Cognitive Deficits After Focal Cerebral Ischemia in Mice

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Background and Purpose—The interpretation of cognitive data in many experimental stroke studies is problematic because middle cerebral artery occlusion (MCAO) is associated with sensorimotor alterations that may become confounding factors in cognitive testing. The purpose of the current study was to determine if it is possible to measure MCAO-induced cognitive deficits by using short durations of ischemia that do not result in alterations in sensorimotor behavior in mice.

Methods—Male C57/Bl6 mice were subjected to 60 or 90 minutes of intraluminal MCAO or sham surgery. In the first cohort of animals (n=12/group), locomotor activity, balance, and coordination were evaluated 2 weeks after surgery. In a second cohort of animals (n=10/group), the effects of 60 minutes of MCAO on subsequent learning and memory were assessed with a step-down passive avoidance task beginning 1 week after surgery. In a third cohort of animals (n=8 to 10/group), training in a passive avoidance task was completed before 60 minutes of MCAO, then retention of the task was assessed 1 week after surgery. In all animals, infarction size was determined after 14 days of reperfusion with use of cresyl violet staining and quantitative image analysis.

Results—There was no significant difference in infarction volume in the cerebral cortex or caudoputamen after 60 versus 90 minutes of MCAO. However, there was a significant increase in latency to move 1 body length in the 90-minute MCAO group compared with the 60-minute MCAO and sham groups. In 2 additional cohorts of animals, 60-minute MCAO was associated with a deficit in the acquisition and retention of a passive avoidance task regardless of whether the task training occurred before or after MCAO.

Conclusions—Long-term cognitive deficits can be induced in mice by using a short duration of MCAO (60 minutes) that does not result in concomitant sensorimotor deficits. (Stroke. 2000;31:1939-1944.)

Key Words: behavior ■ histology ■ middle cerebral artery occlusion ■ stroke, experimental

Stroke is the most common cause of permanent disability among people in the United States and is associated with a high incidence of sensory, motor, and cognitive deficits.1,2 The transient middle cerebral artery occlusion model (MCAO) that was originally developed in rats by Tamura and colleagues3 is considered to be a reliable and reproducible rodent model of cerebral ischemia.4 Thus, the pathohistological consequences of this MCAO model have been studied extensively. In addition, because the ultimate goal of experimental ischemia research is to improve the functional outcome of humans recovering from stroke, several preclinical studies have examined MCAO-induced sensorimotor and cognitive deficits in rats.

Clinical studies indicate that some humans exhibit robust sensorimotor recovery within the first 3 months after stroke.5,6 Rapid spontaneous recovery of sensorimotor function also has been reported in rats subjected to MCAO, which makes long-term assessment of preclinical therapies aimed at improving ischemia-induced sensorimotor deficits difficult to interpret.7,8 In contrast, MCAO-induced deficits in cognitive function in rats appear to remain fairly stable. Several different tasks have been used to assess cognitive function in rats subjected to MCAO including active avoidance, passive avoidance, spontaneous alternation in a T maze, the radial arm maze, and the Morris water maze. Of these tasks, passive avoidance has most often and consistently revealed MCAO-induced cognitive deficits.8–20 In addition, the ability of the passive avoidance test to identify functional improvement in the absence of any discernible change in histological outcome after MCAO suggests that infarct size is not a reliable indicator of functional outcome in cerebral ischemia and that passive avoidance may be an important tool in screening therapeutic agents in preclinical stroke studies.8,11,16

Much less is known about the functional consequences of experimental stroke in mice than rats. However, the increased availability of transgenic and knockout mice over the past several years has led to an increase in the use of mice in

