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Choice, Methodology, and Characterization of Focal Ischemic Stroke Models

The Search for Clinical Relevance

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Summary

To develop novel neuroprotective or neurorestorative agents for clinical application, the appropriate selection and characterization of preclinical focal stroke models is required to provide confidence in predicting therapeutic efficacy. Compelling evidence for novel therapies derived from the pathological and functional consequences of models of cerebral ischemia in the rat (and higher species) is an essential prerequisite before large expensive clinical trials are begun. This chapter provides an overview of focal ischemic models, with an emphasis on objective functional assessment of pathological mechanisms and efficacy of novel therapeutic strategies. The ability to predict functional consequences from structural abnormalities is a critical theme that can be extrapolated from the preclinical to the clinical setting, in that certain brain regions are inextricably linked to specific behavioral functions. This underlying approach is highly relevant, as monitoring the dynamic pathological and functional changes attributed to focal stroke will reveal new insights into novel mechanisms and targets that play a role in the evolution of cell death and impaired function. The utility of novel genomic technologies that are aligned with methods to determine structure–function relationships in preclinical models will facilitate a greater understanding of the pathophysiological process and potentially generate new targets that may ultimately be used to predict or offer clinical benefit.

Key Words

Focal cerebral ischemia; middle cerebral artery occlusion; ischemic stroke; behavior; functional impairments; functional recovery; neuroprotection; neurorestoration; neuroregeneration; animal models.

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1. Introduction and Review

Stroke or focal cerebral ischemia is a leading cause of death and permanent disability for which there is currently no effective treatment; hence a large unmet medical need exists. In most western populations, 0.2% of the population (2000 per million) suffer a stroke each year ***(1)***, of whom one-third die over the next year, one-third remain permanently disabled, and one-third make a reasonable recovery ***(2)***. The purpose of this chapter is to highlight the utility of animal models of focal stroke and to identify strategies with the objective of clinical relevance/application in mind. The chapter attempts to outline:

1. Appropriate choice of animal models to identify novel pathophysiological mechanisms and therapeutic efficacy.
2. Appropriate methods of characterization that will elucidate functional and pathological changes predictive of human stroke, and how one could attempt to extrapolate to the clinical situation using this information.

Methods for inducing middle cerebral artery occlusion (MCAO) in rats via the intraluminal suture technique are explained in detail in the Materials and Methods sections, as well as valid and robust objective behavioral tests to quantify the functional impairments.

In terms of putting the above in the context of novel target identification for stroke, stroke genomic methodologies will be alluded to (which are discussed further in accompanying chapters), in order to glean comprehensive multifactorial in vivo information to help translate preclinical data to the clinical setting.

1.1. Anatomical Considerations

The middle cerebral artery (MCA) in humans is the largest branch of the internal carotid artery (ICA) and is considered the artery most often occluded following a thromboembolic obstruction. The etiology of vessel occlusion in humans relates to a thrombotic cause as a result of a local arteriopathy ***(3,4)***, or an embolic cause through circulating emboli passing from the ICA to the MCA to occlude the vessel because of its proximity and size and the proportion of blood to the area. The MCA can be anatomically divided into four main segments: M1, M2, M3, and M4. The M1 segment comprises the main MCA trunk from which approx 12 deep penetrating vessels, the lenticulostriate arteries, arise in two groups: the larger lateral and smaller medial. From the M2 segment, situated in the sylvian fissure, arise the two main divisions of the MCA and all cortical branches that subsequently comprise the M3, or opercular, segment. Once over the cortical surface, the M4 segment is formed. The cerebral cortex, basal ganglia, and internal capsule are supplied by the MCA and its small penetrating branches. These regions are especially prone to infarction.

Focal infarcts of the MCA territory represent at least 25% of first-time ischemic strokes, although some studies have associated up to 80% of cerebral infarcts with ischemic damage to the MCA territory ***(5,6)***. This area is susceptible because the small penetrating arteries to the brain are not supported by a good collateral circulation, and therefore occlusion of one of these arteries is likely to cause uncompromised infarction ***(7,8)***. The stroke "lesion" can therefore be considered to consist of a central core of densely ischemic tissue (the focus) and of "perifocal" or "penumbral" areas with less dense ischemia ***(9–12)***. The focus, usually encompassing the lateral part of the caudate putamen or striatum (part of the basal ganglia) and the adjacent neocortex, represents tissues that depend heavily on the perfusion from the occluded MCA by end-arterial branches. The periphery of the stroke lesion and the perifocal areas are perfused by the anterior cerebral (ACA) and posterior cerebral arteries (PCA), most of which are leptomeningeal. Clearly, in all but the most central parts of the lesion, perfusion depends on the adequacy of the collateral circulation.

However, human ischemic stroke is seldom permanent; it usually involves some degree of vessel recanalization and spontaneous reperfusion. Spontaneous, or drug-induced (fibrinolytic) reperfusion has the potential to be detrimental (beyond the therapeutic window), as well as beneficial. To elucidate the benefits associated with reperfusion-related tissue salvage or the pathological processes associated with reperfusion injury, it is important to characterize not only the severity and distribution of ischemia but also the effects of the extent and duration of reperfusion on outcome.

The vascular territories of the major cerebral arteries supplying the cerebral cortex, subcortical structures, cerebellum, and brainstem in humans are relatively well mapped. It has been stated that, depending on the location of the infarct, clinical syndromes vary in stroke patients, implying that functional impairment is a feature contingent on the degree of compromised flow within a particular portion of the vascular territory ***(13)***. Hemiparesis, or muscle weakness to one side of the body, is the most common deficit after stroke, affecting more than 80% of patients acutely and more than 40% chronically ***(14)***. The clinical manifestations of MCA infarction depend on the location of the occlusion. Therefore, distinct clinical syndromes are associated with the involvement of the main proximal arterial trunk, the lenticulostriate arteries, and the superior and inferior divisions of the MCA.

The limitations of large-scale human studies become more apparent when one tries to consider events immediately following the insult and before irreversible brain injury. Elucidation of the cause–effect relationships in the progression of the infarct pathology requires immediate access to localized events in the affected area to acquire insight into the ischemic process. Frequently, the

stroke patient is admitted many hours after the symptoms have developed and generally only postmortem tissue or cerebrospinal fluid is available for biochemical analysis of the events. Because very limited human studies are feasible, investigations of the mechanisms and endogenous agents involved in the cascade of events that lead to brain injury following an ischemic insult have been primarily (or at least initially) pursued in experimental models of cerebral ischemia. To yield novel information on pathophysiological mechanisms that may contribute to the cell-death process(es) or impairments in regeneration, it is necessary to select and characterize animal models of focal stroke using appropriate methodologies, to facilitate the development of new and improved therapeutic strategies for human stroke.

1.2. Appropriate Choice of Animal Models of Focal Ischemia

Recently a report was published that outlined recommendations for standards regarding preclinical neuroprotective and restorative drug development, involving a roundtable of key worldwide academic, clinical, and industrial experts from the stroke research area (the Stroke Therapy Academic Industry Roundtable [STAIR]) ***(15)***. It was highlighted that robust rodent models would be "early, go no-go" predictors of therapeutic efficacy for agents and that functional assessment over a longer period should be implemented to investigate the long-term benefit of an approach in order to improve extrapolation to the clinical setting. Furthermore, STAIR ***(15)*** has encouraged the development of nonhuman primate models as a necessary intermediate step for predicting the efficacy of treatment strategies before they enter the clinic. Primate models are important not only for evaluating clinically relevant functional outcome but also for scaling up dosing regimens from rodents in order to improve the definition of dose and duration of drug administration for sustained efficacy. Significant progress has been made in developing clinically relevant primate models of focal stroke ***(16–18)***, which provide a greater level of confidence in progressing up the phylogenetic tree from rodents to humans when novel targets are developed further for large-scale clinical trials ***(19–21)***. Furthermore, developments in nonhuman primate transgenesis ***(22)*** and comparative genomics via microarray analysis ***(23)*** in disease models will provide an even more compelling link between basic research and clinical application for neurological diseases, such as stroke. This chapter focuses on rodent models of focal stroke, which are routinely used world-wide and more accessible to identify novel mechanisms and targets for drug development.

