

Neuroscience

A Novel Model of Transient Occlusion of the Middle Cerebral Artery in Awake Mice

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Background: Stroke is one of the leading causes of death and disability worldwide. As a consequence, several excellent rodent models have been developed to gain insight into the pathophysiology of stroke and testing the efficacy of neuroprotective interventions. However, one potential problem is that albeit roughly 80% of strokes occur in awake patients, all existing murine stroke models employ anesthesia. Moreover, epidemiological studies have shown that stroke injury is more severe in the minority of patients that suffer stroke while asleep. To better mimic the clinical situation, it is therefore preferable to analyze the pathobiology of experimental stroke in awake animals.

New Method: This model of transient middle cerebral artery occlusion (MCAO) in awake mice is based on insertion of an intraluminal suture via the external carotid artery during isoflurane anesthesia. Isoflurane is discontinued during the 60 min MCAO while cerebral blood flow is continuously monitored using laser doppler in the awake unrestrained mouse.

Results: Sixty minutes of awake MCAO model reliably induced infarction in striatum and cortex (relative infarct volume is 24.34% of contralateral hemisphere volume; absolute infarct volume is 47.94mm³).

Comparison with Existing Methods: The animals in this method are awake during the one hour occlusion period, which may provide a more translational research approach than existing methods that apply anesthesia during the occlusion.

Conclusions: Since the state of brain activity likely affects stroke injury and most anesthetics have neuroprotective effects, this model more accurately mimics the condition during which the majority of human ischemic stroke occurs.

Stroke | MCAO | Transient MCAO | Awake Mouse

Introduction

In US, stroke is the fifth leading cause of death and every 40 seconds an individual suffers from a stroke (<http://www.cdc.gov>). Considerable efforts have been dedicated into developing rodent models of focal stroke that recapitulate most, if not all the pathological hallmarks of focal ischemic injury in the human brain (Durukan and Tatlisumak, 2007). The reproducibility of the models and the ability to significantly reduce stroke injury across multiple independent labs, by for example administration of NMDA receptor antagonists or scavengers of oxygen radicals, provided initially considerable optimism in the field (Hoyte et al., 2004; Slemmer et al., 2008). It was therefore a tremendous disappointment when translation of these and other therapeutic strategies failed in clinical trials. Despite decades of experimental stroke studies, we still have only a few therapeutic alternatives to offer stroke victims (Brott and Bogousslavsky, 2000; Moskowitz et al., 2010). Only a minority of stroke victims qualifies for receiving intravascular treatment with tissue plasminogen activator (tPA) or endovascular mechanical clot removal (Brott and Bogousslavsky, 2000; Campbell et al., 2015). Many reasons for the failure of clinical trials exist, including the timing of treatment, effective dose, species and age difference (Cheng et al.,

2004). However, one aspect that so far not has been considered is that the traditional rodent models employ anesthesia and therefore not accurately mimic the clinical situation. From epidemiological studies, it is known that the incidence of ischemic stroke varies with the season of the year, possibly as a consequence of temperature and weather (Hong et al., 2003), but also as a function of the 24 hrs day-night cycle (Hill and Newcommon, 2003; Lago et al., 1998; Marsh et al., 1990). The circadian pattern of stroke is likely regulated by clock genes, as well as the state of brain activity (sleep vs. wakefulness) (Moldovan et al., 2010; Qureshi et al., 1997). Most strokes occur during the morning hours or in the period between 6am-12pm (Chaturvedi et al., 1999; Serena et al., 2003; Wroe et al., 1992). However, several reports have shown that the severity of stroke is worse when the artery occlusions occur during sleep (Jimenez-Conde et al., 2007; Kim et al., 2011; Nadeau et al., 2005). Thus, current rodent models of stroke, in which the animals are anesthetized during the MCA occlusion do not accurately replicate the majority of strokes (75-80%) which occur during wakefulness (Serena et al., 2003; Wroe et al., 1992). In this study, we have developed a model of transient MCAO in which the mice are awake during the 60 min ischemic period and afterward. Isoflurane anesthesia is only administered during the short surgery.

