



STUDY PROTOCOL

REVISED Stage 1 Registered Report: Effect of deficient phagocytosis on neuronal survival and neurological outcome after temporary middle cerebral artery occlusion (tMCAo) [version 3; referees: 2 approved]

Julius V. Emmrich ¹, Jonas J. Neher^{2,3}, Philipp Boehm-Sturm^{1,4}, Matthias Endres^{1,5}, Ulrich Dirnagl ^{1,5,6}, Christoph Harms^{1,6}

¹Department of Experimental Neurology and Center for Stroke Research, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany

²Department of Cellular Neurology, Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany

³German Center for Neurodegenerative Diseases (DZNE), Tübingen, Germany

⁴NeuroCure Cluster of Excellence and Charité Core Facility 7T Experimental MRIs, Charité – Universitätsmedizin Berlin, Berlin, Germany

⁵German Center for Neurodegenerative Diseases (DZNE), Berlin, Germany

⁶QUEST – Center for Transforming Biomedical Research, Berlin Institute of Health (BIH), Berlin, Germany

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Abstract

Stroke is a major cause of death and disability worldwide. In addition to neuronal death resulting directly from energy depletion due to lack of blood supply, inflammation and microglial activation following ischemic brain injury has been increasingly recognized to be a key contributor to the pathophysiology of cerebrovascular disease. However, our understanding of the cross talk between the ischemic brain and the immune system is limited. Recently, we demonstrated that following focal ischemia, death of mature viable neurons can be executed through phagocytosis by microglial cells or recruited macrophages, i.e. through phagoptosis. It was shown that inhibition of phagocytic signaling pathways following endothelin-1 induced focal cerebral ischemia leads to increased neuronal survival and neurological recovery. This suggests that inhibition of specific phagocytic pathways may prevent neuronal death during cerebral ischemia. To further explore this potential therapeutic target, we propose to assess the role of phagocytosis in an established model of temporary (45min) middle cerebral artery occlusion (tMCAo), and to evaluate neuronal survival and neurological recovery in mice with deficient phagocytosis. The primary outcome of this study will be forelimb function assessed with the staircase test. Secondary outcomes constitute Rotarod performance, stroke volume (quantified on MR imaging or brain sections, respectively), diffusion tensor imaging (DTI) connectome mapping, and histological analyses to measure neuronal and microglial densities, and phagocytic activity. Male mice aged 10-12 weeks will be used for experiments.

Keywords

stroke, neuroinflammation, phagocytosis, phagoptosis, middle cerebral artery occlusion, MCAo, microglia

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- 1 **Stuart M. Allan** , The University of Manchester, UK
- 2 **Ádám Dénes**, Hungarian Academy of Sciences, Hungary

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Corresponding authors: Julius V. Emmrich (julius.emmrich@charite.de), Christoph Harms (christoph.harms@charite.de)

Author roles: **Emmrich JV:** Conceptualization, Methodology, Project Administration, Resources, Writing – Original Draft Preparation, Writing – Review & Editing; **Neher JJ:** Conceptualization, Methodology, Resources, Writing – Review & Editing; **Boehm-Sturm P:** Investigation, Methodology, Writing – Review & Editing; **Endres M:** Conceptualization, Methodology, Project Administration, Writing – Review & Editing; **Dirnagl U:** Conceptualization, Methodology, Project Administration, Resources, Supervision, Writing – Review & Editing; **Harms C:** Conceptualization, Methodology, Project Administration, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

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REVISED Amendments from Version 2

Due to unforeseen circumstances and prior to the completion of data collection, we would like to make the following minor changes to the protocol:

1. The longest survival time for animals in this study will be 21 days. In previous versions of this protocol, the survival time has been reported inconsistently. A survival time of 28 days was erroneously reported twice. This oversight has now been corrected.
2. Control animals will be injected with control peptide c(RADIV), which is inactive at the vitronectin receptor, instead of phosphate-buffered saline solution (PBS). Inactive control peptide is a more suitable control than PBS as it will enhance validity and reproducibility of results. In previous versions of this protocol, we did not consider using an inactive peptide because of the high costs. Following publication of the Registered Report, we identified a supplier that provides inactive control peptide at affordable costs.

See referee reports

death and the comparatively less hypoperfused ischemic penumbra, where cells may be stressed and dysfunctional but viable¹. Within the ischemic penumbra, the fate of individual neurons results from a complex interplay of numerous biochemical and cellular events, which may lead to irreversible cell death or survival and tissue repair. Among those, activation of microglia, the primary immune effector cells of the brain, or recruited macrophages is a key feature of the pathophysiology of cerebral ischemia. Apart from their potential to release a number of pro- and anti-inflammatory and cytotoxic molecules, activated microglia demonstrate many characteristics of macrophages, including amoeboid-like morphology, capacity to migrate, antigen presentation, and phagocytic activity^{2,3}. Consequently, one of the main effector functions of microglia is the phagocytic removal of cell debris or dying cells. In these cases phagocytosis is beneficial, because it prevents the disintegration of apoptotic cells and induces an anti-inflammatory response in microglia, thereby contributing to tissue homeostasis and repair (for review see Brown & Neher, 2014)⁴.

