Cortical Selective Neuronal Loss, Impaired Behavior, and Normal Magnetic Resonance Imaging in a New Rat Model of True Transient Ischemic Attacks

Sohail Ejaz, PhD*; Julius V. Emmrich, MD*; Stephen J. Sawiak, PhD; David J. Williamson, PhD; Jean-Claude Baron, ScD

Background and Purpose—New-definition transient ischemic attacks (TIAs) are frequent but difficult to diagnose because magnetic resonance imaging (MRI)-negative by definition. However, hidden underlying cell damage might be present and account for the reported long-lasting cognitive impairment after TIAs. Most prior rodent models of true TIA targeted the striatum or have not been fully characterized. Here we present the MRI, behavioral, and quantitative cell changes characterizing a new rodent model of true TIA targeting the more behaviorally relevant cerebral cortex.

Methods—Fifteen-minute distal middle cerebral artery occlusion was performed in 29 spontaneously hypertensive rats allowed to survive for 7 to 60 days. Behavior was assessed serially using both global neurological and fine sensorimotor tests. Diffusion- and T2-weighted MRI was obtained 20 min postreperfusion and again 7 to 60 days later, and then changes in neurons and microglia were quantified across the middle cerebral artery territory using immunohistochemistry.

Results—No MRI changes or pan-necrosis were observed at any time point, but patchy cortical selective neuronal loss affected 28/29 rats, regardless of survival interval, together with topographically congruent microglial activation that gradually declined over time. The Neuroscore was unchanged, but there was marked contralateral sensorimotor impairment, still recovering by day 28.

Conclusions—Our new rodent model mimicking true cortical TIA is characterized by normal MRI, but consistent cortical selective neuronal loss and microglial activation and long-lasting sensorimotor deficits. By causing selective neuronal loss, TIAs and silent microemboli might affect neuronal reserve, thereby increasing long-term cognitive impairment risk. Selective neuronal loss and microglial activation might represent novel therapeutic targets that could be detectable in vivo after TIAs using appropriate imaging tracers. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.114.007581.)

Key Words: inflammation ■ MCA occlusion ■ MRI ■ transient ischemic attack

Most transient ischemic attacks (TIAs) are secondary to transient arterial occlusion from upstream blood clot. According to their new tissue-based definition, TIAs are characterized both clinically by focal neurological symptoms of ischemic origin lasting <24 hours and radiologically by a lack of topographically congruent changes on diffusion-weighted imaging (DWI) or FLAIR magnetic resonance imaging (MRI) performed within 2 weeks of the clinical event. Normal imaging in true TIAs, which account for 20% to 50% of all clinically diagnosed TIAs, makes them difficult to differentiate from nonischemic transient focal neurological events; hence, their diagnosis rests entirely on clinical skills. Up to 45% of all DWI-negative transient focal neurological events are eventually not classified as genuine TIAs.

The lack of detectable changes on structural MRI in TIAs does not, however, rule out the presence of microscopic damage, such as selective neuronal loss (SNL) and microglial activation (MA). Recently, impairment in subtle cognitive tests after TIAs, despite normal neurological examination, has been repeatedly reported, suggesting underlying tissue damage. Interestingly, positron emission tomography imaging using appropriate tracers is able to detect SNL and MA in vivo. It is therefore of translational importance to develop rodent models of MRI-negative TIAs to investigate whether these histopathologic changes effectively occur and, if so, characterize their behavioral counterparts.

Previous work using brief proximal middle cerebral artery occlusion (MCAo) in rats has documented the occurrence of SNL and MA, despite normal MRI. However, this model primarily targets the striatum, whereas in the clinical setting, TIAs tend to preferentially affect the cortex. Furthermore, cortical damage is more likely to result in long-lasting...
behavioral changes. Three previous studies using brief (<1 hour) distal temporary MCAo (tMCAo) targeting the cortex all reported pure SNL. However, none documented pure SNL as a consistent outcome nor addressed its behavioral correlates. Furthermore, they all used relatively early time points for histopathologic assessment, which may be suboptimal given the possibility of slow infarct maturation.

Here we validated and characterized a novel rodent model of cortical TIA using distal tMCAo in spontaneously hypertensive rats (SHRs) by means of (i) MRI performed both soon after reperfusion and after several days; (ii) serial behavioral assessment; and (iii) quantitative assessment of SNL and MA throughout the MCA territory after survival delays ≤60 days.

Materials and Methods

This study was performed in accordance with the legislation of UK Animals Scientific Procedures Act 1986 and after approval by the Cambridge University Ethical Review Panel. This included the requirement to keep the number of animals to a minimum, yet sufficient to obtain meaningful results.