In theory, there are two types of models that address the mechanistic and therapeutic aspects of focal cerebral ischemia. If the objective of the study is to understand precisely the event following an ischemic insult and to identify endogenous mediators that may participate, then there is a need to use a model

in which variables such as duration and extent of ischemia can be controlled and a reproducible outcome guaranteed. Moreover, if the aim of the investigation is to determine the efficacy of a particular therapeutic intervention then importance should be placed on incorporating into the model design conditions that may be observed in humans, e.g., reperfusion. In practice, most models in current use have been developed to try and satisfy both ideals or at least provide an acceptable compromise. It is well recognized that "rodent" models provide a useful controlled environment to study the mechanisms involved in ischemic pathology as well as an environment in which to assess potential therapeutic interventions. However, many differences exist between the models and the clinical setting, and extrapolation of the results from the models to humans should be carried out with utmost caution. The most widely used models of focal cerebral ischemia can be broadly categorized into two types, namely, permanent MCA occlusion (pMCAO) and transient MCA occlusion (tMCAO) which are described next.

1.3. Models of Focal Cerebral Ischaemia

1.3.1. Unilateral Permanent Electrocoagulation of the MCA

Ligation of the *distal* portion of the MCA in the Sprague-Dawley rat using a frontoparietal approach to produce a focal ischemic lesion was developed nearly 30 yr ago ***(24)***. Although this method produced infarcts in several layers of the cortex (2–5 mm in diameter), infarcts of a reproducible size were not always demonstrated, nor was damage involving the striatum. Refinement of this method was undertaken by Tamura et al. ***(10,11)***. Using a subtemporal craniectomy, these investigators were able to gain access to the more *proximal* portions of the MCA. The coronoid process of the mandible and zygoma were removed, and a burr hole was opened lateral to the foramen ovale. Once the dura had been opened, the MCA could be visualized through the burr hole. Electrocoagulation of the main trunk of the MCA was conducted just medial to the olfactory tract, at a point proximal to the origin of the lateral lenticulostriate branches that supply the lateral portion of the anterior basal ganglia. This refinement produced an area of damage in the MCA territory that involved the cortex and striatum and also had a low mortality. With further slight variation in the surgical approach, involving preservation of the zygomatic arch, the MCA was still accessed and occluded, without compromising recovery from surgery. Lesion size examined many hours after the initial insult from this modified approach was equivalent to that obtained from the original Tamura approach ***(25–27)***. In addition, bisecting or severing the coagulated MCA has been demonstrated to be necessary to ensure blood flow cessation completely, as coagulation alone has been shown to make the thrombus unstable and hence

reduce the homogeneity in size of the infarction ***(28)***. Step-by-step methods for conducting the permanent distal electrocoagulation of the MCA are comprehensively explained in the Chapter 3.

An excellent study ***(29)*** confirmed that electrocoagulation of the MCA proximal to the lenticulostriate branch was associated with lesions in the striatum. Furthermore, it was necessary to extend the length of the occlusion proximal to the inferior cerebral vein to elicit consistent, reproducible infarcts involving the cortex and striatum. The significant contribution of the lenticulostriate branches and other branches of the MCA to perfusion of the cortex and striatum in rats as well as humans has been demonstrated ***(30–34)***. As there may be variation in the length of the vessel occluded, there has been considerable disparity (47–330 mm^3) in the volume of the hemispheric damage induced by proximal lesions ***(26,29,35)***. In more recent years, a hemispheric infarct volume of approx 100–150 mm^3 is consistently reported following proximal electrocoagulation of the MCA in normotensive rats ***(36)***. The extent and location of the lesion reflect the severity of the neurological deficit in both animal models and the stroke patient. The infarct volume of 100–150 mm^3 in a rodent would represent a large hemispheric infarct observed in humans.

Although this permanent model of MCAO has provided a well-controlled and reproducible environment to study the pathology and novel treatments applicable to ischemic stroke, like any model there are some drawbacks. The most obvious relate to the need for surgical craniectomy under anesthesia and the permanent nature of the occlusion. Indeed, exposure of the brain to air during craniectomy may alter intracranial pressure and blood–brain barrier permeability ***(32,37)***. Exposing the brain to occlude the MCA can lead to thermal damage from drilling and dessication of the tissue around the crainectomy site. Frequent irrigation with saline during drilling and expedient surgery can reduce this damage. Furthermore, cauterizing the proximal MCA may cause damage to autonomic nerves around the MCA, and autoregulation of the cerebral blood flow (CBF) may be lost ***(38)***. In addition to being a clinical strategy, reperfusion of a previously ischemic area is observed in humans spontaneously, through resolution of the emboli; however, it cannot be incorporated into the study design of permanent MCAO. Reperfusion presents its own clinical and therapeutic challenges, and other models have been developed to investigate the consequences of reperfusion of ischemically compromised tissue.

1.3.2. Unilateral Intraluminal Thread Occlusion of the MCA

The introduction of a model that allows both permanent (p) and transient (t) MCAO in the rat without craniectomy has heralded a new era in the experimental study of focal cerebral ischemia and reperfusion injury.

An intraluminal occlusion method with subsequent reperfusion was pioneered by Koizumi et al. ***(39,40)***, to study the progression of edema with

reperfusion. These authors described the introduction of a suture into the ICA at the bifurcation of the common carotid artery (CCA) and external carotid artery (ECA) following ligation of the latter vessels. This suture was advanced intraluminally beyond the origin of the PCA and past the origin of the MCA. At this level, the intraluminal device prevents blood flow to the MCA from the ICA and PCA, with the help of restricting any anterograde blood flow from ligation of the CCA. The greatest advantage of this model is the ease with which recirculation can be instigated. Recirculation can be initiated by simply withdrawing the thread and re-exposing the origin of the MCA. In Koizumi's technique, the ipsilateral CCA is ligated, as is the ECA, so recirculation is achieved by the complete circle of Willis via a retrograde flow to the MCA. This technique has led several investigators to conclude that this is a feasible method of inducing reversible ischemia. This method also obviates what are perceived as the surgically more demanding and, some would construe, damaging aspects of the subtemporal exposure of the MCA developed by Tamura et al. ***(10,11)***. The utility of this model to examine reperfusion injury has been fully exploited, and its suitability for inducing pMCAO has been used by a number of groups to reconcile the temporal profile of events between this method and the original Tamura surgical technique.