Introduction

Stroke is the second leading cause of death and a major cause of disability worldwide. Following a sudden interruption of the blood supply, there are two major regions of injury within the brain: the ischemic core, where severe hypoperfusion causes rapid cell

Microglial phagocytosis is closely controlled by the expression of specific cell surface ligands. For the phagocytic removal of host cells, specific ligands, so-called ‘eat-me’ signals, need to be displayed on their surface⁵ (Figure 1). Among a wide variety of ligands, exposure of phosphatidylserine (PS) is the best-described

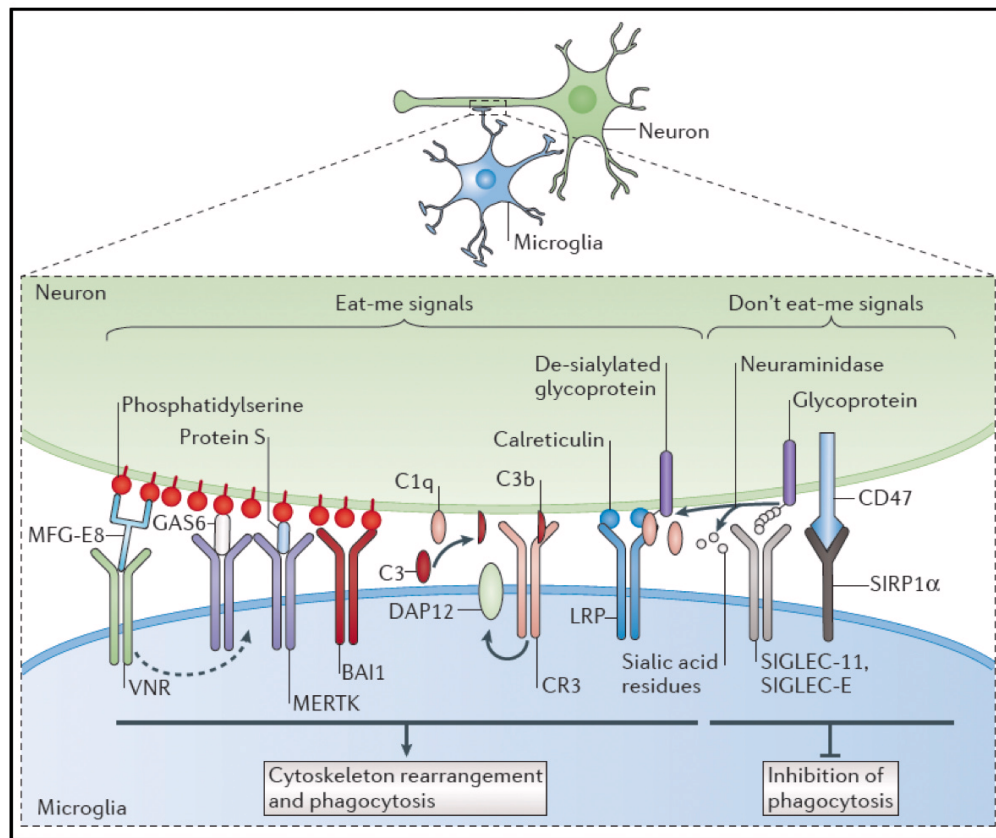


Figure 1. Signalling pathways implicated in the phagocytosis of neurons and neuronal structures. Microglial phagocytosis of neurons is regulated by the neuronal presentation and microglial recognition of ‘eat-me’ (left) and ‘don't eat-me’ (right) signals. [Figure and legend reproduced with permission from: Brown GC & Neher JJ. *Microglial phagocytosis of live neurons*. Nat Rev Neurosci 2014⁴].

and most commonly observed eat-me signal. Importantly, PS externalization of neurons can occur reversibly in response to cellular stress that is not sufficient to induce cell death. In particular, stressed but viable neurons can reversibly externalize PS when exposed to non-toxic levels of oxidative or nitrosative metabolites (as may be released by microglia during inflammation)⁶⁻⁸, low levels of glutamate⁹, or growth-factor withdrawal¹⁰. In the presence of microglia this PS exposure leads to neuronal engulfment and death, whereas in the absence of microglia, these stressed neurons are able to re-internalise PS and survive^{6,9}. Neuronal PS can be recognised by different microglial receptors, leading to a cascade of intracellular events that eventually results in the uptake of the 'eat-me' signal-exposing neuron¹¹. PS can be recognised directly through microglial transmembrane receptors or indirectly through binding of soluble opsonins, such as milk fat globule EGF-like factor 8 protein (MFG-E8, also known as lactadherin or SED1)¹². MFG-E8, a glycoprotein produced by microglia and astrocytes during inflammation, simultaneously engages exposed PS and the microglial vitronectin receptor (the heterodimeric integrin $\alpha_v\beta_{3/5}$), thereby activating phagocytosis^{12,13}. Similar to MFG-E8, two other opsonins, growth arrest specific gene 6 (Gas6), and Protein S, which also bind to exposed PS on neurons, are in turn recognized by the membrane protein Mer receptor tyrosine kinase (MerTK)¹⁴. Of note, MerTK can also be activated downstream of the vitronectin receptor, indicating convergence of the MFG-E8 and MerTK pathways.

