Long-Lasting Functional Disabilities in Middle-Aged Rats with Small Cerebral Infarcts

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Recommendations from experts and recently established guidelines on how to improve the face and predictive validity of animal models of stroke have stressed the importance of using older animals and long-term behavioral–functional endpoints rather than relying almost exclusively on acute measures of infarct volume in young animals. The objective of the present study was to determine whether we could produce occlusions in older rats with an acceptable mortality rate and then detect reliable, long-lasting functional deficits. A reversible intraluminal suture middle cerebral artery occlusion (MCAO) procedure was used to produce small infarcts in middle-aged rats. This resulted in an acceptable mortality rate, and robust disabilities were detected in functional assays, although the degree of total tissue loss measured 90 d after MCAO was quite modest. Infarcted animals were functionally impaired relative to sham control animals even 90 d after the occlusions, and when animals were subgrouped based on amount of tissue loss, MCAO animals with only 4% tissue loss exhibited enduring neurological–behavioral impairments relative to sham-operated controls, and the functional impairments in the group with the largest infarcts (20% tissue loss) were more severe than the functional impairments in the rats with 4% tissue loss. These results suggest that this model, using reversible MCAO to produce small infarcts and long-lasting functional–behavioral deficits in older rats, may represent an advance in the relatively higher-throughput modeling of stroke and its recovery in rodents and may be useful in the development and characterization of future stroke therapies.

Key words: MCAO; stroke; functional disabilities; behavioral deficits; age; preclinical models

Introduction

During the last decade, research on cerebral ischemia identified numerous mechanisms that contribute to cell death, and this progress in terms of the basic science produced a wave of optimism that an effective neuroprotective agent could be developed for the treatment of stroke. Many clinical candidates were identified on the basis of the results obtained in *in vitro* and *in vivo* preclinical models. However, the initial optimism that effective neuroprotective agents could be developed faded when many large and expensive clinical trials all failed to detect therapeutic effects, and it is now widely recognized that there were weaknesses in the preclinical models that were used to identify clinical candidates (Gladstone et al., 2002; Fisher and Ratan, 2003). As a result, new guidelines have been established that address how preclinical models should be used to identify new clinical candidates for stroke (STAIR, 1999).

Occlusive (ischemic) strokes are the most common type of stroke, comprising >80% of all strokes (American Heart Association, 2001). The intraluminal suture procedure for middle cerebral artery occlusion (MCAO) in rats closely approximates this type of stroke (Longa et al., 1989; Belayev et al., 1996), it has been widely accepted as a preclinical model for producing reliable ce-

rebral infarcts after permanent or temporary occlusion in rats, and it has been used frequently to assess potential neuroprotective agents. Typically, these experiments are conducted with young animals, usually producing very large infarcts, and infarct volumes 24 hr after the occlusion are the principal endpoint. Recommendations from experts and recently established guidelines on how to conduct preclinical research to identify clinical candidates suggest that to improve the utility of animal stroke models, infarcts should be produced in older animals, and potential efficacy should be determined on the basis of behavioral–functional effects after longer survival times (Millikan, 1992; STAIR, 1999).

The assessment of functional disabilities in aged animals after experimental stroke has been limited, primarily because older rats often have high mortality rates after MCAO (Futrell et al., 1991; Hachinski et al., 1992; Wang et al., 1995, 2003), and although young rats have lower mortality rates, it is difficult to detect reliable, robust, long-lasting functional deficits in young rats (DeVries et al., 2001). In rat models of other types of brain damage, such as Parkinson's disease, it has been possible to identify and develop behavioral tests that can detect reliable, long-lasting functional deficits in aged subjects even when they have small brain lesions that fail to produce detectable deficits in younger subjects (Schallert, 1983, 1988; Lindner et al., 1997a, 1999). Therefore, the objectives of the present study were as follows: (1) to determine whether the reversible intraluminal suture procedure could be adapted to produce MCAOs in middle-aged

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rats with an acceptable mortality rate and (2) to determine whether long-lasting, robust functional disabilities could be detected in those middle-aged rats. If successful, such a model could have considerable utility in assessing the long-term benefit of potential stroke therapeutics under conditions more closely emulating those seen in human stroke trials.

Materials and Methods

Rats

All experimental procedures were reviewed and approved by the Bristol-Myers Squibb Institutional Animal Care and Use Committee before the study was initiated. Male Sprague Dawley rats (10-month-old males; Harlan Sprague Dawley, Indianapolis, IN) were received in the vivarium ($n = 50$), where they were housed two per cage and maintained at 22°C on a 12 hr light/dark cycle. From the time of their arrival (6 months before surgery), the rats were maintained on a restricted feeding schedule of 15 gm/d standard rat chow (Rodent Lab Chow 5001; Purina, Richmond, IN) except for the week before and after surgery, when they were fed *ad libitum*. At the time of surgery, rats were randomly assigned to one of the following groups: (1) sham occlusion controls; (2) MCAO, 60 min; (3) MCAO, 120 min. After surgery, rats were housed singly, and those with body weights <90% of presurgery weight were supplied with Froot Loops (Kellogg's, Battle Creek, MI), transit gel (Purina), and subcutaneous saline injections, in addition to 15 gm of rat chow. To ensure that rats were motivated for food reward at the time of behavioral testing, uneaten food was removed 18 hr before behavioral test sessions. If body weight dropped >20% from the time of surgery, rats were given daily subcutaneous saline injections in addition to *ad libitum* food, and if their body weights did not begin to recover on that regimen, they were removed from the study and killed with CO₂.

