks, Lambda, Bregma, and medial suture. With the drill installed on the stereotactic frame, position the drill tip at the Bregma as the starting point. Move 2 mm posterior from the Bregma and move ~2.4 mm laterally to the medial suture.

3.5. Drill a 0.5 mm diameter hole for the catheter at this position. Gently puff away any bone dust.

Note: It is important that the dura mater remains intact during the drilling. To ensure this, 1) use a drill that can be installed on the stereotactic frame, 2) inspect the hole frequently during the drilling, and 3) drill down in small steps—if too much pressure is applied to the skull, the drill tip will continue into, and damage the brain when the skull is fully penetrated.

3.6. Drill 3 further holes (~1.3 mm in diameter) for the anchor screws a few millimeters away from the first hole. Gently blow bone dust away.

Note: Select anchor screw locations that provide enough space for the catheter top (~2 mm in diameter) and the anchor screw tops (~1 mm).

3.7. Remove the bone dust by irrigation with around 1 - 2 mL of sterile phosphate-buffered saline (PBS) or physiological saline using a syringe. Clean the skull. Tighten the anchor screws by 2 - 3 full turns.

Note: The anchor screws are necessary to stabilize the set-up by holding the dental cement, and, thereby, the catheter, in place. Whilst tightening an anchor screw, ensure that it is not easily removable by gently lifting it upwards with forceps. Since the implantation of the catheter itself causes tissue trauma, additional dura injury whilst drilling for, or tightening the anchor screws will lead to multiple traumatic injuries, and possibly hamper the comparability within a group. Tighten the anchor screws first and insert the catheter last.

3.8. Insert the 2-mm length catheter through the first hole, perpendicular to the skull surface. While still holding the catheter in, apply a little dental cement and let it polymerize with a brief (~5 s) exposure to the dental curing light to stabilize the catheter, allowing the use of both hands in the next step.

CAUTION: While working with a dental curing light, avoid looking directly at the tip, or at the light reflected from the application area, as the high intensity of this light can cause retinal damage. Use appropriate protective goggles.

3.9. Apply more dental cement around the catheter, anchor the screws, and solidify the dental cement with the dental curing light (~15 - 30 s). Confirm the hardening of the cement with the tip of a forceps.

4. Closing of the Wound and Antagonization of Anesthesia

4.1. Close the head skin with resorbable sutures, anterior and posterior to the catheter.

Note: Since there will be a bumpy set-up over the skull at the end of the implantation, do the wound closure accordingly. Lifting up the skin too much will result in discomfort for the animal.

4.2. Inject the antidote mixture subcutaneously (1.5 mL/kg of body weight) using a 1 mL syringe with a 26 G needle.

4.3. Administer enrofloxacin (2.5%) by a subcutaneous injection (7.5 mg/kg of body weight) (prophylactic antibiotic treatment).

5. Post-operative Care and Medication

5.1. Return the animal to the modified cage and keep it under observation for 1 - 3 h, with an application of infrared light to avoid hypothermia.

5.2. Repeat the enrofloxacin treatment (7.5 mg/kg of body weight) the day after the surgery. Administer carprofen (1 mg/mL) for pain relief by a subcutaneous injection (3 mL/kg of body weight) the day after the surgery.

6. Preparation of Immunization Mixture (at the Earliest 14 Days after Catheter Implantation)

Note: Place the syringes on ice during the preparation procedure.

6.1. Connect two 10 mL Luer lock tip glass syringes to the short arms of a 3-way stopcock and close the third outlet with the long arm.

6.2. Ensure the connections are secure and leak-free: add approximately 4 mL of sterile PBS to the open syringe while holding piston 2. Insert piston 1 and push both pistons back and forth whilst checking for leakage. If no leakage occurs, discard the PBS and remove piston 1 again.

6.3. Pipette 1 mL of IFA and 50 µg of $rMOG_{1-125}$ together and adjust the mixture to a final volume of 2 mL with sterile PBS (pH 7.4) in a suitable tube.

Note: Due to losses of emulsion on the tips or walls of syringes during the preparation, prepare a larger volume than intended for the administration. It is similarly more practical to prepare for more than 1 animal at once.

6.4. Place the diluted IFA and $rMOG_{1-125}$ mixture in the open syringe. Insert the piston gently whilst maintaining a loose pressure on the opposite piston (**Figure 3A**).

6.5. Emulsify the inoculum by driving it from one syringe to the other by pushing the pistons back and forth, until it is white and viscous (**Figure 3B**).

6.6. Fix a 1 mL Luer lock syringe to the open short arm of the 3-way stopcock and fill it with inoculum (**Figure 3C**). Distribute all inoculum to 1 mL syringes. Keep it on ice until the injection. Administer the mixture on the day of the preparation.

7. Immunization

7.1. Inject 200 µL subcutaneously at the tail base under a temporary isoflurane anesthesia using a 21 G needle.

Note: Administer the injection slowly as the solution is viscous.

8. Determination of Antibody Titers

8.1. Withdraw ~200 µL of blood 4 weeks after the immunization in order to determine the anti-MOG antibody titers.

8.2. Coat the wells of a 96-well plate with MOG (5 µg/mL in PBS) and incubate them for 1 h at 37 °C.

8.3. Block the plate with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature.

8.4. Incubate the plate with rat serum (1:50) and standard it for 2 h at 37 °C. Wash the plate 3x with 200 µL of PBS/Polysorbate 20.

8.5. Incubate the plate with horseradish peroxidase-conjugated anti-rat IgG secondary antibody (1:10,000). Wash the plate 3x with 200 µL of PBS/Polysorbate 20.

8.6. Add 100 µL of peroxidase substrate solution per well and incubate it for 20 - 30 min in the dark at room temperature.

8.7. Measure the optical density at a 405 nm wavelength and calculate the antibody titer from the optical density using a standard curve.

9. Intracerebral Cytokine Injection

9.1. Adjust the length of the connector cannula (2 mm). (See **Figure 4** for the preparation steps.)

9.2. Fill a 1 mL syringe with the cytokine mixture (500 ng/µL of TNF-alpha, 300 U/µL of recombinant rat IFN-gamma in sterile PBS). Connect the syringe to a connector cannula. Fill the cannula with the cytokine mixture. Avoid any bubbles.