Several types of coated or uncoated sutures ***(39,41–46)*** and rat strains ***(47,48)*** have been used in studies utilizing the intraluminal thread approach, and it has become increasingly clear that these factors significantly influence outcome. In the original Koizumi method, the 4/0 suture was coated with silicone at the distal 5 mm (0.25–0.30 mm in diameter, for animals weighing 280–350 g) to provide a soft and malleable coating, which gently dilates the vessels through which it passes. This leads to a greatly reduced risk of vessel perforation and a much tighter fit (reducing the incidence of subarachnoid hemorrhage), preventing any residual blood flow around the thread. However, more recently the reproducibility and reliability of Koizumi's silicone-coated suture method has been tested further, illustrated by the fact that coating the thread makes the diameter of the occluder more consistent and offsets minor variations of commercially prepared suture diameters, which may ultimately affect infarct volume ***(49)***. It has been debated whether a coated filament with a larger diameter caused a more complete vessel occlusion with lower residual CBF and better consistency of ischemic lesion volume, without incurring subarachnoid hemorrhage ***(45,49)***. Also, in the original Koizumi model, the suture was inserted via the CCA rather than the ECA ***(42)***, which was permanently ligated to fix the thread in place and therefore to provide a more homogeneous infarct size (by reducing the variability of the ipsilateral collateral supply). Therefore it has been argued that Koizumi's technique is the method of choice ***(50)*** in generating a reliably noninvasive proximal MCAO approach with consistent reductions in CBF.

Although there appears to be a tremendous potential in using this model to investigate the deleterious effects of reperfusion injury such as edema and the microvasular alterations that might contribute to such pathology, there are several drawbacks to this model. Even though mechanical damage to the vascular smooth muscle via external compression is obviated, the extent to which the lumen of the vessel (in particular the endothelial cells) is damaged by this procedure has not been fully assessed. In addition to the mechanical consequences of denudation of the endothelial cells *per se*, the loss of the contribution of the endothelium-derived products to cerebrovascular tone may complicate analysis of the results. Although the intraparenchymal vessels would not be affected directly by the mechanical damage from the intraluminal device, there are concerns regarding the influence this damage would have locally on the permeability of the blood–brain barrier and whether it would provide a source of emboli that occlude more distant vessels. The relatively high mortality rate with extended durations of MCAO (70% with 3 h of MCAO) is disappointing and most likely reflects the increased edema and brain swelling; pathology also observed clinically with delayed reperfusion ***(7,51–53)***. Nevertheless, this intraluminal thread model has been used to establish correlates between pathological tissue changes observed post mortem and the changes using novel imaging technology with shorter and permanent durations of MCAO with appropriate survival times. Differences between lesion evolution via magnetic resonance imaging (MRI) have also been noted between the Koizumi and Zea Longa approaches, suggesting that infarct expansion may be simulated differentially by these contrasting methods and represent different populations of stroke patients appropriately ***(54)***. This provides valuable information to correct clinical use of this technology.

1.4. Functional Consequences of MCAO in Rodents

Although the pathophysiological features of MCAO in rats has been extensively investigated, until recently an examination of the behavioral consequences has received less attention. A description of the functional correlates of ischemic damage is important, as the principle goal of any stroke therapy in humans is the restoration of normal behavioral function of the patient. An appraisal of the behavioral deficits, which are objectively quantified in an animal model of stroke, allows a realistic association between specific pathophysiological mechanisms and specific behavioral impairments.

Most of the behavioral work that has been conducted using the rat pMCAO electrocoagulation technique has focused on simple reflex and motor function during the early phase of infarction, i.e., during the first 24 h ***(29)***. Indeed, neurological examinations based on posture and hemiparesis ***(29)***, the motor screen test ***(55)***, the balance beam test ***(55–57)***, the limb placing test ***(58)***, and

the prehensile-traction test ***(59)***, have been widely used to assess outcome because of their simplicity and the fact that they have been developed according to clinical criteria. Although these tests include many parameters or grades to determine total deficits, each parameter tends to be relatively crude. There also tend to be problems such as quantification and objectivity for measurement in the chronic phase, as testing conditions and individuals can vary to different degrees over time. Clearly there is a need to develop fine, objective, and quantitative methods of assessment over time in animal models of MCAO in order to standardize reliable baselines to the severity of ischemia and to be confident in predicting therapeutic efficacy. Few studies have attempted to extend behavioral assessment to determine the chronic consequences of tMCAO after the intraluminal thread technique, even though this surgical approach is commonly used in experimental studies of stroke. In studies that have been extended over time, using alternative surgical procedures for both pMCAO and tMCAO, simple tests of somatosensory and motor function (postural reflex, bilateral sticky label/tactile extinction, beam walking, rotarod) have shown spontaneous recovery ***(60–64)***. Indeed, the bilateral sticky label test is an objective test developed by Schallert and Upchurch ***(65)*** that quantifies the latency to contact and remove sticky tape/labels simultaneously presented on the forepaws of rats. Typically, MCAO produces elevated latencies in contacting and removing tactile stimuli from the contralateral forepaw ***(60,61,63,64)*** and has similarities to the tactile extinction observed in human stroke patients ***(66)*** and the contralateral neglect syndrome arising from damage to the parietal cortex in humans ***(67)***. Although this test shows reproducible impairments within the acute to subacute phase following MCAO, recovery of variable degrees has been demonstrated within the chronic phase of testing (at 1 mo). Clearly this is a problematic issue, as results from the experimental literature suggest either that rodents have a greater capacity for recovery of function after stroke than humans or that the behavioral tests are insensitive or inappropriate for assessing function over time.

Alternatively, tests of skilled motor function, such as the staircase test, originally developed by Montoya et al. ***(68)***, has demonstrated stable and persistent (up to 3 mo) impairments in retrieving food pellets with the contralateral forepaw ***(64,69–71)*** as well as the ipsilateral forepaw ***(64,70,71)***, following MCAO in rats. The staircase test provides a highly objective measurement of independent skilled paw use, requiring the rat to exert precise motor control over each paw in order to grasp and retrieve pellets. Furthermore, the number of attempted but displaced pellets can also be recorded, allowing an assessment of motivation owing to the appetitive nature of this task. Therefore the staircase test provides a good approximation of one of the most pronounced long-term deficits in human stroke patients, finger dexterity.

Therefore a number of behavioral tests have sufficient sensitivity to detect functional impairments, although application on a widespread basis in experimental stroke studies has not been apparent. In several cases relationships between histological outcome and behavioral tests have not been performed; in others, correlations between histological outcome and functional outcome have produced conflicting results ***(69,72–74)***. Correlating total lesion size with functional impairment only makes the assumption that the degree or extent of damage is associated with the behavioral deficit. In addition, this assumption does not attempt to identify specific structures that may fulfil the specific functions being probed by the behavioral task. The importance of selective lesion studies in animals has established the crucial relationship between specific or regional brain damage and specific impairments in behavioral performance. The behavioral tasks are designed to yield information concerning distinct components of behaviour that are potentially disrupted by the experimental lesion. Furthermore, Gavrilescu and Kase ***(13)*** have stated that, depending on the location of the infarct, clinical syndromes vary in stroke patients, implying that functional impairment is a feature of compromised flow within a particular portion of the vascular territory. Moreover, Gavrilescu and Kase ***(13)*** highlighted the role MRI and computed tomography scans play in improving our understanding of correlations between the anatomical substrates recruited by the infarction process and the neurological status of the individual stroke patient. Therefore, specific anatomical substrates recruited by the infarction process, rather than lesion size *per se*, may be the critical determinants of behavioral impairment following focal cerebral ischemia.