Overall Study Design

SHRs were elected because this strain is best representative of the clinical stroke population and because among rat strains it shows both the largest and least variable infarct size after MCAo, including with the distal clip model. We chose a 15-min distal MCAo duration based on previous literature and own previous work (Methods in the online-only Data Supplement). Two successive series of experiments were performed, involving altogether 29 young adult (3–6 months old) male SHRs (n=300 g; Charles River, UK). In the first (Part 1), we documented consistent cortical SNL without MRI or histopathologic evidence of infarction up to day 60, and in the second (Part 2), we replicated this outcome and characterized its behavioral correlates.

Part 1 used 23 consecutive rats, including 7 left to survive 28 days for the main study; 3 rats each to 45 or 60 days, to ensure lack of delayed infarct maturation; and 5 rats each to 7 and 14 days as to examine early presence of SNL. NeuN, OX42, and GFAP immunohistochemistry was performed to assess changes in neurons, microglia, and astrocytes, respectively. In Part 2, 6 consecutive SHRs were used; behavior was assessed serially until killing at 28 days; and immunofluorescence using NeuN and IB4 was performed to assess SNL and MA, respectively.

Common Procedures

Anesthesia

Experiments were performed in freely breathing animals under 2% isoflurane and 1:3 O2/N2O mix. Body temperature was monitored with a rectal probe and strictly maintained at 37.0°C ausing a heated pad throughout all procedures. SaO2 and heart beat were continuously monitored using a pulse-oximeter and were within physiological limits throughout.

MCAo

Distal microclip left tMCAo was performed as detailed previously. The clip was removed after 15 mins and the wound closed. Reperfusion was visually confirmed on clip removal by MRI angiography in a subset of rats (see below). We have previously reported consistent recanalization on time of flight-MRI angiography using this model. To avoid additional procedural complications that may affect tissue outcome, including blood loss from femoral cannulation, arterial blood pressure was not measured here and is known to be already significantly elevated (>170 mm Hg) by 3 months of age in SHRs.

MRI

MRI was performed at day 0, ≈20 min postreperfusion, and again immediately before perfusion-fixation in Series 1 and at 14 days in Series 2 subjects. The protocol included T2-weighted and DWI scans in all rats and MRI angiography in a subset of Part 1 rats. MRI data analysis was visual, although an ancillary analysis of MRI signal looking for potential subtle markers of SNL was also performed (Methods in the online-only Data Supplement).

Histopathology

On the final experimental day, perfusion-fixation was performed and the brain cryosectioned as detailed previously and in Methods in the online-only Data Supplement. Ischemic lesions were classified into 3 main subtypes, namely pan-necrosis, partial infarction, or SNL (Methods in the online-only Data Supplement).

Part 1: Specific Procedures

Immunohistochemistry

Immunohistochemistry for NeuN, OX42, and GFAP and standard Cresyl Violet staining were performed as previously detailed.

Quantitative Assessment of Ischemic Changes

In animals allowed to survive 28, 45, or 60 days post MCAo, formal cell counting was performed as described elsewhere in whole regions-of-interest (ROIs) spanning the MCA territory on both sides, using a template of 44 prespecified symmetrical ROIs (derived from Paxinos’ anatomic atlas) belonging to 8 predetermined coronal sections, as described in detail elsewhere. See Methods in the online-only Data Supplement for details.

For each ROI, changes in neuron and activated microglia density were expressed as percentage relative to the contralateral side. In addition, for each rat, a whole hemisphere average neuronal loss or MA, to be referred to as CellDens_Total below, was calculated as the weighted mean percentage change (normalized by the area of the ROI) across all 44 ROIs for NeuN and OX42, respectively.

Part 2: Specific Procedures

Behavior

Behavioral effects were assessed with both García’s Neuroscore, performed the day before surgery and at postoperative days 1, 7, 14, 21, and 28, and the more sensitive modified sticky label test (mSLT), performed the day before surgery and at postoperative days 1, 3, 7, 11, 14, 18, 21, 25, and 28. See Methods in the online-only Data Supplement for details.

Histopathology

Immunofluorescence was implemented using NeuN and IB4 antibodies to map neuronal loss and MA, respectively (Methods in the online-only Data Supplement). Quantitative assessment was performed on the same set of 8 coronal sections described earlier, according to procedures detailed in Methods in the online-only Data Supplement. Briefly, on whole-brain immunofluorescence sections of the 6 rats presented in random order and without knowledge of the affected side, 2 experienced raters independently delineated using computer mouse any areas with lack of NeuN immunoreactivity or increased IB4 staining, and total lesion volume across the whole hemisphere, to be referred to as Vol_total below, was computed for each side separately.