9.3. Mount the syringe onto the programmable syringe pump and program it to inject 0.2 µL/min (**Figure 5A**). Start the pump and keep it working in order to avoid an air bubble formation at the tip of the cannula.

Note: The injection speed must take into account the inner diameter of the specific syringe used; thereby, the syringe diameter has to be registered during the pump set up.

9.4. Anesthetize the rat with isoflurane in a chamber (~2 min, mixed with 2 L/min of oxygen) and then sustain the anesthesia through a mask (mixed with 1.5 L/min of oxygen) (**Figures 5B** and **5C**).

9.5. Remove the catheter cap with the inlet. Insert the connector cannula into the catheter and screw and tighten it (**Figures 5D** and **5E**).

Note: Do not overtighten it, as this will destroy the upper tip of the catheter.

9.6. Allow the injection to proceed for 10 min (the total volume of injection being 2 µL). Stop the pump. Leave the cannula inside the catheter for 20 min to allow the injected volume to fully diffuse.

9.7. Unscrew the connector cannula and remove it slowly to avoid a vacuum effect.

9.8. Reattach the catheter cap with the inlet and screw it. Allow the animal to recover from the anesthesia in a cage.

REPRESENTATIVE RESULTS:

Cortical demyelination could be assessed at different time points after a cytokine injection by immunohistochemistry for proteolipid protein (PLP) (**Figure 6**). **Figure 6A** shows intact PLP immunoreactivity at day 15 in a MOG-immunized control animal that received only sterile PBS through the implanted catheter. On day 1 after the cytokine injection, demyelination could already be detected in MOG-primed animals, albeit only in the close vicinity of the catheterized area (**Figure 6B**). The PLP immunoreactivity stays intact in the contralateral cortex 1-day post-cytokine injection. On day 3, a gradual increase in the loss of the PLP immunoreactivity, which spreads in the ipsilateral cortex (**Figure 6C**), could be observed. Contralateral cortical demyelination could also be detected at day 3 (**Figure 6D**), but it is rather restricted to the area beneath the anchor screws, possibly due to a low-flow area of interstitial fluid caused by the anchor screws[43]. The absence of a similar observation in the PBS-injected control animals excludes the possibility of trauma-induced demyelination stemming from the anchor screw.

Between days 9 - 15, demyelination affects large parts of the cortex of both hemispheres (**Figures 6E**, **6F**, and **6G**). This coincides with an observation of slow behavior, though without a statistically significant decrease in motor skills in a rotarod test[43]. The cortical demyelination is sustained for up to 30 days post-cytokine injection in both hemispheres (**Figures 6H** and **6I**) with only a partial remyelination. **Figure 6J** shows a quantification of PLP loss in the cortical grey matter after the intracerebral cytokine injection. It should be noted that PLP immunoreactivity has not yet been assessed after periods longer than 30 days; thereby, the instantaneous resolution of remyelination, if there is any, remains to be assessed by further experimentation. A second

administration of cytokine mixture through the implanted catheter 30 days after the first injection results in marked brain atrophy at day 15 (**Figure 7**).

FIGURE AND TABLE LEGENDS:

Figure 1: Preparation of the catheter. (**A** and **B**) The guide cannula and the dummy cannula (catheter cap with inlet) are assembled and screwed. (**C**) Then the catheter is cut to 2 mm in size with the help of a scalpel. The microscopic observation showed that the usage of scissors for that purpose distorts the circular shape of the cannula tip, and, thereby, must be avoided.

Figure 2: The implantation of the catheter. (**A**) The surgery starts with a longitudinal incision and removal of the periosteum. (**B, C**) This panel shows the marking of the place for the catheter at 2 mm posterior from Bregma and 2.4 mm lateral to the right from the sagittal suture; as well as the places for the holes intended for the three anchor screws with an appropriate distance from the catheter and Lambda. (**D**) After drilling the catheter hole (a 0.5 mm diameter, with a round drill tip) and the holes for the anchor screws (1.3 mm-diameter with a twisted drill tip), the anchor screws are tightened. (**E, F**) Then the catheter is inserted and the whole setup is stabilized with polymerizing dental cement. (**G**) The wound is stitched with two or three knots anterior and posterior to the catheter. B = Bregma; L = Lambda; C = Catheter; S1, S2 and S3 = places for the holes for the three anchor screws. The scale bars = 1 cm.

Figure 3: Preparation of rMOG/IFA emulsion. (**A**) The mixture of rMOG, PBS, and IFA is emulsified by pressing the inoculum from one syringe to another by pushing the pistons back and forth, (**B**) until it is white and viscous. (**C**) Subsequently, the inoculum is distributed to 1 mL syringes for the injection. 5 µg of rMOG is used in 200 µL of PBS/IFA mixture to sub clinically immunize one rat; however, due to the losses at the tips and walls of the syringes during the preparation, a larger volume should be prepared.

Figure 4: Preparation of the connector cannula. (**A** - **D**) These panels show how a connector and an internal are assembled with a 2-mm size template guide cannula. (**E**) The internal is cut to the same size as the guide cannula with the help of a scalpel (**F**) and the template guide is then unscrewed. (**G**) The other end of the connector cannula is fixed to a 1 mL syringe, which contains the injection mix, with a 20 G needle. The scale bars = 3 cm.

Figure 5: Intracerebral injection. (**A**) A programmable syringe pump is adjusted for a 2 µL/min injection speed, and the 1 mL syringe filled with cytokine mix (or sterile PBS for the controls) is mounted to the pump. (**B**) The animal is first anesthetized in the chamber using 5% isoflurane with a 2 L/min oxygen flow and then (**C**) the anesthesia is sustained through the mask using 2.5% isoflurane with a 1.5 L/min oxygen flow. (**D**) The catheter cap with the inlet (the dummy cannula) is screwed off and the injection cannula is inserted through the implanted catheter. Since the volume of the injection is very small, the investigator should be cautious to avoid air bubbles at the tip of the cannula. For that reason, it is important to start the insertion while the pump is in operation and only when there is a growing liquid bubble at the tip. The extra volume will not go into the brain anyway, as it breaks down on top of the catheter before the insertion. (**E**) Then the

connector cannula is tightened, and the pump is let to operate for 10 min. After 10 min of injection, the pump is stopped, and the cannula is left inside for 15 - 20 min to allow for the diffusion of the injected volume to the interstitial fluid. The scale bars = 5 cm, unless indicated otherwise.

