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TITLE

Modeling stroke in mice: transient middle cerebral artery occlusion via the external carotid artery

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KEYWORDS:
stroke, brain ischemia, animal model, middle cerebral artery, transient

SUMMARY:
Different models of middle cerebral artery occlusion (MCAo) are widely used in experimental stroke research. Here, an experimental stroke model of transient MCAo via the external carotid artery (ECA) is described. This model aims to mimic the human stroke, in which the cerebrovascular thrombus is removed due to spontaneous clot lysis or therapy.

ABSTRACT:
Stroke is the third most common cause of death and the main cause of acquired adult disability in developed countries. Still, to date, therapeutic options are limited to a small proportion of stroke patients within the first hours after stroke.Novel therapeutic strategies are extensively being investigated, especially to prolong the therapeutic time window. These current investigations include the study of important pathophysiological pathways after stroke, such as post-stroke inflammation, angiogenesis, neuronal plasticity, and regeneration. Over the last decade, there is a growing concern about the low reproducibility of experimental results and scientific findings between/among independent research groups. To overcome the so-called "replication crisis", detailed standardized models for all procedures are urgently needed. As an effort within the "ImmunoStroke" research consortium (https://immunostroke.de/), a standardized transient MCAo mouse model is proposed. This model allows the complete restoration of the blood flow when removing the filament, simulating the therapeutic or spontaneous clot lysis that occurs in a large proportion of human strokes. In this video, the surgical method of this "filament" stroke model and functional analysis tools are demonstrated.

INTRODUCTION

Stroke is one of the most common causes of death and disability worldwide. Although, there are mainly two distinct forms of stroke, ischemic and hemorrhagic, 80%–85% of all stroke cases are ischemic[1]. Only two treatments are currently available for ischemic stroke patients: pharmacological treatment with recombinant tissue plasminogen activator (rtPA) or mechanical thrombectomy. However, due to the narrow therapeutic time window and multiple exclusion criteria, only a select number of patients are eligible to benefit from these specific treatment options. Over the last two decades, preclinical and translational stroke research has been centered on the study of neuroprotective approaches, but all compounds that reached clinical trials have so far show no improvements for the patient [2].

As an in vitro model cannot properly model brain interactions and the systemic pathophysiological mechanisms during a stroke, animal models are essential for preclinical stroke research. Mimicking all aspects of human ischemic stroke in a single animal model is not yet feasible, since ischemic stroke is itself a complex and heterogeneous disease. For this reason, different ischemic stroke models have been developed in different species. Brain ischemia due to photothrombosis of cerebral arterioles or by permanent distal occlusion of the Middle Cerebral Artery (MCA) are common models that induce small and locally defined lesions in the neocortex[3,4]. Yet, the probably most commonly used stroke model is the so-called "filament model", in which a transient MCA occlusion is achieved. This model consists of a transient introduction of a suture filament into the internal carotid artery until the origin of the MCA, resulting in a sharp reduction of the cerebral blood flow and the subsequent large infarction of subcortical and cortical brain regions[5]. Although most stroke models mimic occlusions of the MCA[6], the "filament model" enables us to precisely delimitate the ischemic interval depending on the reperfusion time point. Reperfusion by filament removal mimics the human clinical scenario, in which there is a restoration of the cerebral blood flow after spontaneous or therapeutic (rtPA or mechanical thrombectomy) of a clot. Different modifications of this

"filament model" have been described to date. In the most common approach, firstly described by Longa *et al*. in 1989[5], a silicon-coated filament is introduced via the common carotid artery (CCA) and advanced along the internal carotid artery (ICA) into the Circle of Willis, where it blocks the origin of the MCA[7]. Although being a very commonly used approach, this model does not allow the complete restoration of the blood flow during the reperfusion, since the CCA is permanently ligated after removing the filament.

Over the past decade, an increasing number of research groups have been interested in modeling stroke in mice by using the "filament model". However, the huge variability of this model and the lack of standardization of the procedures, among others, are some of the reasons behind the high variability and poor reproducibility of the experimental results and scientific findings reported so far[2,8]. A potential cause of the currently "replication crisis", so the low reproducibility among research laboratories, is the non-comparable stroke infarct volumes between research groups even using the same experimental methodology[9]. Indeed, after conducting the first preclinical randomized controlled multicenter trial study[10], we could confirm that the lack of sufficient standardization of this experimental stroke model and the subsequent outcome parameters was the main reason for the current failing reproducibility between preclinical studies from independent laboratories[11]. Such drastic differences in the resulting infarct sizes despite supposedly using the same stroke model justifiably pose not only a threat to confirmatory research, but also for scientific collaborations due to the lack of robust and reproducible models.

In light of these challenges, we aimed to develop and describe in detail the procedure for a standardized transient MCAo model as it is used for the collaborative research efforts within the "ImmunoStroke" research consortium (https://immunostroke.de/), which aims to understand brain-immune interactions underlying the mechanistic principles of stroke recovery. Additionally, histological and related functional methods for analysis of stroke outcome in this model are presented. All methods are based on standard operating procedures already developed and used in all research laboratories within the ImmunoStroke consortium.

PROTOCOL:

Ethics statement

The experiments reported in this video were conducted following national guidelines for the use of experimental animals, and the protocols were approved by the German governmental committees (Regierung von Oberbayern, Munich, Germany). 10 weeks old, male C57Bl/6J mice were used. The animals were housed under controlled temperature (22 °C ± 2 °C), with a 12h light-dark cycle period and access to pelleted food and water *ad libitum.*

1. Preparation of the material and instruments

1.1. Connect the heat blanket to maintain the operation area warm and maintain constant mouse body temperature during anesthesia (37 °C).

