Chapter 5

Focal Ischemia Models: Middle Cerebral Artery Occlusion Induced by Electrocoagulation, Occluding Devices, and Endothelin-1

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Abstract

This chapter covers established rodent models of middle cerebral artery occlusion (MCAO) where ischemia is induced by electrocoagulation of the artery, occluding devices applied to the artery, or application of the peptide endothelin-1 to the artery to induce vasospasm. Electrocoagulation induces a permanent occlusion of the artery, but the other models can be modified to induce permanent or transient MCAO.

All of the models involve some degree of cranial surgery, so the importance of aseptic technique is highlighted as is the importance of monitoring and maintaining the animals' physiology under anesthesia. Mortality rates are generally low in models which require a craniectomy. Since these models are used for both short- and long-term survival studies, some details of postoperative care are also included.

Key words Electrocoagulation, Diathermy, Endothelin-1, Compression, Microaneurysm clips, Tamura model, Brint model, Middle cerebral artery occlusion

1 Introduction

Models of MCA occlusion (MCAO) were first developed in primates in the 1930s [1] and later refined and scaled down for use in rodents in the 1970s and early 1980s [2, 3]. The subtemporal approach with MCAO by electrocoagulation, published by Akira Tamura and colleagues in Glasgow [3], has emerged as the standard method for permanent proximal MCAO in rats and has been cited over 1200 times since its publication in 1981. Its success is based on reproducible ischemia, infarction, and low mortality. Cerebral blood flow (CBF) is reduced to below 25 mls 100 g^{-1} min^{-1} within MCA territory, ischemic damage is evident within the cortex and dorsolateral striatum by 4 h post-occlusion, and infarct size (corrected for brain swelling) is maximal by 24 h. Subsequent variations in the model include:

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1. Restricting infarction to the cortex by sparing the lenticulostriate branches and applying electrocoagulation more distally [4]. This variation is also less technically demanding.
2. Tandem occlusion of the MCA and ipsilateral common carotid artery (ICCA) to improve reproducibility of infarction [5, 6].
3. Replacing electrocoagulation with occluding devices such as microaneurysm clips, hooks, ligature snares, or compression devices [7–10], which in addition provide the opportunity to induce transient ischemia of defined duration followed by reperfusion.
4. Replacing electrocoagulation with application of the peptide endothelin-1 to induce vasospasm [11–13].

The size and reproducibility of infarction induced by the original Tamura model have also been investigated in aged versus adult rats and in different strains, including those with recognized stroke risk factors such as the spontaneously hypertensive rat, spontaneously hypertensive stroke-prone rat, and the streptozotocin-induced diabetic rat [14]. This chapter provides an overview of these models and the basic equipment and consumables needed to set them up.

2 Equipment and Materials

2.1 Laboratory Equipment

1. A good operating microscope is essential when setting up rodent stroke models which require surgical exposure of the MCA (*see* **Note 1**).
2. Equipment is required for sterilizing surgical instruments (e.g., autoclave). In some labs I have seen commercial equipment designed for sterilizing dental tools or hairdressers' scissors used. When operating on a number of animals in a single session, tips of instruments can be sterilized with bench top glass bead sterilizers (instrument tips should be cleaned and then inserted into the heated beads (>200 °C) for ~15 s, allowing time to cool before contacting tissues).
3. Sterile medical surgical drapes (e.g., Vet Tech Solutions Ltd, UK). Sterile instruments and consumables are placed on sterile drapes to maintain sterility during surgical procedures.
4. Homeothermic heating blanket (e.g., Harvard) or thick cork board (e.g., Pyramid Innovation Ltd, UK) and heating lamp to maintain body temperature under anesthesia. Extra vigilance is required when using heating lamps. Monitor body temperature continuously to avoid the possibility of overheating the animal.
5. Rectal temperature recording system (e.g., Physitemp). Fine needle probes for insertion into temporalis muscle are also available to gauge brain temperature during surgery.