Materials and Methods

Animals

C57BL/6J male mice (12 weeks old, 25-30g, Jackson Laboratory) were used in all experiments. The experiments were approved by the Institution of Animal Care and Use Committee of University of Rochester and efforts were taken to minimize the number of animals used.

Middle Cerebral Arterial Occlusion

Preparation for MCAO surgery

A 7-0 monofilament suture (Harvard Apparatus, Holliston, MA) was cut into 20 mm segments. The tip of each segment was embedded with silicon (Heraeus Kulzer, Inc., Armonk, NY). The diameter of the tip was measured using a micrometer (Applied Image Inc., Rochester, NY). We used a suture with a final tip diameter of 0.22-0.23 mm for mice with body weight in the range of 25-30 g. All surgical tools (Retractor; SuperCut Iris scissors,

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straight; Dumont forceps, fine tip, 45° bent tips; Vannas microscissors, straight; micro vessel clip, all from World Precision Instruments, Inc., Sarasota, FL, USA.) were sterilized by autoclaving (minimum 121 °C, 15 PSI, for 15 min). Sterile cotton tips (Fisher Scientific International, Inc. Pittsburgh, PA), Gauze sponges (Fisher Scientific International, Inc. Pittsburgh, PA), 70% ethanol, and sterile 0.9% saline were prepared. The surgery table was sanitized using 70% ethanol.

Real time monitoring cerebral blood flow and blood pressure

The mouse was anesthetized with 2% isoflurane Henry Schein, Dublin, OH) in 30% O₂ using the V-10 Anesthesia system (Smith Medical PM, Inc., St Paul, MN). Following induction of anesthesia, isoflurane was reduced to 1.5%. Then, the mouse was placed in prone position on a heating pad. The fur was shaved around the head and neck (Oster Golden A5). Eye lubricant ointment was applied to both eyes in order to prevent corneal injury. A 0.8-1cm incision was made in the skin overlying the calvarium. The skin was pulled laterally and skull exposed. A laser doppler probe (MT B500-0 Straight Microtip; 0.5mm diameter, Perimed AB, Sweden) was firmly glued with cyanoacrylate. perpendicularly to the surface of the right parietal skull (2 mm posterior and 5 mm lateral from the bregma) and connected to a laser Doppler flowmetry (PeriFlux System 5000, Perimed AB, Sweden) to continuously monitor blood flow in the MCA territory. The mouse was then turned to supine position. One femoral artery was cannulated for continuously monitoring of mean arterial blood pressure (MABP); the cannula was tunneled under the skin and exited through an incision in the lower back and connected to pressure monitor BP-1 (World Precision Instruments, Inc., Sarasota, FL, USA). The femoral wound was closed before the starting of MCAO, reopened at the end of MCAO and cannula withdrawn. All surgical fields including the exposed skull were infiltrated with 0.25% Bupivacaine.

MCAO surgery

Induction of MCAO was modified from the original method described by Longa and co-workers (Longa et al., 1989). Briefly, mouse fore- and hind paws were taped to the operating platform. Teeth were tied to a pillar to make operating area flat. A rectal probe was inserted, and body temperature was monitored and maintained between 36.5-37.5 °C using a Gaymar homeothermic temperature system (Stryker Corporation, Kalamazoo, MI). The fur on the ventral neck region was shaved to expose the skin, which was disinfected by three applications of 70% ethanol. Under a stereo dissecting microscope (Leica MZ8, Leica Biosystems®, German), a 1 cm long midline incision was made on the neck. The surgical field was exposed by retractors and then, the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were dissected free from surrounding nerves and fascia. ECA was further dissected distally as much as possible and tied by a 7-0 silk suture. 7-0 silk sutures were then tied loosely around the ECA stump. To relieve pain and discomfort in the postoperative period, 0.25% Bupivacaine was applied to all exposed regions. A microvessel clip was applied at the bifurcation of the CCA into the ECA and ICA. Then the ICA was closed by another micro vessel clip in order to prevent retrograde flow. A small incision was cut at the end of ECA stump by Vannas micro scissors. The coated suture was inserted into the incision and advanced until it reached the clip. The two silk sutures were tightened around the lumen enough to avoid