Surgical procedure

At the time of surgery, the rats (16 months of age and 422 ± 5.1 gm) were anesthetized initially with a mixture of 5% halothane, 30% oxygen, and 70% nitrous oxide in an induction chamber and maintained thereafter with a 70:30% nitrous oxide:oxygen mixture containing 1–1.5% halothane delivered via a face mask. Body temperature was monitored throughout the surgical procedure by a rectal thermometer, and the animals were maintained at normal body temperature ($37 \pm 1^\circ\text{C}$) via a heating blanket controlled by the thermometer. A subcutaneous, periorbital temperature probe was also inserted into the left temporalis muscle to give an indirect measurement of brain temperature, and cranial temperature was maintained at $37 \pm 1^\circ\text{C}$ using a heating lamp.

Rats received 60 or 120 min occlusions of the left MCA via a cervical carotid approach using the intraluminal suture technique described in detail previously (Longa et al., 1989; Belayev et al., 1996). The ventral neck region was exposed, and a midline incision was made. The common carotid, external carotid, and internal carotid arteries were exposed by blunt dissection under an operating microscope. The arterial branches of the external carotid artery (superior thyroid, occipital, lingual, and maxillary arteries) were exposed and divided using diathermy forceps, leaving a stump of ~2–3 mm in length. An arteriotomy was made in the external carotid artery stump, and the heat-blunted suture was advanced into the lumen of the internal carotid artery and passed into the intracranial circulation to lodge in the narrower lumen of the proximal anterior cerebral artery, where mild resistance was felt (~20–22 mm distal to the carotid bifurcation), thereby occluding the origin of the MCA. Restoration of MCA blood flow was achieved by complete withdrawal of the suture under halothane anesthesia after either 60 or 120 min. Sham-operated animals underwent the same surgical procedure without suture insertion. Anesthesia was discontinued with the onset of reperfusion, and the rats regained consciousness and righting reflex under observation in an incubator (23–25°C) for 1 hr. Rats were then housed singly for the remainder of the study.

Acute neurological assessment

The experimenter performing the surgeries assessed motor and behavioral changes 1 hr after the surgery using a five point scale as follows: 0, no neurological deficit (normal); 1, failure to extend right forepaw (mild); 2,

decreased resistance to lateral push (mild to moderate); 3, circling or walking to the right (moderate); and 4, loss of walking or righting reflex (severe). Chronic functional assessments were performed throughout the duration of the experiment as described below.

Quantification of tissue loss

Ninety days after MCAO, rats were perfusion-fixed with 4% paraformaldehyde in PBS. Briefly, the animals were reanesthetized with a mixture of 3% halothane in 30% oxygen and 70% nitrous oxide and placed in a supine position. The thorax was opened through a midline incision, and an oral gavage needle was inserted into the ascending aorta via the apex of the left ventricle and clamped in position. The right atrium was incised, and 0.9% heparinized saline (10 IU/ml) was infused at a pressure equal to the mean arterial blood pressure (90–100 mmHg) until the effusate from the right atrium was bloodless (~50 ml). Rats were then perfused with 200 ml of 4% paraformaldehyde in PBS at the same pressure and decapitated. The brains were stored *in situ* in 4% paraformaldehyde for 24 hr, removed, stored in 4% paraformaldehyde for 24 hr, and then cryoprotected in 30% sucrose in PBS at 4°C until preparation for histopathology.

To assess infarct localization and volume, the brains were frozen, and serial coronal sections (20 μm) were cut at 12 equidistant planes (1 mm apart, covering the entire forebrain), between approximately +12.7 and +1.7 mm anterior to the interaural line (Paxinos and Watson, 1986). These sections were stained with cresyl violet and captured using a computer-interfaced light microscopic workstation with a high-resolution digital camera (25 $\times$ final magnification). For each section and hemisphere, the total area as well as the areas of the cerebral cortex and striatum were obtained using Neurolucida contour tracing software (MicroBrightfield, Williston, VT). Entire hemisphere area measures did not include infarct-induced holes, obvious necrotic tissue, or ventricles. The volume of each structure was estimated using the Cavalieri method (Gundersen et al., 1988) (i.e., it was calculated as the product of the sum of the areas and the distance between sections). Contour tracing and volumetric analyses were conducted by an investigator who was blind to the experimental treatment of the animals.

Data analyses

Statistical analyses were conducted using SAS-PC (SAS Institute, Cary, NC), including ANOVAs with procedures for general linear models, with options for repeated measures where appropriate and planned contrasts between groups. Correlation coefficients were also computed. Data are presented as means $\pm$ SEM.

Chronic functional assessments

General. Rats were handled and habituated to the testing procedures and then tested until stable baselines were obtained on all measures. Rats were then tested repeatedly in a battery of functional tests as described below, beginning 24 hr after the occlusion and then again once every 10–14 d for 90 d. To reduce variability, the data were pooled and presented as the means at days 1, 10, 30, 60, and 90 after MCAO. All assessments of chronic functional deficits were conducted by an experimenter blind to the treatment classification.