6. Transparent anesthesia induction chamber for use with gaseous anesthetics. Chambers with scavenging function are recommended to protect the investigator from anesthetic gases.
7. Rodent Ventilator (e.g., Ugo Basile, Harvard).
8. Cool light source (e.g., swan neck flexible light source) to provide illumination without heat.
9. Fur shaver (e.g., Wahl Sterling 2 Plus Trimmer).
10. Dental drill, handpiece, and dental burrs (e.g., Fine Science Tools Worldwide and Wright Cottrell, UK). Make sure the handpiece is slim enough to use within the restricted space between the animal and operating microscope (e.g., NSK FX65M handpiece used with Komet Round Diamond Burs (K801104016P5), both available from Wright Cottrell, UK.)
11. Bipolar diathermy unit with finest-tipped diathermy forceps available (e.g., Aesculap, part of Downs Surgical, Eschmann)
12. Blood pressure transducer connected to a monitoring system (e.g., Biopac) for continuous readout of blood pressure and heart rate (particularly important for prolonged stroke experiments conducted under general anesthesia).
13. A blood gas analyzer and plasma glucose analyzer allow the investigator to record and maintain physiology within normal limits while the animal is under anesthesia.
14. A stereotaxic frame is required for intraparenchymal injection of endothelin-1. Gaseous anesthesia can be delivered to rats and mice via mask adaptors (e.g., from Harvard Apparatus) which fit onto the stereotaxic frame. Anesthesia is delivered locally at the nostrils and then, via an exit tube, is routed back to a closed circuit for recycling or connection to a scavenging system.
15. A micromanipulator is needed for the Brint [6], Kaplan [8], and Morancho [10] models where an 80 μm diameter stainless steel wire is inserted under the MCA to raise it off the surface of the cortex [6, 8] or a blunted 30G needle is used to directly compress the artery [10].

2.2 Surgical Tools and Consumables

1. Disinfectants for operating table (e.g., Distel/Trigene wipes or equivalent cleaning agent for medical surfaces), surgical instruments (e.g., Medistel and Reprochem), and skin (e.g., chlorhexidine- or iodine-based soap such as Hibitane, Betadine®, or Topionic).
2. Clean (or disposable) lab coat (or surgical greens), face mask, cap, and gloves.
3. Scalpel, with size 22A blade (e.g., Maersk Medical, Sheffield, UK).
4. Watchmaker's forceps, curved and straight (e.g., Downs Surgical, Sheffield, UK).

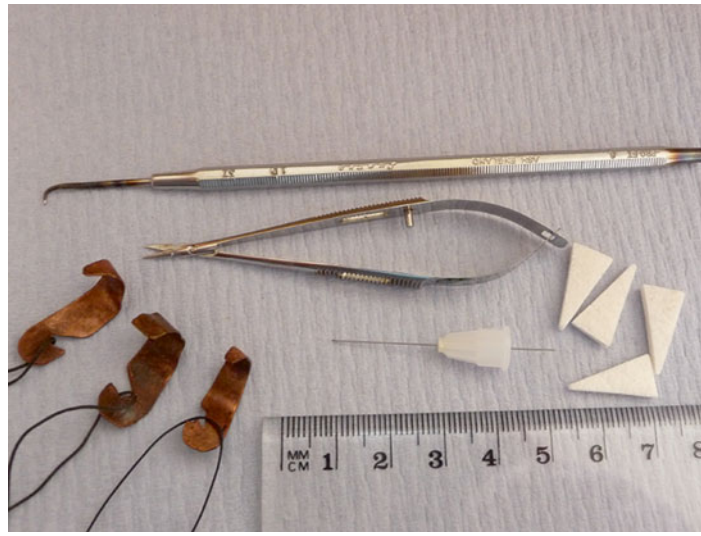

Fig. 1 Surgical tools used for MCAO: dural hook, spring-loaded microscissors, handmade copper retractors, 30-gauge dental needle, and *arrowhead* swabs