Figure 6: PLP immunoreactivity in coronal brain sections. (**A**) This panel shows a control brain (MOG-primed) with a PBS injection (day 15), which does not result in a cortical demyelination. (**B**) As early as day 1 post-cytokine injection, demyelination is apparent in the catheter area. (**C**) A broader loss of PLP immunoreactivity is observed in the ipsilateral cortex at day 3, (**D**) as well as at the contralateral side. Widespread loss of PLP immunoreactivity is observed in both hemispheres at day 15, as (**E**) this panel shows the ipsilateral cortex and (**F**) this panel shows the contralateral cortex. (**G**) An overview of both hemispheres is given in showing the widespread loss of PLP at day 15. At day 30, as (**H**) this panel shows the ipsilateral cortex and (**I**) this panel shows for contralateral cortex, there is still remarkable demyelination, but also some remyelinated areas could be observed. (**J**) This panel shows the quantification of the demyelination (PLP loss in mm^2/hemisphere). 1.5 - 2 µm coronal brain sections were used for the PLP detection with MS anti-PLP with a dilution factor of 1:500. The sections were counterstained with hematoxylin for cell nuclei. For detailed information on the immunohistochemistry, see Ucal *et al.*[43]. Panel **G** and **J** were modified from Ucal *et al.*[43].

Figure 7: Brain atrophy after second cytokine injection. (**A**) This panel shows a control brain (MOG-primed) with a PBS injection (day 15). (**B**) At day 15 after the first cytokine injection, a second injection leads to brain atrophy within 15 days. 1.5 - 2 µm coronal brain sections were used for the PLP detection with MS anti-PLP with a dilution factor of 1:500. The sections were counterstained with hematoxylin for cell nuclei. For detailed information on immunohistochemistry, see Blakemore[37]. The scale bars = 500 µm.

Figure 8: Modified cages to prevent catheter removal by the animal. In the standard cages, the food holder space on the grid is located closer to the cage bottom, creating a risky narrow space that increases the chance of the catheter to tangle with the grid and, thereby, its removal. To avoid this, the cage has to be modified. This narrow space was blocked with a transparent plane, allowing the observance of the animal. The food has to be given inside the cage in these modified cages.

DISCUSSION:

Our method uses DA rats. The adaptation to mice will likely require the use of a smaller catheter and screws. It should also be borne in mind that the disease course, the inflammatory response, and the extent of demyelination might differ from what is presented here if a different species/strain is used. Such differences have been observed with classical EAE models using different strains of mice. MOG_{92-106} of rat origin, for instance, resulted in primary progressive or secondary progressive EAE in A.SW mice, whilst it induced relapsing-remitting EAE in SJL/J mice[46]. Animals of the same strain should, therefore, be used. Gender differences in EAE manifestation have also been reported in various previous studies[24]. The occurrence of such gender effects

might well be expected for the protocol described here yet remains to be validated in further experiments.

The intraperitoneal (IP) administration of an anesthetics mixture comprising 0.02 mg/mL of fentanyl, 0.4 mg/mL of midazolam, and 0.2 mg/mL of medetomidine is used for the surgical intervention. Adult male DA rats weighing 270 - 300 g require around 0.4 - 0.6 mL of this mixture (*i.e.*, ~1.5 mL/kg) to induce an anesthesia lasting 60 - 90 min. Following the surgery, the anesthesia is antagonized by a subcutaneous injection of an antidote comprising 0.07 mg/mL of flumazenil and 0.42 mg/mL of atipamezole in physiological saline (0.9% NaCl). A dose of 1 - 1.5 mL/kg antagonizes the anesthesia within 5 min. Alternatively, the animals can be allowed to wake up spontaneously upon a physiological washing out of the anesthetics, but in that case, the animals would need to be kept under observation until they are fully conscious.

Other anesthesia options frequently used for animal surgeries, such as an IP injection of ketamine and xylazine[47] or sodium pentobarbital[48], or an inhalation of volatile anesthetics like isofluorane[49] and halothane[50], can also be considered for the surgery presented here. It is critical, however, to choose an anesthetic agent that does not interfere with the intended downstream intervention(s).

During the immunization and intracerebral cytokine injection, 5% isoflurane is used for the anesthesia. The model described here was established using rats, and the experimental details listed are, thus, specifically applicable to the rat. The catheter implantation coordinates were selected to enable the simultaneous analysis of possible white matter changes (the catheter tip in the corpus callosum). Whilst the catheter insertion site can be varied with respect to the anteroposterior and lateral position, the selection of the central sulcus requires the avoidance of damage to the superior sagittal sinus.

A further feature of the described method is the equivalent demyelination of both ipsi- and contralateral hemispheres, possibly resulting from the carriage of the injected cytokine mixture to the subarachnoid space by the physiological flow of the interstitial fluid from cortical regions[51]. The injection mode, and not the location of the catheter, therefore, causes demyelination throughout the cerebral cortex, and the choice of right or left parietal cortex should, thus, be immaterial in this regard.

The protocol uses a 26 G catheter, which is small enough to avoid extensive traumatic injury and large enough to avoid an increased rate of clogging of the catheter tip over the long course of the experiment. Certainly, the implantation and the presence of the catheter itself cause an astrocytic and microglial activation, also in the control animals receiving only the catheter implantation; however, this is minor when compared to the cytokine-injected animals.[43] To avoid any interference with subsequent analyses, we used MRI-compatible catheters made of poly-ether-ether-ketone (PEEK).

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

in the pattern and extent of demyelination in the cortex. Therefore, a modification of the catheter length might be considered 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.

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

Following the catheter implantation, it is important to single out the animals in the modified (preferably high top) cages in order to avoid catheter removal (**Figure 8**). Animals might also unscrew the catheter cap with the inlet, although this rarely happens. The animals should be observed daily and removed caps should be replaced with fresh ones, to avoid catheter tip blockage in the absence of an inlet, and to ensure an accurate delivery into the parenchyma following the intracerebral injection. The animals are immunized at the earliest 2 weeks after the catheter implantation to allow the healing and closure of the blood-brain barrier.