Traditionally, phagocytosis has been regarded to occur secondary to a target cell being dead or dying. However, accumulating evidence suggests, that during neuroinflammation or cerebral ischemia phagocytes can also eat viable neurons, and thereby induce cell death (for review see Brown & Neher, 2014)⁴. This form of cell death resulting from the cell being phagocytosed has been termed 'phagoptosis'¹⁵, with the defining characteristic that inhibition of phagocytosis prevents cell death (Figure 2). Using a rodent model of focal cerebral ischaemia induced by stereotactic microinjection of the vasoconstrictive peptide endothelin-1 (ET-1) into the striatum or sensorimotor cortex of rats or mice, respectively, we previously found that the phagocytic proteins MFG-E8 and MerTK were transiently upregulated by microglia within the ischaemic area peaking at 3–7 days after insult. Animals deficient for MFG-E8 or the microglial phagocytic receptor MerTK had reduced brain atrophy and improved neurological function. While the number of microglial cells and the levels of inflammatory mediators were indistinguishable between genotypes, microglia from *Mfge8* and *Mertk* knockout animals showed reduced phagocytosis of neurons⁹. In conclusion, these results suggest that deficiency of MerTK or MFG-E8 blocks phagocytosis of neurons by microglia and thereby prevents engulfment-induced neuronal death. However, the observed behavioural benefits among phagocytosis-deficient animals were moderate at best and the ET-1 ischemia model may have confounding effects on neuroinflammation and neuronal survival as ET-1 receptors are also expressed by neurons, astrocytes, and microglia^{16,17}.

We therefore propose to investigate how phagocytosis and specific phagocytic signalling pathways contribute to the pathophysiology

of stroke, by using an established model of focal cerebral ischemia. We will perform histological, biochemical, and behavioural analyses of phagocytosis-deficient wildtype mice and homozygous *Mfge8* and *Mertk* knockout mice, and use pharmacological inhibition of the MFG-E8 receptor to assess whether phagocytosis is beneficial or detrimental for neuronal survival and neurological function following temporary (45min) middle cerebral artery occlusion (tMCAo). In these animals, we will test:

- 1) Whether phagocytic deficiency is beneficial or detrimental for neurological function; and
- 2) Whether phagocytic microglia and recruited macrophages contribute to neuronal and/or synaptic loss following cerebral ischemia and if this is beneficial or detrimental for tissue recovery.

By pre-registering this study we strive to foster transparency about our aims, study design, and analysis plan, thereby strengthening the robustness and accountability of our data.

Methods

Animals, housing and husbandry

All animal experiments will be performed in accordance with local regulations, and have been approved by the Berlin governmental authorities (Landesamt für Gesundheit und Soziales, LaGeSo), approval number G057/16.

Male C57BL/6NCrl mice will be derived from Charles River at the age of 8 weeks. Phagocytosis-deficient *Mertk* (Jax: B6;129-*Mertk*^{tm1Gr1/J}) and *Mfge8* (from C. Théry, INSERM 932, France)¹⁸ knockout mice will be derived from The Jackson Laboratory and Hertie Institute for Clinical Brain Research, respectively, and bred locally. Male homozygous *Mertk* and *Mfge8* knockout mice and their homozygous wildtype littermates will be used in experiments at the age of 10 – 12 weeks. Animals will be group-housed with *ad libitum* access to food and water and cages will be equipped with environmental enrichment tools (red transparent plastic nest box and brown paper towels). Animals will be kept in specific pathogen free (SPF) conditions under a 12 h light/dark cycle (lights on: 8am; lights off: 8pm). Room temperature will be maintained at 22 ± 1°C.

Methods to prevent bias

Animals will be randomized using the GraphPad calculator tool (<http://www.graphpad.com/quickcalcs/randomize1.cfm>) by a researcher who is not involved in the surgical procedure, behavioural, histological, biochemical or MRI analysis. Animals will be allocated to blocks. Blocks will be randomized for a given day of surgery (10 animals) and for a given week of experiments (30 to 40 animals) or 3 – 4 surgery days so that MCAo/sham procedures and drug treatment are randomly allocated to animals and performed in a randomized order. Information on genotype and treatment group allocation will be concealed from experimenters until the end of the study. Behavioral, histological, biochemical and MRI analysis will be performed by researchers who are not involved in the surgical procedure.