The experimental literature relating to animal studies of stroke has provided an extensive body of evidence to suggest that intact cortical regions surrounding an infarct, as well as contralateral regions, may contribute to the restitution of function following brain injury. Furthermore, changes in axonal outgrowth and synaptogenesis, detected by immunohistochemistry (growth-associated protein-43 and synaptophysin, respectively), have been demonstrated within penumbral and contralateral regions that parallel functional recovery following distal pMCAO in SHRs ***(75)***. In addition, recent advances in pharmacological interventions that amplify these cellular events such as amphetamine ***(76)***, basic fibroblast factor ***(77,78)***, nerve growth factor (NGF) ***(79)***, and anti-Nogo-A ***(80)***, as well as osteogenic protein-1, that has been postulated to enhance new dendritic sprouting ***(81,82)***, have been associated with significantly improved outcome in forelimb and hindlimb tests following focal brain injury in rats. These studies also demonstrate that the enhancement of recovery of function does not necessarily depend on the reduction of total infarct volume but rather on the reorganization of the remaining intact brain. This evidence suggests that the time window for enhancing stroke recovery is potentially

much longer than that for reducing infarct volume and implies that recovery-promoting drugs for stroke may have effective time windows of several days or even weeks after the onset of ischemia.

The next sections document appropriate materials and methods to conduct the intraluminal thread MCAO surgical approach (via the modified Koizumi technique) in rats with the aim of assessing animals on objective behavioral tests. This approach can provide useful insights into mechanisms that play a role in spontaneous recovery of function and persistent impairments, ultimately to gauge the efficacy of novel therapeutic strategies.

2. Materials

2.1. Behavioral Testing

1. Parcel tape.
2. Beam-walking apparatus (100-cm horizontal beam).
3. Staircase test apparatus.
4. Coco Pops (Kelloggs, UK).
5. Stopwatches/stop clocks.

2.2. Surgical Procedure

1. Halothane (Concord Pharmaceuticals, UK) in N_2O/O_2.
2. Hibiscrub (Schering Plough Animal Health, UK).
3. Fur shaver.
4. Homeothermic heating blanket with rectal probe (Harvard).
5. Saline.
6. Scalpel.
7. Autoclaved cotton buds.
8. Triangular arrowhead swabs.
9. Watchmaker forceps.
10. Surgical retractors.
11. Aneurysm clips.
12. Microscissors.
13. 3-0 Nylon monofilament (Ethicon, UK).
14. Silicone sealant.
15. Sutures.
16. Operating microscope.
17. Blood gas analyzer.

2.3. Postoperative Care

1. Baby Food (Farleys, UK).
2. Complan (Complan Foods, UK).
3. Weighing boats.
4. Soft bedding.

2.4. Preparation for Brain Removal

1. Halothane (Concord Pharmaceuticals, UK) in N_2O/O_2.

or Pentoject (Animal Care, UK).

2.5. Perfusion-Fixation for Histopathology

1. Heparin (CP Pharmaceuticals, UK).
2. Saline.
3. Paraformaldehyde (Sigma).
4. Perfusion pump.

2.6. Processing Brains for Histopathological Interrogation

1. Rat Brain Matrix
2. Processing and paraffin-embedding center (Shandon Citadel 1000 processor and Shandon Histocentre 2 embedding center).
3. Paraffin microtome.
4. Microtome blades.
5. Water bath for paraffin sections.
6. Microscope slides.
7. Absolute alcohol.
8. Distilled water.
9. Cresyl violet.
10. Luxol fast blue.
11. Histoclear.
12. Histomount.
13. Cover slips.
14. Image analyzer.

3. Methods

3.1. Animal Housing and Preparation for Behavioral Testing and Postoperative Care

Adult male Sprague-Dawley rats (Charles River, UK; 300–350 g weight at time of surgery) are typically housed singly or in groups of two (*see* **Note 1**) and maintained under a 12-h light/dark cycle with water *ad libitum*.

Food is typically restricted during pretraining and at 7 d post MCAO to facilitate performance on the staircase test, which is an appetitively motivated task. The feeding regimen is controlled so that animals gain weight at a rate of 3–5 g per week, maintaining animals at 85–90 % of their free feeding weight. Animals are provided with food pellets *ad libitum*, together with a mixture of baby food and Complan in appropriate water provided in small weighing boats, on the floor of the cage, from 6 h to 6 d post MCAO to increase postoperative weight and improve recovery. Soft bedding should be introduced to the cage

following surgery to keep the animals warm and to facilitate recovery. Rats are initially trained on the following behavioral tasks, prior to surgery: bilateral sticky label test, beam walking and the staircase test.

3.2. Behavioral Training and Testing Following Surgery

3.2.1. Bilateral Sticky Label Test

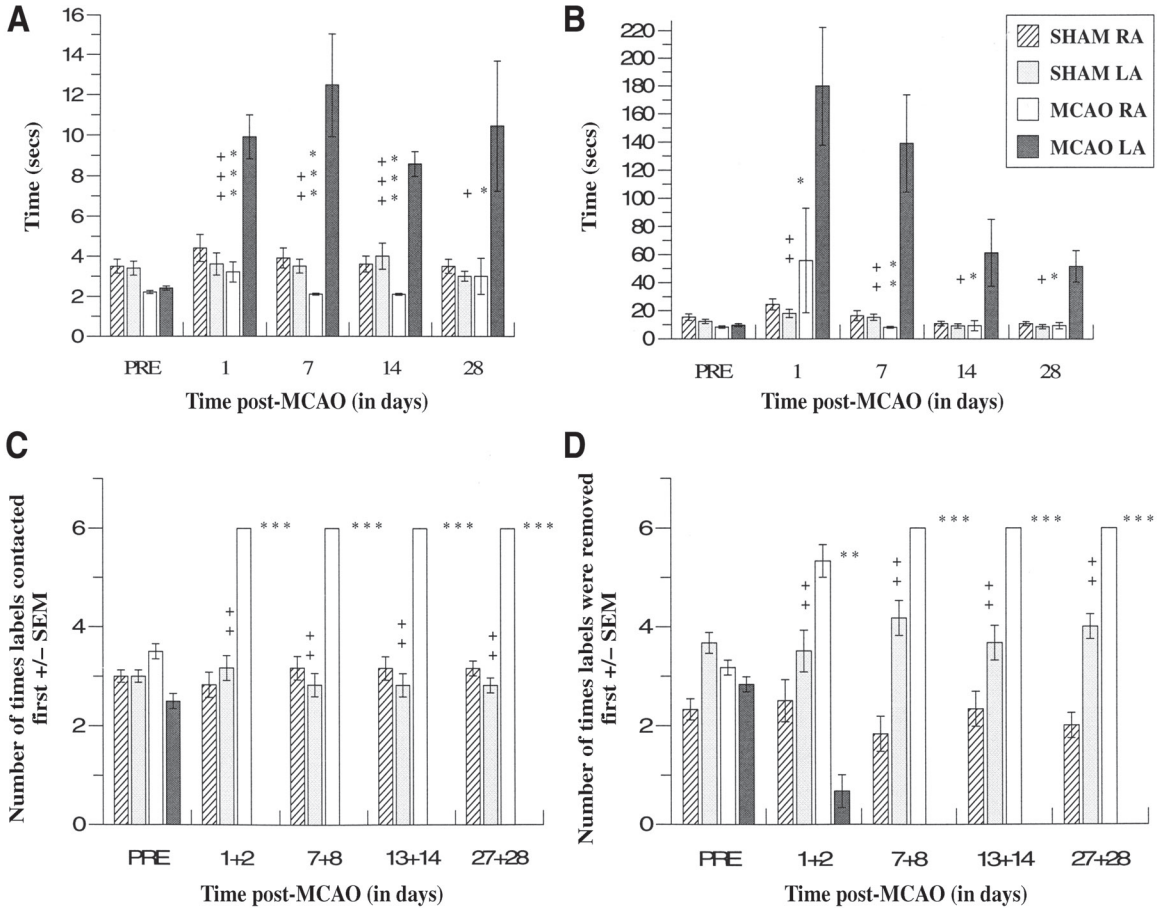
The bilateral sticky label test ***(65)*** is used to quantify contralateral neglect/ipsilateral bias and model tactile extinction to double-simultaneous stimulation (DSS), which is observed in human stroke patients ***(66)***. Bilateral stimulation of the radial aspect of the forearm is achieved by placing thin strips of brown parcel tape (1.5 × 4 cm) firmly around each animal's wrists, so that they cover the hairless part of the forepaw. In rare instances in which the sticky label comes partially or completely off, without the animal having attempted to remove the tape with its mouth, the trial should be repeated again.