1.2. Autoclave scissors, forceps, prepare ethanol 70% solution and dexpanthenol eye ointment, and have on-hand several pieces of cotton, , and 5-0 coated braided polyester suture. Prepare a 1 mL syringe with 0,9% saline solution (without needle) to maintain the animal's incision site hydrated. Prepare the anesthesia gas (100% O_2 + isoflurane).

1.3. Prepare a holder for the laser Doppler probe by cutting the tip of a 10 µl pipet tip (3-5 mm length)

2. Preparation of the laser Doppler

2.1. Inject analgesia to the mouse 30 min before surgery (4 mg/kg Carprofen und 0,1 mg/kg Buprenorphine, intraperitoneally)

2.2. Anesthetize the mouse by placing it into the induction chamber with an isoflurane flow rate of 4% until the cessation of the spontaneous body movement and vibrissae.

2.3. Transfer the mouse into the operation area and place it in a prone position with its nose into the anesthesia mask. Maintain isoflurane concentration at 4% for another minute, then reduce it and keep it at 2%.

2.4. Gently insert the rectal probe to monitor the temperature throughout the surgical procedures. Set the associated feedback-controlled heating pad for maintaining the mouse body temperature at 37 °C.

2.5. Apply dexpanthenol eye ointment on both eyes.

2.6. Disinfect the skin and hair surrounding the left eye and ear with a disinfectant.

2.7. Cut the scalp between the left ear and the eye (1 cm long) to expose the skull bone.

2.8. Cut and retire the temporal muscle to visualize the MCA beneath the skull.

2.9. Fix the outside part of the tip holding the laser Doppler probe/fiber on top of the left MCA with glue and close the skin over it, so the skin is glued as well. Apply 2-3 drops of hardener glue to speed the process. Make sure that the laser Doppler fiber is not glued and can be easily removed from the tip holder at any time.

3. Transient MCAo model (occlusion)

3.1 Turn the mouse into the supine position. Put the snout into the anesthesia cone and fix the paws with tape.

3.2 Desinfect the skin and hair surrounding the chest and make a 2-cm-long midline incision in the neck.

3.3 Use forceps to pull the skin, submandibular gland and the sternomastoid muscle apart. Use retractors to expose the surgical field and find the left common carotid artery (CCA). Dissect the CCA free from connective tissue and surrounding nerves (without harming the vagal nerve) and do a transient ligation before the bifurcation.

3.4 Dissect the external carotid artery (ECA) and tie a permanent knot at the most distal visible part. Place another suture under the ECA, close to the bifurcation, and prepare a loose knot to be used later.

3.5 Dissect the internal carotid artery (ICA) and place a microvascular clip on it, 5 mm over the bifurcation. Make sure not to damage the vagal nerve.

3.6 Cut a small hole into the ECA between the tight and the loose ligations be careful not to cut the entire ECA.

3.7 Introduce the filament and advance it towards the CCA. Tight the loose ligation in the ECA around the lumen to momentanely secure the filament in that position and avoid bleeding when removing the microvascular clip.

3.8 Remove the microvascular clip and insert the filament through the ICA until reaching the origin of the MCA by detecting a sharp reduction (>80%) in the cerebral blood flow as measured by the laser Doppler, and fix the filament in this position by further tightening the knot around the ECA. (When the filament goes to the appropriate direction it advances smoothly and no resistence is found)

3.9 Record laser Doppler values before and after filament insertion.

3.10 Remove the retractor and relocate the sternomastoid muscle and the submandibular gland before suturing the wound. Remove the laser Doppler probe and place the animal in a recovery chamber at 37 °C for 1h (until filament removal).

4. Transient MCAo model (Reperfusion)

4.1. Anesthetize the mouse by placing it into the induction chamber with an isoflurane flow rate of 4% until the cessation of spontaneous body movement and vibrissae.

4.2. Apply dexpanthenol eye ointment on both eyes.

4.3. Transfer the mouse to the operation area and place it in a supine position with its snout in the anesthesia mask. Maintain isoflurane concentration at 4% for another minute, then reduce it and keep it at 2%. Fix the animal´s paws with tape.

4.4. Insert the laser Doppler probe into the probe holder.

4.5. Remove the wound suture, use forceps to pull the skin, the submandibular gland and the sternomastoid muscle apart. Use retractors to expose the surgical field.

4.6. Lose the suture on the ECA, the one that tights the filament, and gently pull the filament. Make sure not to damage the silicone-rubber coating of the filament during the removal.

4.7. Tightly tie the ECA suture.

4.8. Confirm the increase of the cerebral blood flow in the laser Doppler device (>80% of the initial value before reperfusion).

4.9. Record laser Doppler values before and after filament removal.

4.10. Open the transient ligation before the bifurcation from the CCA.

4.11. Remove the retractor and relocate the sternomastoid muscle and the submandibular gland before suture the wound and place the animal in a recovery chamber at 37 °C for 1 h to recover from anesthesia.

4.12. After recovery, the mice are returned to their cages in a temperature-controlled room.

4.13. Take care of the animals by adding wet food pellets and hydrogel in small Petri dishes on the cage floor until day 3 after surgery.