5. Dural hook and dental needles, 30 gauge (e.g., Terumo, Tokyo, Japan, see Fig. 1).
6. Surgical retractors for retracting skin and muscle when exposing the MCA (*see* **Note 2** and Fig. 1).
7. Surgical scissors for cutting sutures and Vannas Spring-loaded microscissors (e.g., Downs Surgical, UK) for cutting through MCA at the end of electrocoagulation to ensure complete occlusion (see Fig. 1).
8. Rat (or neonatal) laryngoscope and a stethoscope are very useful aids for carrying out oral intubation in rats.
9. Intubation tube for artificial respiration (for standard adult rat, Quick-cath 16G or Anicath 16G/45mm IV cannula, Millpledge).
10. For clip-induced MCA occlusion, the smallest available microaneurysm clips with lowest closing pressure and microaneurysm clip applicator (e.g., Codman AVM micro-clips with 10 g closing pressure).
11. Hamilton syringe for administration of small (μl) volumes of endothelin-1.
12. Local anesthetics (e.g., lidocaine, bupivacaine, and ropivacaine) and analgesics (e.g., buprenorphine, paracetamol, meloxicam, carprofen, ketoprofen, and butorphanol) to limit pain and suffering and maximize animal welfare post-stroke.
13. Atropine sulfate to control bronchial secretions under anesthesia.
14. Sterile saline for irrigating surgical sites, cooling drill tip during craniectomy, post-surgery subcutaneous injections to prevent dehydration, and heparinized saline for flushing vascular cannulae.

15. Bone wax to stem bleeding from bone during drilling.
16. Eye drops and ointment to prevent dry eye (e.g., Viscotears liquid eye gel, Novartis, and Lacri-lube, Allergan).
17. Cotton buds, gauze pads, and triangular arrowhead swabs (John Weiss & Son cat. no. 0111002, *see* **Note 3** and Fig. 1) to remove blood and excess fluid from craniectomy site.
18. Absorbable sutures (e.g., USS/DG sutures, Tyco Healthcare) for closing wounds after surgery.
19. Adhesive tape (e.g., masking tape, *see* **Note 4**).

3 Methods

3.1 Set-Up and Sterilization of Surgical Instruments

Surgery to induce cerebral ischemia should be carried out in as sterile an environment as possible. The operating room and operating table should be cleaned and disinfected. All equipment, surgical instruments, suture swabs, sterile saline, heparinized saline (10 units/ml), and other consumables required for surgery should be set up in advance on sterile surgical drapes. Surgical instruments should be autoclaved or sterilized by some other means, prior to the start of surgery.

3.2 Anesthesia, Intubation, and Animal Preparation for Surgery

Gaseous anesthesia is more controllable, enabling the investigator to alter depth of anesthesia quickly, for example, isoflurane administered in medical air (or N_2O/O_2, 70:30) or other suitable anesthetic (gaseous or injectable) (*see* **Note 5**). When using gaseous anesthesia, an adequate scavenging system is essential to protect the investigator from exposure to anesthetic gases. Anesthesia is first induced in a transparent induction chamber (usually with ~4–5% isoflurane in 70:30 N_2O/O_2), and once the animal is unconscious, it is transferred to a face mask delivering ~1.5–2% isoflurane or intubated for artificial respiration. Fur should be shaved from all incision sites and the skin cleaned with antiseptic prior to the start of surgery. This is best done away from the operating table to avoid any loose hairs getting into surgical sites. For adequate surgical anesthesia, the animal should be unresponsive to toe pinch, and the corner of a sterile saline-soaked swab should induce no blink reflex when applied to the eye. For animals anesthetized on a face mask, watch the animal's respiratory movements to ensure anesthesia is not too deep. Rat paws should stay a healthy pink color. Body temperature should be continuously monitored (e.g., with rectal probe) and maintained at 37 °C. Without a blink reflex, there is the potential for the eye to become dry and damaged during surgery. This can be avoided by closing the eye and securing it with a thin strip of adhesive tape or a temporary stitch. Alternatively, Viscotears or Lacri-lube ointment can be applied to the eye to prevent drying during surgery.