Animals are given three bilateral stimulation trials daily, each lasting up to a maximum of 5 min, and the following parameters are recorded: latency to contact label on left and right forearm (in seconds); latency to remove left and right label on forearm (in seconds); order of contact (total number of times left and right forearms are contacted first); and order of removal (total number of times left and right label on forearms are removed first). Care is taken in each trial to apply each stimulus with equal pressure and to randomize the order of application (left vs right). Latency data are assessed on a daily basis, and order of contact and removal data are represented as the mean ± SEM of two consecutive days, so that a bias analysis can be carried out, i.e., order of contact and removal determined, according to established protocols ***(16,64)***.

Animals are trained on this task until a stable baseline for latency and order of contact are established over a period of a week. Final preoperative measurements are carried out over a period of another week prior to surgery to confirm that no bias in order of contact or removal exists prior to surgery, so animals are matched for ability (handedness). This test can be applied daily post surgery, e.g., from 1 to 28 d post MCAO, or at specific time-points post MCAO. Results generated from this test up to 28 d post MCAO can be expressed typically in the format of **Fig. 1** ***(64)***, showing a sustained impairment in the latency to contact and remove the contralateral label and an ipsilateral bias following MCAO.

3.2.2. Beam Walking

Beam walking ***(58,64,83)*** is used as a measure of hindlimb coordination via distance travelled across an elevated 100-cm beam (2.3 cm in diameter, 48 cm off the floor).


A
Time (secs)
PRE
1
7
14
28
Time post-MCAO (in days)
B
SHAM RA
SHAM LA
MCAO RA
MCAO LA
Time (secs)
PRE
1
7
14
28
Time post-MCAO (in days)
C
Number of times labels contacted first +/– SEM
PRE
1+2
7+8
13+14
27+28
Time post-MCAO (in days)
D
Number of times labels were removed first +/– SEM
PRE
1+2
7+8
13+14
27+28
Time post-MCAO (in days)

Rats are systematically trained to walk along the elevated beam from start to finish, with the aim of completing the task by 3 min. The total time spent (up to 3 min) on the beam is also recorded as a measure of coordination. A safe/target location, e.g., a flat box, is placed at the end of the beam so that the rat is motivated to cross the beam and complete the task. On occasions rats are "prodded," defined as a gentle tap on the rump delivered with a soft pencil eraser, to facilitate movement across the beam during the training phase (*see* **ref.** ***83*** and **Note 2**).

Each rat is trained twice daily for a maximum of 3 min per trial, and an acquisition curve can be constructed to demonstrate that rats can learn this task to achieve a stable baseline prior to surgery, e.g., typically within 5 d. Following surgery, each testing day can be represented as the mean of two daily sessions ***(64)*** (**Fig. 2**). This task can be utilized daily post surgery, e.g., from 1 to 28 d post MCAO or can be used at specific time-points post MCAO (*see* **Note 3**). This test identifies a functional impairment relating to the hindlimb within the first 7 d, which has been shown to recover with time (*see* **Fig. 2**) ***(64)***. Functional recovery on this test at 28 d post MCAO also correlates with the recovery of MRI tissue signatures and tissue salvage within the "penumbral" hindlimb cortex (*see* **Figs. 5** and **6** and **ref.** ***64***).

3.2.3. Staircase Test

The staircase test ***(64,68,70)*** is used to measure skilled independent forelimb paw reaching, i.e., pellet recovery is only possible with the left paw from the left stair and with the right paw from the right stair. Pellet recovery is not performed under visual guidance but by using tactile and possibly olfactory cues. Apart from the top two steps of the six-step stairway, from which a few rats use their tongues in the early stages of training, retrieval of a food pellet is only possible with a coordinated grasping action using all digits of the forepaw.

Fig. 1. Mean latency (s ± SD) to contact **(A)** and remove **(B)** adhesive labels placed around the right (ipsilateral) and left (contralateral) forearms by middle cerebral artery occlusion (MCAO) and sham control animals. Total numbers of times that the right (ipsilateral) and left (contralateral) labels were contacted **(C)** and removed **(D)** first by MCAO and then by sham control animals are also depicted (mean ± SEM) in 2-d blocks across the time-course. RA, right forearm; LA, left forearm; *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, MCAO (LA) significantly different from respective MCAO ipsilateral forearm (RA). +, $p < 0.05$, ++, $p < 0.01$, and +++, $p < 0.001$, MCAO (LA) significantly different from respective sham control contralateral forearm (LA). Post hoc statistical tests were computed following a significant repeated measures ANOVA ***(64)***. (Reproduced with permission from **ref.** ***64***.)

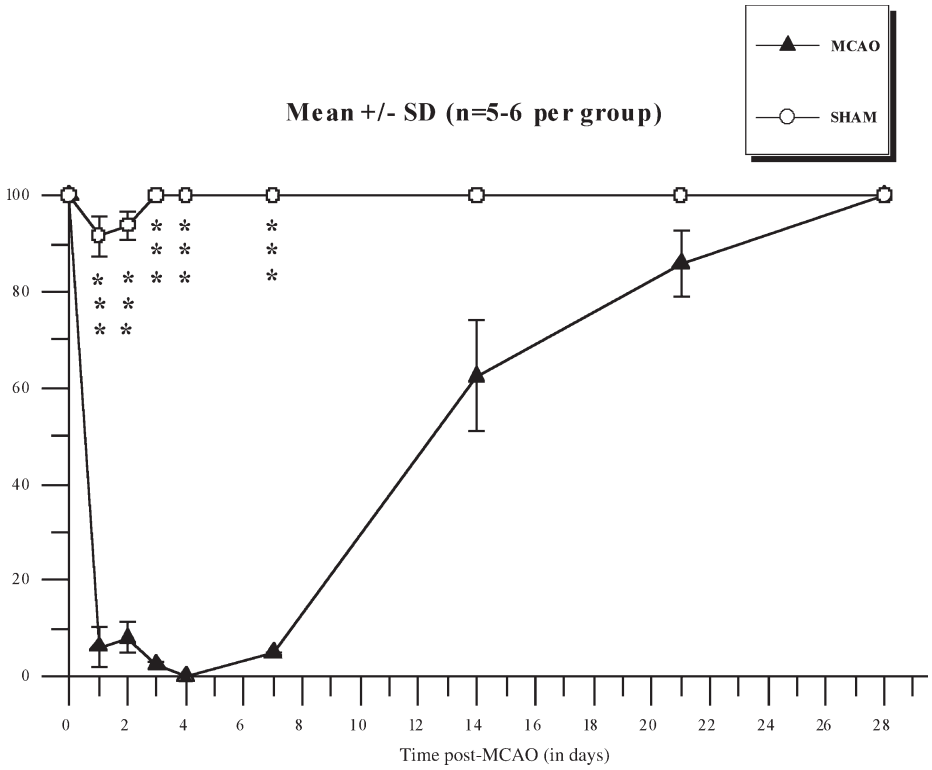


Fig. 2. Mean distance (cm ± SD; n = 5–6 per group) travelled across a 100-cm elevated beam by middle cerebral artery occlusion (MCAO) and sham control animals across the time-course. ***, $p < 0.001$, significantly different from sham control group. Post hoc statistical tests were computed following a significant repeated measures ANOVA. (Reproduced with permission from **ref**. ***64***.)