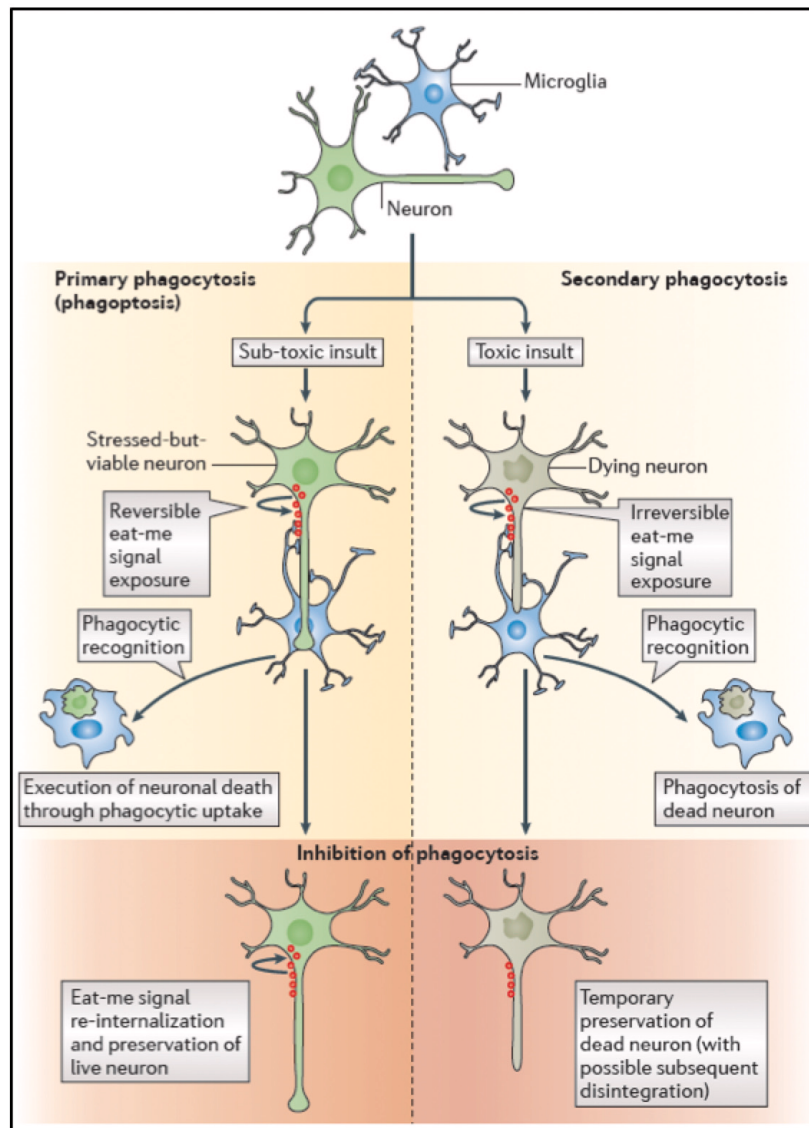


Figure 2. Phagocytosis and phagoptosis. Recent data indicate that phagocytosis can execute the death of viable neurons during development, inflammation, and neuropathology. This form of cell death is called phagoptosis, which means that cell death is caused by the cell being phagocytosed, with the defining characteristic that inhibition of phagocytosis or phagocytic signalling prevents cell death. Experimentally distinguishing between primary phagocytosis (that is, phagoptosis) and secondary phagocytosis (that is, the phagocytosis of a cell dying by apoptosis or necrosis) is possible through inhibiting phagocytosis, which in the first case will leave live cells, whereas in the second case it will leave dead cells (at least temporarily before their disintegration). [Figure and legend reproduced with permission from: Brown GC & Neher JJ.; Nat Rev Neurosci 2014⁴].

Exclusion criteria

Animals will be excluded from this study if: i) infarct volume is not detected and/or non-middle cerebral artery territory ischemia (such as cerebellar infarction) is detected on MRI imaging at 24h following cerebral ischemia; ii) pellet-reaching performance in the Staircase test differs by more than 1.2 standard deviations from the mean performance of the corresponding genotype and the treatment group at the end of the conditioning phase.

Anesthesia

All animals will be induced with 2% isoflurane in oxygen and nitrous oxide (ratio 0.3/0.7) and maintained with 1% isoflurane during surgical or imaging procedures.

Temperature monitoring and body weight measurement

Temperature will be maintained at $37 \pm 0.5^\circ\text{C}$ throughout the surgery or imaging procedures using a homeothermic feedback

system. In addition, following cerebral ischemia body temperature and weight will be measured once daily. Temperature measurements will be obtained non-invasively using subcutaneous radio-frequency identification (RFID)-transponders (IPTT-300; Bio Medic Data Systems). Transponders will be implanted by subcutaneous injection at least one week prior to the induction of cerebral ischemia.

Temporary filamentous middle cerebral artery occlusion

Mice will be subjected to 45 minutes filamentous temporary middle cerebral artery occlusion (tMCAo), and will be killed for histological and biochemical analyses after 3, 5, or 21 days, respectively. The filamentous tMCAo model will be performed using Dirnagl *et al*'s method (doi: [10.1038/npre.2010.3492.2](https://doi.org/10.1038/npre.2010.3492.2)) as implemented in our laboratory¹⁹⁻²¹. For pain relief, Bupivacaine gel is topically applied to the wound, and the wound is temporarily closed with an adaptive suture. Following tMCAo animals will have access to mashed chow placed on the cage floor in a petri dish for up to 24 hours. There will be no additional fluid replacement.

Inhibition of phagocytic signaling

For inhibition of phagocytic signaling mice will be treated with EMD121974 (Peptide Special Laboratories, GmbH), a commercially available vitronectin receptor-antagonist. EMD121974 or inactive control peptide cRGDFV (Peptide Special Laboratories, GmbH) will be administered daily by intraperitoneal injection for 7 days after tMCAo. The first dose of EMD121974 or control peptide (30 mg/kg) is administered intraperitoneally 6 h after tMCAo, followed by daily doses of 10 mg/kg, respectively.