Surgical intubation is recommended for terminal experiments where the animal is anesthetized throughout the experiment and nonsurgical (oral) intubation for recovery experiments. An intubation tube size of 16 gauge is recommended for rats of 250–350 g. A specialized rat (or neonatal) laryngoscope and a stethoscope can aid oral intubation in the rat.

As a rough guide, ventilator settings for the rat are a tidal volume of 3–4 mls and a respiration rate from 45 (surgical tracheostomy) to 60 (nonsurgical, oral intubation) breaths per minute. Investigators should optimize artificial ventilation by adjusting settings to achieve stable blood pressure and blood gases within the normal range. With prolonged periods of anesthesia, a mucus plug can build up at the end of the intubation tube, compromising ventilation. A length of fine polythene tubing attached to a 5 or 10 ml syringe can be used to aspirate and withdraw any mucus buildup at the tube tip and/or atropine administered to reduce bronchial secretions (*see* **Note 6**). At the end of the surgical procedure, allow animals to regain spontaneous respiration before removing the intubation tube. Oral intubation is more challenging in mice but is possible. If your first attempt to intubate in either rats or mice is unsuccessful, you can try a second time, but stop after a failed second attempt so as not to cause any traumatic injury to the airway. If oral intubation is not possible, delivery of gaseous anesthesia via a face mask in a freely breathing animal can be considered as an alternative for recovery experiments (*see* **Note 7**).

3.3 Induction of Focal Ischemia: (i) Models That Involve a Craniectomy and Exposure of the Middle Cerebral Artery

Once stable anesthesia has been achieved, the animal is placed in the lateral position (on its side) and can be secured to the cork board or homeothermic blanket with masking tape. Always occlude the MCA on the same side of the brain for each study as there is some evidence that infarct size may vary in different hemispheres [15]. The fur between the left eye and ear should have been shaved and disposed of and the area swabbed with antiseptic prior to positioning the animal. Local/regional analgesia is recommended to control pain following surgery. Inject the local anesthetic into the subcutaneous space ("line block") below the planned incision line prior to surgery. Ropivacaine is a good choice of local anesthetic. It has a similar duration of action as bupivacaine but a wider safety margin (if there is inadvertent intravenous inoculation). It is purchased as Naropin (0.2% solution) and should be administered at the same dose as bupivacaine (1–2 mg/kg) with a maximum dose of 4 mg/kg.

A skin incision is made midway between the eye and the ear with a scalpel; the temporalis muscle is divided, separated, and retracted using retractors (see Fig. 1); and any tissue attached to the bone is scraped away. At this point a fine brain temperature probe (e.g., type IT-21 tissue-implantable thermocouple microprobe, Physitemp Instruments, Clifton, NJ, USA) can be inserted into the temporalis muscle to give a continuous assessment of brain temperature. The MCA is easier to locate in mouse as it can be

seen through the thinner skull. In the rat, the bone can be thinned using a saline-cooled dental drill (*see* **Note 8**) to aid exact location of the MCA. A circular craniectomy to expose the MCA is then made. In the original descriptions of proximal MCAO in the rat, the zygoma was removed to improve access to the proximal MCA. This should be avoided if possible as it will affect the animal's ability to eat on recovery. Once complete, the circle of thinned bone can be carefully teased off using watchmaker's forceps, a dural hook, or a dental needle (see Fig. 1). The dura is then carefully pierced and torn using a sterile 30-gauge dental needle (with the tip bent with pliers to form a hook) to reveal the middle cerebral artery. For a diagram of the craniectomy site and exposed MCA, see Figure 3 in ref [3]. Useful landmarks are the white myelinated fibers of the olfactory tract, which run rostro-caudally along the bottom of the craniectomy (if not visible, gently push the brain away from the ventral aspect of the craniectomy site with a blunt probe to see more ventrally), and the inferior cerebral vein which runs parallel to the olfactory tract, crossing the MCA more dorsally (see Fig. 2a). Although the craniectomy site represents a potential risk for infection, with good aseptic technique, stroke models which require a craniectomy are associated with very low