mobility of the suture. The ECA stump and the inserted suture were pulled down together to make them align towards the ICA. The clip from the bifurcation was removed and the filament gently advanced 9-10 mm into the ICA until a mild resistance is felt. Meanwhile, a 90% drop in cerebral blood flow value confirmed the origin of MCAO was occluded. The incision on the neck was closed and the incision on the head was closed around the laser doppler probe. The total duration of surgery was about 30-45 min. Once the MCA was occluded, the anesthesia was discontinued. The mouse was placed into a 35 °C transparent box and allowed to move freely, while blood flow was continuously monitored in the MCA territory by the laser doppler. After 60 minutes, the mouse was re-anesthetized with 2% isoflurane and the filament was withdrawn to allow reperfusion. The laser Doppler probe was removed and the incision sutured. The closing steps took around 10 mins. Anesthesia was then discontinued and the mouse was placed in a transparent heated box for recovery. After 2 hours of recovery, the animal was returned to their home cages. Carprofen was given twice daily for the next twenty four hours to mitigate inflammation and associated pain.

TTC (2,3,5-triphenyltetrazolium chloride) staining and quantification of the infarct volume.

The mice were 24 hrs after the 1 hr MCA occlusion anesthetized with Ketamine/Xylazine and decapitated. Their brains were removed and coronally sectioned at 1mm interval by a vibratome (leica microsystems, Inc). The brain slices were incubated for 15 min in a 2% solution of TTC at 37°C, fixed with 4% paraformaldehyde (PFA) and photographed using an Olympus szx12 microscope (New York / New Jersey Scientific Inc, Middlebush, NJ). The infarct volume was quantified using image J program, by summing the infarcted areas in all sections and multiplying by the slice thickness (1 mm). To correct for edema, the volume of infarction was adjusted by the calculation: Corrected Infarct Volume (CIV), % = [contralateral hemisphere volume - (ipsilateral hemisphere volume - infarct volume)]/contralateral hemisphere volume x 100.

Results

Surgical procedure and transient MCAO time line

We excluded mice that experienced excessive bleeding at any point during the procedure, as well as mice of which the duration of surgery exceeded 45 mins. A schematic diagram of surgical procedure is depicted in Fig 1A. The procedure was modified from the initial report by Longa and co-workers (Longa et al., 1989). During the 60 min MCAO occlusion in awake mice, it was important to minimize the mice's discomfort, but also that the arterial occlusion was maintained for the 60 min without interruption. Timeline diagram for awake MCAO is shown in Fig 1B.

Awake MCAO model generates stable infarcts in striatum and dorsolateral cortex

Cerebral blood flow and blood pressure were continuously monitored before occlusion, during the entire duration of MCA occlusion (60 min), and for a few minutes after removal of the filament occluding the MCA. Cerebral blood flow promptly dropped by 91.8% ± 4.9 % after occlusion of MCA and remained low for the duration of 60 min artery occlusion (Fig 2A).