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stroke research. Several studies have compared the effects of varying durations of MCAO on histological outcomes in mice, but little is known regarding the long-term functional consequences of experimental stroke in this species. There are many similarities in the mechanisms underlying behavior in rats and mice, but it is not always possible to extrapolate the effects of experimental manipulations on behavior in rats to mice. Therefore, it is necessary to evaluate in mice whether MCAO induces deficits in sensorimotor function and passive avoidance, as it has been shown to do in rats. In addition, because alterations in sensorimotor function (particularly locomotor activity) and anxiety are common confounding factors identified in cognitive testing, it also is necessary to determine the threshold beyond which increases in MCAO duration result in alterations in anxiety and sensorimotor function. The goal of the current study was to identify a length of MCAO time in mice that was not associated with potentially confounding alterations in sensorimotor or anxiogenic-like behavior (experiment 1) but did induce a cognitive deficit, as measured by passive avoidance (experiment 2 and experiment 3). Taken together, the data from this study will provide evidence to support or disprove the hypothesis that mice are an appropriate model in which to study cerebral ischemia–induced alterations in cognitive function.

Materials and Methods

This study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research and under protocols approved by the Animal Care and Use Committee of the Johns Hopkins University. Adult male C57/B6 mice (21 to 27 g, Charles River, Mass) were housed individually and maintained on a 14:10 light/dark cycle. The animals were allowed ad libitum access to tap water and rodent chow throughout the study.

Surgery

Mice were anesthetized with 1% to 1.5% halothane in oxygen-enriched air delivered by a face mask, then subjected to 60 or 90 minutes of intraluminal MCAO or sham surgery as previously described. Briefly, unilateral MCAO was achieved by introducing a 6-0 nylon monofilament into the right internal carotid artery through the external carotid artery, then positioning the filament tip for occlusion at a distance of 6 mm beyond the internal carotid artery–pterygopalatine artery bifurcation. Then, the wound was cleaned with a dilute alcohol solution (10% ethanol) and allowed to dry before testing each animal. The individual conducting the behavioral assessments was not informed of experimental group assignment.

In experiment 1, sensorimotor tests and the elevated plus-maze were conducted 13 days after MCAO (60 minutes, n=12; 90 minutes, n=12) or sham surgery (n=12). Each sensorimotor test was conducted 3 times, whereas the elevated plus-maze was conducted once per animal. Descriptions of the individual tests are provided below.

Initiation of Walking

The experimental animal was placed on a flat surface, and the time for the animal to move 1 body length was recorded.

Turning in an Alley

The experimental animal was placed facing the back wall of an alley (3 cm wide with walls 15 cm high). The amount of time (up to 2 minutes) required for the animal to turn around and face the open end of the alley was recorded. This test was used as a measure of coordinated muscle control.

Hanging Wire

The experimental animal was suspended by its forelimbs on a wire stretched between 2 posts 60 cm above a foam pillow. The time (in seconds) until the animal fell was recorded (a score of zero was assigned to animals that fell immediately and a score of 60 was assigned to animals that did not fall). This task was used as a measure of grasping ability and forelimb strength.

Locomotor Balance and Coordination

The experimental animal was placed at the center of a horizontal wooden pole (2 cm in diameter) that was elevated 75 cm above the substrate. A large pillow was placed under the screen. Latency to fall was recorded.

Visual Placement

The experimental animal was suspended by its tail and slowly lowered toward a bench top. If the animal extended both of its forelimbs toward the surface, then the animal was assigned a +; if the animal extended only 1 forelimb toward the surface, then the animal was assigned a −; if neither forelimbs were extended toward the surface, then the animal was assigned a 0.

Elevated Plus-Maze

The experimental animal was placed in the center of an elevated plus-maze with 2 open arms and 2 closed arms (75×24 cm). The closed arms were constructed of black-tinted Plexiglas with walls 15 cm high. The maze was mounted 75 cm above the floor on a tripod. Choice behavior was observed for 5 minutes and the number of visits to each arm and the time spent in each arm, as well as the time spent in the central area, was recorded. Incidents of grooming, rearing, and number of fecal boli produced also were recorded. This task was used as a measure of anxiogenic-like behavior. The elevated plus-maze exploits the natural tendency of rodents to prefer enclosed, dark spaces to open, brightly lit spaces when anxious.