Magnetic resonance imaging, stroke volumetry, and connectomics

Magnetic resonance imaging (MRI) will be performed at 24 h and 21 days following tMCAo using a 7 Tesla rodent scanner (BioSpec 70/16AS, Bruker BioSpin, Ettlingen, Germany) with a 16 cm horizontal bore magnet and a 9 cm (inner diameter) shielded gradient with an H-resonance frequency of 300 MHz and a maximum gradient strength of 300 mT/m. For imaging, a quadrature volume resonator with an inner diameter of 86 mm for excitation and a decoupled mouse head quadrature surface coil for signal reception (both Bruker) will be used. Data acquisition and image processing will be performed with the Bruker software Paravision 6.0.1. and custom MATLAB (MathWorks, Natick, MA, USA) scripts. Both, at 24 h and 21 d, a rapid acquisition with relaxation enhancement (RARE) T2-weighted (T2w) sequence will be used for anatomical imaging (repetition time TR=3500, echo time TE=33 ms, RARE factor 8, 4 averages, 32 contiguous coronal slices, slice thickness 0.5 mm, field of view FOV=1.92 × 1.92 cm², matrix MTX=192×192, total acquisition time TA=5:36 min). At 21 d, in addition to anatomical imaging, diffusion tensor imaging (DTI) using a Stejskal-Tanner 4-shot spin echo EPI pulse sequence will be performed (TR/TE=3500 ms/28.5 ms, 1 average, geometry identical to the T2w image, MTX=96×96, high angular resolution diffusion (HARDI) encoding scheme with 60 diffusion directions, b=1000 s/mm², five b=0 images, diffusion duration δ =2.6 ms, diffusion separation Δ =8.5 ms, TA=15:10 min). Maps of fractional

anisotropy and mean/axial/radial diffusivity (FA, MD, AD, RD) will be calculated and the DTI connectome of each mouse will be reconstructed using whole brain fiber tracking in DSI studio (<http://dsi-studio.labsolver.org/>) as described previously²². The stroke lesion will be segmented on T2w images 24 h post-surgery using Analyze 10.0 software (AnalyzeDirect, Overland Park, KS, USA) by selecting hyperintense areas of ischemic tissue by an experienced researcher and a mask will be exported. The 24 h lesion mask, and 21 d DTI parameter maps will be coregistered to the Allen mouse brain atlas using their corresponding anatomical T2w images in the MATLAB toolbox ANTX (<https://github.com/philippboehmsturm/ANTX/>)²³⁻²⁵. Since the registration includes nonlinear terms, lesion volumes are effectively corrected for edema when measured in atlas space. Edema-corrected lesion volume and DTI parameters will be measured in all regions of the atlas and in a volume of interest encompassing the lesion territory at 24 h. Values will be compared using two sample t-test using false discovery rate post-hoc correction. ANTX will also be used to coregister a modified atlas with less brain structures (MRMNeAt) to the DTI connectome and a graph theoretical analysis will be performed using the brain connectivity toolbox followed by comparison of scalar network parameters as described previously^{22,26}.

Modified DeSimoni neuroscore

DeSimoni's neuroscore²⁷, a composite of general behavioral alterations and focal motor, sensory, reflex, and balance deficits, will be performed at days 1, 2, 7, 14 and 21 following cerebral ischemia as implemented in our laboratory¹⁹. In brief, general health and behavioral alterations and specific focal deficits will be scored separately and subsequently added to form a summation score. The maximum score is 43 points, with more points meaning more deficits.

Rotarod

The Rotarod, a widely used test of motor coordination and learning, will be performed at days 2, 7, and 14 following cerebral ischemia as implemented in our laboratory¹⁹. The best run of three runs per time point will be used for statistical analysis.

Staircase

The Staircase test will be performed daily for 7 days before and up to 21 days after cerebral ischemia for conditioning and testing skilled forelimb motor function, respectively. Mice will be placed in a Plexiglas holding box with an attached baited double staircase. Food rewards are presented bilaterally on a descending double staircase with each of the 8 steps containing a food-well loaded with 20 mg sucrose pellets (Sandown Scientific). During testing, the animal is required to ascend onto a central base with one staircase on either side, designed such that only the ipsilateral paw can reach a given staircase. A lip on the platform edge prohibits the animal from dragging the pellet up alongside the platform, thus requiring it to be grasped and brought up and around the platform edge. The pellets in lower wells are more difficult to grasp than those in wells higher on the staircase, thus providing objective measures of maximum forelimb extension and grasping skill. Outcome is measured as the number of pellets grasped and eaten with each forepaw. Animals are tested once daily for 20 minutes per session.