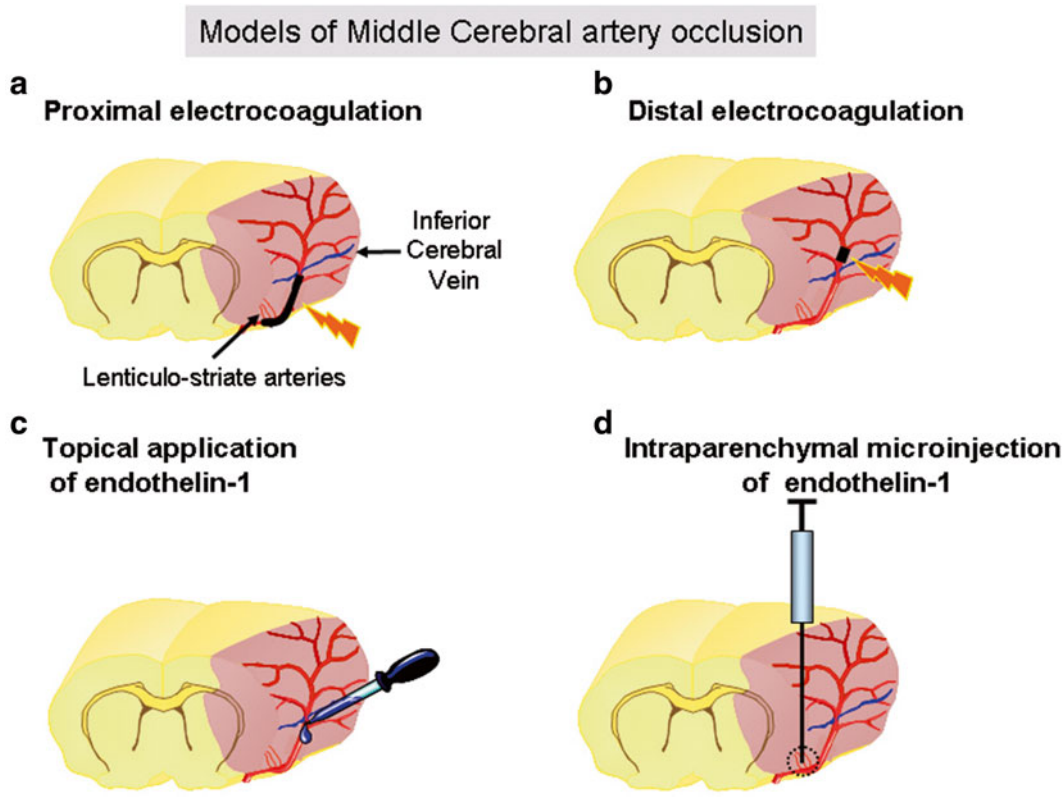

Fig. 2 Illustrations of MCAO models

or no mortality as the craniectomy provides space for the brain to swell and prevents increases in intracranial pressure in the brain. Edema and brain swelling correlate with infarct size and increase over the first 24–48 h post-stroke. In closed skull models (e.g., intraluminal filament model), significant brain swelling can occur within this period, leading to increased intracranial pressure and significant mortality.

The middle cerebral artery can be occluded by a number of different approaches. This chapter describes MCAO induced by electrocoagulation (diathermy occlusion); mechanical devices such as hooks, clips, and needles; and the vasoconstrictor endothelin-1 (ET-1). Methodology papers (including video clips of surgery) are available for a number of these models in the *Journal of Visualized Experiments* (JoVE) [16–20].