In experiment 2, a passive avoidance paradigm was used to assess cognitive function. The chamber (25×10×12 cm) consisted of an electrified grid (2.5 mA) that covered half of the box and an unelectrified, raised (2.5 cm), Plexiglas platform that occupied the other half of the box. Avoidance training began 1 week after MCAO (60 minutes, n=10) or sham surgery (n=10). On 2 consecutive days (reperfusion days 7 and 8), the animal was placed in the chamber on the electrified grid. Once the animal reached the platform, the session began and continued until the animal remained on the platform for 5 consecutive minutes without stepping down onto the electrified grid. The number and timing of “step-downs” that occurred during the training sessions was recorded for each animal and used to determine how well the animals were acquiring the avoidance task. On reperfusion day 9, the animal’s ability to retain the task was determined by placing the animal in the chamber on the unelectrified platform. The session ended when the animal stepped down onto the electrified grid or 5 minutes passed without any step-downs. Avoid-
ance of the electrified grid during the entire 5 minutes session indicated successful retention of the passive avoidance task. Latency to initiate movement also was recorded on day 7, as described above.

In experiment 3, the animals were trained to avoid the electrified grid on 3 consecutive days, as described above. The number and timing of the “step-downs” that occurred during the training sessions were recorded for each animal and used to determine how well the animals were acquiring the avoidance task. On day 4, the animal’s ability to retain the task was determined by placing the animal in the chamber on the unelectrified platform. The session ended when the animal stepped down onto the electrified grid or 5 minutes passed without any step-downs. Avoidance of the electrified grid during the entire 5-minute session indicated successful retention of the passive avoidance task. Only animals that exhibited successful acquisition and retention of the passive avoidance task were included in the remainder of the study. MCAO (n=10) and sham surgeries (n=8) were performed the day after the retention test. Seven days after surgery, a second retention test was performed with the same criteria as described above.

Motoric behavior also was assessed immediately after the retention test on day 7 after surgery. Initiation of walking was assessed as described above. General locomotor activity over a 5-minute test period was assessed with Digiscan photocell activity monitors. The apparatus consisted of a clear Plexiglas box (30 cm high×42 cm long×42 cm wide) fitted inside a metal frame that contained 12 equally spaced infrared photocell detectors along 2 adjacent walls of the apparatus. Interruptions in the infrared light sources by the experimental animal were recorded and used as an index of generalized locomotor activity.

Preparation of Brain Tissue and Infarction Analysis
Fourteen days after surgery, the animals in experiments 1, 2, and 3 were deeply anesthetized and perfused with normal saline (10 minutes) followed by neutral buffered 10% formalin (30 minutes). Then, the brains were blocked coronally at the midcerebellar level and embedded in paraffin. Each brain was sectioned from the level of the olfactory bulbs to the cerebellum. Eight evenly spaced coronal sections (10 μm thick and 750 μm apart) were stained with cresyl violet. Images of each section were digitized and the infract measured with an image analysis system (Inquiry, Loats). The relative size of the cortical infract in each section was determined as follows: 100%×[1–(total ipsilateral cortex–cortical infract)]/total contralateral cortex]. The relative size of the caudate putamen infarcts was determined in a similar fashion.

Statistics
One-way ANOVA was used to analyze the infarction and behavioral data. Group differences were considered statistically significant at P<0.05. When appropriate, post hoc comparisons were made with the Tukey test. Behavioral data that did not meet the assumptions of ANOVA (latency to move, latency to fall from the pole, latency to turn in the alley, time spent grooming) were analyzed with Kruskal-Wallis 1-way ANOVA on ranks followed by post hoc analysis with Dunnett’s method. Correlational analyses were performed with Spearman’s rank correlation method.