Quantification of infarct volume, cellular densities, and phagocytosis

Following perfusion with physiological saline and 4% paraformaldehyde (PFA) and cryosectioning of the fixed tissue, brain sections will be stained for cresylviolet or NeuN to label all surviving cells and infarct size will be determined by stereological quantification by a blinded observer on random sets of every 12th systematically sampled 40 μ m thick sections throughout the brain. Analysis will be conducted using the Stereologer software (Stereo Investigator 6; MBF Bioscience) and a motorized x-y-z stage coupled to a video microscopy system (Optronics) as previously described²⁸, with application of the Cavalieri estimator technique²⁹. Neuronal and microglial/macrophagic densities will be quantified using the optical fraction fractionator on sections stained for NeuN and ionized calcium binding adaptor molecule 1 (Iba1), respectively³⁰. To quantify phagocytosis of neurons, high-resolution confocal z-stack images of the peri-infarct area stained for microglia and recruited macrophages (Iba1) and neuronal nuclear antigen (NeuN) will be obtained. Z-stack acquisition will be followed by 3-dimensional reconstruction using Imaris software (Bitplane, UK) and quantifying the percentage of microglia/macrophages that contain NeuN-positive inclusions (as described in detail in 9).

Sample size calculation and statistical analysis

The study is designed with 80% power to detect a relative 25% difference in pellet-reaching performance in the Staircase test. *A priori* power analysis using a repeated measures ANOVA with Tukey's post hoc test under the following assumptions $\alpha = 0.05$, $\beta = 0.2$, mean, SD 20% of the mean determines the number of required experimental units at 17 animals per group. Based on previous results with this model we estimate that 15% of animals will have to be excluded from this study based on insufficient infarct volume (10%) or insufficient pellet-reaching performance in the Staircase test, respectively. Thus, we will use 20 animals per genotype or treatment group, respectively (*Mertk* and *Mfge8* phagocytosis-deficient knockout animals vs. littermate controls and phagocytosis inhibitor-treated animals vs. untreated controls, respectively). Data will be tested for normal distribution using D'Agostino's K^2 test and analyzed by 1-way analysis of variance (ANOVA) or in case of measuring the effects of 2 factors 2-way ANOVA with posthoc Holm-Sidak adjustment for p values. For DTI parameter maps, lesion volumes, and network analysis metrics a false discovery rate to control for multiple comparisons and Type I errors will be used. If only 2 groups

are compared, unpaired, two-tailed Student's t test will be used. Nonparametric functional data will be compared using Kruskal-Wallis test with posthoc Dunn multiple comparison test. Survival will be compared for the effect of genotype or treatment using a log-rank Mantel-Cox test. P values ≤ 0.05 are considered statistically significant.

Study design

Following tMCAo, animals will be killed for histological and biochemical analyses after 3, 5, or 21 days, respectively. Functional, MRI, and histological outcome data of phagocytosis-deficient *Mertk* and *Mfge8* knockout animals and littermate controls will be assessed using a block design (i.e. all animals will be tested before data analysis is performed). Outcome data of phagocytosis inhibitor-treated animals vs. untreated controls will be assessed using a group sequential design with 4 stages (k=4, sample size at stage 1, 2, and 3, respectively: 14 animals (7 animals vs. 7 animals), sample size at stage 4: 12 animals (6 animals vs. 6 animals), for details see 31). To save animals and money, testing will be terminated following unblinded interim data analysis at each stage based on the following stopping criteria: stage 1) none or detrimental effect of phagocytosis inhibitor on neuronal and/or synaptic survival and tissue recovery at day 5 following tMCAo; stages 2–4) detrimental or beneficial effect of phagocytosis inhibitor on pellet-reaching performance in the Staircase test following tMCAo (i.e. mean pellet-reaching performance differs by more than 20% from one standard deviation of the mean pellet-reaching performance of the control group).

Study timeline

The study will be conducted within 12 months, following successful peer review of this Stage 1 Registered Report submission.

Competing interests

No competing interests were disclosed.

Grant information

Funds for transgenic animal breeding will be provided by the German Federal Institute for Risk Assessment and parts of the study will be supported by grants of the Federal Ministry of Education and Research (BMBF) for the Center for Stroke Research Berlin to UD, CH and PBS and of the Berlin Institute of Health, QUEST Center to UD.

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Ádám Dénes

MTA Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary

I find it a great idea to share the design of the proposed studies with the scientific community. In general, the aim of the research is great and the methods to be used are largely appropriate. The authors deserve the credit for providing details on given experimental objectives, including randomisation, blinding, power calculation and description of the experimental procedures, which all increase the transparency and credibility of these studies.

Specific points:

1. It is stated that the studies will be completed in 12 months, which is doable given that all mouse strains are available and breeding on site. Based on what is described in the methods, it remains unclear how long it will take to establish breeding colonies from the phagocytosis-deficient strains derived from The Jackson Laboratory and how the phenotype of these will be compared to the control animals, which will be obtained from another vendor (Charles River). For such studies comparing different KO lines, it would be useful to ensure the identity of the background ideally by establishing heterozygous breeding colonies and use littermates for the experiments. C57BL/6 (and other) mouse strains from different vendors could show markedly different inflammatory or phagocytic responses that could influence the interpretation of the results.
2. In addition to using Mfge8 KO mice, the pharmacological inhibition of the microglial MFG-E8 receptor is also proposed in the methods. If phagocytic signaling will be blocked by intraperitoneal administration of the antagonist, will this regimen effectively reach areas of the brain where there is no major BBB injury? Details on brain penetration and pharmacokinetics of the drug (if known) would be useful.
3. The phagocytosis-deficient lines and pharmacological inhibitors will likely change phagocytic responses in all tissue macrophages, including perivascular macrophages in the brain (the latter will possibly be influenced by the pharmacological treatment earlier than microglia). Unless the authors plan sophisticated studies on discriminating microglial actions from that of blood-borne phagocytes that may be recruited into the affected brain areas, it may be more useful to talk about microglia /macrophages throughout. Iba1 is not an ideal marker either to discriminate brain microglia /macrophages of different origin.
4. Several excellent sensory-motor tests are planned for functional assessment, among which the staircase requires extensive conditioning and testing. Has the same battery of tests been successfully used in similar MCAo studies previously? Extensive testing might lead to overtraining of mice and a