Results

There was no instance of micro- or macro-infarcts, hemorrhage, SNL, or MA in the unaffected hemisphere in any subject of any group.
There was no evidence of pan-necrosis or partial infarction in the occluded hemisphere in any subject. However, patchy or layer-wise cortical SNL was present at both low and high resolution in all 7 rats, not consistently involving a specific cortical layer. Typical for recent SNL, MA consistently matched the areas of SNL, together with more diffuse astrocytosis on GFAP (Figure 1). These findings were confirmed by cell counting showing similar regional pattern of SNL and MA involving the cortical MCA territory and reaching statistical significance in several ROIs (Figure IA and IB in the online-only Data Supplement). Accordingly, there was a significant ($P<0.01$) negative correlation between NeuN and OX42 density changes across the 44 ROIs (Figure IC in the online-only Data Supplement). The CellDens_Total values for NeuN and OX42 across the sample (Figure 2A) were significantly reduced and increased, respectively, relative to the control side ($P<0.0001$ for both).

Figure 1. Representative histological sections (bregma+1 mm) from 1 rat demonstrating cortical selective neuronal loss with topographically congruent microglial activation and astrocytosis 28 days after 15 min middle cerebral artery occlusion (MCAo). A-D, Displayed are \times 10 NeuN, cresyl violet (CV), OX42 and GFAP images, respectively. The middle row shows \times 50 and the left row \times 100 magnification of the insets shown on the \times 10 images. Layer-wise arranged patches of variously severe neuron loss are present on NeuN (red arrows), with consistent but less clear findings on CV. Patches of activated microglia, topographically congruent with the selective neuronal loss (SNL) areas, are present on OX42 (blue arrows), whereas GFAP reveals conspicuous, more diffuse astroglial reaction in the same areas.

Figure 2. A, Mean (and SEM) CellDens_Total, that is, the weighted percent cell density change averaged across the 44 regions-of-interest (ROIs; $n=6$ as NeuN staining was inadequate for ImageJ analysis in 1 rat) for NeuN and OX42, showing highly significant NeuN loss and increased OX42 binding 28 days after 15 min middle cerebral artery occlusion (MCAo). B and C, Same results for the 45 days and 60 days survival groups, respectively ($n=3$ per group), showing statistically significant NeuN loss and increased OX42 binding at both time points. Note the essentially stable NeuN value relative to the markedly declining OX42 value over time.
No instance of infarction was found in any rat. At 45 days, no definite SNL, MA, or astrocytosis were identified at low magnification, though consistently present at high magnification. In no rat survived 60 days could definite MA or astrocytosis be identified even at high magnification, despite the presence of a few small SNL patches in each rat (Figure 3).

Cell counting showed roughly similar NeuN reductions at both 45 and 60 days, both ≈2-fold less prominent than at day 28, whereas OX42 values showed a sharp ≈6-fold decline from day 28 to day 60 (Figure II in the online-only Data Supplement). Accordingly, NeuN CellDens_Total values on the affected side were highly significantly reduced at both time points (Figure 2B and 2C). The corresponding OX42 values were also significantly increased at both time points, but marginally so for the day 60 value. This is also illustrated in the scatterplots of NeuN versus MA cell counts, showing as compared with day 28 a flattening of the OX42 values as opposed to persistently reduced NeuN values over time, with their correlation losing statistical significance (Figure III in the online-only Data Supplement).

Seven and 14 Days Survival Group
Definite SNL and MA were present in all subjects for both time points, adopting a regional distribution similar to that observed in the day 28 group (see Figure IV in the online-only Data Supplement for illustration). Because the aim of studying these earlier time points was to ensure that definite SNL was already present, cell counting was not performed.

MRI
MRI angiography, obtained in 3 animals from the 28 days group, consistently showed full recanalization. No DWI or T2-weighted changes were visible either acutely or at follow-up in any subject (Figure 4). Quantitative data are presented in the Table in the online-only Data Supplement.
Discussion
Fifteen-minute distal MCAo in SHRs resulted in cortical SNL in 28/29 subjects, with no evidence of associated infarction in any subject either on MRI or at postmortem performed as late as day 60. Because pure SNL was already present at day 7, it does not represent a delayed phenomenon; however, longitudinal studies would be worth performing to determine the precise time course of postischemic SNL. Consistent with the histopathologic literature on SHR,21,22 there was no evidence in this study of SNL or microinfarcts, nor of any MRI changes, in the contralesional hemisphere, so the observed changes can be confidently ascribed to the MCAo. However, SHRs are known to develop brain vasculature changes secondary to chronic hypertension that make them particularly susceptible to ischemic injury.17,18,21,22

Our model mimics true TIA not only as no MRI changes were detected at any postreperfusion time point, but also because neurological examination was normal throughout. However, finer behavioral tests disclosed significant sensorimotor dysfunction still incompletely recovered at 4 weeks. Finally, MA was consistently associated with SNL, although declined over time, and represents another salient consequence of brief focal ischemia.