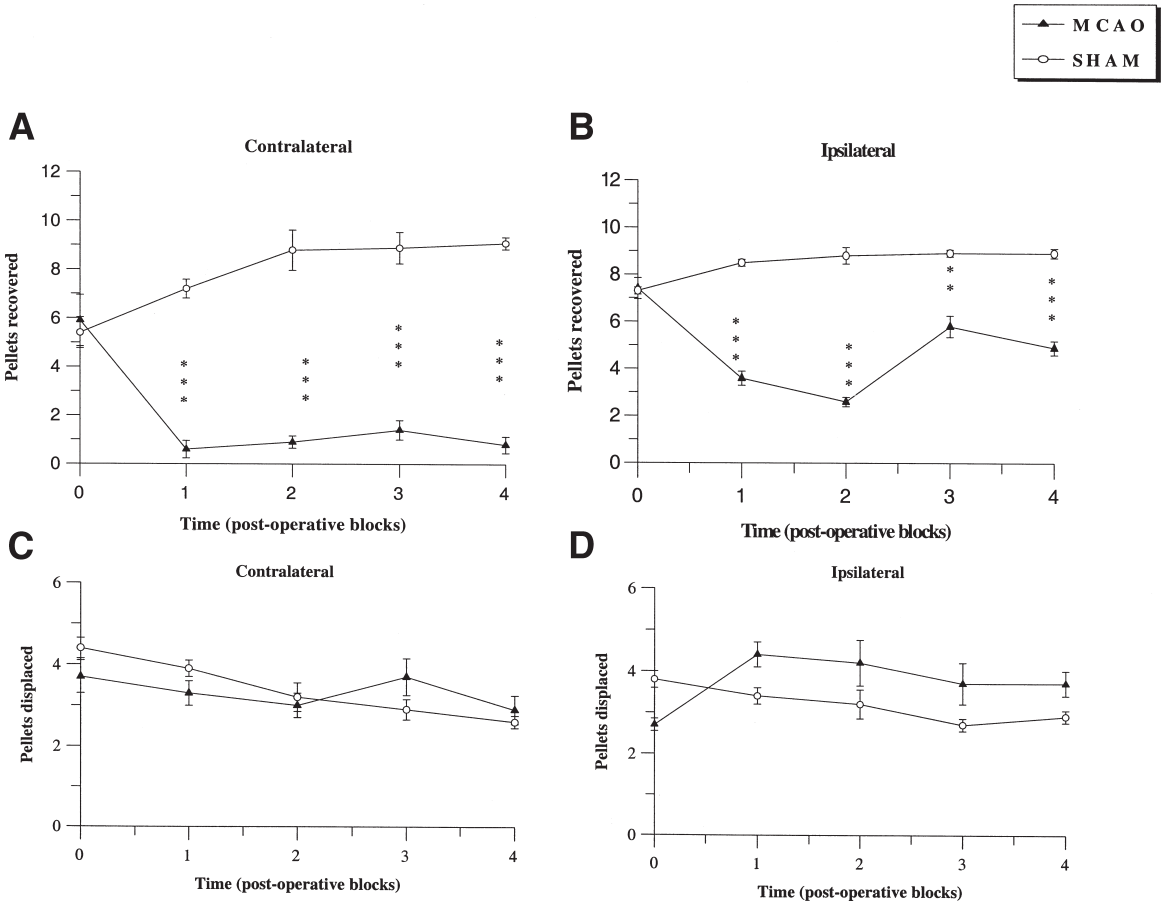
Animals are placed on a mild food-restricted diet during pretraining to provide motivation for food rewards. Animals are then introduced into a Perspex enclosure (300 × 67 × 95 mm, long × wide × high) attached to a holding box. A central plinth (190 × 20 × 48 mm) is positioned between the entry aperture and the front wall. Two removable staircases are positioned, either side of the plinth, by insertion through the front wall. Each stair consists of six steps, each measuring 14 × 17 × 6 mm with a hemispheric cup (11.5 mm in diameter). Between the back stair and the entrance aperture a *V*-shaped barrier is positioned on the floor of the box, so food pellets displaced from the steps can be held without the animal getting hold of them, i.e., pellet recovery is only possible with the left paw from the left stair and with the right paw from the right stair. Animals are given two 5-min sessions daily in the staircase testing box,

with approx 10 min between tests. Each step of the stairs is baited with one chocolate food pellet. Each animal had to retrieve as many pellets as possible (maximum of 6 pellets a side per session are typically available, i.e., a total of 12 pellets a side for any one day). At the end of the test period, the stairs are removed, and the animals are returned to their home cage.

Performance is scored as the number of pellets recovered from each stair and the number of pellets displaced but not recovered. Rats are trained over a period of 3 wk on this task, with a criterion of at least six pellets recovered from each side and no more than four pellets displaced per side per day over three consecutive days. Each data point is represented in blocks, i.e., the mean of three consecutive days of testing, from pre- to postoperative blocks, as employed by Marston et al. ***(70)*** and Virley et al. ***(64)***. This test is typically conducted from 7 d post MCAO, in order to give the animals enough time for recovery before food restriction is reintroduced (owing to the appetitive nature of this test). This test demonstrates a significant impairment in both the contralateral and ipsilateral forepaw retrieval following MCAO, without affecting levels of motivation. *See* **Fig. 3** for a description of typical data (from **ref. *64***).

3.3. Surgical Procedures and Confirmation of Successful MCAO

1. After successful training and an overnight fast, animals are randomly assigned to receive either tMCAO (e.g., 90 min) via the intraluminal thread technique by adaption of the method originally described by Koizumi et al. ***(39)*** or sham surgery, for example. This duration of MCAO is appropriate for assessing long-term function in rats, as opposed to longer durations of, or permanent, occlusion via the intraluminal thread method (*see* **Note 4**).
2. Surgical procedures can be performed under halothane (2/1 mixture of N_2O/O_2) anesthesia.
3. Following exposure of the right CCA, through a midline cervical incision, sterile silk sutures are looped around the common and internal carotid arteries.
4. An aneurysm clip is placed across the common carotid and blood flow in the internal carotid, can be temporarily be arrested using a further clip.
5. The 3/0 nylon monofilament thread, its leading 5-mm end coated with silicone rubber (diameter of 0.30–0.32 mm), is then introduced carefully via a right common carotid arteriotomy and carefully advanced along the ICA, beyond the looped suture so it can be tied followed by removal of the clip on the ICA.
6. The silicone thread is then advanced until its tip is positioned 1 mm beyond the origin of the right MCA (rMCA), as verified by a slight resistance. This is typically 19–22 mm distal to the carotid bifurcation.
7. The filament is tied in place with suture thread, and the aneurysm clip across the CCA is removed.
8. The wound is then sutured closed, and the animal is allowed to recover from anesthesia, typically in an incubator.