4.14. Inject analgesia every 12h for 3d after surgery (4 mg/kg Carprofen und 0,1 mg/kg Buprenorphine).

5. Sham operation

5.1. Perform all procedures identically to the operation described above, including the ligation of the arteries and the introduction of the filament (Steps 1-3.7).

5.2. Remove the filament immediately after its insertion. Then, place the animal in the recovery chamber for 1h.

5.3. After that time, place the animal into the operation area again and remove the transient ligation of the CCA to ensure a full cerebral blood flow restoration

5.4. Suture the wound and place the animal in a recovery chamber at 37 °C for 1 h to recover from anesthesia. After recovery, the mice are returned to their cages in a temperature-controlled room.

5.5. Take care of the animals by adding wet food pellets and hydrogel in small Petri dishes on the cage floor until day 3 after surgery.

5.6. Inject analgesia every 12h for 3d after surgery (4 mg/kg Carprofen und 0,1 mg/kg Buprenorphine).

6. Neuroscore

6.1. Perform the Neuroscore always at the same time of the day and Use surgical clothes to keep a “neutral smell”.

6.2. Mice rest 30 mins in the room with an “open” cage before the test

6.3. Observe each item for 30s (**Table 1-2**).

7. Intracardiac perfusion

7.1. Prepare a 20 mL syringe containing PBS-heparin (2U/mL) and place it 1 m above the bench to facilitate/ensure gravity-driven perfusion. (OPTIONAL: Intracardiac perfusion can be also done with 4% PFA. To that end, prepare a 20 mL syringe containing 4% PFA in PBS, pH: 7,4).

7.2. Inject 100 µl of ketamine and xylazine (120/16 mg/kg body weight, respectively). Wait 5 min and corroborate cessation of spontaneous body movement and vibrissae.

7.3. Fix the animal in a supine position and disinfect the abdominal body surface with ethanol 100%.

7.4. Make a 3-cm-long incision into the abdomen, cut the diaphragm, the ribs and sternum to completely visualize the heart.

7.5. Make a small incision in the right atrium and insert the perfusion cannula into the left ventricle.

7.6. Perfuse with 20 mL PBS-heparin.

7.7. After perfusion, decapitate the animal and remove the brain.

7.8. Freeze the brain on powdered dry ice and store them at -80 °C until further use.

8. Infarct volumetry

8.1 Cryosectioning: Cut the brains serially on a cryostat to 20 µm thick sections every 400 µm on slides. Store the slides at −80 °C until use.

8.2 Cresyl violet (CV) staining

8.2.1 Prepare the staining solution: Mix 0.5 gr of CV acetate in 500 mL H_2O. Stir and heat (60 °C) until crystals are dissolved. Let the solution cool and store it in a dark bottle. Reheat to 60 °C and filter before every use.

8.2.2 Dry the slides at room temperature for 30 min. Then place them in 95% ethanol for 15 min, in 70% ethanol for 1 min, and afterward in 50% ethanol for 1 min.

8.2.3 Place the slides in distilled water for 2 min, refresh distilled water, and place them in again for 1 min. Afterward, place the slides in the pre-heated staining solution for 10 min at 60 °C. Wash the slides twice in distilled water for 1 min.

8.2.4 Place the slides in 95% ethanol for 2 min. Then place them into 100% ethanol for 5 min, refresh the 100% ethanol and place them in again for 2 min. Afterward, cover the slides with a mounting medium.

8.2.5 Analysis (**Fig.4C**)
Scan the slides and analyze the indirect infarct volume by the Swanson method [13] to correct for edema:

(Ischemic area) = (ischemic region)-((ipsilateral hemisphere)-(contralateral hemisphere))

REPRESENTATIVE RESULTS

The model that is described here is a modification of the commonly used "filament" stroke model that consists of introducing a silicon-coated filament through the ECA to transiently block the origin of the MCA. After removing the filament, only the ECA is permanently occluded, allowing a complete blood restoration in the CCA and ICA (**Fig.1**). Besides, it is described a method for measuring the cerebral blood flow during both occlusion and reperfusion procedures by fixing a cannula connected to the laser Doppler probe at the skull over the MCA territory.

Because the blood flow in the CCA is restored after removing the filament, complete reperfusion of the brain occurs (**Fig.2**), similar to the situation observed after successful mechanical thrombectomy in human patients. The mortality rate during the surgery is less than 5% when performed by trained surgeons. At these early time points, animals generally present severe postural and movement deficits, general weakness and loss in body weight[14]. These severe deficits are transient and after approximately 1 week the animals show improved activity and deficits are more specific for focal neurological symptoms.

Behavioral deficits after MCA occlusion were assessed by the composite Neuroscore[12]; general and focal deficits were measured 24 h and 3 d after surgery. The general Neuroscore has 5 items (**Table 1**), including the evaluation of the fur, ears, eyes, posture and spontaneous activity, with a maximum score of 18. The focal Neuroscore comprises 7 items (**Table 2**), including the evaluation of body symmetry, gait, climbing, circling behavior, forelimb symmetry, compulsory cycling and whiskers response, with a maximum score of 28. This composite scale ranges from 0 (no deficits) to 46 (severe impairments) Stroke animals presented a significant change in the composite and focal Neuroscore but not in the general Neuroscore when compared to sham animals (**Fig.3**).