3.4 Electrocoagulation of MCA

The MCA is permanently occluded with this approach, and the size and location of ischemic damage can be controlled by altering the length and proximal/distal portion of the artery that is electrocoagulated. A short (e.g., 2 mm) occlusion of MCA, distal to the inferior cerebral vein, will induce ischemia confined to the cortex (Fig. 2b), while a longer and more proximal occlusion incorporating the lenticulostriate branches will induce cortical and subcortical ischemia (Fig. 2a). Sufficient current should be applied at the tips of the diathermy forceps to ensure there is no prospect of any remaining blood flow through the artery while minimizing damage to the underlying brain surface (*see* **Note 9**). When electrocoagulation is complete, the occluded part of the artery should be cut with spring-loaded microscissors to confirm complete occlusion. Sham-operated control animals undergo the same procedure up to and including removal of the dura. This model has been established in rats [3], mice [21], cats [22], and miniature pigs [23].

3.5 Electrocoagulation of MCA Combined with Ipsilateral Common Carotid Artery Occlusion

Modifications to the MCA electrocoagulation model have been developed to simplify the occlusion procedure, increase throughput, and improve reproducibility in infarct size. The severity of the ischemic insult can be increased by inducing hypotension, occluding the carotid arteries, and using strains with poor collateral supply such as the spontaneously hypertensive (SHR) and spontaneously hypertensive stroke-prone (SHRSP) rat. Brint and colleagues [6] combined a single-point distal MCAO with permanent ipsilateral common carotid artery occlusion (ICCAO) which produced a very reproducible cortical infarct in spontaneously hypertensive rats. Following ICCAO and exposure of the MCA, a fine (80 μm) stainless steel wire hook, inserted under the MCA just distal to the inferior cerebral vein, is elevated using a micromanipulator to raise the artery away from the underlying cerebral cortex. The artery in contact with the hook is then cauterized and severed by heating the wire with an electrocautery device, thus causing minimal damage to the underlying tissue.

3.6 Mechanical Occlusion of the MCA with Microaneurysm Clips, Ligatures, Hooks, and Direct Compression

The permanent MCAO/tandem ICCAO model in SHR rats [6] was further developed to produce a model of transient focal ischemia confined to the cortex [8]. A Silastic loop was used to reversibly occlude the ICCA, and the hook elevated the MCA sufficiently off the cortical surface to stem flow. This was checked by a continuous laser Doppler flowmetry readout from the cortical surface to allow readjustment of hook height, using the micromanipulator, during the period of ischemia. After the designated period of ischemia, recirculation was achieved by releasing the Silastic loop around the ICCA and lowering the MCA off the hook. The exposed brain and artery were bathed in warmed saline prior to and during ischemia to prevent desiccation.

Microaneurysm clips and ligatures have also been used successfully to permanently or transiently occlude the MCA [9, 24, 25] and are often accompanied by tandem ICCAO to improve reproducibility of infarction. Microaneurysm clips are too small to apply by hand and have to be loaded into a special applicator for attachment to the MCA. Some difficulties can be encountered when using these devices to induce transient MCAO, as removing microaneurysm clips after a period of ischemia (without inducing any damage) can be more challenging than applying the device (*see* **Note 10**). Mechanical compression of the M1 parietal branch of the MCA can also be used to induce distal MCAO in mice [10]. A laser Doppler probe, placed on the surface of the M1 parietal branch bifurcation, provides a flow readout, and compression of the blood vessel is applied until a reduction in CBF of ≥80 % is achieved.

The one issue with these techniques is the increased variability in ischemia and infarct size when only a single point on the MCA is occluded. Reproducibility can be improved by occluding more than 1 point along the MCA, combining MCAO with hypotension or ICCAO, and/or using strains with poor collateral supply. However, when considering models for testing neuroprotective compounds, it is worth considering that steps taken to improve reproducibility in infarct size might also result in less penumbral (potentially salvageable) tissue being available for rescue.