Forelimb adduction. Forelimb adduction was quantified by observing the rats for 5 min periods in a Plexiglas cylinder (20 cm diameter, 40 cm height). The cumulative time that each forepaw was adducted while the other paw was in contact with the floor or walls of the cage for postural support or locomotion was recorded (Lindner, 1990; Schallert et al., 1990; Lindner et al., 1995, 1997b).

Bracing test. The ability to make postural adjustments was assessed in the present study as reported previously (Schallert et al., 1979). The experimenter placed one hand along the side of the rat and gently pushed the rat laterally for 90 cm at ~20 cm/sec on a smooth stainless-steel surface. Normally, rats will adjust their posture as they are forced laterally, making numerous forelimb movements. The number of steps or forelimb adjustments made with the forelimb on the side in which the rat was being moved was recorded. Each trial included moving the rat two times in each direction. The means from the two test sessions were analyzed.

Tactile adhesive-removal test. Somatosensory function was assessed as reported previously (Schallert et al., 1982, 1983). Pairs of circular adhe-

sive papers (circular “dots” for color-coding file folders; surface area, 113.1 mm²; Maco, Hillside, NJ) were affixed to the distal–radial areas of each forelimb. The forepaws were held apart and away from the animal’s mouth while it was returned to its home cage. The latencies to remove the stimuli with the mouth were recorded for the stimuli on each forelimb. The maximum cutoff latency was 3 min, and rats received three trials per session, with a 1–2 min intertrial interval.

Placing test. Somatomotor function was measured in the placing test, which assesses the rat’s ability to make directed forelimb movements in response to sensory stimuli (Marshall, 1982). Rats were held so that their limbs were hanging unsupported. They were then raised to the side of a table so that their whiskers made contact with the top surface with the length of their body parallel to the edge of the tabletop. The data were scored as the number of times the rat successfully raised its forelimb to the tabletop in 10 trials for each forelimb.

Forelimb akinesia test. Ability to initiate movements with the affected forelimb was assessed in the forelimb akinesia test. The experimenter held the rat so that it was standing on one forelimb and allowed to move on its own. The number of steps taken with the usable forelimb was recorded during a 30 sec trial for each forelimb as described previously (Schallert et al., 1992).

Rotorod. Motor–ambulatory function was assessed with a rotorod (Economet 0207-001L; Columbus Instruments, Columbus, OH). Rats were shaped to ambulate on the rotorod to avoid a 35 cm fall and then tested with three acceleration speeds over 60 sec periods: 5–10, 5–20, and 5–40 rpm. Latencies to fall off or the 60 sec cutoff were recorded for analysis.

Staircase test. Fine motor control of the affected forelimb and paw was assessed in the staircase test with an apparatus constructed according to the dimensions reported previously (Montoya et al., 1990, 1991; Abrous and Dunnett, 1994). Each rat was placed on a narrow central platform and allowed to reach down into a trough on the left side of the central platform with its left forepaw only and on the right side with its right forepaw only, to retrieve and eat 45 mg food pellets. Inside the trough on either side of the rat were food pellets, placed four per well, with six wells on each side; each well was located 13 mm deeper and 14 mm farther caudal than the previous well, in staircase manner. The apparatus was constructed so that the four pellets on the top stair were directly accessible by mouth, but pellets from lower stairs had to be grasped, held, and lifted with the forepaws; pellets could not be retrieved by pushing or sliding them without grasping them. Rats were trained to perform the staircase task before the surgery, then given one 10 min trial on every test day. The average number of pellets eaten per side was recorded as the dependent measure.

Bar pressing. Forelimb speed and endurance were assessed with a fixed-ratio (FR) bar-pressing task using operant boxes (Coulbourn Instruments, Allentown, PA) equipped with two levers, one on either side of a central food bin. Rats were first trained to press the lever using a continuous reinforcement schedule (FR1), in which one 45 mg food pellet (Formula A/I; PJ Noyes, Lancaster, NH) was awarded for each lever press. After acquiring the FR1 schedule, rats were shaped to perform the more difficult food-reward schedule, which required five presses for each food reward (FR5), and were then tested with the FR5 schedule during 20 min sessions as reported previously (Cousins et al., 1993; Salamone et al., 1993).

Results

Mortality rates

Two of the 50 rats died during the 6 month period before surgery, and one of the nonoccluded control rats died over the subsequent 3 month testing period. In an initial experiment, 8 of the first 10 rats given MCA occlusions died within the first 24 hr after recovering from the anesthetic. To reduce the mortality rate, subsequent occlusions were produced with noncoated suture material. After using a noncoated suture for the intraluminal occlusions, only 7 of 29 occluded rats died, either within 24 hr of the occlusion, after recovering from the anesthetic ($n = 5$), or because their body weight fell >20% within a few days of surgery and they

had to be killed ($n = 2$). The final analyses included nine sham-occlusion controls, 14 rats with 60 min MCA occlusions, and 10 rats with 120 min occlusions.

Tissue loss

Tissue volumes could not be accurately quantified on five brains because of freezing artifacts, so the histology data from these brains were not included in the analyses of tissue loss. Three of these brains were from control rats and two were from occluded rats. Although the section quality was insufficient to precisely quantify degree of tissue loss in these two occluded rats, it was apparent that they suffered very little tissue loss, and they had little or no behavioral deficits. Therefore, the behavioral data for these two rats were included in the analyses of the behavioral data, and they were included in the subgroup of rats with small occlusions (see below). Excluding the behavioral data for these two animals had no significant effect on the pattern of the results.