Serum anti-MOG antibody titers should be measured after the immunization. A dose-response experiment showed that 5 µg of MOG_{1-125} (in IFA) provided sufficient immunization within 4 weeks in adult male DA rats. A titer of 5,000 µg/mL and higher would be sufficient, but will certainly depend on several factors, including the MOG preparation and the animal strain and, thus, will have to be determined individually. It is important to avoid excessively high antigen doses potentially resulting in a classical EAE phenotype with paralyzed hind limbs even before the cytokine injection.

Each animal is immunized with 5 µg of recombinant myelin oligodendrocyte glycoprotein ($rMOG_{1-125}$) emulsified in 200 µL of incomplete Freund's Adjuvant (IFA). Since some of the emulsion is lost within the syringe during the preparation, it is advisable to prepare more than this amount for each animal. We used recombinant MOG (1-125 from the N-terminus of rat MOG), which was expressed in *Escherichia coli* and was then purified to homogeneity by chelate chromatography, dissolved in 6 M urea, and dialyzed against PBS to obtain a physiological preparation[52,53]. Commercially available MOG may, however, also be used.

Other antigen preparations, such as MOG_{1-116}, MOG_{35-55} or $PLP_{139-151}$ are used in various EAE models, and antigen and animal strain differences are known to induce distinct disease phenotypes in these models[20]. These antigen preparations were not tested in DA rats and, if used in preference to $rMOG_{1-125}$, might induce a disease phenotype or histology results differing from what is presented here.

A connector cannula the same length as the catheter is prepared prior to the intracerebral injection. This can be done by assembling it with a template catheter and cutting it to the same size (2 mm in length) (**Figure 4**). It is important that the connector cannula be air bubble-free during the cytokine injection—because the injection volume is only 2 µL, even a tiny air bubble at the cannula tip will significantly reduce the volume of the liquid successfully delivered into the brain. This is achieved by keeping the pump running and inserting the cannula only when a growing drop of injection liquid is present at the tip. Following the cannula insertion, the connector is screwed to the catheter while avoiding overtightening so as not to damage the upper tip of the catheter, which will make it difficult to recap after the injection. An injection speed of 0.2 µL/min is used to avoid injection-induced trauma. Moreover, a slow injection, combined with a 20 min waiting period after the injection, ensures the diffusion of the injected liquid into the interstitial fluid and an effective draining into the CSF. The cannula is then removed slowly to avoid a vacuum effect.

The reported method includes surgical intervention and, therefore, requires staff able to perform stereotactic survival surgery. Personnel in direct contact with the animals should have taken the appropriate animal experimentation courses. The remainder of the protocol can be carried out by competent lab members.

The method is intended to produce inflammation-triggered demyelination of the cerebral cortex and does not reproduce all features of human MS (*e.g.*, the occurrence of focal inflammatory white matter lesions, which is a hallmark of human MS).

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DISCLOSURES
The authors have nothing to disclose.

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Figure 1

Figure 2

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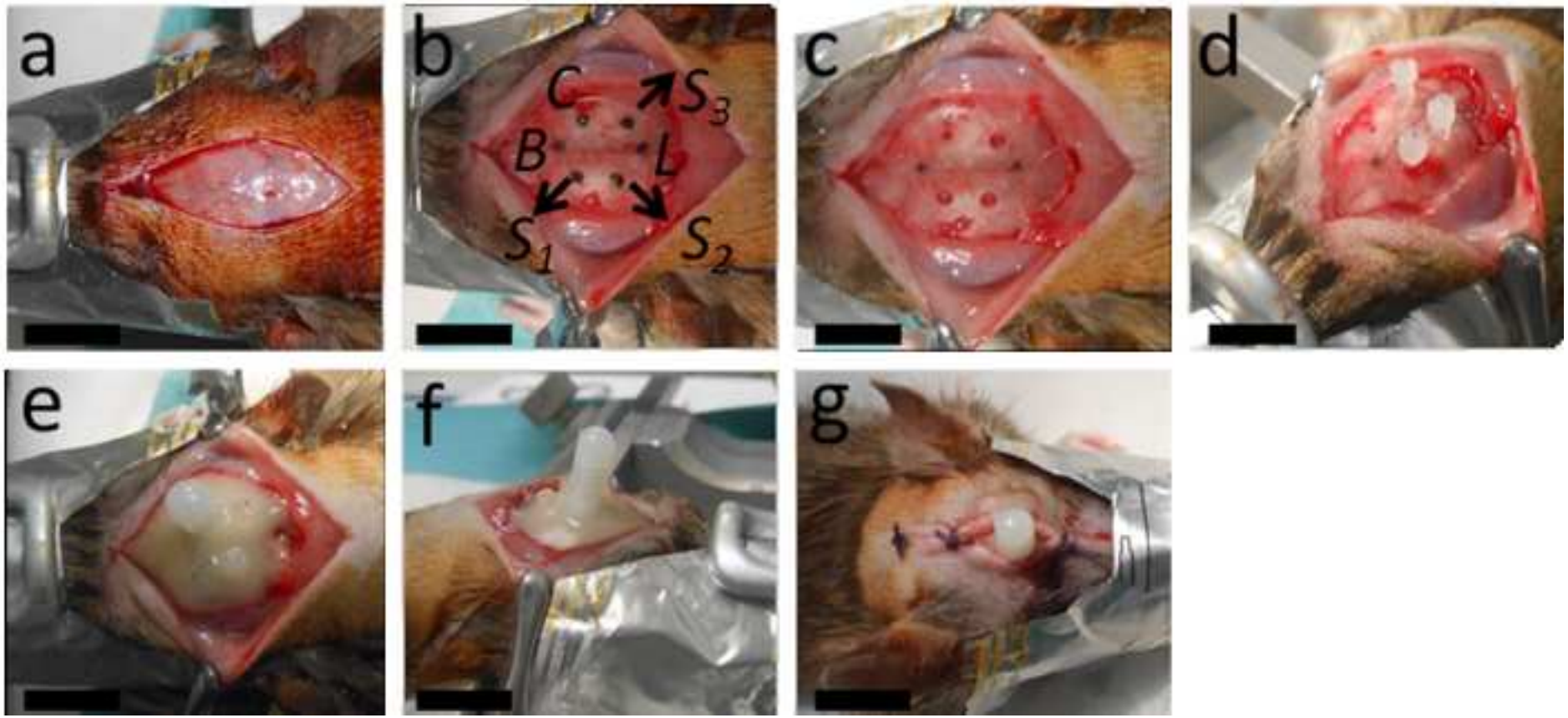