MCAO
SHAM
A
Contralateral
Pellets recovered
Time (post-operative blocks)
B
Ipsilateral
Pellets recovered
Time (post-operative blocks)
C
Contralateral
Pellets displaced
Time (post-operative blocks)
D
Ipsilateral
Pellets displaced
Time (post-operative blocks)

9. A saline injection is administered to provide fluid replacement as a result of surgery.
10. Sham surgery is achieved by introducing the thread into the ICA, followed by rapid removal.
11. The rMCA is occluded for e.g., 90 min, after which each rat is briefly reanesthetized with halothane (2/1 mixture of N_2O/O_2), and the thread is withdrawn (not completely removed) to the CCA to permit retrograde blood flow to the rMCA, via the complete circle of Willis, and hence reperfusion.
12. The thread is then cut at the point of the CCA, and the wound is sutured closed.
13. Throughout all surgical procedures, rectal temperature is monitored and maintained at 37 ± 1°C (mean + SD), with a heated electrical blanket.
14. Arterial blood samples can be obtained just prior to inserting the intraluminal thread in both MCAO and sham-operated control animals for assessment of blood gas status (pH, PCO_2, PO_2, HCO_3^-).
15. Successful occlusion is verified by applying the Bederson Neurological Scoring System (grades 0–3) after 60 min of occlusion and on reperfusion. Typically the ischemic deficit can be crudely assessed using the following grading system:

    Grade 0 = no deficit.
    Grade 1 = failure to extend contralateral forepaw properly.
    Grade 2 = decreased grip of contralateral forelimb while tail is gently pulled.
    Grade 3 = spontaneous circling or walking to the contralateral side.

    This simple neurological scoring system can also be used throughout the extended time-course post MCAO and historically is a commonly used grading system that assesses neurological function in experimental rodent stroke models over time.

3.4. Histological Procedures

1. At the desired end point of the study e.g., 28 d post MCAO, both MCAO and sham animals, for example, are terminally anesthetized and transcardially perfusion-fixed with heparinized saline followed by 4% paraformaldehyde.
2. Brains are then removed (*see* **Fig. 4** for superficial changes indicative of cavitation at 28 d post MCAO), immersed in fixative, and stored at 4°C for about 7 d.
3. Each brain is then cut into 2-mm-thick coronal blocks for a total of six blocks per animal, using a rat brain matrix.
4. Blocks are then processed for paraffin embedding using a processing and paraffin-embedding center.

Fig. 3. Total number of pellets recovered by contralateral (**A**) and ipsilateral (**B**) forelimbs in preoperative and postoperative blocks. Each block represents the mean ± SEM of six trials over a period of 3 d. Total number of pellets displaced by contralateral (**C**) and ipsilateral (**D**) forelimbs. **, $p < 0.01$, ***, $p < 0.001$, significantly different from the respective sham control groups. Post hoc statistical tests were computed following a significant repeated measures ANOVA. (Reproduced by permission from **ref**. ***64***.)

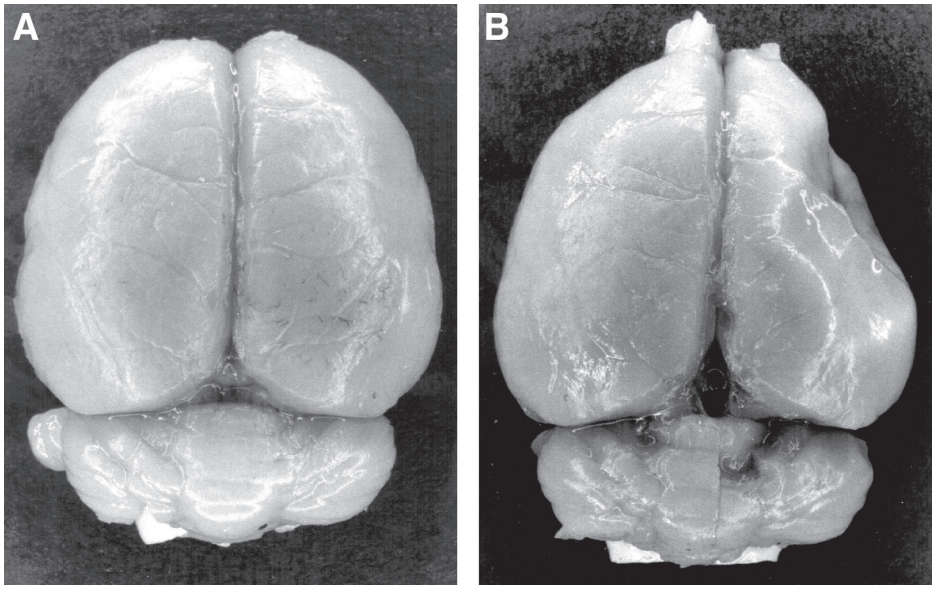


Fig. 4. Dorsal surface profile of **(A)** sham and (**B**) MCAO rat brain at 28 d post-surgery. Note the extensive cavitation on the MCAO rat brain that is attributable to MCA territory infarction.

5. Once coronal blocks have been paraffin-embedded, 10-μm-thick paraffin sections are typically cut using a paraffin microtome and floated out onto a water bath. Once they are flat and crinkles are no longer visible, they are carefully floated onto microscope slides and dried for subsequent staining with Cresyl Violet (Nissl) and Luxol Fast Blue, to delineate regions of gray and white matter loss (**Fig. 5**), respectively, using an appropriate image analysis system. Detailed methods of histological staining and analysis of neuropathology are outlined in other chapters.

3.5. Summary

At the dawn of the new millenium, we are now in a better position to use more sophisticated technology to establish novel treatment strategies that may translate into a clinically realistic benefit. The rigorous assessment of treatment strategies in preclinical animal models of stroke over an extended time-course, in both rodents and nonhuman primates, is a primary objective to predict true therapeutic value before embarking on clinical trials. It is clear that a multimodal approach is of considerable benefit when one is assessing the consequences of central nervous system injury in animal models, as correlations among behavior (**Figs. 1–3**), serial MRI (**Fig. 6**, and Chap. 8), and histological outcome (**Fig. 5**) or genomic information from the same animal can

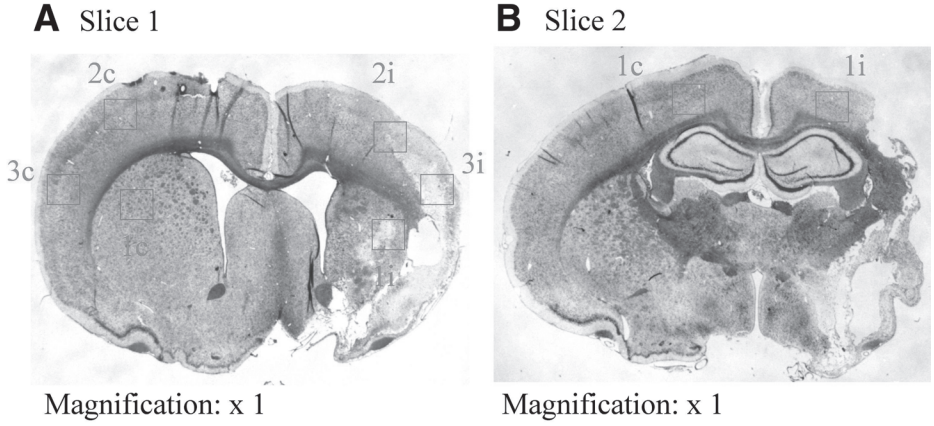


Fig. 5. Photomicrographs of cresyl violet- and Luxol Fast Blue-stained sections depicting ROIs from the same MCAO rat at 28 d post-surgery used for MRI analysis (*see* **Fig. 4**). (**A**) Slice 1 = –0.3 mm from bregma, showing ROI: 1i, ipsilateral caudate putamen (CPU); 1c, contralateral CPU; 2i, ipsilateral forelimb cortex (FLC); 2c, contralateral FLC; 3i, ipsilateral lower parietal cortex (LPC); 3c, contralateral LPC. (**B**) –1.8 mm from bregma, depicting the hindlimb cortex (HLC). 1i, ipsilateral HLC; 1c, contralateral HLC. (Reproduced with permission from **ref. *64***.)