Rodent TIA Model
Striatal SNL after proximal tMCAo has been extensively reported.27 Across studies, striatal SNL—occasionally associated with mild cortical SNL—regularly follows ≤20 min MCAo in the rat, whereas longer occlusions consistently cause striatal infarcts.27 Two previous articles only have been published as rodent models of TIA.28,29 In one,29 striatal and occasional cortical microinfarcts were present 24–72 hours after 5 to 10 minutes proximal MCAo in Sprague–Dawley rats, but the definition used for microinfarcts is questionable because key criteria to distinguish pan-necrosis from SNL, such as coagulation necrosis, astrocytosis, and MA, may not develop that early. Additionally, this outcome was present in 9/32 subjects only, and neither MRI nor behavior were assessed. In the other study,28 mice subjected to proximal MCAo lasting 5 to 12.5 mins consistently exhibited cortical selective neuronal necrosis on H&E performed at 24–72 hours together with normal MRI and Neuroscore. However, postmortem assessment was again too early, whereas neither quantitative immunohistochemistry nor finer behavioral tests were performed. The latter have to date been reported in a single study where rats subjected to 20 min proximal MCAo showed impaired SLT performance followed by full recovery occurring between days 7 and 21, associated with normal MRI but striatal selective neuronal necrosis and milder cortical selective neuronal necrosis at postmortem.8

Although the above reports are useful as models of TIAs resulting from proximal MCA occlusion, they do not model cortical TIAs, and more generally, cortical SNL is never extensive following proximal MCAo unless striatal infarction also occurs.27 Using 45 min distal MCAo in SHRs, isolated cortical SNL was present in a subset of rats but the majority showed clear infarcts,14,15 indicating this duration is inadequate for a consistent outcome. Brief distal MCAo was previously assessed in a study on Wistar rats focusing on
recurrent ischemic insults. A single 40 min MCAo episode caused mild selective neuronal necrosis on H&E and normal MRI on day 7, but without significant SNL or MA on immunohistochemistry. In addition, outcome consistency was not reported, nor behavior assessed.

In the present study, visual identification of SNL was increasingly difficult as time elapsed, paralleled by an ≈2-fold smaller cell loss at 60 as compared with 28 days. Chance finding as a result of small samples rather than delayed tissue collapse likely explains this observation because cell counting was performed in whole symmetrical ROIs. However, even though neuronal repopulation is elusive after cortical infarcts, migrating neuroblasts might have penetrated SNL patches given the sparse glial scar.

**Behavioral Effects**

The unaltered Neuroscore in our study is consistent with the absence of infarction, which induces clear Neuroscore deficits in SHRs, whereas the markedly impaired mSLT matches SNL predominantly involving S1/2. Deficits on SLT are thought to reflect a mix of hemi-sensory neglect and extinction, forepaw motor deficit, and impaired somatosensory integration (Methods in the online-only Data Supplement). Among several tests, only the SLT was affected in mice subjected to permanent distal MCAo. Sensorimotor deficits after proximal MCAo more specifically reflect cortical relative to striatal damage. This differential effect probably explains why in Sicard’s 20 min proximal MCAo study, where only marginal cortical SNL was present, impaired SLT had fully recovered before day 21, as compared with still recovering at day 28 here.

Although a sham group was not included, young adult SHRs are not known to develop sensorimotor impairment over time, whereas longitudinally assessed mSLT performance does not show habituation in sham rats. Furthermore, the time course of changes does not fit with an MCAo-unrelated spontaneous decline as the day 28 score showed significant recovery relative to previous time point. Nevertheless, SHRs do develop histopathologic and behavioral stigmata of accelerated brain aging over time, which could have contributed to the behavioral effects of MCAo by reducing the brain reserve.
Inflammation

Using OX42, a monoclonal antibody to CD11b strongly expressed by activated microglia/macrophages, inflammation was found to be consistently associated and topographically congruent with SNL, though declined over time and was barely detectable at day 60, consistent with literature. 27 That MA is consistently associated with SNL is well established. Strong topographical congruence and quantitative correlation between SNL and MA have been previously reported on both days 14 and 28 after distal MCAo in SHRs. 14, 15 It is therefore plausible that MA, which is initially triggered by neuronal injury, might exacerbate SNL. 36 However, intervention studies to date have been largely inconclusive, which may be as a result of the confounding prorepair effects of MA. 36 Detailed longitudinal studies in our model might help clarify these complex inter-relationships.