Figure 3

Click here to download Figure 3. MOG Preparation_300dpi.tif

Figure 4

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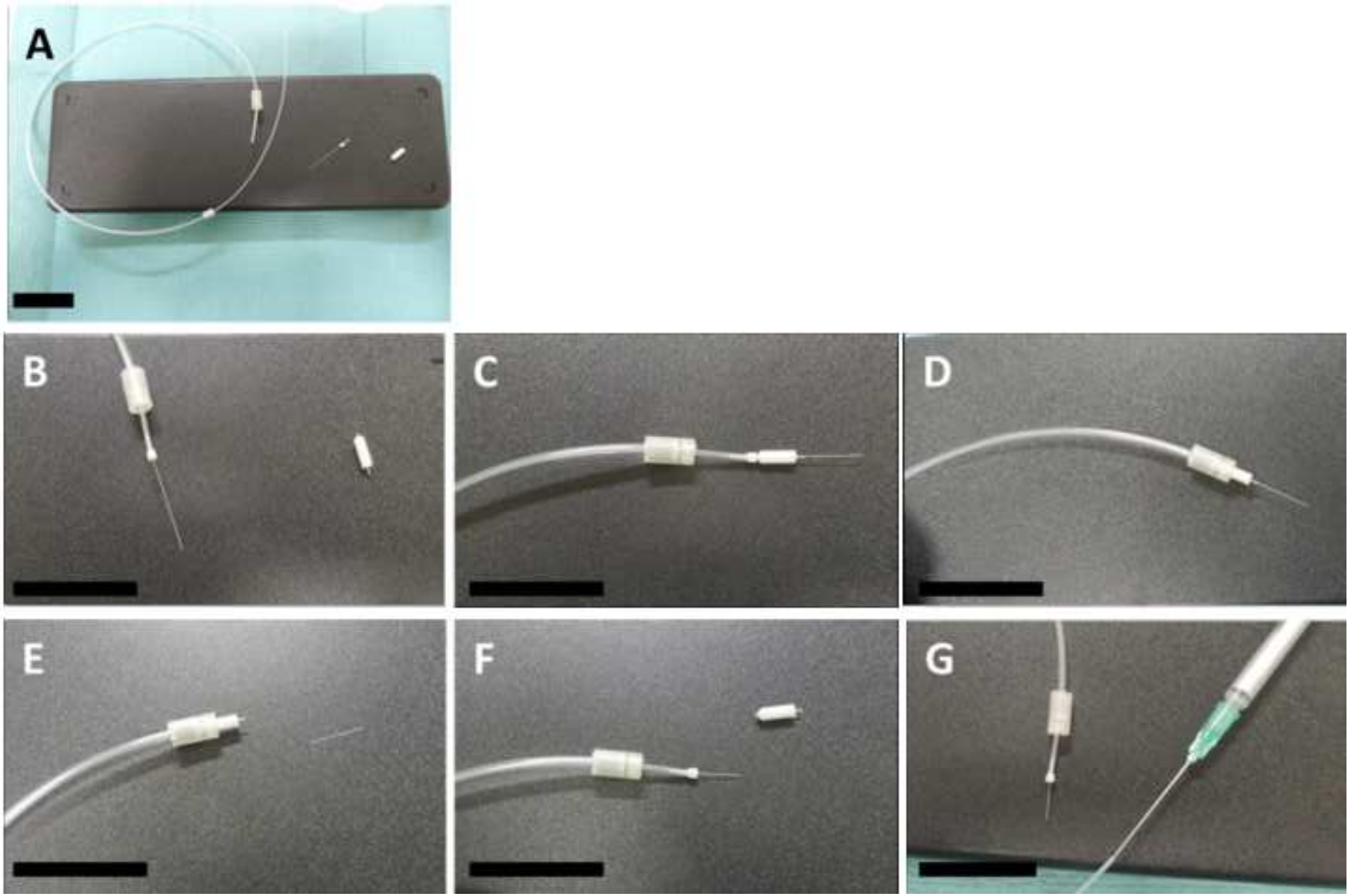