reduction of differences in performance between experimental groups in spite of the expected changes in the number of surviving neurons.

The notes above merely aim to help the interpretation of these excellent studies rather than suggesting extensive changes in experimental design, particularly regarding costly breeding and determination of microglial-specific effects.

Have the authors pre-specified sufficient outcome-neutral tests for ensuring that the results obtained can test the stated hypotheses, including positive controls and quality checks?

Yes.

Is the rationale for, and objectives of, the study clearly described?

Yes

Is the study design appropriate for the research question?

Yes

Are sufficient details of the methods provided to allow replication by others?

Yes

Are the datasets clearly presented in a useable and accessible format?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 09 Nov 2017

Julius Emrich, Charité-Universitätsmedizin Berlin, Germany

We would like to thank the reviewer for appreciating the aim of this registered report and the helpful comments. Where appropriate the manuscript has been revised following the reviewer's remarks.

Point 1: We agree with the reviewer that the description of breeding procedures lacked some detail. Heterozygous breeding colonies for phagocytosis-deficient mouse strains have already been established in our laboratory. Homozygous *Mertk* and *Mfge8* knockout offspring will be used in experiments. Homozygous wildtype littermates will serve as controls. C57BL/6NCrI mice, which will be obtained from Charles River, will be used for inhibition experiments.

Point 2: Several pharmacokinetic studies have shown penetration of EMD121947 (cilengitide) through the intact blood brain barrier. An excellent study on the pharmacokinetics of cilengitide in mice and men by Dolgos et al. can be found here: www.ncbi.nlm.nih.gov/pmc/articles/PMC4804314.

Point 3: We thank the reviewer for bringing up this important point. We do not plan to discern effects of deficient phagocytosis between microglia and blood-derived macrophages. As suggested by the reviewer, we have modified the wording throughout the manuscript.

Point 4: We appreciate the reviewer's concern that excessive training might have a negative impact on behavioral outcomes. Both tests have been established in our laboratory and have been used in parallel for MCAo studies. Several other groups have also used a combination of these tests following MCAo in mice (see for example: <http://www.jneurosci.org/content/24/27/6209>). It is well conceivable that training in one test might have an influence on the results of the other. The staircase and Rotarod tests assess skilled forelimb function (i.e. number of pellets grasped and eaten with each forepaw) and general motor coordination, respectively. Thus, we believe that the additional information which is gained by assessing two distinctly different motor skills outweighs a potential confounding effect.

Competing Interests: None.

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Stuart M. Allan 

Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, UK

The rationale behind the study is well explained and on the most part the methods are described in full. Importantly the authors provide full details of experimental design, including power calculations and planned statistical analyses. They also propose the use of sequential design which is very unusual in preclinical research, but can be used more widely.

Overall there are no major concerns regarding the planned study but the report would benefit from additional detail and clarification in a few places as detailed below:

1. The authors do not specify what will be their primary endpoint and what will constitute secondary analyses. The study is powered on the Staircase test which would imply that this is being taken as the primary outcome. The title and abstract should be amended to clarify this, and the latter should also have details of the secondary outcomes that are being assessed.
2. The abstract would benefit from additional details on the age and sex of mice used.
3. Page 2: The authors indicate no grants were involved in supporting the work. It is possible to clarify therefore exactly how the work was supported, since presumably the funds had to come from somewhere.
4. Page 5: Clarify if a homeothermic feedback system used to maintain body temperature.
5. Page 5: Clarify if modified diet provided to aid post-stroke recovery and on similar lines if fluid replacement instigated.
6. Page 5: Clarify why systemic analgesia not used.

7. Page 5: Clarify how dosing regimen decided on.
8. Page 5: Clarify why only male mice being used.
9. Page 6: For rotarod clarify why best run being used rather than average.
10. Page 6: Please specify endpoints (days post MCAo) when animals are perfused.
11. Page 7: day 5 has not been previously specified as an endpoint.

Have the authors pre-specified sufficient outcome-neutral tests for ensuring that the results obtained can test the stated hypotheses, including positive controls and quality checks?

Yes

Is the rationale for, and objectives of, the study clearly described?

Yes

Is the study design appropriate for the research question?

Yes

Are sufficient details of the methods provided to allow replication by others?

Partly

Are the datasets clearly presented in a useable and accessible format?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 09 Nov 2017

Julius Emrich, Charité-Universitätsmedizin Berlin, Germany

We would like to thank the reviewer for the insightful and constructive remarks. All points have also been addressed in the revised version of this article.