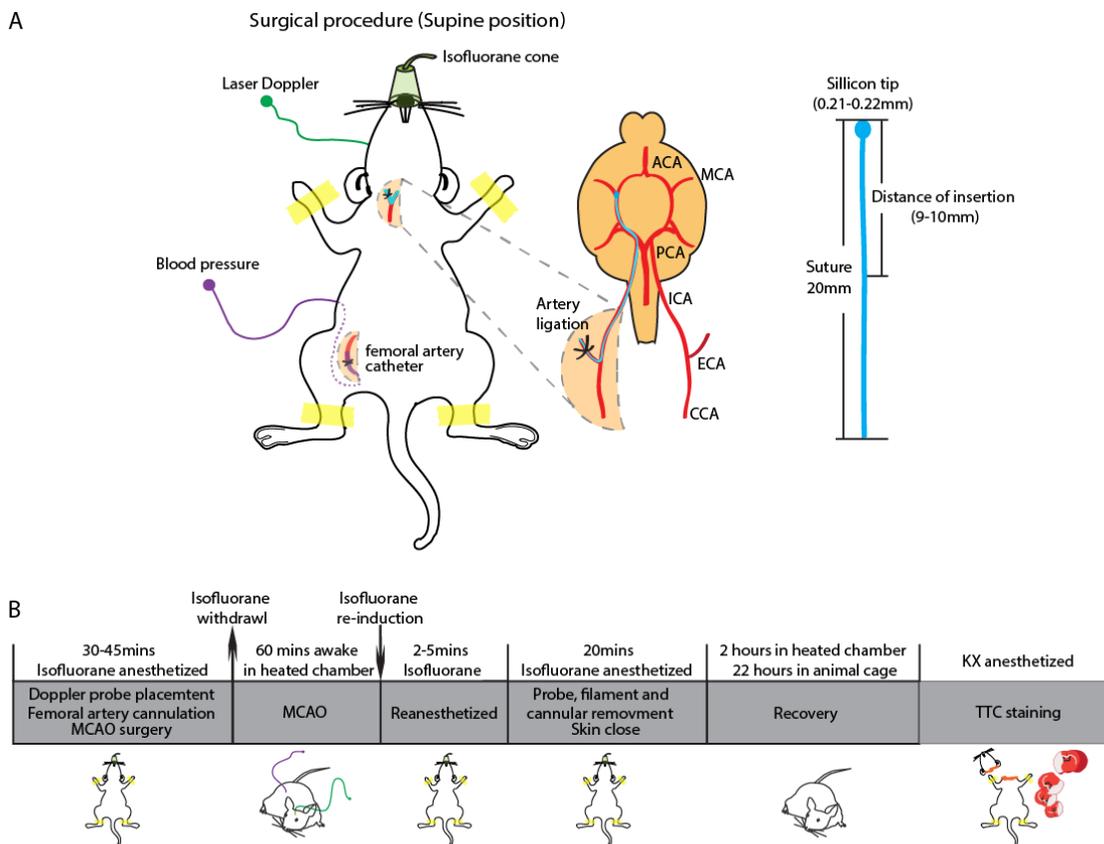


Figure 1. Schematic diagram of surgical procedure and transient MCAO time line in subjected mice. (A) Summary diagram of awake MCAO surgical procedures. The monofilament suture was inserted from external carotid artery (ECA) into the internal carotid artery (ICA) and pushed forward for a distance of 9-10 mm beyond the bifurcation of common carotid artery (CCA) until a minor resistance is felt. Blood flow in the MCA territory was continuously recorded by laser doppler. One femoral artery was cannulated for recording of mean arterial blood pressure (MABP). (B) Timeline of awake MCAO. The duration of surgery took 30-45 mins. Once the filament was correctly inserted in the trunk of the MCA, cerebral blood flow dropped by >90%. The incision on the neck was closed and the isoflurane anesthesia was discontinued. The animals were returned to a heated cage (Deluxe reusable animal Cages, Tecniplast Inc., West Chester, PA) while blood flow in the MCA territory and MABP were continuously monitored. After 60 minutes, the mice were re-anesthetized with 2% isoflurane for another 20 mins for removing MCA filament, withdrawing femoral artery catheter, closing the skin and cutting the Doppler probe. Anesthesia was then stopped and the mouse was placed into the transparent box again for recovering. 2 hours later, the mice were returned to their home cage. 24 hrs after MCAO, the mice are re-anaesthetized with ketamine/xylazine. When the mice were unresponsive to stimulation they were quickly decapitated. Their brains were removed and coronally sectioned for TTC staining and imaging.