Figure 5

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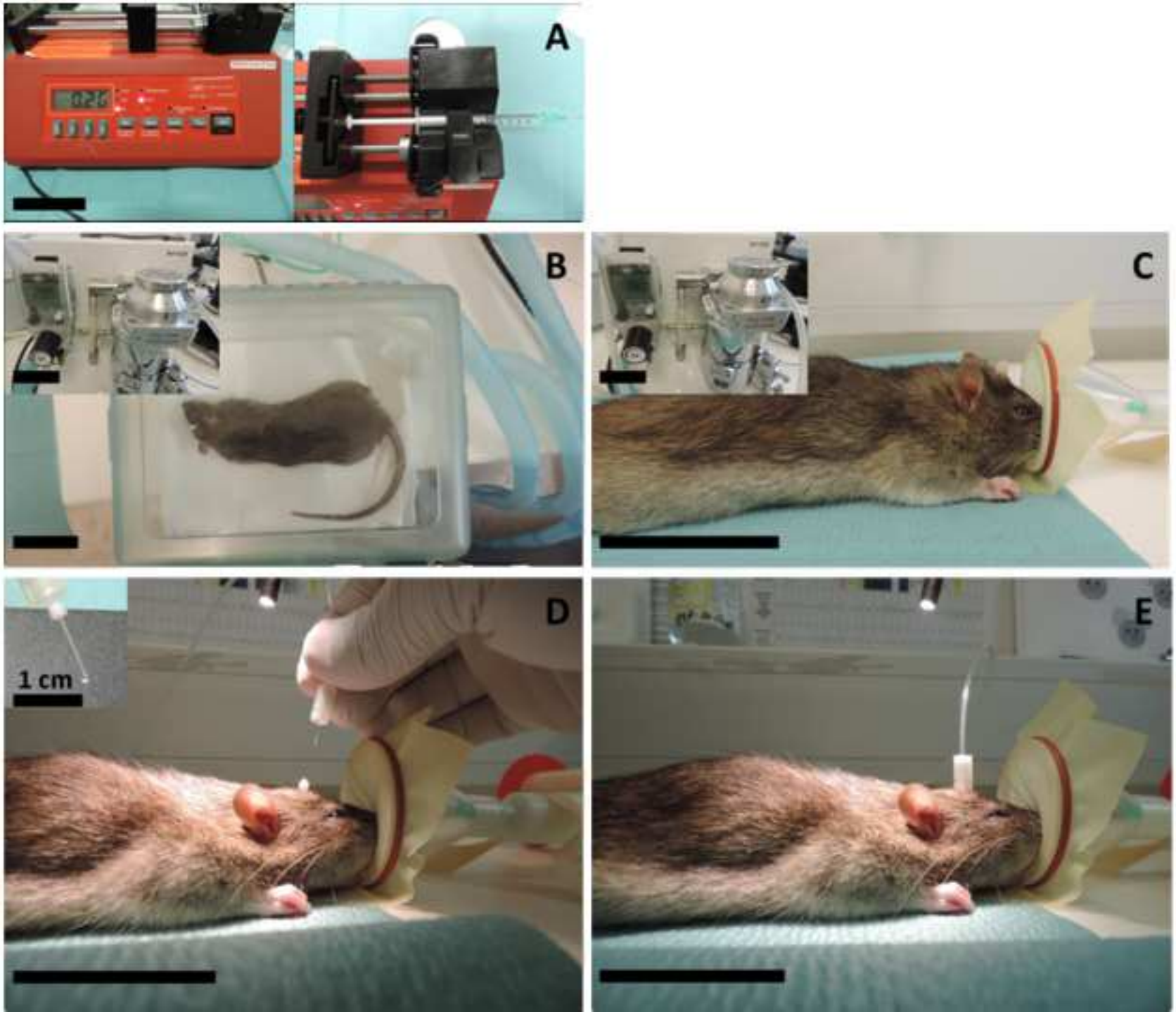

Figure 6

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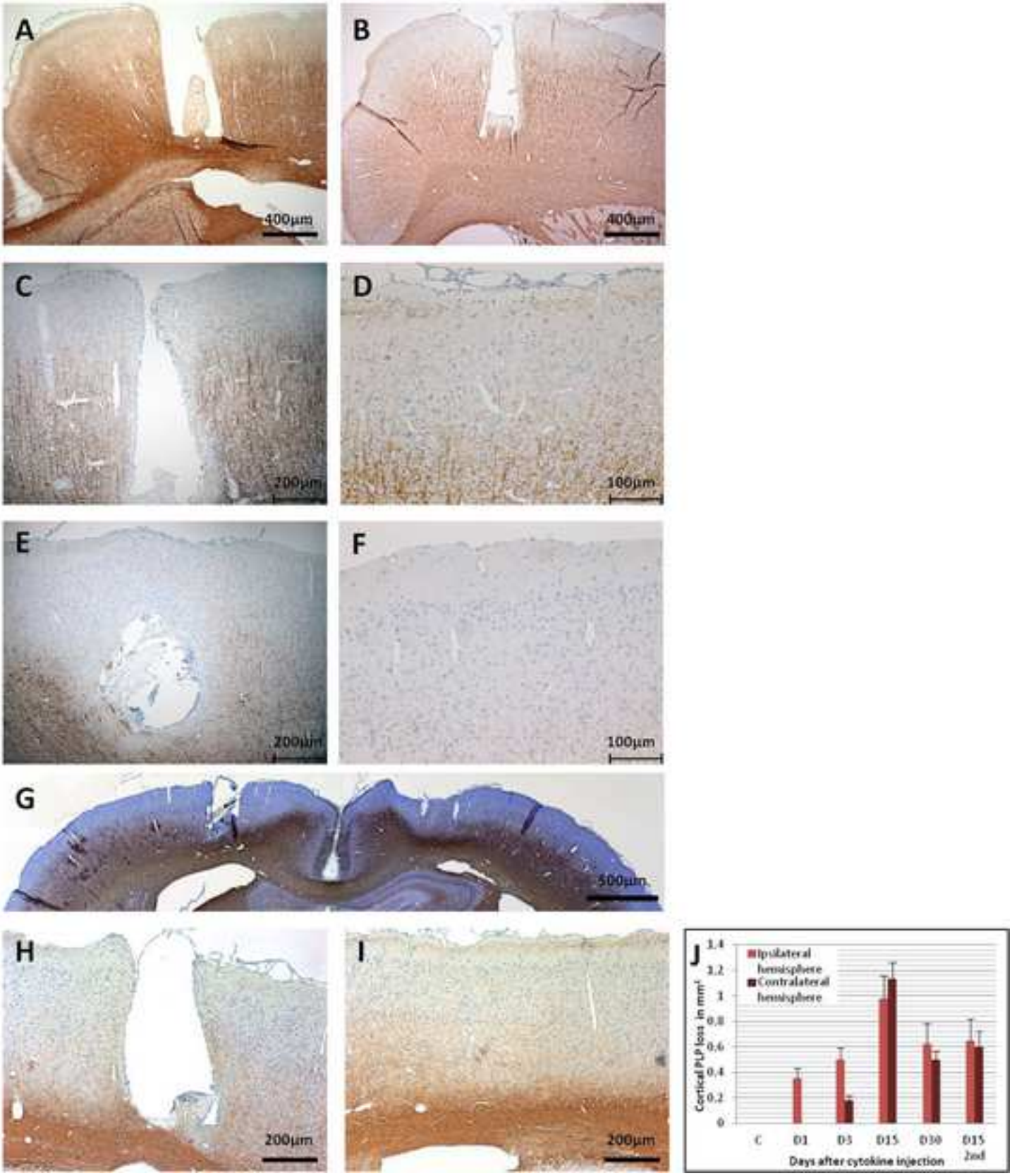