Relative to other studies in the literature, the sizes of the infarcts measured 90 d after MCAO were small, and there was variability and some overlap in the size of the infarcts between the animals that were occluded for 60 min and those that were occluded for 120 min. The focus of this study was on characterizing chronic behavioral deficits after MCA occlusions. Therefore, first and foremost, we were interested in whether animals receiving MCAO had significant functional deficits relative to sham-operated control animals. Second, we were interested in whether there was a significant correlation between any possible long-term functional deficits and amount of tissue loss. Therefore, data were initially analyzed for behavioral deficits in MCAO animals versus sham controls. The data were then analyzed with rats grouped according to infarct size (rather than according to occlusion duration). There were six rats with tissue loss of 15–27%, and the rest of the occluded rats had tissue loss of <10%, so the animals were divided into the following groups: (1) sham occlusion controls with no tissue loss ($n = 9$), (2) rats with small infarcts and tissue loss of 4% (0–10%) of the hemisphere ($n = 18$), and (3) rats with larger infarcts and tissue loss of 20% (15–27%) of the hemisphere ($n = 6$), relative to the nonoccluded hemisphere. (As stated above, because of problems with staining, the data for histological analyses included six controls, 16 rats with 4% tissue loss, and six rats with 20% tissue loss.) As shown in Figure 1, rats in the larger infarct group had major damage to the striatum, globus pallidus, and nearby areas as well as damage to the cortex (perirhinal and other ventral cortical regions). In a subset of animals with large infarcts, damage to the internal capsule ($n = 3$), amygdala ($n = 4$), and hippocampus ($n = 1$) was also evident. Clearly, necrotic tissue could not be found in the cortex in the small infarct group.

Volumetric analyses of the cortex, striatum, and entire hemisphere showed that MCAO in the present study did not affect the volumes in the contralateral hemispheres, but there was significant tissue loss in the hemisphere ipsilateral to the occlusion. In all occluded animals combined, cortical volumes were decreased by 4% (9 mm³), striatal volumes were decreased by 18% (6 mm³), and the volume of the entire hemisphere was decreased by 8% (41 mm³) relative to those structures in the contralateral hemisphere. In the group with the smallest infarcts, there was a nonsignificant 1–2% tissue loss in the ipsilateral cortex relative to the cortex in the contralateral hemisphere ($F_{(1,15)} = 4.14$; $p = 0.06$) (Fig. 2A), there was significant tissue loss in the striatum with a 12% reduction compared with the striatum on the contralateral side ($F_{(1,15)} = 23.24$; $p = 0.0002$) (Fig. 2B), and there was a small (4%) but significant degree of tissue loss in the entire

Figure 1. Representative photomicrographs of infarcts of the 20% tissue loss (*A*) and 4% tissue loss (*B*) groups. The infarcted area is indicated by the thick black lines in *A* and the arrows in *B*. The thinner black lines delineate the division between the hemispheres. Each section is from a different animal and is representative of the infarct at that coronal plane. Numbers indicate approximate coordinates in millimeters anterior to the interaural line. Scale bar, 1 mm. CTX, Cortex; STR, striatum. *C*, Higher-magnification view of the region of striatal infarct shown in *B* reveals clear gliosis and no definable neurons. *D*, A comparable region in the contralateral striatum showing a normal mixture of glia and neurons. Scale bar, 50 μ m.

Figure 2. Volumetric measures of cortex, striatum, and the entire hemisphere for rats in the control group (CTL; $n = 6$), rats with 4% tissue loss of the entire hemisphere ($n = 16$), and rats with 20% tissue loss of the entire hemisphere ($n = 6$). *A*, Volumes of neocortex in hemisphere ipsilateral and contralateral to occlusion. *B*, Volumes of striatum in hemispheres ipsilateral and contralateral to occlusion. *C*, Volumes of entire hemisphere ipsilateral and contralateral to occlusion. Volumes of occluded structures that are significantly smaller than the contralateral side are indicated by asterisks. Data are presented in text and figures as means $\pm$ SEM.

hemisphere ($F_{(1,15)} = 32.62$; $p = 0.0001$) relative to the contralateral hemisphere (Fig. 2*C*). In the rats with the larger infarcts, there was significant tissue loss in the ipsilateral cortex of 11% relative to the contralateral cortex ($F_{(1,5)} = 25.22$; $p = 0.004$) (Fig. 2*A*), there was a significant degree of tissue loss in the ipsilateral

Figure 3. Tests discontinued 30 d after surgery because of insufficient sensitivity to MCA occlusions. *A*, Bracing, which is the number of postural adjustments with affected forelimb while being pushed sideways for 90 cm. *B*, Akinesia, which is the number of steps initiated spontaneously with the affected forelimb during 30 sec trials. *C*, Rotorod, which is latency to fall from accelerating rotorod (5–40 rpm during 60 sec trial). Control rats ($n = 9$), rats with 4% tissue loss ($n = 18$), and rats with 20% tissue loss ($n = 6$) are shown.

striatum of 38% relative to the striatum on the contralateral side ($F_{(1,5)} = 13.21$; $p = 0.02$) (Fig. 2*B*), and there was significant tissue loss in the entire ipsilateral hemisphere of 20% relative to the contralateral cortex ($F_{(1,5)} = 124.49$; $p = 0.0001$) (Fig. 2*C*).