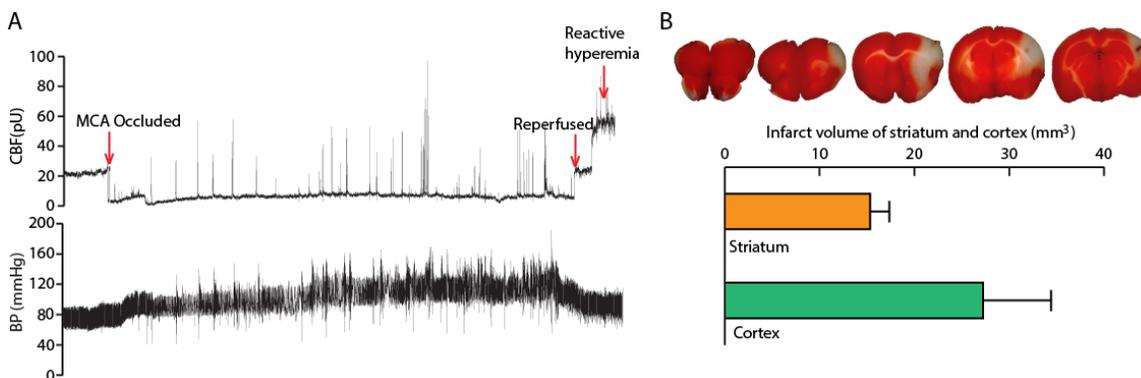


Figure 2. Representative traces of cerebral blood flow (laser doppler) and mean arterial blood pressure as well as images of TTC and quantification of the ischemic lesions. (A) The cerebral blood flow dropped by 90.0 % ± 3.0 % when the MCA was occlusion and remained low during the 60 min artery occlusion. Removal of the filament occluding the MCA resulted in hyperemia (242 % ± 28.9 %, n=6). Mean blood pressure (MBP) remained stable ~ 100mmHg. In coronal sections prepared 24 hours after awake MCAO, clear ischemic lesions were evident in striatum and dorsolateral cortex. Infarct volume corrected for edema in the ischemic hemisphere and normalized to whole brain was 24.34 ± 3.9 %, and absolute infarct volume is 47.94 ± 7.75 mm³. The infarct volume of cortex and striatum is 27.24 ± 6.21 and 15.34 ± 2.02 mm³, n=6.

A dramatic hyperemia was noted during the reperfusion ($242 \pm 28.9\%$, Fig 2A) but one limitation of the awake MCA occlusion model was that we could not record blood flow for an extended recovery period as the incisions needed to be closed prior to withdrawal of anesthesia. Mean blood pressure (MBP) remained stable around 100 mm Hg during the whole process. Twenty four hrs after 60 mins of MCAO, clearly delineated infarct were evident in striatum and dorsolateral cortex (Fig 2B). Corrected infarct volume of whole brain was $24.34 \pm 3.9\%$ of contralateral hemisphere, and absolute infarct volume is $47.94 \pm 7.75 \text{ mm}^3$; the infarct volume of cortex and striatum is 27.24 ± 6.21 and $15.34 \pm 2.02 \text{ mm}^3$ (n=6).

Discussion

In this study we described a novel model of transient middle cerebral artery occlusion in awake mice. The mice were anesthetized with isoflurane during all surgical procedures, including occlusion of the MCA by inserting a filament in the proximal trunk of the artery. Local anesthetics were applied to all exposed surfaces before the surgical incisions were closed. Isoflurane was then discontinued and the animals were returned to a chamber where they could freely move around. Cerebral blood flow was continuously monitored during the 1 hr awake MCAO after which isoflurane was administered again for a few minutes to allow removal of filament occluding the MCA and the femoral catheter. The surgeries were adapted from the classical model of MCA occlusion in anesthetized mice first described by Longa and co-workers (Longa et al., 1989). The key topics that are necessary to consider when discontinuing anesthesia during MCAO are: (1) that the awake mice are comfortable during the MCAO while awake, and also (2) that the occlusion of the middle cerebral artery is stable and not interrupted. It is therefore necessary to apply local anesthetic and closely monitor cerebral blood flow in the MCA territory by a laser Doppler probe glued to the skull. Analysis of the infarct volume on TTC stained coronal sections showed that occlusion of MCA in awake mice induced ischemic lesion that were fairly comparable to those evoked by MCAO in anesthetized mice. Future studies will assess the effect of the state of brain activity, i.e. awake vs anesthesia on the severity of ischemic injury. It is here important to note, that a comparison of ischemic injury in awake vs anesthesia is complicated by the fact that most anesthesia trigger a significant drop in blood pressure. A prior study compared awake and isoflurane anesthesia in rats (Sakai et al., 2007). The study found that isoflurane reduced MABP by 20-25 mmHg compared with awake MCAO in rats. Epinephrine was given to increase MABP in the isoflurane anesthetized animals. The analysis showed that isoflurane highly significantly reduced the severity of ischemia corresponding to a 50% decrease in the size of the ischemic lesion (Sakai et al., 2007). The study differed also from ours by that the rats were restrained and that 40% oxygen was provided during the 50-80 min MCA occlusion.