Figure 7

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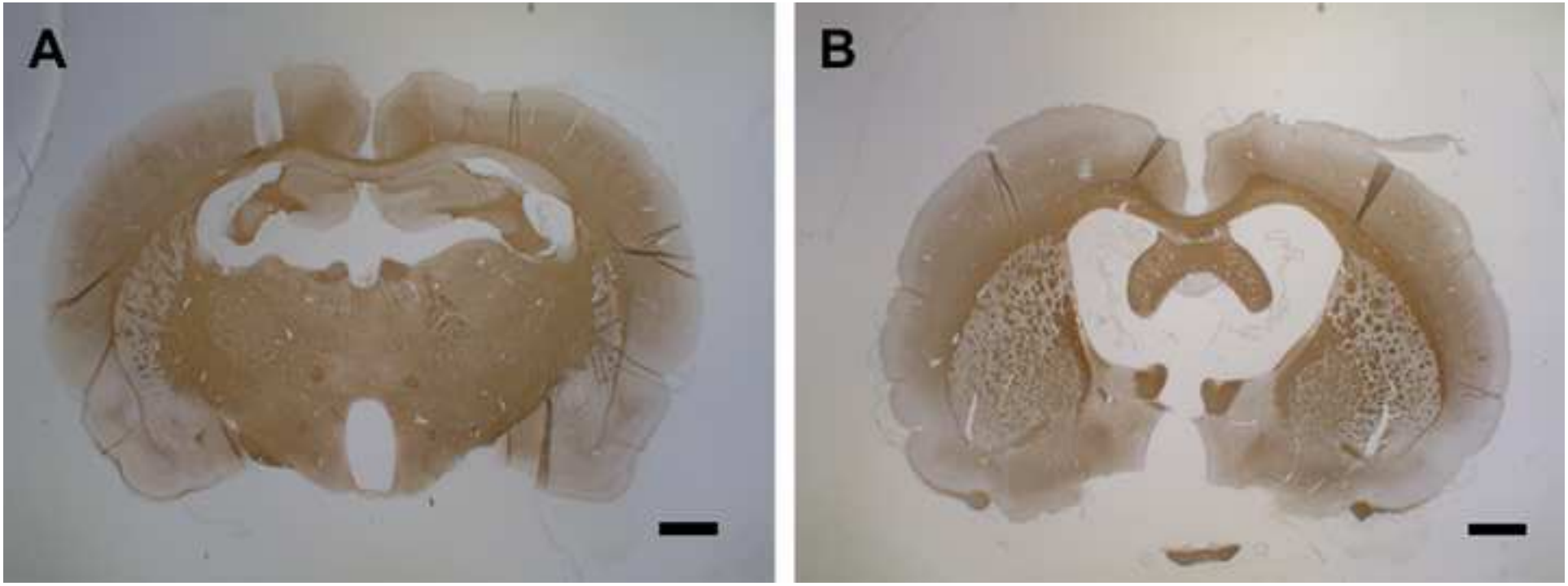

Figure 8

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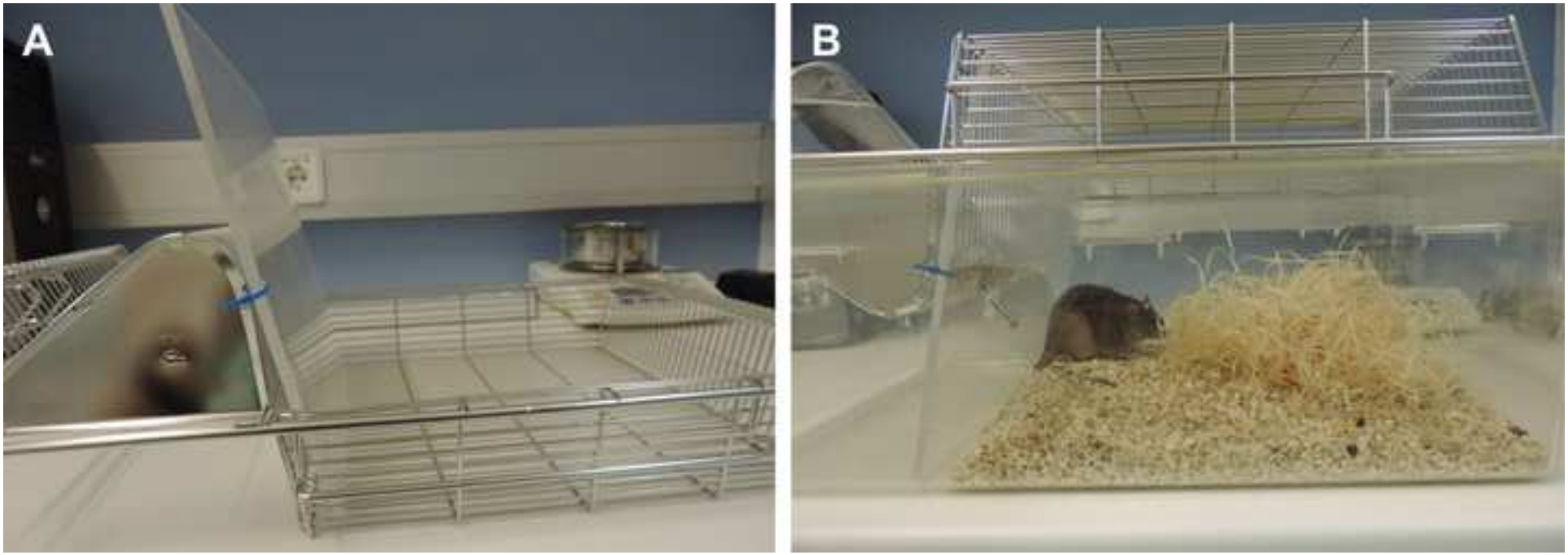

Materials Table

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Name of Material/ Equipment	Company	Catalog Number
Adult male Dark Agouti rats (300 ±25 g)		
Fentanyl	Hameln pharma plus, Germany	
Midazolam	ERWO Pharma, Austria	50039017
Medetomidin	Orion Pharma, Finland	
Flumazenil	Roche, Switzerland	
Atipamezol	Orion Pharma, Finland	
10% povidone-iodine complex	Mundipharma, Austria	
Dental cement	Heraeus Kulzer, Germany	6603 7633
Physiological saline solution	Fresenius Kabi, Austria	
Phosphate buffered saline (PBS)	Sigma-Aldrich, Germany	P3813
Isofluorane	AbbVie, Austria	
Lubricating eye drops	Thea Pharma, Austria	
70% EtOH	Merck, Germany	1070172511
2.5% enrofloxacin	Bayer, Germany	
carprofen	Pfizer, USA	
Tween-20	Sigma-Aldrich, Germany	P9416
Pentobarbital	Richter Pharma, Austria	
Interferon gamma	PeproTech, USA	400-20
Tumor necrosis factor alfa	R&D Systems, USA	510-RT-050/CF
$rMOG_{1-125}$	own product at the Centre of Molecular Medicine, Karolinska Institute, Sweden	
Anti-MOG antibody	Ana Spec/Kaneka Corporation, Japan	AS-555157
Incomplete Freund's adjuvant	Sigma-Aldrich, Germany	F5506