Results

Experiment 1
There was no significant difference between the effects of 60 versus 90 minutes of MCAO on infarction volume in the cortex (9.9±4.1% versus 8.8±3.8% of the contralateral cortex, respectively; F(1,23)=0.04, P>0.05) or caudate putamen (15.3±4.0% versus 20.8±4.0%, of the contralateral caudate putamen, respectively; F(1,23)=0.94, P>0.05) that was infarcted. As expected, sham surgery did not result in infarcted tissue. Sixty and 90 minutes of MCAO also resulted in similar neurological scores at the time of occlusion (2.1±0.2 and 2.2±0.2, respectively; F(1,23)=0.11, P>0.05).

Duration of MCAO also had an effect on latency to move 1 body length (Table). Animals subjected to 90 minutes of MCAO demonstrated a significant increase in latency to move 1 body length compared with the 60 minutes of MCAO and sham groups, which did not differ significantly from each other (H=19.24, df=2, P<0.05). However, there was no effect of MCAO on other measures of sensorimotor function (Table), including latency to fall from the pole (H=0.49, df=2, P>0.05) or the wire (F(2,35)=0.17, P>0.05) or in latency to turn in an alley (H=0.11, df=2, P>0.05). All animals in every group exhibited positive visual placement scores. MCAO also failed to have an effect on anxiety-like behavior as measured by behavior in the elevated plus-maze (data not shown). The percent entries into the open arms of the elevated plus-maze (F(2,35)=0.24, P>0.05), duration of grooming behavior (H=1.31, df=2, P>0.05), incidence of rearing (F(2,35)=1.52, P>0.05), and total number of arm entries (F(2,35)=1.67, P>0.05) were similar among treatment groups.

Experiment 2
When measured 1 week after surgery, there was no significant difference in latency to move 1 body length between animals subjected to 60 minutes of MCAO versus sham (F(1,18)=0.53, P>0.05). However, on the first day of training, animals in the MCAO group stepped down onto the electrified grid more than twice as many times as the animals in the sham group before reaching the criterion for successful acquisition of the task (F(1,18)=17.81, P<0.05; Figure 1). On the second day of training, all animals successfully avoided the electrified grid except 1 animal in the MCAO group, which stepped down onto the electrified grid once during the training session. Compared with the sham group, the MCAO group also demonstrated a deficit in retention of the passive avoidance task measured 24 hours after the second training session (F(1,18)=4.45, P<0.05; Figure 2).

Infarction volume in the cortex and caudate of animals after 60 minutes of MCAO was 10.4±3.6% and 20.5±2.5% of the contralateral region, respectively. No damage was observed in the sham-operated animals. The data points from 1 animal in the sham group were excluded from the analyses because the animal was inadvertently placed on the electrified grid while the current was turned off.

Experiment 3
As one would expect, before surgery there were no significant differences between the MCAO and sham groups in acquisition of the passive avoidance task on training day 1 (F(1,17)=0.73, P>0.05), training day 2 (F(1,17)=1.14, P>0.05)
or training day 3 ($F_{1,17}=0.79, P>0.05$). In addition, during retention testing before surgery, 100% of the animals in both experimental groups avoided the electrified grid. Therefore, no animals were removed from the study for not successfully acquiring or retaining the passive avoidance task.

The mice were then retested for their retention of the passive avoidance task 7 days after MCAO or sham. Ten percent of MCAO animals and 50% of sham animals successfully retained the passive avoidance task for 7 days after surgery. MCAO animals also exhibited a shorter latency to step down onto the electrified grid during the retention test than did sham animals ($H=8.3, df=1, P<0.05$; Figure 3). In contrast, there were no significant differences between MCAO and sham animals in latency to move 1 body length ($H=0.44, df=1, P>0.05$) or generalized locomotor activity ($F_{1,17}=2.08, P>0.05$).

