Research report

Using the behavioral flexibility operant task to detect long-term deficits in rats following middle cerebral artery occlusion

Melissa M.M. Andrews\textsuperscript{a,b}, Sarah Peruzzaro\textsuperscript{a,b}, Shelby Raupp\textsuperscript{b,c}, Jordin Wilks\textsuperscript{b,c}, Julien Rossignol\textsuperscript{a,b,d}, Gary L. Dunbar\textsuperscript{a,b,c,e}, \textsuperscript{*}Corresponding author.

\textsuperscript{a}Field Neurosciences Institute Laboratory for Restorative Neurology, Central Michigan University, Mount Pleasant, MI 48859, United States
\textsuperscript{b}Program in Neuroscience, Central Michigan University, Mount Pleasant, MI 48859, United States
\textsuperscript{c}Department of Psychology, Central Michigan University, Mount Pleasant, MI 48859, United States
\textsuperscript{d}College of Medicine, Central Michigan University, Mount Pleasant, MI 48859, United States
\textsuperscript{e}Field Neurosciences Insit., 4677 Towne Centre Rd. Suite 101 Saginaw, MI 48604, United States

\textbf{A B S T R A C T}

Stroke is a leading cause of death and disability and currently only has one FDA approved pharmacological treatment (tissue plasminogen activator), which is only administered to a fraction of stroke patients due to contraindications. New treatments are desperately needed but most treatments fail in clinical trials, even after showing benefit in animal models of stroke. To increase the translatability of animal stroke research to humans, sensitive functional measures for both the acute and chronic stages in animal models of stroke are needed. The objective of this study was to determine the sensitivity of certain behavioral tasks, up to seven weeks following occlusion of the middle cerebral artery (MCAo) in rats. A battery of behavioral tasks, including rotord, cylinder, and limb-placement, was conducted weekly for seven weeks. Also, a behavioral flexibility operant task was introduced at the end of the study to measure cognitive deficits. All functional outcome measures showed significant differences between stroke and control groups, indicating that these tasks are sensitive enough to detect deficits in a long-term MCAo study in rats. This provides useful information for those trying to increase translatability in their own stroke research by providing long-term sensitive testing paradigms in a relevant stroke model.

\textbf{1. Introduction}

Stroke is a cerebrovascular disease in which a blood vessel within the brain or leading to the brain becomes blocked or ruptures. Although the frequency of stroke has been declining, it is still a leading cause of death and long-term disability [1]. There are limited treatment options following stroke which include surgery and tissue plasminogen activator. Because of the contraindications of these treatments, less than 10\% of stroke patients benefit from these interventions [2,3]. Although other treatments have been shown to be beneficial in animal stroke models, none have proved to be consistently effective in phase III clinical trials [4].

This lack of translatability may, in part, be due to the lack of long-term functional testing in rodent stroke models. It has been suggested that functional outcome measures be assessed at both the acute and chronic (30+ days) stages of stroke [5,6]. However, functional testing at these later time points is difficult and can be complicated by spontaneous recovery [7]. In addition, there are only a few tests that are sufficiently sensitive to detect chronic stroke deficits.

One underutilized option for detecting long-term functional deficits is operant conditioning. Since humans can have a vast array of deficits, ranging from motor and sensory, to cognitive deficits such as learning and memory, inhibitory control, and cognitive flexibility [8–10], a sensitive operant task, capable of detecting long-term cognitive deficits, could prove useful in translational research. Operant conditioning involves modifying behavior by manipulating environmental consequences, which can be conducted efficiently by using operant conditioning chambers, whereby rats learn to press levers in a specified

\textbf{A B B R I E V I A T I O N S}:

ANOVA, analysis of variance; FR, fixed ratio; HSD, Honest significant difference; MCAo, middle cerebral artery occlusion; PBS, phosphate-buffered saline; PFA, paraformaldehyde

\textsuperscript{*}Corresponding author.

E-mail addresses: andre2mm@cmich.edu (M.M.M. Andrews), peruz1st@cmich.edu (S. Peruzzaro), raupp1sm@cmich.edu (S. Raupp), wilks1j@cmich.edu (J. Wilks), rossi1j@cmich.edu (J. Rossignol), dunba1g@cmich.edu (G.L. Dunbar).

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manner to earn reinforcers (such as sucrose pellets or sweetened liquids). Because these procedures can be automated, substantial amounts of data can be gathered in an efficient and objective manner. In addition, work in our lab and those of others have indicated that operant techniques can be sufficiently sensitive to detect subtle, long-term deficits up to 21 weeks following stroke [11–14].