Horse radish peroxidase conjugated anti-rat IgG secondary antibody	Ana Spec/Kaneka Corporation, Japan	AS-555157
Bovine serum albumin	Sigma-Aldrich, Germany	A9576
Peroxidase substrate solution	Vector Laboratories, USA	SK-45000
Stereotactic frame	David Kopf Instruments, USA	
Catheters, MRI suitable	PlasticsOne, USA	8IC315GPKXXC
Dummy cannulas	PlasticsOne, USA	8IC315DCNSPC
Plastic screws, MRI suitable	PlasticsOne, USA	8L080X093N01
Connector cannula	PlasticsOne, USA	8IC313CXSPCC
Screw driver with 2mm tip-size		
Drill with flexible shaft extension	Proxxon, Germany	NO 28 472, NO 28 706, NO 28 620,
Drill bit, round, 0.5 mm	Hager & Meisinger, Germany	REF310 104 001 001 009
Drill bit, twisted, 1.3 mm	Hager & Meisinger, Germany	REF350 104 417 364 013
Scalpel	Braun, Germany	BB510
Scalpel handle	Fine Science Tools, Germany	91003-12
Cotton tip applicator	Henry Schein Medical, Austria	900-3155
Surgical scissors	Fine Science Tools, Germany	14101-14, 14088-10
Surgical forceps	Fine Science Tools, Germany	11002-12, 11251-35
Bulldog clamps	Fine Science Tools, Germany	18050-35
Homoeothermic blanket	TSE systems, Germany	
Infrared Lamp	Beurer, Germany	616.51
Dental curing light	Guilin Woodpecker Medical, China	
Absorbable suture	Johnson & Johnson, Belgium	V792E
Programmable syringe pump	World Precision Instruments, USA	AL-1000
Exam gloves		
Surgical gown		
Electric Shaver	Aesculap, Germany	GT420
Volatile anesthetic vaporizer	Rothacher Medical, Switzerland	CV 30-301-D
Oxygen source for volatile anesthetic vaporizer	Air Liquide, Austria	19,113

Volatile anesthesia chamber	Rothacher Medical, Switzerland	PS-0347
Anesthesia mask for rats	Rothacher Medical, Switzerland	PS-0307-A
1 ml syringe	Codan, Denmark	REF 62.1612
26 Gauge needle for injection	Braun, Germany	4657683
20 Gauge needle for cytokine injection and immunization	Braun, Germany	4657519
Luer lock tip glass syringes	Poulten & Graf, Germany	7.140-37
3 way stopcock	Becton Dickinson, Sweden	394600
96-well plate	Thermo Fisher Scientific, USA	442404
Plate reader	Cole-Parmer, USA	EW-1396-00
37°C incubator	Kendro, Germany	50042301
Micropipettes	Gilson, USA	F167350

	Comments/Description
as Fentanyl-Citrate, 50 µg/ml 5 mg/ml	
as Medetomidin hydrochloride, 1mg/ml 0.1 mg/ml as Atipamezol hydrochloride, 5 mg/ml	
0.9% NaCl	
Absolute ethanol was diluted in ddH_2O for preparation of 70% v/v	
Prophylactic antibiotics	
Painkillers, 50 mg/ml	
pentobarbital sodium, 400 mg/ml	
Recombinant rat myelin oligodendrocyte glycoprotein, amino acids 1-125 from the N-terminus, also commercially available: AnaSpec, AS-55152-500, USA	
Standard from ELISA-Kit; Ana Spec/Kaneka	

Secondary Antibody from ELISA-Kit; Ana Spec/Kaneka Corporation

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Author(s): M. Üçal*, M.T. Haindl*, M.Z. Adzemovic, M. Zeitelhofer, U. Schäfer, F. Fazekas, S. Hochmeister

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Signature:	S. Hochmeister
Date:	17.1.2018

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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).

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."

RESPONSES TO THE EDITORIAL COMMENTS

Graz, 08.04.2018

Dear Editor,

Authors are thankful for the editorial comments and are pleased to implement the respective changes that serve for the improvement of the manuscript titled "**Rat Model of Widespread Cerebral Cortical Demyelination Induced by Intracerebral Injection of Pro-Inflammatory Cytokines**".

Author responses to the editorial comments are given under each comment separately starting with a subtitle "**Authors**" and continuing with a red font.

With kind regards,

Authors of the manuscript JoVE57879.

Editorial comments:

1. The editor has formatted the manuscript as per journal's style. Please retain the same.

Authors: No format changes implemented in the manuscript. However, the word document may be subjected to some format changes as opened in a different PC (e.g. some of the lines are automatically changed to the formatting by German language). In addition, EndNote frequently reformats the font and spacing automatically in the References section and this might as well come into occurrence once again when the revised document is opened for further editorial purposes.

2. Please address specific comments marked in the manuscript.

Authors: Changes have been implemented in the revised document. For the specific responses to the respective comments, please see the document. Please note that that the MSWord might change the responses to the comments into a different format (e.g. displaying it as another "comment", instead of a "response comment").

3. Please submit each figure as a vector image file to ensure high resolution throughout video production. If submitting as a .tif or .psd, please ensure that the image is 1920 x 1080 pixels or 300 dpi.

Authors: All Figures are now revised to be of 300 dpi print quality.

4. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 60, 63-67.

Authors: Authors wish to thank for this remark. These mistakes come into occurrence since similar introductory sentences are read and written several times in different occasions by the authors. Lines that significantly overlap with a previous report are now revised (lines 64-67). Lines 60-64, on the other hand, contain the definition and prevalence of the disease, where a partial overlap with several previously published works is pretty unavoidable, although we tried our best to refrain.

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