Infarction analysis revealed that 60 minutes of MCAO in experiment 3 resulted in infarctions that consumed 21.0±3.4% of the contralateral cortex and 22.5±3.9% of the contralateral caudate putamen. As expected, there were no infarctions detected in any of the sham animals.

**Discussion**

MCAO is a reliable and reproducible rodent model of cerebral ischemia in humans that has been demonstrated to result in sensorimotor and cognitive deficits in rats as reviewed above. The MCAO technique has been modified for use in mice, but the mouse studies have focused primarily on characterizing the histopathological rather than long-term functional outcomes of MCAO in this species. The current study confirms that MCAO is associated with long-term deficits in the initiation of movement and cognitive function in mice, as it is in rats. In addition, the study demonstrates that it is possible to detect MCAO-induced cognitive deficits in mice that do not exhibit potentially confounding alterations in sensorimotor behavior.

Experiment 1 compared the effects of 60 and 90 minutes of MCAO on histology and several measures of sensorimotor and anxiogenic-like behaviors after 2 weeks of reperfusion. There was no significant difference between 60 and 90 minutes of cerebral ischemia in male C57/bl6 mice produce a largely sublethal injury with a small core of infarcted tissue and variable penumbral size of injured but not dead cells. In our laboratory, prolonged MCAO of 120 minutes produces a substantially larger infarction volume (eg, 46% of contralateral striatum and 24% of contralateral cortex) than 60 or 90 minutes of MCAO. Therefore, durations of vascular occlusion greater than 90 minutes are required before a significantly larger infarction volume is achieved than with 60 minutes of occlusion. Others also have reported little difference in the histological pattern of infarction or injury volume of mice subjected to MCAO durations of >60 minutes.

Despite the lack of a histological difference after 60 versus 90 minutes of cerebral ischemia, animals subjected to 90 minutes of MCAO in experiment 1 exhibited a significantly longer latency to initiate movement than either the 60-minute
MCAO or sham groups when tested after 13 days of reperfusion (Table). MCAO also has been shown to increase latency to move in rats.28 Although a significant correlation between infarct size and latency to move was not reported in the current study, it is likely that this behavioral effect is due to subcortical injury resulting from the ischemia. Damage to the striatum, and specifically the caudate putamen, previously has been associated with alterations in generalized locomotor activity, skilled motor, and sensorimotor control.29–32

Mice subjected to MCAO did not exhibit deficits in other measures of sensorimotor or anxiogenic-like behavior compared with sham animals (Table). Skilled motor coordination and balance, as measured by latency to turn in an alley and ability to balance on a pole, respectively, were not altered by MCAO in mice. As described above, skilled motor movement can be affected by damage to the caudate putamen29–32 and deficits in ability to balance on a rotating pole after MCAO have been reported in rats.8,35 MCAO also did not influence anxiety as measured by the elevated plus-maze. Although damage to the cortex and caudate putamen is not typically associated with increases in anxiety, it was necessary to rule out the possibility of MCAO causing a nonspecific increase in anxiety, which could then affect performance in cognitive testing.

On the basis of the deficit in latency to move, it was concluded that 90 minutes of MCAO is not suitable for precise evaluation of complex poststroke behavioral outcomes without risk of confounding lethargy or sensorimotor disturbance. In contrast, absence of an effect of 60 minutes of MCAO on sensorimotor or anxiogenic behavior suggests that it is an appropriate duration of cerebral ischemia to use in additional behavioral testing. Therefore, in experiments 2 and 3, a duration of 60 minutes of MCAO was used to assess the effects of cerebral ischemia on learning and memory in passive avoidance paradigms. In experiment 2, acquisition and retention of the passive avoidance task was assessed after animals had been exposed to cerebral ischemia or sham surgery. In experiment 3, animals acquired the passive avoidance task before surgery, then were tested for their ability to retain the task several days after MCAO or sham. Training animals in the passive avoidance task before surgery allows one to assess the effects of MCAO on task retention (experiment 3), whereas training animals after surgery allows one to assess the effects of MCAO on both task acquisition and retention, but not independently (experiment 2).