MRI Correlates

Consistent with all previous reports, 8, 9, 13, 14, 37 SNL and MA were not associated with any visually detectable DWI lesions or infarct-type T2-weighted hyperintense lesions on early postreperfusion and follow-up MRI, respectively. Ancillary analysis confirmed the lack of significant acute DWI or follow-up T2 signal changes; interestingly, however, there was a small (∼5%) but significant acute-stage apparent diffusion coefficient reduction (Table in the online-only Data Supplement), probably as a residual of the preceding severe cortical ischemia. Our model therefore effectively mimics true TIA, which is defined by lack of corresponding lesion on early and delayed standard diffusion and FLAIR sequences. 3 As we designed our study to match these criteria, we cannot exclude that cortical SNL might be detectable on specific MRI sequences or as transient changes on standard sequences. For instance, in one study, striatal T2 hypersignal was present 24 hours and 1 and 2 weeks after 60 min proximal MCAo in Wistar rats, but had vanished at week 10, 37 though reflected severe, near complete SNL as opposed to mild cortical SNL here. Of note, the T2-weighted sequence used here has been previously shown to clearly depict pathologically proven cortical infarcts. 14

Clinical Implications

Our findings have clinical relevance. First, that DWI-negative TIAs may involve hidden cortical damage points to potential long-term, equally hidden consequences on brain functions, especially in case of multiple TIAs. Thus, similar to our findings, subtle cognitive impairment not apparent with standard neurological scales and lasting days to weeks have been documented after TIAs. 3– 5 The high frequency of both multiple silent DWI lesions 38 and equally silent microemboli 39 in this population might lead to extensive cortical SNL, even in case of single clinical TIA, in turn affecting the brain’s plastic reserve and potentially contributing to worse deficits/recov- ery in case of stroke, as well as vascular cognitive impairment and old-age dementia in association with Alzheimer pathology. 27 Accordingly, TIAs are known to be associated with an increased long-term risk of cognitive decline and dementia. 3, 4 Independent from or in addition to this classic diminished reserve mechanism, neuronal death might also directly trigger Aβ deposition and facilitate Alzheimer pathology. 40, 41 An effect possibly enhanced in SHRs given their naturally occurring accelerated brain aging. Thus, SNL and MA might represent therapeutic targets in patients with TIAs. Second, given the neuronal damage, the original TIA symptoms might resurface under psychological or pharmacological stress disrupting local reorganization. 42 Finally, relevant to both diagnosis and research, both cortical SNL and MA could be readily detectable after clinically unclear transient focal neurological event using specific positron emission tomography ligands. 6, 7
Sources of Funding
European Community Grant (EUSTROKEHealth-F2-2008-2022131). J.V. Emmrich was funded by a Theracur Stiftung grant and D.J. Williamson by a Medical Research Council collaborative grant (G0600986).

Disclosures
None.

References


Cortical Selective Neuronal Loss, Impaired Behavior, and Normal Magnetic Resonance Imaging in a New Rat Model of True Transient Ischemic Attacks
Sohail Ejaz, Julius V. Emmrich, Stephen J. Sawiak, David J. Williamson and Jean-Claude Baron

*Stroke*. published online February 10, 2015;
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://stroke.ahajournals.org/content/early/2015/02/10/STROKEAHA.114.007581

Data Supplement (unedited) at:

http://stroke.ahajournals.org/content/suppl/2015/02/10/STROKEAHA.114.007581.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/
Supplemental Material

1. Supplemental Methods

1.1. Procedures common to Parts 1 and 2

tMCAo duration
Although the optimal distal MCAo duration to reliably obtain pure cortical SNL in SHRs is unknown, the available literature using proximal MCAo in Sprague-Dawley rats indicates that occlusion times between 15 and 30 minutes regularly induce striatal SNL, with cortical SNL occasionally present as well (see Baron et al for review). In two previous studies, 45min distal tMCAo in SHRs induced pure cortical SNL in less than half the subjects, and infarcts in the rest. Considering the above data and following preliminary experiments using 30 and 22min MCAo, we finally elected a 15min occlusion time.

MRI
Part 1: MRI was acquired whenever feasible immediately following reperfusion (~20min after clip removal; minimum time required to clean and seal the wound then position the animal inside the bore), and again systematically several days later. Anesthesia used 1.5% isoflurane in 0.3 l/min O2 and 0.7 l/min N2O mix. Body temperature was kept at 37 ± 1°C with an integrated heating system, and a pressure probe to monitor the respiration. Images were acquired using a 4.7T Bruker BioSpec 47/40 system (Bruker, BioSpin GmbH, Ettlingen, Germany) with a 2cm surface coil used for signal reception. Structural imaging was performed with a T2-weighted RARE sequence (TR/TE 3500/36ms, ETL 8, slice thickness 1mm, in plane resolution 0.156mm). Diffusion weighted images (DWI) were acquired using an EPI sequence (TR/TE 3000/35ms, 35 directions b = 1000s/mm2, slice thickness 1.5mm, in plane resolution 0.312mm). In addition, standard MRA of the circle of Willis was performed in a subset of rats to confirm consistency of MCA recanalization with this model. It was performed with a fast low-angle shot sequence with flow compensation (TR/TE 12/3ms using two averages covering a field of view of 40mm with a matrix of 256 yielding a planar resolution of 156µm in 64 slices of 0.7mm with an overlap of 0.15mm between consecutive slices).