Use of anesthesia complicates interpretation of data collected in experimental rodent stroke models and is likely reducing the success of clinical translation. For example, use of anesthesia provide neuroprotective effects by affecting hemodynamics by reducing blood pressure (Conzen et al., 1992; Hug et al., 1993; Seyde and Longnecker, 1984), and metabolism (Newberg and Michenfelder, 1983; Schlunzen et al., 2012). Also, almost all anesthetics profoundly inhibit thermoregulation (Fiedler, 2001). Induction of anesthesia usually induces core hypothermia (Matsukawa et al., 1995), which profoundly can affect ischemic

outcome. For example, a $2.8 \pm 0.5^\circ\text{C}$ reduction in core temperature nearly abolished hippocampal CA1 neuronal death after 3 hrs of forebrain ischemia (Busto et al., 1987; Matsukawa et al., 1995). The hypotension induced by anesthesia can improve ischemic outcome (Reich et al., 2005) (Miura et al., 1998). The substantial reduction in cerebral oxygen consumption during anesthesia may also reduce the severity of the ischemic insult (Turner et al., 2005). Moreover, several anesthetics can independently of these physiologic effects protect against ischemic injury. For example, propofol and thiopental have antioxidant properties which inhibit lipid peroxidation and reduce release of radical oxygen species (ROS) (Harman et al., 2012). Isoflurane has been shown to improve neurological recovery after stroke regardless of the presence or absence of hypotension during the ischemic insult (Homi et al., 2003). Ketamine, which is also broadly used, provides significant neuroprotection, due to its inhibition of N-methyl-D-aspartate (NMDA) receptors (Hoyte et al., 2004).

Epidemiological studies have showed that most strokes strike between 6 a.m and noon (Wroe et al., 1992), whereas only ~20% of all strokes occur during nighttime. Stroke-on-awakening patients are more likely to suffer worse outcome, although this finding has been contradicted (Jimenez-Conde et al., 2007; Kim et al., 2011; Moradiya and Janjua, 2013; Nadeau et al., 2005). However, it is clear that several factors, including blood pressure, cortisol and catecholamine levels differ between awake and natural sleep or anesthesia (Constantinople and Bruno, 2011; Engquist et al., 1977; Redwine et al., 2000; Sakai et al., 2007; Schibler, 2006; Xie et al., 2013). Based on this reasoning, an awake murine MCAO model will better mimic the clinical situation. An ideal awake model should totally avoid anesthetic from the beginning to the end. However the pain inflicted by the surgery was in this study prevented by short lasting use of anesthesia. We used a volatile anesthetic, isoflurane, which due to its rapid action promoted that the mice woke up immediately when isoflurane administration was discontinued.

The transient occlusion of MCA in awake mice induced robust reactive hyperemia. The magnitude and duration of hyperemia observed here in awake MCAO ($251 \pm 49.5\%$ of basic CBF) is significantly higher than in traditional MCAO in anesthetized animals (as for example $134.78 \pm 49.5\%$ of basic CBF) (Yang et al., 2002). A similar phenomenon was noted in an awake model of focal ischemia (MCAO) in monkeys (167.77% of basic CBF in one monkey and 200% of basic CBF in another monkey) (Jones et al., 1981). Reactive hyperemia can contribute to tissue injury (Tsuchida et al., 1997) and may therefore in part contribute to the neuroprotective effects of anesthesia (Yang et al., 2002).

Conclusions

In sum, we here describe a novel model of occlusion of the middle cerebral artery in awake behaving mice. The model replicates stroke in the clinic better since the use of anesthesia is minimized. Since translational efforts intended to reduce the severity of stroke injury so far have been disappointing, this new model of stroke in awake mice will facilitate treatment of this devastating disease.

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Abbreviations

MCAO, middle cerebral artery occlusion; MCA, middle cerebral artery; CCA, the right common carotid artery; ECA, external carotid artery ; ICA, internal carotid artery; MBP, mean blood pressure

Authors' contributions

LX collected experimental data, analyzed data, made figures and drafted the manuscript. HK performed the animal surgery. MN designed the study and revised the manuscript.

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