Although these lesions were fairly small, the pattern of the lesions was similar to what has been reported in previous studies (Rogers et al., 1997), with approximately twice as much tissue loss in the cortex as in the striatum. With the smallest lesions, statistically significant tissue loss was not detectable in the cortex, similar to results in other studies (Rogers et al., 1997), but this may be because the limit of detection with the large cortical structures is larger than for the striatum. In the animals with the smallest lesions, both the cortex and striatum were ~ 3.0 mm³ smaller in the occluded hemisphere than in the contralateral hemisphere, but this difference was only statistically significant in the striatum.

Functional deficits

After recovering from the anesthetic, all occluded rats were circling or walking to the right and received a score of 3 on the acute neurological assessment during the first hour after the occlusion. One day after MCAO, many of the occluded rats were drooling, the fur under their mouth and on their neck was wet, and many of them were still circling. Signs of stress were evident for the first few days after MCAO by the large amounts of red Harderian gland secretions around their eyes and nostrils. Body weight dropped in occluded rats, but these changes were transient, peaking 2 weeks after MCAO, with a maximum loss of 6% for all occluded animals combined.

After 7–10 d, rats were no longer drooling or circling, their food intake quickly returned to baseline levels, and gross inspection of the rats in their home cages failed to detect any apparent behavioral deficits. Consistent with these gross observations of the rats, several of the behavioral tests failed to detect significant behavioral deficits. Although some transient deficits were de-

Figure 4. Performance on tests of forelimb function that were sensitive to MCAO. *A*, Forelimb adduction. *B*, Tactile adhesive test of forelimb somatosensory function. *C*, Placing test of forelimb somatomotor function. *D*, Staircase test of forelimb and paw fine motor control. *E*, Fixed-ratio bar pressing test of forelimb motor speed and endurance. Control rats ($n = 9$), rats with 4% tissue loss ($n = 18$), and rats with 20% tissue loss ($n = 6$) are shown.

tected, it was apparent that the tests for forelimb bracing, akinesia, and the rotorod test were not detecting significant, long-lasting behavioral deficits (Fig. 3). For that reason, these tests were discontinued 30 d after the surgeries, and the data for these tests were not included in additional analyses.

Forelimb adduction

Results of the forelimb adduction test reliably detected individual differences among rats with occlusions, Cronbach's α reliability coefficient was 0.86, and the test of forelimb adduction was sensitive to the effects of MCAO. Even 90 d after MCAO, rats in the sham-occlusion control group held their right paw adducted for 18.4 ± 3.9 sec during the 5 min observation periods, whereas MCAO rats as a group adducted their affected paw 36.2 ± 5.3 sec. The main effect for tissue loss (control, 4% tissue loss, and 20% tissue loss) was statistically significant ($F_{(2,30)} = 6.36$; $p = 0.005$) (Fig. 4*A*).

Tactile adhesive test

Results of the tactile adhesive test reliably detected individual differences among rats with occlusions, Cronbach's α reliability coefficient was 0.85, and the tactile adhesive test of somatosensory function also detected deficits, especially initially after MCAO. Although there was substantial recovery of function on this test, 90 d after MCAO control rats responded in 15.2 ± 4.9 sec, whereas all MCAO rats combined responded in 29.2 ± 7.8 sec. The main effect of tissue loss (control, 4% tissue loss, and 20% tissue loss) was statistically significant ($F_{(2,30)} = 4.41$; $p = 0.02$) (Fig. 4*B*).

Placing

Results of the placing test reliably detected individual differences among rats with occlusions, Cronbach's α reliability coefficient was 0.93, and the placing test of somatomotor function still detected deficits 90 d after MCAO, with controls responding 6.5 ± 1.4 times and all MCAO rats combined responding 3.7 ± 0.8 times. The rats with 4% tissue loss had smaller deficits than the rats with 20% tissue loss; the main effect for tissue loss (control, 4% tissue loss, and 20% tissue loss) was statistically significant ($F_{(2,30)} = 3.38$; $p = 0.04$) (Fig. 4*C*).

Staircase test

Results of the staircase test reliably detected individual differences among rats with occlusions, and Cronbach's α reliability coefficient was 0.92. The staircase test of fine motor control was sensitive to the effects of the occlusion throughout the 90 d of testing after MCAO, with controls retrieving 16.3 ± 1.3 pellets compared with 7.8 ± 1.1 pellets for all MCAO rats combined (Fig. 4*D*). The main effect for tissue loss (control, 4% tissue loss, and 20% tissue loss) was statistically significant ($F_{(2,30)} = 16.72$; $p = 0.0001$) (Fig. 4*D*).

FR5 bar presses

Results of the bar pressing test reliably detected individual differences among rats with occlusions, and Cronbach's α reliability coefficient was 0.92. The number of bar presses during 20 min operant sessions in which the rats could earn a food reward

after every five bar presses (FR5) was sensitive to the effects of MCAO even 90 d after MCAO, with controls pressing 1342 ± 123 and all MCAO rats combined pressing the bar 809 ± 115 times. The main effect for tissue loss (control, 4% tissue loss, and 20% tissue loss) was statistically significant ($F_{(2,30)} = 15.33$; $p = 0.0001$) (Fig. 4*E*).