In Part 2, MRI was systematically acquired both ~20min following reperfusion and just before killing at 14 days. The same sequences as in Part 1 were acquired, save for MRA.

MRI data analysis
The aim of our study was to model the clinical situation of ‘true’ TIA where standard clinical MR images are read visually. Accordingly, hyperintense lesions were assessed visually on the immediate post-reperfusion DWI images as markers of severe ischemia, and more importantly hyperintense lesions on the follow-up T2-weighted images were sought as markers of infarction. We have previously reported using exactly the same acquisition parameters that our T2-weighted sequence is highly sensitive to histopathological infarcts following distal MCAo in SHRs. For the sake of completeness, and even though looking for MR markers of SNL and MA was outside the aim of this study and MR data acquisition was not optimized for quantitative assessment, we additionally obtained quantitative data for acute DWI and follow-up T2-weighted images. Given the patchy nature of SNL and MA as identified on very thin brain sections as compared to the spatial resolution of MRI, and the lack of validated software to co-register these two image modalities in 3D, we opted for a systematic assessment of the MR data within identical cortical areas across all subjects. To this end, we used the DARTEL image registration tool of SPM8 (Wellcome Trust Institute for Neurology, UCL, London) adapted with the SPMMouse toolbox. Default values calculated for the rat brain by the toolbox...
were used for the image segmentation required for DARTEL and correction for receiver coil inhomogeneity. The primary and secondary somatosensory cortex are the areas most targeted by our distal MCAo model, and were mapped on the mean template image produced by DARTEL based on our previously published cytoarchitectonic ROI template, and used to extract signal values from acute DWI and follow up T2 on both sides of the brain. A paired t-test on the left-right percent signal difference was then used to assess statistical significance. For completeness, the same ROI analysis method was applied on the apparent diffusion coefficient (ADC) images obtained acutely.

**Perfusion-fixation and brain cryosectioning**

The experimental protocol was terminated by intraperitoneal injection (30mg/100g) of sodium pentobarbitone, as detailed previously. Brains were post fixed in 4% paraformaldehyde overnight (4°C) and then immersed into 30% sucrose solution (0.1 M PB, pH 7.4) for at least 3–4 days for cryoprotection. Cryosectining was carried out across the MCA territory, from the level of the forceps minor to the superior colliculi (Bregma 3.7 to -6.80mm) as detailed previously.

**Visual assessment of ischemic lesions**

Ischemic lesions were classified into three main subtypes, namely: i) pan-necrosis (tissue necrosis with absence of neurons, microglia and astrocytes; dissolved extracellular matrix; presence of cavitations and tissue loss); ii) partial infarction (same as pan-necrosis but only mild volume loss, relatively preserved extracellular matrix, no or few small cavitations, presence of dense activated microglia and elongated astrocytes); and iii) SNL (patchy loss of neurons with preserved tissue structure and presence of activated microglia (i.e., adopting spherical morphology) and elongated astrocytes matching SNL).

**1.2. Part 1 specific procedures**

**Quantitative assessment of ischemic changes**

For each animal, eight coronal stained sections spanning the MCA territory (bregma: +2.70, +1.00, -0.26, -0.92, -2.12, -3.14, -4.52, and -6.04 mm) were selected and were digitized as described previously. Quantitative data describing the regional distribution and severity of IHC changes were obtained in a set of pre-specified symmetrical cytoarchitectonic ROIs derived from Paxinos and Watson’s stereotaxic atlas of the rat brain applied to each captured section, as described previously. This ROI template covered the MCA territory and comprised 44 cytoarchitectonically-defined ROIs, including 39 cortical, 4 caudate/putamen and one thalamus ROIs. The resulting digitized maps (x1.6) of individual animals at corresponding coronal levels were mapped onto this template using Adobe Photoshop as detailed previously.

The precise methodology used for cell counting has been described elsewhere. Briefly, using Photoshop CS software (Adobe, CA, USA) each ROI was contrast-enhanced using the Auto Contrast command and background noise was reduced using Unsharp-Mask, Despeckle, and High-Pass filters. ImageJ software (National Institutes of Health, Bethesda, USA) batch processing was used to create binary images based on threshold values determined with the Auto Threshold command. Finally, the ImageJ Watershed algorithm was applied to separate overlapping cells, and cell numbers for each ROI were quantified based on average (± 1SD) values for size and circularity using the Particle Analysis command for neuronal and microglial cells, respectively.
For descriptive purposes, statistical significance for each ROI was tested by paired t-test comparing cell numbers on the affected vs unaffected side across the sample of rats. In addition, for each rat a whole hemisphere average neuronal loss or microglial activation, to be referred to as CellDens_Total, was calculated as the weighted mean percentage (normalized by the area of the ROI) across all 44 ROIs for NeuN and OX42, respectively. Again, statistical significance within each group was tested by paired t-test. Two-tailed p<0.05 was considered significant.