Infarct volumetry was also performed using cresyl violet staining of coronal serial brain sections 24 h after stroke induction. The infarct volume mean was 61.69 mm3, representing 48% of the affected brain hemisphere (**Fig.4**). When performed by a trained surgeon, the variability of this stroke model is low, with a coefficient of variation of 6%. The lesion area includes the somatosensory and motor cortex as well as subcortical structures such as the striatum (**Fig.4**).

FIGURE AND TABLE LEGENDS

Table 1: General Neuroscore.Animals received between 0 and 4 points, depending on the severity, for each of the five general deficits measured. The scores on the different areas are then summed to provide a total general score ranging from 0-18.

Table 2: Focal Neuroscore.Animals received between 0 and 4 points depending on the severity,

for each of the seven general deficits measured. The scores on the different areas are then summed to provide a total focal score ranging from 0-28.

Figure 1: Scheme for the access and intraluminal MCA occlusion. The filament (dotted line) is inserted between the proximal and distal suture knots in the ECA , and advanced along the ICA until it reaches the origin of the MCA (see insert). Once in place, the ECA is ligated with a suture to fix the filament. ACA anterior cerebral artery, BA basilar artery, CCA common carotid artery, ECA external carotid artery, ICA internal carotid artery, MCA middle cerebral artery, PCA posterior communicating artery, PTG pterygopalatine artery. This figure has been modified from Jackman *et al.* 2011[15].

Figure 2: Blood flow during occlusion and reperfusion. Blood flow is registered before and after filament insertion and before and after filament removal, where the reduction of the blood flow during the occlusion and the restoration of the blood flow during the reperfusion was observed. Every color represents one animal.

Figure 3: Neuroscore for functional deficits after tMCAo. A. Total, **B.** focal and **C.** general Neuroscore before, 24h and 3d after tMCAo. Open bars: sham; dark grey bars: tMCAo. BL=before tMCAo. n=10 per group. *p<0.05.

Figure 4: Volumetric infarct analysis and infarct outcome 24h after tMCAo. A. Representative cresyl violet stained coronal brain sections every 400 µm at 24h after tMCAo. Dashed lines demarcate the lesion area. **B.** Analysis of infarct volume of 10 brains (each dot representing one individual brain) 24h after tMCAo. The horizontal red line represents the mean (61.69 $mm3^3$), error bars indicate standard deviation (3.78 mm3). **C.** Representative picture for infarct volume calculation from a cresyl violet coronal section. Blue=Contralateral hemisphere. Red=Ipsilateral hemisphere. Pale striped area= Ischemic region.

DISCUSSION

The present protocol describes the experimental stroke model of transient MCAo by introducing a silicon-coated filament through the ECA until the origin of the MCA. This stroke model is one of the most commonly used stroke models due to the possibility to achieve arterial reperfusion after a delimitated occlusion period. Thus, can be regarded as a translationally relevant stroke model.

The “filament” model as presented in this video has some advantages compared other previously described stroke models, including the fact of not needing craniotomy and achieving complete reperfusion of the occluded vessel. However, the complexity of the surgery could be considered as a limitation, since it includes an invasive surgery and a precise manipulation of the different arteries very close to the trachea and the vagal nerve. Also, the long exposure of the animal to anesthetics might be a critical factor to also take into account, as the impact of anesthetics on neuroprotection and stroke outcome has already been well documented[16]. Although this complex surgical procedure cannot be achieved as brief as other described brain ischemia

models, it can be completed in approx. 20 min when performed by a trained surgeon.

In contrast with previously described "filament" stroke protocols[17], the method here described also allows the measurement of the cerebral blood flow during both, occlusion and reperfusion phases. Monitoring the blood flow during repefusion might be an important parameter for preventing stroke repefusion injury[18], which in clinics is known to cause deleterious consequences in patients that underwent pharmacologic or endovascular interventions for recanalization of the thrombosed vessels. Despite discrepancy exist between the consequences of cerebral blood flow restoration after MCAo[19] , it has been described that the variability of blood flow restoration after stroke can influence the pathophysiological and biochemical events in brain, as well as the infarct volume and the neurological deficits of stroke mice[20]. Therefore, in the model here described, a complete blood flow restoration and its recording are requirements to guarantee reproducible infarcts among mice, specially when aiming at conducting translational stroke studies.

The overall mortality during the surgery here described is less than 5% and is mainly caused by anesthesiology complications, bleedings, or sacrifice due to pre-defined exclusion criteria. In contrast, this stroke model presents a moderate mortality rate within the first 24h-48h after stroke induction, which might increase the number of animals needed per experiment to ultimately achieve a proper cohort of stroke mice. In terms of infarct volume, this model induces large infarcts, with approx. 50% of the hemisphere affected by the ischemia. It also produces brain swelling, overall affecting different regions of the brain, including cortical and subcortical regions.

To warrant a low variability and a high reproducibility of this stroke model, In addition, we suggest that the following exclusion criteria be taken into account : 1)Operation time longer than 20 min; 2)More than 20% of blood flow reduction when CCA ligated (step 3.3); 3)Reduction of blood flow during occlusion below 80% of the initial pre-occlusion value, and 4)Increase of blood flow 10 min after repefusion rate below 80% compared to the pre-reperfusion value. For an experienced and trained surgeon, no animals are excluded due to the operation time. However 10-15% of the animals show a 20% reduction of the blood flow when the CCA is ligated and 5-10% do not have an adequate reduction or increase of the blood flow during occlusion or reperfusion, respectively. Therefore, the success rate after excluding animals according to these criteria is around 75-85%.