A relatively sensitive operant task for detecting deficits in stroke is the behavioral flexibility task, developed by Linden et al. [13]. This task is inexpensive and easy to administer and translates nicely to cognitive flexibility deficits observed in human stroke patients. In this procedure, mice or rats are offered two levers within an operant chamber for which 5 lever presses on the active lever delivers a pellet. Each time a pellet is earned, the active lever alternates between the two available levers. Linden et al. [13] demonstrated a deficit in mice given the middle cerebral artery occlusion (MCAo) at three weeks prior to testing. The MCAo mice earned fewer reinforcers and made more incorrect lever presses than control mice.

The present study utilized the behavioral flexibility task, along with more commonly used assessments (i.e., the rotorod, limb-placement, and cylinder tasks) for a 7-week period following unilateral MCAo in rats. We assessed whether a 60-minute occlusion would be sufficient to affect the behavioral measures on these tasks, given that this duration should produce measurable deficits, yet spare sufficient amounts of salvageable tissue for assessing potential treatment effects [15,16].

2. Materials and methods

2.1. Subjects

Twenty male Sprague Dawley rats bred onsite from breeders obtained or purchased directly from Charles River (Wilmington, MA) were used for this study. Rats were group housed with food and water available ad libitum within the home cage until day 24, when rats were single housed and given daily supplemental feeding throughout the remainder of the study, with water available ad libitum (Fig. 1). All rats weighed between 250–300 g at the time of surgery and no between-group differences in body weights were observed, thereafter. All rats were housed on a reversed day/night cycle with lights on at 2000 h and off at 0800 h, with all testing occurring during the dark part of the cycle. Rats were randomly assigned to receive either a sham surgery (no MCAo, n = 10) or a 60-minute MCAo (n = 10). Randomization was chosen to alleviate any systematic differences between groups. All procedures in this study were approved by the Institutional Animal Care and Use Committee at Central Michigan University.

2.2. MCAo surgery

At 250–300 g of weight, rats underwent the MCAo surgery following the procedures of Longa et al. [17]. Briefly, rats were anesthetized using a mixture of O2 and isoflurane. A small incision in the neck exposed the carotid arteries. The external carotid artery was cauterized. At that point, if rats belonged to the sham group they were sutured closed and placed into recovery. For the 60-minute MCAo group, microvascular clips were placed on the common and internal carotid arteries and a silicon coated nylon filament (0.37 mm in diameter; Doccol, MA) was inserted into the external carotid artery. The clips were removed, and the filament maneuvered through the internal carotid artery to block blood flow at the MCA. The filament was left in place for 60-minutes. Since no differences or significant paw preferences were observed in the baseline testing of the cylinder task, occlusions were made in the same (right) hemisphere for each rat to maintain consistency. Once the filament was removed, the wound was sutured, and the rat was closely monitored for post-operative recovery.

2.3. Behavioral testing

2.3.1. Baseline performances and training

The timeline for behavioral testing is depicted in Fig. 1. Prior to any surgical manipulation, baseline performance was assessed on the cylinder-, rotorod-, and limb-placement-tests. Preceding the rotorod baseline, rats were trained to maintain balance on an accelerating rotorod (beginning at 4 rpm, with the rod accelerating at 0.2 rpm/second) for at least 2 min. At post-operative day 2, all rats began behavioral testing. Behavioral testing occurred weekly throughout the study at approximately the same time of day.

2.3.2. Cylinder test

The cylinder test was utilized to detect forelimb-use asymmetry [18]. The rat was placed in a clear plexiglass cylinder, 39 cm high, with a diameter of 21 cm (PLAS BY LABS, Lansing, MI) and forepaw placements were counted. A paw placement was defined as a forepaw touching the side of the apparatus while the rat balanced on its two rear paws. Sessions concluded once a combined total of 20 left and right paw placements were made or when 10 min had elapsed, whichever occurred first. To account for right, left, and both paw placements, the following formula was used to calculate a laterality index [19]:

\[
\text{Laterality Index} = \frac{\text{Left paw placements} - \text{Right paw placements}}{\text{Total left, right, and both paw placements}}
\]

Cylinder testing occurred at approximately 0830 h on day 2, 9, 16, 23, 30, 37, and 44 (Fig. 1).

![Diagram of experimental timeline.](image-url)
**2.3.3. Limb-placement test**

The limb-placement test (a variation of [20]) was employed to assess asymmetry in limb placement. All testing occurred on an elevated surface with 90° corners between the top and sides of the structure (i.e. a table top). The rats underwent experimenter manipulations and the left and right paws were scored on a 0-2 scale. If the limb was not placed, it was scored as a 0, complete and immediate placing was scored as a 2, and a 1 was given for anything in between such as displaced, it was scored as a 0, complete and immediate placing was scored as a 2, and a 1 was given for anything in between such as displaced, or falling (Fig. 2). The total score was calculated with a maximum score of 16 points. The limb-placement test took place at approximately 0930 h on day 2, 9, 16, 23, 30, 37, and 44 (Fig. 1).