3.7 Topical Application of Endothelin-1 to MCA

Application of the peptide endothelin-1 (ET-1) to blood vessels induces a potent and prolonged vasoconstriction [26] capable of blocking flow and inducing downstream ischemia [11]. Following removal of the dura, exposure of the MCA, and puncture of the arachnoid membrane on either side of the blood vessel (to improve peptide access), topical application of ET-1 (25 μl of 10^{-7}–10^{-4} M) constricts the artery sufficiently to block blood flow (Fig. 2c). The higher the concentration of ET-1 applied, the more severe and prolonged the ischemia and the larger the infarct, which has both a cortical and subcortical component, similar to proximal MCAO. As the effect of the peptide wears off, the MCA diameter returns to normal and blood flow is gradually reestablished [12]. Therefore,

with this approach, ET-1 induces a transient MCAO (*see* **Note 11**). This model was developed in the rat but should work in mouse and has been set up in marmosets [27].

3.8 Induction of Focal Ischemia: (ii) Intraparenchymal Injection of Endothelin-1

A more prolonged focal ischemia can be induced by injecting ET-1 directly into brain tissue. Using a stereotaxic frame, ET-1 can be targeted to any neuroanatomical site within the brain, inducing discrete localized ischemic lesions in gray [10] or white matter [28, 29]. Sharkey and colleagues [13] used this approach to target the proximal MCA by injecting ET-1 (120 pmol in 3 μl) stereotaxically into the piriform cortex (Fig. 2d). This approach was technically less challenging than surgical exposure of the MCA and induced a reproducible and prolonged ischemia in MCA territory. [^{14}C]iodoantipyrine autoradiography revealed reductions in CBF of ~85 % in MCA territory 3 h after ET-1 injection (personal communication J Sharkey). This is in comparison to topical ET-1 application to the exposed MCA (Fig. 2c) where there was evidence of partial reperfusion within 60 min of ET-1 application [12].

3.9 Post-operative Care

Animals regaining consciousness following MCAO surgery should be carefully monitored and their recovery recorded. In terms of behavioral changes, up to 100% of animals exhibit circling and transient mild hemiparesis affecting the contralateral limbs, although these symptoms are less apparent in distal compared with proximal MCAO models. Lethargy, altered consciousness, hunching, and piloerection may be exhibited for up to 24 h postoperatively in up to 100% of animals. Post-op pain relief should be administered. Follow local guidelines and consult your local vet for advice on type, dose, and frequency of administration. Recommended analgesics include: buprenorphine, paracetamol, meloxicam, carprofen, ketoprofen, and butorphanol. Care should be taken in selecting an analgesia regime as certain analgesics may affect outcome measures such as performance in behavioral tests, inflammatory processes, and lesion volume.

Subcutaneous fluids should be administered acutely (5 mls/kg sterile saline for rat and 10 mls/kg for mouse, split between two sites) following surgery to prevent dehydration and animals kept in a warm environment (e.g., 28 °C) until fully conscious. Continue administering subcutaneous fluids until the animal is drinking normally, and provide animals with softened rodent chow and/or baby food, Complan, etc. to encourage feeding and limit weight loss. Introducing any new forms of diet prior to stroke surgery improves the animal's willingness to eat this diet after stroke.

Animals should first be housed individually to allow recovery from stroke and wound healing. Thereafter, group housing is recommended. Fresh bedding allows the investigator to check that the animal is urinating and defecating normally. Body weight should be recorded daily and any wounds checked for potential

infection or removal of stitches. Weight loss of >20 % is beyond the accepted severity limit, and euthanasia should be considered, particularly if movement, inquisitiveness, and grooming are absent. If failure to drink persists beyond 48 h or failure to eat persists beyond 72 h, the animal should be euthanized.

The intracranial temporal approach can affect the jaw alignment in rats. With prolonged survival periods, animals may need to be continually supplied with softened diet. Teeth overgrowth can become a problem, and teeth may require filing or clipping at regular intervals throughout the survival period if the animals do not eat dry diet or use chew blocks. Teeth clipping should be done under brief anesthesia if required. Chromodacryorrhea with unilateral loss of blink reflex and dry eye (up to 100 %) are often associated with intracranial MCAO and can be prevented and controlled by taping or suturing the ipsilateral eye shut during surgery. Eye ointment (e.g., chloromycetin, Orbenin) and/or drops (e.g., Viscotears, Lacri-lube) can be applied to the eye to limit this adverse effect.