1.3. Part 2 specific procedures

Behavioral assessment

Animals were single-housed on a 12-hour light/dark cycle and had free access to water and standard rodent chow. Training/testing was performed in the light phase and animals were left in their housing cages during sessions. Animals received daily handling for at least 4 days before baseline testing to ensure accurate behavioural results. The modified sticky label test (mSLT) was performed one day before surgery and postoperatively animals were tested at days 1, 3, 7, 11, 14, 18, 21, 25, and 28. Neurological examination was carried out the day before surgery and at postoperative days 1, 7, 14, 21, and 28 using Garcia’s Neuroscore.

Garcia's Neuroscore consists of motor, sensory, reflex, and observational tests to evaluate neurological deficits following MCAo in rats. It is scored on a scale from 3 to 18 (normal: 18; maximal deficit: 3), i.e. the lower the score the worse the deficit.

mSLT: Subtle sensorimotor dysfunction following MCAo was assessed using the mSLT as previously described. This test is consistently sensitive to subtle ischemic damage even when the Neuroscore is normal and, contrary to the standard version, uses a non-removable tape. As a result, non-stroked rats spend most of the 30s sessions trying to remove it, with no habituation effect over time. A small patch of paper tape (2.5cm long, 1.0 cm wide) was wrapped around the animal’s wrist contralateral to the ischemic insult such that the tape sticks to itself and that the fingers protrude from the sleeve formed. The rat is placed into its home cage and the time spent attending to the stimulus is recorded. Animals are given five sessions per day, each observation period lasting for a maximum of 30s. After each trial the tape is removed and animals are given a resting time of ≥3mins. mSLT performance is calculated by dividing the time attending to the stimulus by 30s, expressing the fraction of the observation period that the animal spends attending to the tape. The best two ratios on each day are averaged. In order to reduce noise, all daily time-points for each week were collapsed into a single value. The results of the final day of pre-surgery training served as baseline for assessment of post-MCAo performance. Following stroke, sensorimotor deficits make the rat spend less time attending to the tape than normally. Deficits on the SLT are thought to reflect a mix of subtle sensorimotor impairments including hemi-sensory neglect and extinction, forepaw motor deficit and impaired somatosensory integration.

Using SPSS (version 15, SPSS Inc.), the mSLT data were assessed for a main effect of Time (within-subject factor) using repeated-measures analysis of variance (ANOVA). If applicable, post-hoc multiple comparisons were then conducted using Holm-Bonferroni corrected t-tests to determine differences between performance at each post-stroke time-point and baseline. Results were considered to be statistically significant if 2-tailed p < 0.05.

Immunofluorescence (IF)

IF was implemented using anti-NeuN and anti-IB4 antibodies to map neuronal loss and microglial activation, respectively. Free-floating sections were incubated for 2hrs at room temperature in PBS plus 5% normal goat serum and 0.3% Triton X-100. Sections were incubated with anti-NeuN antibody (Millipore Bioscience Research Reagents, 1:500) and Alexa Fluor 488-labeled isolectin-B4 (Sigma, 1:500) overnight, 4°C, washed, and incubated
with goat anti-mouse-Cy3 antibody (Jackson ImmunoResearch, 1:150) for 2 hrs at room temperature. Sections were washed, mounted on gelatine-coated slides, dried for 15 minutes on a heating block (40°C) and coverslipped using FluorSave reagent (Calbiochem).

**Quantitative assessment of ischemic lesions**

Assessment of ischemic changes was carried out on the same set of eight coronal sections spanning the MCA territory detailed above. Stained sections were captured via whole-slide scanning on an Ariol SL-50 automated scan station (Applied Imaging, Santa Clara, USA) using an Olympus BX61 microscope at 20x magnification. Composite images depicting whole brain sections were stitched automatically from individual frames and digitized. Then, without knowledge of the affected side, two experienced raters (SE and JCB) independently delineated using the computer mouse any area with lack of NeuN immunoreactivity or increased IB4 staining. The whole-brain scans of the six rats were presented in random order on a high-resolution monitor screen. The cross-sectional surface area encompassed within the traced regions on each section was then measured using ImageJ software. Whole lesion volume across adjacent sections was estimated using the planimetric Cavalieri method (area of region of interest x 1/2 x mean slice thickness or area of region of interest x mean slice thickness for one or more than one neighbouring sections affected by immunohistochemical changes, respectively) 15. For each rat, the total volume across all ROIs, to be referred to as Vol_total, was then calculated.