Composite behavioral score

To maximize the sensitivity of the behavioral measures to the effects of the infarcts, the five tests that detected statistically significant effects were converted into standardized scores, and the average of those standardized scores was computed. That composite score detected statistically significant differences between the sham-occluded controls and all MCAO rats combined (Fig. 5*A*). There were also significant differences between the controls and the rats with 4% tissue loss at every point, from 1 to 90 d after surgery, and between the rats with 4% tissue loss and 20% tissue loss at every time point (Fig. 5*B*). In addition, among the rats given MCAO, the composite behavioral score 90 d after the occlusions was significantly correlated with percentage of tissue loss ($r_{(22)} = -0.62$; $p = 0.002$).

Occlusion duration

Because of the variability in these small infarcts and the fact that we were primarily concerned with the relationship between behavioral–functional measures and lesion size, we chose to present the data by grouping the animals in terms of the size of the infarcts. However, because the animals received either 1 or 2 hr occlusions, we also present the data, very briefly, by grouping the animals with respect to occlusion period just for reference. Tissue loss in the striatum, cortex, and entire hemisphere is greater for animals with 2 hr occlusions than for those with 1 hr occlusions (Fig. 6), and the behavioral deficits are greater for rats with 2 hr occlusions as well, although there were detectable behavioral deficits even in the rats with 1 hr occlusions (Fig. 7).

Discussion

Stroke is the leading cause of long-term disability among adults (Bonita, 1992; Bonita et al., 1994) and the third leading cause of death (Minino et al., 2002), with total costs of \$50 billion per year in the United States (National Heart, Lung, and Blood Institute,

Figure 5. Composite behavioral score combining the five behavioral measures in Figure 4. *A*, All MCAO rats combined. *B*, MCAO rats divided into subgroups with 4% tissue loss and 20% tissue loss. Deficits are evident on day 1 even in the sham occlusion group. Both occluded groups are significantly impaired throughout the 90 d testing period. *Significantly different from control. **Significantly different from 4% tissue loss group.

Figure 6. Volumetric measures of cortex, striatum, and entire hemisphere for rats grouped by occlusion duration: Control group (CTL; $n = 6$), rats with 1 hr occlusions ($n = 12$), and rats with 2 hr occlusions ($n = 10$) are shown. *A*, Volumes of neocortex in hemisphere ipsilateral and contralateral to occlusion. *B*, Volumes of striatum in hemispheres ipsilateral and contralateral to occlusion. *C*, Volumes of entire hemisphere ipsilateral and contralateral to occlusion. Volumes of occluded structures that are significantly smaller than the contralateral side are indicated with asterisks.

2002). Stroke is principally a disease of the elderly; older stroke survivors are more likely to be disabled by a stroke, and their disabilities tend to be more severe (Loewen and Anderson, 1990; Westling et al., 1990; Wyller, 1998). There are now ~4 million stroke survivors with neurological deficits in the United States, most of them over the age of 65 (Gresham et al., 1979; Kelly-Hayes et al., 1998), and as many as 750,000 new strokes can be expected in the United States each year (Williams et al., 1999). Only a single approach to poststroke treatment has been shown to provide clinical benefit, clot dissolution in ischemic stroke exemplified by the actions of tissue plasminogen activator (Na-

Figure 7. Composite behavioral scores with groups on the basis of occlusion duration combining the five behavioral measures that were sensitive to the infarcts. Both occluded groups were significantly impaired throughout the 90 d testing period. *Significantly different from control. **Significantly different from 1 hr occlusion group.

tional Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). This treatment is used in only a small percentage of the stroke population because of the need to administer the compound within a relatively short time after stroke onset, and because of the potential for increased risk of hemorrhage. Clearly, additional approaches, if successful, could greatly impact this large patient population with a significant unmet medical need.

The results of the present study suggest that a rodent model of stroke can be developed using MCAO in older rats, with significant, long-lasting functional-behavioral deficits and acceptable mortality rates. According to recently established guidelines and recommendations from experts in the field, this model may have more face and predictive validity for assessing the therapeutic potential of novel treatments than acute measures of infarct volume in young rats (Millikan, 1992; STAIR, 1999). In our experiments, middle-aged rats (16 months of age) were given 60 or 120 min MCA occlusions. This resulted in very high mortality rates (80%) until the procedure was modified, and a non-poly-L-lysine-coated intraluminal suture was used. This change in the surgical procedure successfully reduced the mortality rate to acceptable levels (24%) and probably accounted for the small size of the end-stage infarcts measured at the time of killing (Belayev et al., 1996). Volumetric measures revealed ventricular enlargement and tissue loss in the occluded hemisphere. In all occluded animals combined, the volume of the entire hemisphere was decreased by 8%; in rats with smaller infarcts, the volume of the entire hemisphere was decreased by 4%; in rats with larger infarcts, the volume of the entire hemisphere was decreased by 20%.

A major focus of the present study was to examine a battery of sensitive behavioral tests of neurological function and integrity under the assumption that these small infarcts, which would not be expected to produce detectable deficits in young rats, might produce detectable deficits in these older rats. After recovering

from the acute effects of the occlusion, no behavioral deficits were apparent during gross examination of the rats in their home cage. Consistent with the gross observations, three of the eight behavioral tests included in the present experiment (i.e., bracing, akinesia, and rotorod) were discontinued after 30 d because of a lack of sensitivity to the effects of the occlusion. Some of those tests detected transient behavioral deficits that were only apparent 1–10 d after the occlusions. Five behavioral tests detected statistically significant deficits in the occluded rats [i.e., (1) forelimb adduction, (2) the tactile adhesive removal test of somatosensory function, (3) the placing test of somatomotor function, (4) the staircase test of fine motor control, and (5) the bar pressing test of motor speed and endurance]. Combining the data from these five tests into a composite behavioral score produced a measure that detected statistically significant deficits throughout the 90 d test period. This composite behavioral score discriminated between the sham controls and the group with 4% tissue loss.