4 Notes

1. When purchasing an operating microscope, discuss with the company the optimal working distance you require between the animal and the microscope to have adequate access for surgery. We have found that an angled binocular tube with 10 or 12.5× eyepieces provides the surgeon with a more comfortable seated position when operating for prolonged periods. Also consider a second, monocular eyepiece which can be fitted to some operating microscopes to allow trainees to observe the surgery.
2. Specialized retractors for rodent brain surgery can be purchased commercially (e.g., Small Animal Retractor System, Harvard Apparatus) or home made. In our lab we cut them to size from thick copper sheet, file them to shape (see Fig. 1), and keep them in place by connecting to surgical silk or rubber bands which are secured to a cork board with poster board pins.
3. Arrowhead swabs are incredibly useful tiny sponges which absorb blood and CSF from the craniectomy site to improve visualization of the MCA.
4. Masking tape is extremely useful for securing the anesthetized animal to the cork board or heated operating table. It is also useful for taping equipment cables to the table to avoid accidental movement of anything around the animal which might result in an intubation line or blood vessel cannula being inadvertently dislodged.
5. Isoflurane is a safer and less toxic alternative to halothane, which is gradually being phased out. N_2O/O_2 (70:30) is use-

ful as N_2O provides analgesia during surgery but must be used with a scavenging system.

6. Aspiration to clear mucous plugs from intubation cannula. Mark off the required length of fine polythene tubing needed to just clear the end of the intubation cannula, and connect it to a hypodermic needle attached to a 5 or 10 ml syringe. Quickly disconnect the intubation cannula from the ventilator, push the fine tubing down to just clear the end, aspirate, withdraw the fine tubing, and quickly reconnect the intubation cannula to the ventilator. Additionally, if you have problems with bronchial secretions and mucus plugs, consider a subcutaneous injection of atropine sulfate (0.05 ml of a 600 μg/ml solution).
7. When animals are artificially ventilated, the expired gases can be removed by connecting the ventilator exit pipe to the scavenging system. In freely breathing animals, double-tube systems with integrated face masks are available where anesthesia is delivered down an inner tune and removed by negative pressure via the surrounding outer tube.
8. Uncontrolled bleeding from muscle can be stopped using electrocoagulation forceps; bleeding from the bone can be stemmed by applying bone wax. Drilling the bone generates heat which can damage underlying tissue and disrupt the blood-brain barrier, thus producing surgery-induced artifacts. The bone and drill burr can be kept cool by spraying a steady stream of sterile saline (e.g., from a syringe with a hypodermic needle attached) onto the bone during drilling. Applying circular, lateral not downward pressure, when making the craniectomy, will avoid the possibility of the drill burr breaking through the bone and damaging the underlying cortex.
9. Flushing the MCA with saline and dipping forceps in saline before application help to prevent them from sticking to the artery during electrocoagulation. Start proximally and work distally, first with short zaps of electrocoagulation and, once the artery is constricted, with longer bursts of electrocoagulation. If the lenticulostriate branches of the MCA are to be occluded, they should be occluded first. Electrocoagulation induces heat; regularly replacing the saline that collects within the craniectomy site will ensure that the temperature does not rise above 38 °C. Keep the tips of the electrocoagulation forceps scrupulously clean at all times during surgery to avoid sticking. Polishing the tips after use will also help to minimize forceps sticking to the artery. We use Duraglit Metal Polish Wadding. Check with the manufacturer of the electrocautery equipment for their recommended product.
10. When clips or ligatures are applied to the proximal MCA (e.g., at the level of the lenticulostriate branches) for 1–2 h, ensuing

tissue swelling can hinder visualization and access to the device, making it very difficult to remove without damaging the artery.

11. Reproducibility of the ET-1 technique relies on consistency in the potency of the endothelin-1. The lyophilized peptide should be made up to the required concentration, aliquoted out into single-use vials, and frozen at –80 °C. A fresh aliquot should be thawed for each experiment and not refrozen.

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