In addition, animals are examined daily after MCAo (body weight, temperature and basic physiological behavior) to control for sickness behavior, pain or discomfort.. Besides this general care, several tests for specific behavioral analysis after focal brain ischemia have been also developed. Despite all the different available tests to evaluate sensorimotor dysfunction, such as the Rotarod test [22], Sticky label test [23], Corner test [24] or the Cylinder test [25].Here animals submitted to this stroke model were evaluated for focal as well as general deficits because stroke also induces cytokine-sickness behavior independent of focal (sensory or motor) deficits[26].

Taken together, the “filament” stroke model here described is a valuable model for basic and translational stroke research. This model as a standardized stroke model is proposed to be used to harmonize stroke models across laboratories.

ACKNOWLEDGMENTS

We thank all our collaboration partners of the Immunostroke Consortia (FOR 2879, From immune cells to stroke recovery) for suggestions and discussions. This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany’s Excellence Strategy within the framework of the Munich Cluster for Systems Neurology (EXC 2145 SyNergy – ID 390857198) and under the grants LI-2534/6-1, LI-2534/7-1 and LL-112/1-1.

DISCLOSURES

The authors have no competing interests to disclose.

REFERENCES:

1 Donnan, G. A., Fisher, M., Macleod, M. & Davis, S. M. Stroke. *Lancet.* **371** (9624), 1612-1623, (2008).

2 O'Collins, V. E. *et al.* 1,026 experimental treatments in acute stroke. *Ann Neurol.* **59** (3), 467-477, (2006).

3 Tureyen, K., Vemuganti, R., Sailor, K. A. & Dempsey, R. J. Infarct volume quantification in mouse focal cerebral ischemia: a comparison of triphenyltetrazolium chloride and cresyl violet staining techniques. *J Neurosci Methods.* **139** (2), 203-207, (2004).

4 Zhang, Z. *et al.* A new rat model of thrombotic focal cerebral ischemia. *J Cereb Blood Flow Metab.* **17** (2), 123-135, (1997).

5 Longa, E. Z., Weinstein, P. R., Carlson, S. & Cummins, R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke.* **20** (1), 84-91, (1989).

6 Carmichael, S. T. Rodent models of focal stroke: size, mechanism, and purpose. *NeuroRx.* **2** (3), 396-409, (2005).

7 Engel, O., Kolodziej, S., Dirnagl, U. & Prinz, V. Modeling stroke in mice - middle cerebral artery occlusion with the filament model. *J Vis Exp.* 10.3791/2423 (47), (2011).

8 Dirnagl, U. *et al.* A concerted appeal for international cooperation in preclinical stroke research. *Stroke.* **44** (6), 1754-1760, (2013).

9 McNutt, M. Journals unite for reproducibility. *Science.* **346** (6210), 679, (2014).

10 Llovera, G. *et al.* Results of a preclinical randomized controlled multicenter trial (pRCT): Anti-CD49d treatment for acute brain ischemia. *Sci Transl Med.* **7** (299), 299ra121, (2015).

11 Llovera, G. & Liesz, A. The next step in translational research: lessons learned from the first preclinical randomized controlled trial. *J Neurochem.* **139 Suppl 2** 271-279, (2016).

12 Clark, W. M., Lessov, N. S., Dixon, M. P. & Eckenstein, F. Monofilament intraluminal middle cerebral artery occlusion in the mouse. *Neurol Res.* **19** (6), 641-648, (1997).

13 Swanson, G. M., Satariano, E. R., Satariano, W. A. & Threatt, B. A. Racial differences in the early detection of breast cancer in metropolitan Detroit, 1978 to 1987. *Cancer.* **66** (6), 1297-1301, (1990).

14 Lourbopoulos, A. *et al.* Inadequate food and water intake determine mortality following stroke in mice. *J Cereb Blood Flow Metab.* **37** (6), 2084-2097, (2017).

15 Jackman, K., Kunz, A. & Iadecola, C. Modeling focal cerebral ischemia in vivo. *Methods Mol Biol.* **793** 195-209, (2011).

16 Kitano, H., Kirsch, J. R., Hurn, P. D. & Murphy, S. J. Inhalational anesthetics as neuroprotectants or chemical preconditioning agents in ischemic brain. *J Cereb Blood Flow Metab.* **27** (6), 1108-1128, (2007).

17 Rousselet, E., Kriz, J. & Seidah, N. G. Mouse model of intraluminal MCAO: cerebral infarct evaluation by cresyl violet staining. *J Vis Exp.* 10.3791/4038 (69), (2012).

18 Rha, J. H. & Saver, J. L. The impact of recanalization on ischemic stroke outcome: a meta-analysis. *Stroke.* **38** (3), 967-973, (2007).

19 Liu, J. R. *et al.* Transient filament occlusion of the middle cerebral artery in rats: does the reperfusion method matter 24 hours after perfusion? *BMC Neurosci.* **13** 154, (2012).