A previous study in young rats with 4% tissue loss failed to detect behavioral deficits, even when testing was conducted 24 hr after the occlusion (Rogers et al., 1997). In another study in which MCAO resulted in 8.8% tissue loss, behavioral deficits were detected, but they were only transient, and were no longer significant after 7 d (Zhang et al., 2000). Long-lasting behavioral deficits in young rats have only been detectable after 18–30% tissue loss (Kawamata et al., 1996, 1997, 1998, 1999; Bland et al., 2000, 2001; Hudzik et al., 2000; Modo et al., 2000), whereas in the present study, long-lasting deficits were detected in middle-aged rats with as little as 4% tissue loss. In a previous study that assessed behavioral deficits in aged rats after MCAO, the infarcts were reportedly small and produced no statistically significant behavioral deficits (Andersen et al., 1999). The difference between that study and our results may be attributable to the choice of behavioral tests; they used the rotorod test of ambulatory function, general measures of activity levels, and several tests of cognitive function such as the Morris water maze, olfactory learning, and social interaction tests. The results of the present study confirm that the rotorod is not sensitive to small infarcts in aged rats, and other studies have shown that tests of cognitive function are not very sensitive to unilateral damage in rats (Sutherland et al., 1983; Boissard et al., 1992; Kraemer et al., 1996; Lindner et al., 1998). The tests included in the present study that were most sensitive to these small infarcts were shown previously to be sensitive to unilateral striatal dopamine depletion or unilateral traumatic brain injury (Schallert, 1988; Salamone et al., 1993; Cousins and Salamone, 1996; Lindner et al., 1997a, 1998).

In addition to the choice of behavioral tests, the age of the animals is also critical. Although older animals are generally more sensitive to neurological damage and exhibit more robust behavioral deficits than younger animals (as discussed above), animals at the upper limit of the age range can exhibit such profound age-related behavioral deficits even before any experimentally induced lesions, making it difficult to distinguish between very old animals with infarcts and very old animals without infarcts. For example, a study including animals at 3, 6, 12, 18, and 24 months of age at the time of occlusion reported that they were unable to include the data for the oldest animals in the analyses, because the 24-month-old rats were so impaired before the occlusions that they could not even be trained on the beam-walking task that the authors used in the same way as the animals at all the other ages (Brown et al., 2003). In that study, almost all of the rats at 3 and 6 months of age recovered within 30 d, rats at 12 and 18 months exhibited very small but more persistent deficits in the behavioral task included in the study (beam walking), but ani-

mals at 24 months of age could not be included in the analysis. The 16- to 19-month-old rats included in the present study are within the late middle-age range relevant to clinical stroke populations, and clearly, they are old enough to exhibit behavioral deficits even with very small infarcts, but they are not so old that there is a problem with floor effects because of severe age-related deficits even before surgery.

We did not include young rats in the present study, in part because it is already known that young rats show tremendous plasticity and spontaneous recovery in stroke models, but also because we were not trying to make a comparison between young and old rats. We argue that there is already a consensus that stroke models should be developed in older animals. It is well known that human stroke occurs in late middle age or at older ages, and that there are a whole range of age-related changes in neurochemistry, neuroplasticity, and general physiology that make it clear that it is critical to use older animals when modeling the clinical disorder (Cox, 1983; Futrell et al., 1991; Hachinski et al., 1992; Davis et al., 1995; Sutherland et al., 1996). Experts in the field have commented that the disappointing record for identifying clinically effective drugs in animal models of stroke is attributable at least in part to the fact that preclinical studies are conducted in young animals (Millikan, 1992). One of the most recent reviews conducted by a panel of experts in the field even stated, “It is uncertain if benefit in young, healthy animals can be extrapolated to elderly, sick humans” (STAIR, 1999). Not surprisingly, experts in the stroke field argue that preclinical studies should be conducted in aged animals to increase the clinical relevance and predictive validity of the results (Millikan, 1992). There is no question that older animals are different from young animals and that preclinical stroke models conducted in older animals would have more clinical relevance and predictive validity. The question is whether the high mortality rate seen with older animals can be overcome, while still producing infarcts large enough to produce robust, reliable, long-term behavioral–functional deficits, and that was the question this study was designed to address. In fact, our study demonstrates for the first time that it is possible to produce infarcts in animals within the late middle-age range with an acceptable mortality rate, and that with a battery of sensitive behavioral tests, it is possible to detect robust, reliable, long-term behavioral–functional deficits that are large enough to test potential improvements with novel treatments. Of course, in future studies using aged rats to assess potential therapies, it might be valuable to include a group of young occluded rats as a control group to determine how much functional improvement novel treatments might be expected to produce in aged rats.