provide the necessary tools to predict fruitful neuroprotective or restorative treatment strategies. Furthermore, an assessment of pathophysiological mechanisms from tissues harvested following MCAO using novel stroke genomic technologies (*see* Chaps. 6, 11–13, 15) may break new ground in identifying novel targets. If these novel targets have the potential of being chemically tractable, then they too may be assessed in turn in a study design outlined in this chapter to provide evidence for therapeutic efficacy. Therefore this underlying approach is highly relevant to the clinical situation, as monitoring the dynamic pathological and functional changes over time in preclinical animal models of stroke will ultimately provide a robust test of power for predicting the utility of novel treatments on final outcome in patients.

4. Notes

1. Depending on the hypothesis being tested, which has implications for the study design, rats can be either singly housed for drug interventions, i.e., continuous intravenous drug delivery or group housed (typically two or more). If appetitive behavioral tests are being incorporated into the study design, then weight gain and hence food restriction becomes an issue when two or more animals are grouped together.

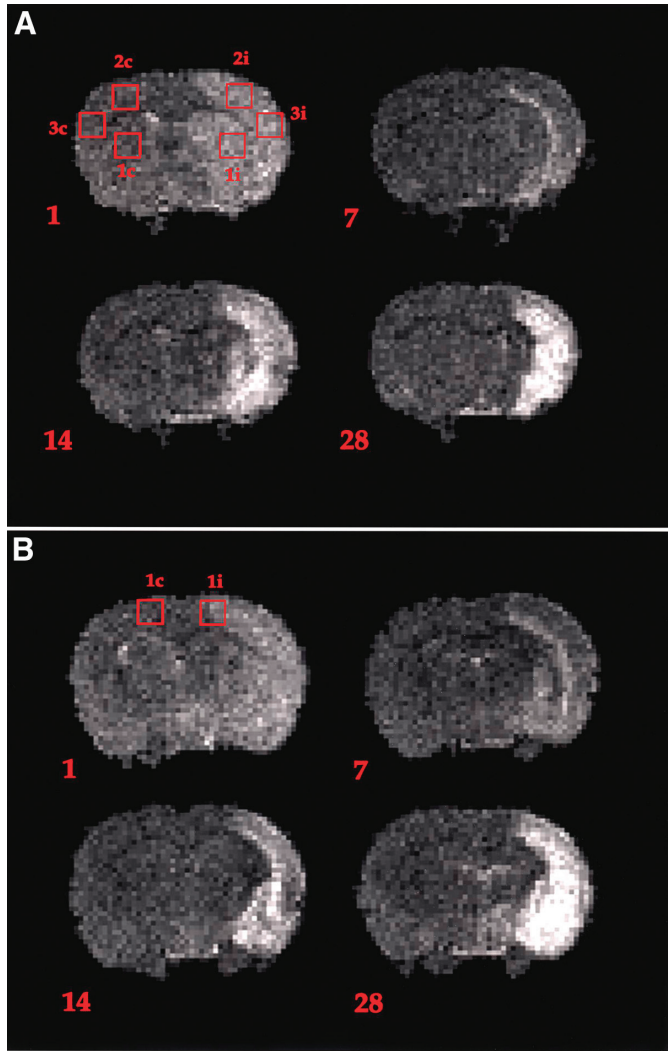


Fig. 6. Representative T2W MR images of an MCAO rat, after surgery. (**A**) Approximately –0.3 mm from bregma, depicting regions of interest (ROI) for analysis of MR tissue signatures at 1, 7, 14 and 28 d post-surgery. 1i, ipsilateral Caudate Putamen (CPU); 1c, contralateral CPU; 2i, ipsilateral ForeLimb Cortex (FLC); 2c, contralateral FLC; 3i, ipsilateral Lower Parietal Cortex (LPC); 3c, contralateral LPC; and (**B**) –1.8 mm from bregma, depicting the HindLimb Cortex (HLC) for analysis across the same time course. 1i ipsilateral HLC; 1c, contralateral HLC. Reproduced by permission of Lippincott Williams and Wilkins (Virley et al., 2000, A temporal MRI assessment of neuropathology following transient MCAO in the rat: Correlations with behaviour. *J. Cereb. Blood Flow Metab.*, **20,** page 566).

2. For pretraining on the beam walking test, on average, animals only require a few gentle "prods" to initiate successful locomotion across the beam. Indeed, "prodded" rats have been shown to attempt to take more steps on the beam, acquiring more task-specific experience, following a sensorimotor cortex lesion, which aided beam walking recovery ***(83)***. It must be stressed that "prodding" should *only* be used during the training phase, in order to train the animals successfully on this task. "Prodding" should therefore *not* be used in the postsurgical assessment of rats performing this task.
3. Testing animals daily post surgery raises a number of key issues that need to be addressed. Johansson and Ohlsson ***(84)*** have demonstrated improvement on the beam walking task using a subjective grading scale, with SHRs subjected to proximal pMCAO and subsequently housed in an enriched environment. The actual compensatory mechanisms that may be stimulated by an enriched environment have not been examined in any great detail, although a study design that assesses MCAO rats tested on a daily basis may induce a stimulating environment to enhance the mechanisms of plasticity in the brain (either ipsilateral or contralateral to the infarct), e.g., to improve hindlimb function. It is important to have appropriate controls in place for any study design that implements a variety of objective behavioral tests to determine whether weekly testing would provide similar stable levels of impairment relative to daily testing. Moreover, recovery of function on the beam walking test needs to be ascertained with appropriate ischemic controls to determine whether recovery was demonstrated as a result of daily testing (familiarization/enrichment), or whether this phenomenon was reproduced with longer intervals between test days.
4. It has been demonstrated that for the intraluminal thread approach in rats, the extent of injury cannot be reduced by reperfusion after 2 h of MCAO ***(43,44,85)***, suggesting that occlusion times of 2 h or more yield lesions equivalent in size to those derived from pMCAO ***(86,87)*** and hence increase the risk of mortality. Alternatively, tMCAO of 90 min, provides a severe enough depression of CBF within the MCA territory to provide ischemic injury as well as allowing the instigation of reperfusion to be beneficial to some regions, consequently improving survival rate ***(43,44)***. Therefore 90 min of tMCAO is a rational duration to investigate the long-term effects of focal cerebral ischemia in rats. This model also closely corresponds to human ischemic stroke, in which reperfusion commonly occurs and clinical outcome can be monitored, particularly in the case of cerebral embolism ***(7)***. Indeed, an ideal stroke model for neuroprotection and/or regeneration studies has a measurably salvageable ischemic penumbra, high reproducibility, and a low mortality rate.

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