20 Sommer, C. J. Ischemic stroke: experimental models and reality. *Acta Neuropathol.* **133** (2), 245-261, (2017).

21 in *Mechanisms of Vascular Disease: A Reference Book for Vascular Specialists* eds R. Fitridge & M. Thompson) (2011).
22 Jones, B. J. & Roberts, D. J. A rotarod suitable for quantitative measurements of motor incoordination in naive mice. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol.* **259** (2), 211, (1968).
23 Bouet, V. *et al.* The adhesive removal test: a sensitive method to assess sensorimotor deficits in mice. *Nat Protoc.* **4** (10), 1560-1564, (2009).
24 Zhang, L. *et al.* A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *J Neurosci Methods.* **117** (2), 207-214, (2002).
25 Schallert, T., Fleming, S. M., Leasure, J. L., Tillerson, J. L. & Bland, S. T. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology.* **39** (5), 777-787, (2000).
26 Roth, S., Yang, J., Cramer, J., Malik, R. & Liesz, A. Detection of cytokine-induced sickness behavior after ischemic stroke by an optimized behavioral assessment battery. *Brain Behav Immun.* 10.1016/j.bbi.2020.11.016, (2020).

Figure 1

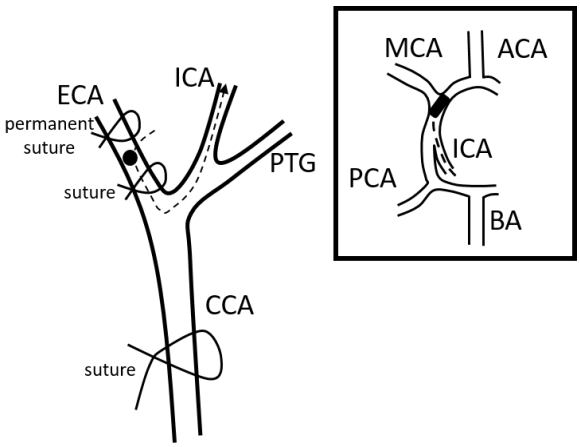
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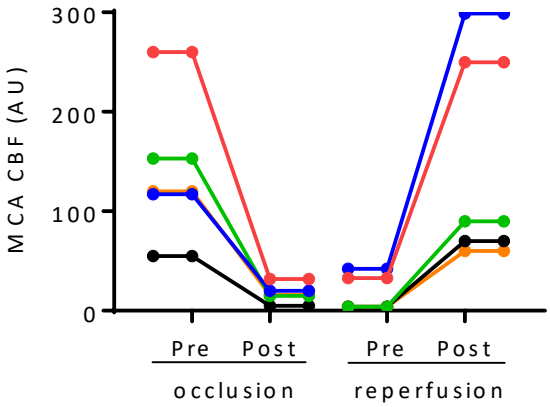
Figure 2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Pre
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occlusion
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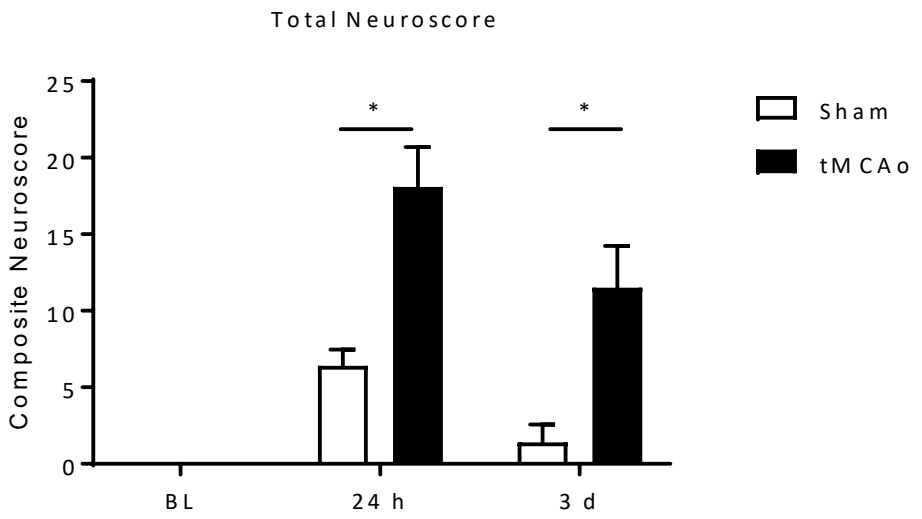
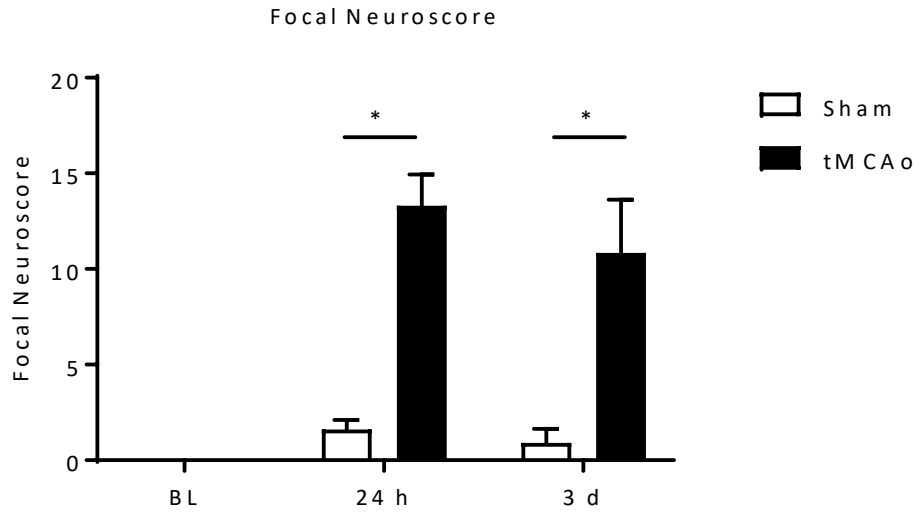
Figure 3