Point 1: The primary outcome measure of this study is functional recovery following tMCAo as determined with the staircase test. The study has been powered accordingly. Secondary outcomes constitute Rotarod performance, stroke volume (quantified on MR imaging or brain sections, respectively), diffusion tensor imaging (DTI) connectome mapping, and histological analyses to measure neuronal and microglial densities, and phagocytic activity.

Point 2: Male mice at the age of 10-12 weeks will be used for experiments. The abstract has been modified accordingly.

Point 3: Funds for transgenic animal breeding will be provided by the German Federal Institute for Risk Assessment and parts of the study will be supported by grants of the Federal Ministry of

Education and Research (BMBF) for the Center for Stroke Research Berlin to UD, CH and PBS and the Berlin Institute of Health, QUEST Center to UD.

Point 4: Temperature will be maintained at $37 \pm 0,5$ °C during surgery or imaging procedures using a closed loop homeothermic feedback system which consist of a heating blanket connected to a rectal probe.

Point 5: Following tMCAo animals will have access to mashed chow placed on the cage floor in a petri dish for up to 24 hours after surgery. There will be no additional fluid replacement.

Point 6: The brain itself is not sensitive to pain. Local delivery of gel comprising anesthetics is sufficient to reduce the pain that originates from the wound and the vessels. Many inflammatory signals are modulated by non-steroid analgesic drugs or opioids. Therefore, we aimed to avoid using drugs that probably affect stroke outcome or inflammatory signaling including phagoptosis.

Point 7: The dosing regimen of EMD121947 (cilengitide), an inhibitor of phagocytic signaling, was decided based on results from previous pharmacokinetic studies in mice and preliminary, unpublished data.

Point 8: Only male mice will be included in this study, which might introduce a potential sex bias. However, including female animals is likely to increase variation, which, in turn, would require the use of additional animals, time, and effort. The number of subjects for this study was limited to the minimum required to obtain scientifically meaningful results and only the use of male mice has been approved by the Berlin Ethical Review Panel (LaGeSo). If results from the pharmacological intervention study will be encouraging, we would consider planning a pre-clinical trial that is powered to study sex differences by EMD121947.

Point 9: We expect that the variation of Rotarod results will be smaller if the best run out of three trials is used rather than the average. If we would calculate averages across three trials, an accidental slip or drop off the rod that is caused by lack of attention or motivation would falsely increase variation. We believe that this approach is best suited to obtain adequate information on the motor coordination of a given animal.

Point 10: As specified on page 5 in the Materials and Methods section, endpoints will be at 3, 5, or 28 days following 45min tMCAo, respectively.

Point 11: As specified on page 5 in the Materials and Methods section, animals will be killed for histological and biochemical analyses after 3, 5, or 28 days after 45min tMCAo, respectively. To improve clarity, this information has now also been included on page 7 (study design).

Competing Interests: None.

Discuss this Article

Version 1

Author Response 09 Nov 2017

Julius Emmrich, Charité-Universitätsmedizin Berlin, Germany

Thank you for this constructive comment.

Animals will be allocated to blocks. Blocks will be randomized for a given day of surgery (10 animals) and for a given week of experiments (30 to 40 animals) or 3 – 4 surgery days so that MCAo/sham procedures and drug treatment are randomly allocated to animals and performed in a randomized order. Treatment allocation will be concealed from the surgeon and scientists involved in behavioral analysis, histology or imaging until all data will be collected.

To save time, animals, and money we opted to assess the effect of drug-induced inhibition of phagocytosis using a group sequential design. Similar to clinical studies, this allows for early termination of experiments in case of efficacy or futility of the administered drug. To ensure independence of the analysis, unblinded interim analysis of the effect of EMD121947 (cilengitide) will be performed at predefined stages by a statistician who is otherwise not part of this study. A potential disadvantage of this approach will be that each next stage can only be started after the outcome of a preceding stage has been assessed and analyzed. But we are keen to find out how this tool can be applied in practice.

Competing Interests: None.

Reader Comment 01 Nov 2017

Lawrence Moon, Wolfson CARD, King, UK

This is a great initiative. Nice to see a stroke paper leading the way.

In the spirit of supporting this endeavour positively, I have a few questions and comments.

It's good that the animals will be randomized (p. 5) but can the authors expand on this: will there be block randomisation for a given day of surgery (e.g., so that the treatments are performed in a randomised order as well as being randomly allocated to animals?)

My comment is that this initiative will help others improve their study design way before the results are published.

For example, I like the fact that the authors plan to use a "group sequential design" and I'll be reading up on this (ref 31) to see if it's suitable for my own work. I'll need to learn how to handle the fact that I'll be "repeatedly peeking at the data" . [Will the authors have a third party do the unblinding and interim data analyses?]

I also like the fact that the exclusion criteria for the staircase are based on 1.2 x standard deviation at the end of the training phase. I can imagine how I'd base this on my sample size calculations in future, so thank you.

I hope that the authors consider continuing this dialogue via this comments channel. It'll be an opportunity for many of us to learn from their example. Thanks!

Lawrence Moon
King's College London, UK

Competing Interests: None.

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