When animals were trained in the passive avoidance task 1 week after surgery, MCAO animals stepped down onto the electrified grid and received approximately twice as many shocks as sham animals before successfully reaching the training criterion of avoiding the electrified grid for 5 consecutive minutes (Figure 1). On the second training day, both MCAO and sham animals avoided the electrified grid after receiving an initial shock on placement into the apparatus. Taken together, these data suggest that MCAO mice are capable of meeting the criterion for successful acquisition of the passive avoidance task but that they require more training than sham animals. In addition, MCAO mice exhibited a deficit in retention of the passive avoidance task compared with sham animals regardless of whether they learned the task before or after being subjected to cerebral ischemia (Figures 3 and 2, respectively). These passive avoidance data in mice confirm and extend prior reports that MCAO in rats impairs the acquisition14,16 or retention18–20 of passive avoidance tasks.

None of the behavioral changes identified in the current study were significantly correlated with infarction size. A lack of congruity between histology and behavior also was reported in a previous study in which no difference in infarction volume was reported for mice subjected to 60 versus 120 minutes of MCAO despite group differences in neurobehavioral recovery within the first 24 hours after ischemia.24 The lack of a correlation between histology and MCAO-induced behavioral alterations is not unusual in cerebral ischemia studies with rats, either.12,17,28,34,35 and may be due to several factors including (1) redundancy of mechanisms underlying behavior,29 (2) recovery of function during the period of time that elapses between surgery and behavioral testing,36 (3) focusing primarily on infarction size rather than distribution of cell death,37 and (4) inability of current histological methods to assess the functional capabilities of neurons that have survived the ischemic insult.38 Therefore, it does not appear that infarction size is an accurate predictor of functional outcome in experimental stroke studies with rats or mice.

In summary, the data from this study suggest that 60 minutes of MCAO in mice results in an impairment of learning and memory without causing concomitant long-term sensorimotor deficits. Taken together, these data establish mice as a suitable alternative to rats for studying the effects of potential stroke therapies on cognitive outcome. In addition, data from experiments 1, 2, and 3 indicate that histological outcome is not a reliable predictor of functional outcome, thereby emphasizing the advantage of including measurement of both of these end points in future preclinical studies.

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References

Hattori and colleagues make an important contribution to the field of experimental stroke-induced brain injury with their behavioral characterization of the cognitive deficits produced by MCAO in mice. Their study provides initial steps for future mechanistic studies that could take advantage of transgenic and/or knockout mice. They have identified a duration of MCAO in 57/BL6 mice that produces cognitive deficits in a passive-avoidance task without significantly altering sensorimotor function. However, one needs to be cautious when extrapolating the results from a single strain of mice to the many other strains available, and especially to those with genetic modifications that may have altered behavior profiles. Nevertheless, this study demonstrates the feasibility of using cognitive behavioral assessment in the study of stroke in mice.

Behavioral assessment of cognitive function after any experimental brain injury can be problematic when the injury also affects motor function. Animal experiments generally make inferences of cognitive function from measures based on motor responses such as locomotion, lever pressing, or head movements. Motor impairments pose less of a problem in the inferences of cognitive function from measures based on motor affects. Animal experiments generally make inferences of cognitive function from measures based on motor responses such as locomotion, lever pressing, or head movements. Motor impairments pose less of a problem in the assessment of cognitive function in brain-injured humans, since psychological testing often relies on verbal or written responses. Because brain injury in animal models characteristically produces behavioral motor deficits, attention to the capacity of these deficits to confound the cognitive interpretation of behavior responses is critical. The use by Hattori and colleagues of multiple behavioral testing procedures provides a sound template for dealing with confounding motor deficits in future studies of cognitive behavior in brain-injured animals.

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