A


Total Neuroscore
Composite Neuroscore
25
20
15
10
5
0
*
*
BL
24 h
3 d
Sham
tMCAo

B


Focal Neuroscore
Focal Neuroscore
20
15
10
5
0
*
*
BL
24 h
3 d
Sham
tMCAo

C

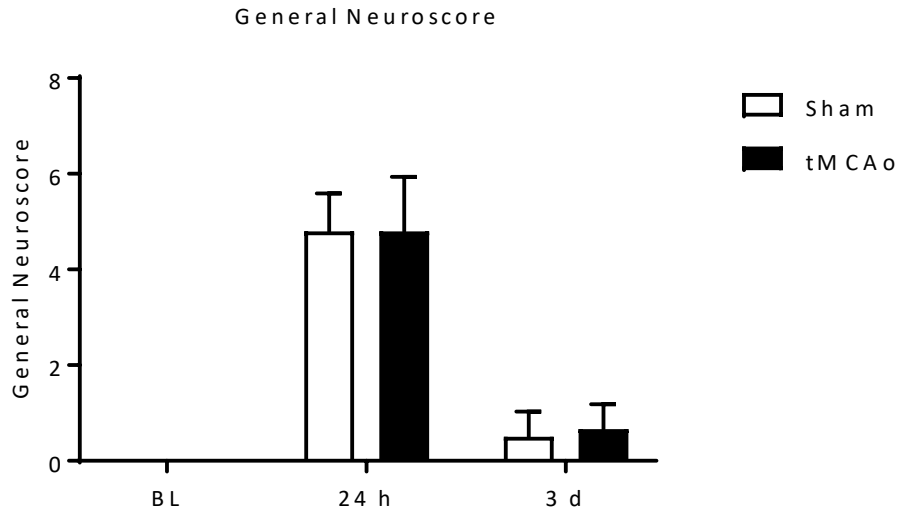

General Neuroscore
General Neuroscore
8
6
4
2
0
BL
24 h
3 d
Sham
tMCAo

Figure 4

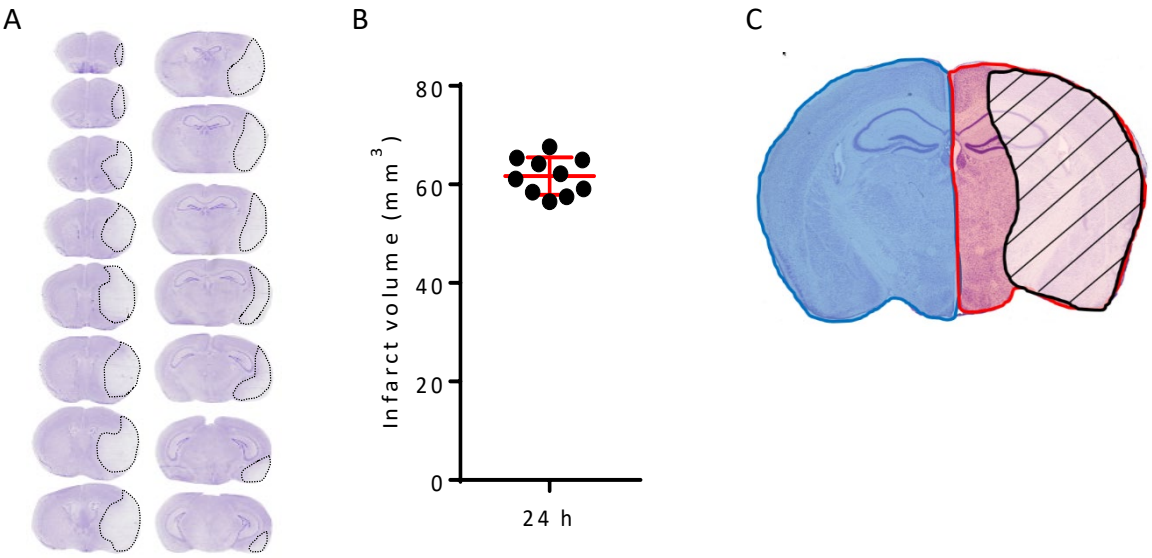


Table 1

		Time-point of scoring	*score*
General Neuroscore	**Hair**	0. Hair neat and clean	
		1. Localized piloerection and dirty hair in 2 body parts (nose and eyes)	
		2. Piloerection and dirty hair in >2body parts	
	Ears (mouse on an open bench top)	0. Normal (ears are stretched laterally and behind, they react by straightening up following noise)	
		1. Stretched laterally but not behind (one or both), they react to noise	
		2. Same as 1. NO Reaction to noise.	
	Eyes (mouse on OBT)	0. Open, clean and quickly follow the surrounding environment	
		1. Open and characterized by aqueous mucus. Slowly follow the surrounding environment	
		2. Open and characterized by dark mucus	
		3. Ellipsoidal shaped and characterized by dark mucus	
		4. Closed	
	Posture (place the mouse on the palm and swing gently)	0. The mouse stands in the upright position with the back parallel to the palm. During swing, it stands rapidly.	
		1. The mouse stands humpbacked. During the swing, it flattens the body to gain stability.	
		2. The head or part of the trunk lies on the palm	
		3. The mouse lies on one side, barely able to recover the upright position.	
		4. The mouse lies in a prone position, not able to recover the upright position.	
	Spontaneos activity (mouse on OBT)	0.The mouse is alert and explores actively	
		1.The mouse seems alert, but it is calm and sluggish	
		2.The mouse explores intermittently and sluggishly	
		3.The mouse is somnolent and numb, few movements on-the-spot	
		4.No spontaneous movements	
	Total score for general scoring **(normal=0 max=18)**		