Cohen’s kappa was used to determine inter-rater reliability of manual lesion contouring. Then, differences in Vol_Total between ipsi- and contralesional sides were assessed using Wilcoxon signed-rank tests. Pearson’s linear correlation was used to assess the relationships between NeuN and IB4 volumes, while the relationship between peak behavioral deficit and Vol_Total was assessed using Kendall’s Tau correlation. 2-tailed p < 0.05 was considered statistically significant.
**Supplemental Table:** Percent (left – right) difference in mean acute-stage (i.e., ~20mins after reperfusion) DWI signal and apparent diffusion coefficient (ADC) values, and chronic-stage T2-weighted MR signal, for the primary (S1) and secondary (S2) somatosensory (S1) cortex regions of interest (see Methods for definition)

<table>
<thead>
<tr>
<th></th>
<th>Acute DWI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Acute ADC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T2 Day 14&lt;sup&gt;b&lt;/sup&gt;</th>
<th>T2 Day 28&lt;sup&gt;b&lt;/sup&gt;</th>
<th>T2 Day 45&lt;sup&gt;b&lt;/sup&gt;</th>
<th>T2 Day 60&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>1.3 ± 6.8 %</td>
<td>-5.5 ± 3.6 %&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-0.9 ± 1.4 %</td>
<td>-0.4 ± 1.3 %</td>
<td>1.6 ± 0.9 %</td>
<td>0.8 ± 1.2 %</td>
</tr>
<tr>
<td>S2</td>
<td>0.0 ± 5.8 %</td>
<td>-5.0 ± 4.1 %&lt;sup&gt;##&lt;/sup&gt;</td>
<td>1.2 ± 1.2 %</td>
<td>1.5 ± 1.8 %</td>
<td>0.6 ± 2.4 %</td>
<td>3.2 ± 1.7 %</td>
</tr>
</tbody>
</table>

<sup>a</sup>: n=6 (Series 2 subjects); <sup>b</sup>: n=3 (Series 1 subjects)

All values not statistically significant except *: p<0.02; and #: p<0.05 (paired t-test against neutral)
Supplemental Figure I: Mean (± SEM; n = 6 rats) percentage change (relative to control side) in NeuN (A) and OX42 (B) for each of the 44 ROIs following 15min distal MCAo, showing a similar regional pattern of changes, involving mainly the auditory, insular, parietal, visual and primary and secondary somatosensory cortex (S1/2, including barrel field). Significant changes for single ROIs are indicated by stars (paired t-test; * = p < 0.05; ** = p<0.01; ***= p<0.001). Region code: Aud–auditory cortex, Caud Put–caudate putamen, Ins–insular cortex, Mot–motor cortex, Piri–piriform cortex, Pariet–parietal cortex, Rhinal–rhinal cortex, Prim Som–primary somatosensory cortex, Sec Som–secondary somatosensory cortex, Prim Som Bar Fld–primary somatosensory cortex barrel field, Thal–thalamus, Vis–visual cortex. The numbers bracketing groups of regions refer the 8 coronal sections used for the quantification (see3, 16 for details). (C): Relationship between NeuN and OX42 relative cell density changes across the 44 ROIs, demonstrating a significant negative correlation (Kendall tau= -0.338, p < 0.01), such that greater MA was associated with increasing SNL.
**Supplemental Figure II**: Mean (± SEM) NeuN and OX42 relative cell density values for each of the 44 ROIs, for the rats survived 45 days (A and B) and 60 days (C and D) (n = 3 per group). Same layout and codes as in Supplemental Figure I. This figure illustrates the persistently reduced NeuN densities at these two late time-points, although overall to a lesser degree than at day 28 (Fig. 2), while the OX42 changes markedly decreased over time from 28 to 45 to 60 days. As a consequence, the topographical matching of NeuN and OX42 disappeared over time.
Supplemental Figure III: Relationship between NeuN and OX42 relative cell density changes across the 44 ROIs, plotted for the 45 days (A) and 60 days (B) survival groups, demonstrating a gradual loss of the relationship between SNL and MA over time largely due to OX42 values markedly decreasing over time, as compared to Day 28 (Suppl. Fig. 2). Neither of these two correlations was statistically significant.
Supplemental Figure IV: Representative NeuN (left) and OX42 (right) brain sections from two rats allowed to survive 7 days (top row) and 14 days (bottom row) following 15min distal MCAo. For each immunostain, the images on the left are x10 magnification, and the higher magnification of the boxes at x50 magnification. Topographically congruent cortical SNL (stars) and MA (arrows) were present in both subjects. The red arrowheads point to the surgical area.
Supplemental references