Another difference that may be important between the methods used in the present study and many of the other studies conducted with preclinical stroke models is the use of scheduled feedings. Several of the behavioral tests included in the behavioral battery were food-reward tasks that required that the animals be motivated to work for a food reward. Therefore, except for the time shortly before and after the occlusions, animals in the present study were maintained on 15 gm/d standard rat chow, which was allotted to them after their behavioral test sessions. This sustained the animals at ~400 gm, which is only slightly less than the 450 gm average weight expected for F-344 rats maintained on *ad libitum* feeding. Although this does not represent a severe degree of caloric restriction, and this restricted feeding schedule did not begin until the rats were 10 months of age, it is well known that animals maintained on restricted feeding schedules have lower mortality rates and increased longevity, and it has been reported that recovery from brain injury is faster and more

complete in food-restricted rats (Grijalva et al., 1976; Schallert and Whishaw, 1978; Schallert, 1989), even in aged rats (Joseph et al., 1983), and that food-restricted rats suffer fewer complications (Grijalva et al., 1976; Luszczowska et al., 1977). Therefore, it is possible that the acceptably low mortality rates seen in the present study may be partly attributable to the use of fairly long-term scheduled feedings. Animals on restricted or daily feeding schedules also tend to be much more active, and it is possible that increased activity levels in these late middle-aged rats, related to the use of feeding schedules, helped to increase the sensitivity of some of the behavioral tests. In other words, increased activity levels related to the scheduled feedings could help to avoid the floor effects sometimes seen with aged rats. For example, if the 24-month-old rats used in the beam-walking study mentioned above (Brown et al., 2003) had been maintained on scheduled feedings, they may have been active enough to be testable using the same task parameters as the younger animals in that study. Future studies using aged rats should consider the influence that daily feeding schedules have on mortality rates, recovery rates, general activity levels, and the potential sensitivity of behavioral tests, and the impact that those effects could have on the outcome of the study, even if the study does not include any behavioral tests that are motivated by food reward.

Although the use of older rats and measures of long-term functional-behavioral deficits have better face validity than the use of young rats and infarct volumes calculated only 24 hr after occlusion, as with any model, whether this represents a real advantage in terms of improved predictive validity remains to be determined empirically. In addition, even if the use of older animals and long-term behavioral end points represents an improvement in the model, there are still other factors that might need to be addressed to optimize the model. For example, the molecular and cellular mechanisms related to permanent ischemia and reperfusion after long-term occlusion may be different from the reversible occlusions used in the present study. Acceptable mortality rates may not be producible with permanent ischemia in older rats, and this may limit the predictive validity of this model for permanent ischemia and reperfusion injury. It may also be necessary or desirable to examine additional age-related diseases and disorders and their potential contribution to the degree of tissue loss, the magnitude of neurological deficits, and the degree of behavioral recovery. For example, the effects of the major risk factors, such as obesity, smoking, high blood pressure, high cholesterol levels, stress, previous history of stroke, and the use of alcohol and other drugs, could all be examined with experimentally controlled manipulations and treatments in older rats. In fact, including these other factors may be critical in accurately determining potential efficacy of novel compounds. Finally, no matter how many improvements are made to this rodent model, it will still be limited by the fundamental differences between humans and rats. For example, humans have more cortical tissue than rats and thus more cortical tissue loss after a stroke, and humans have more functional lateralization, especially for cognitive functions, than rats. For example, humans with unilateral stroke exhibit significant deficits in terms of language and other cognitive functions, whereas rats, because they exhibit less lateralization of function, are relatively unaffected in tests of cognitive function if the damage is limited to only one hemisphere. In the end, these kinds of species differences may represent the upper limit in terms of the ultimate development and optimization of this rodent model.

Stroke patients are primarily aged, and surviving patients have, on average, total tissue loss of <15% of the volume of the

cerebral hemisphere (Brott et al., 1989; Lyden et al., 1994; Kissela et al., 2001; van der Worp et al., 2001). Patients with significantly larger infarcts usually do not survive, and among the survivors, the proportion of patients with functional deficits increases with age (Wyller, 1998). Significant disabilities are observed even among patients with very small infarcts (Saunders et al., 1995; Pantano et al., 1999; Pereira et al., 1999; Saver et al., 1999; Leinonen et al., 2000; Fink et al., 2002). The results of the present study with older rats are in good agreement with the findings in the clinical literature in terms of the size of the infarct and degree of tissue loss and in terms of the presence of functional deficits even in animals with small infarct volumes. Although the need to use larger, gyrencephalic species is clearly recognized, cost and animal availability issues dictate that preclinical tertiary drug assessment for stroke will continue to use rodent species whenever possible. It is therefore imperative to improve the rodent models to enhance face and, hopefully, predictive validity in the characterization of preclinical stroke therapy candidates.

As far as we are aware, this is the first preclinical study to demonstrate that aged rats can be used to produce modest tissue loss and long-term functional deficits. Our results demonstrate that a modified version of the intraluminal MCAO procedure can be performed on aged rats with an acceptable mortality rate, and chronic functional deficits can be detected and monitored reliably in these animals for periods comparable with those used for human poststroke clinical evaluation end points. Although the deficits were still detectable 90 d after MCAO, they were stable as early as 30 d after MCAO, suggesting that it might be possible to maximize throughput for drug screening with this model by assessing potential therapeutic effects within the first 30 d after MCAO. Additional studies could be conducted to further refine and validate this rodent stroke model, but the present results suggest that this model may be useful in the development of neuroprotective treatments to reduce neuronal loss, as well as treatments targeting recovery of function in older patients suffering with chronic functional disabilities.

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