Table2

	Time-point of scoring		*score*
Focal Neuroscore	**Body symmetry** (mouse on OBT, observe the nose-tail line)	0. Normal (Body: normal posture, trunk elevated from the bench, with fore and hindlimbs leaning beneath the body. Tail: straight)	
		1. Slight asymmetry (Body: leans on one side with fore and hindlimbs leaning beneath the body. Tail: slightly bent.)	
		2. Moderate asymmetry (Body: leans on one side with fore and hindlimbs stretched out. Tail: slightly bent).	
		3. Prominent asymmetry (Body: bent, on one side lies on the OBT. Tail: bent)	
		4. Extreme asymmetry (Body: highly bent, on one side constantly lies on the OBT. Tail: highly bent)	
	Gait (mouse on OBT. Observed undisturbed)	0. Normal (gait is flexible, symmetric and quick)	
		1. Stiff, inflexible (humpbacked walk, slower than normal mouse)	
		2. Limping, with asymmetric movements	
		3. Trembling, drifting, falling	
		4. Does not walk spontaneously (when stimulated by gently pushing the mouse walks no longer than 3 steps)	
	Climbing (mouse on a 45° surface. Place the mouse in the center of the gripping surface)	0. Normal (mouse climbs quickly)	
		1. Climbs with strain, limb weakness present.	
		2. Holds onto slope, does not slip or climb	
		3. Slides down slope, unsuccessful effort to prevent fail	
		4. Slides immediately, no effort to prevent fail.	
	Circling behavior (mouse on OBT, free observation)	0. Absent circling behavior	
		1. Predominantly one-side turns.	
		2. Circles to one side, although not constantly.	
		3. Circles constantly to one side.	
		4. Pivoting, swaying, or no movement.	
	Forelimb symmetry (mouse suspended by tail)	0. Normal	
		1. Light asymmetry: mild flexion of contralateral forelimb.	
		2. Marked asymmetry: marked flexion of contralateral limb, the body slightly bends on the ipsilateral side.	
		3. Prominent asymmetry: contralateral forelimb adheres to the trunk.	
		4. Slight asymmetry, no body/limb movement.	
	Compulsory circling (forelimbs on bench, hindlimbs suspended by the tail: it reveals the presence of the contralateral limb palsy)	0. Absent. Normal extension of both forelimbs.	
		1. Tendency to turn to one side (the mouse extends both forelimbs, but starts to turn preferably to one side)	
		2. Circles to one side (the mouse turns towards one side with a slower movement compared to healthy mice)	
		3. Pivots to one side sluggishly (the mouse turns towards one side failing to perform a complete circle)	
		4. Does not advance (the front part of the trunk lies on the bench, slow and brief movements)	
	Whisker response (mouse on the OBT)	0. Normal	
		1. Light asymmetry (the mouse withdraws slowly when stimulated on the contralateral side)	
		2. Prominent asymmetry (no response when stimulated to the contralateral side)	
		3. Absent response contralaterally, slow response when stimulated ipsilaterally.	
		4. Absent response bilaterally	
	Total score for focal deficits (normal=0 max=28)		

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
45º ramp	H&S Kunststofftechnik		height: 18 cm
5/0 threat	Pearsalls	10C103000	
5ml Syringe	Braun		
Acetic Acid	Sigma Life Science	695092	
Anesthesia system for isoflurane	Drager		
Bepanthen pomade	Bayer		
C57Bl/6J mice	Charles River	000664	
Clamp	FST	12500-12	
Clip	FST	18055-04	
Clip holder	FST	18057-14	
Cotons	NOBA Verbondmitel Danz	974116	
Cresyl violet	Sigma Life Science	C5042-10G	
Cryostat	Thermo Scientific CryoStarNX70		
Ethanol 70%	CLN Chemikalien Laborbedorf	521005	
Ethanol 96%	CLN Chemikalien Laborbedorf	522078	
Ethanol 99%	CLN Chemikalien Laborbedorf	ETO-5000-99-1	
Filaments	Doccol	602112PK5Re	
Fine 45 angled forceps	FST	11251-35	
Fine forceps	FST	11252-23	
Fine Scissors	FST	14094-11	
Glue	Orechseln	BSI-112	
Hardener Glue	Drechseln & Mehr	BSI-151	
Heating blanket	FHC DC Temperature Controller		
Isoflurane	Abbot	B506	
Isopentane	Fluka	59070	
Ketamine	Inresa Arzneimittel GmbH		
Laser Doppler	Perimed	PF 5010 LDPM, Periflux System 5000	
Laser Doppler probe	Perimed	91-00123	
Phosphate Buffered Saline PH: 7,4	Apotheke Innestadt Uni Munchen	P32799	
Recovery chamber	Mediheat		
Roti-Histokit mounting medium	Roth	6638.1	
Saline solution	Braun	131321	

Scalpel	Feather	02.001.30.011
Silicon-coated filaments	Doccol	602112PK5Re
Stereomicropscope	Leica	M80
Superfrost Plus Slides	Thermo Scientific	J1800AMNZ
Vannas Spring Scissors	FST	15000-00
Xylacine	Albrecht	