**2.3.4. Rotorod test**

Rotorod testing was used to assess gross motor movement [21]. Rats were placed opposite of rotation on a 7.3 cm diameter rod elevated 70 cm above the floor rotating 4 rpm and accelerating to 40 rpm over 180 s (Columbus Instruments, Columbus, Ohio). Once the rat fell, or was removed, from the rod, the time was recorded as latency to fall. The rat was removed from the rod at 180 s, or if the rat clung to the rod through two rotations. Three trials were conducted each testing day, with a 10-minute inter-trial interval. Rotorod testing occurred at approximately 1600 h on day 2, 9, 16, 23, 30, 37, and 44 (Fig. 1).

**2.4. Operant testing**

On day 24, rats were reduced to approximately 85% of their free feeding weight and were maintained at this weight through the remainder of the study via daily supplemental feedings (Fig. 1).

**2.4.1. Operant apparatus**

Operant chambers were housed inside sound-attenuating cubicles (from Med Associates Inc., St. Albans, VT) which contained fans for feeding weight and were maintained at this weight through the remainder of the study via daily supplemental feedings.

<table>
<thead>
<tr>
<th>TASK</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4 &amp; 5</th>
<th>6</th>
<th>7 &amp; 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rat is lowered toward the ground by the tail</td>
<td>Rat’s view of the ground is obstructed during normal stance</td>
<td>Rat is suspended by head and tail and forepaws touched to the side of the surface</td>
<td>Rat is suspended same as task 3, but each the left and right sides are touched to the surface edge instead of the front</td>
<td>Rat is on surface and pushed forwards towards the edge</td>
<td>Rat is pushed toward the surface edge laterally from each side</td>
</tr>
<tr>
<td>SCORING</td>
<td>2-immediate reaching for ground</td>
<td>1-delayed or unready reaching</td>
<td>0-no-reaching</td>
<td>Scoring same as previous task</td>
<td>Scoring same as previous task</td>
<td>Scoring same as previous task</td>
</tr>
<tr>
<td>SCORING</td>
<td>2-paw maintains contact with the ground</td>
<td>1-paw lifts after delay or falls</td>
<td>0-paw immediately lifts from ground</td>
<td>1-paw placed on surface top after delay or with uncontrolled movements</td>
<td>0-paw never lifts</td>
<td>2-paw grips surface edge to maintain contact with surface</td>
</tr>
<tr>
<td>SCORING</td>
<td>2-paw lifts after delay or falls</td>
<td>0-paw immediately lifts from ground</td>
<td></td>
<td></td>
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**Fig. 2.** Scoring for the limb-placement test.

**2.4.3. Behavioral flexibility task**

Beginning on day 37, rats began the behavioral flexibility operant task. The behavioral flexibility operant task was conducted to assess ability to learn new reinforcement paradigms [13]. During a behavioral flexibility session, reinforcers were earned by completing 5 consecutive lever presses on the active lever (either the left or the right lever as chosen initially at random). No stimulus was associated with the active lever. Responses on the inactive lever had no programmed consequence. At the completion of 5 consecutive active lever-presses, a reinforcer was delivered, and the alternate lever became active. After each earned reinforcer, the active lever was alternated until 40 min elapsed and the session ended. Reinforcers earned, along with active and inactive lever presses, were recorded during each session. Sessions were conducted at approximately 1000 h each day for 7 continuous days.

**2.5. Histology**

Rats were euthanized on day 45 with an overdose (120 mg/kg) of sodium pentobarbital (Fetal Plus, Vortech Pharmaceuticals, Dearborn, MI) and transcardially perfused with 0.1 M phosphate buffered saline (PBS, pH 7.4) and then 4% paraformaldehyde (PFA; dissolved in 0.1 M PBS, pH 7.4). Brains were collected and submerged in 4% PFA over night at 4 °C and then moved to a sucrose solution (30% w/v; Fisher Scientific, Waltham, MA) at 4 °C until the tissue was saturated. Tissue was then frozen with anhydrous methylbutan (Sigma-Aldrich, St. Louis, MO) packed in dry ice and stored at ~80 °C until sectioned. Using a cryostat (Vibratome, St. Louis, MO), brains were sectioned coronally at 20 μm and directly mounted onto positive-charged slides (Thermo Fisher Scientific, Waltham, MA). Thirteen equally spaced regions of interest were collected throughout the striatum, beginning at approximately +2.20 mm from bregma and ending at approximately -4.16 mm from bregma.

Hematoxylin and eosin (H&E) staining was utilized to visualize the damaged area of the brain. The tissue was heat-fixed to the slide at ~90 °C for 2.5 min. Slides were brought back to room temperature and then submerged for 60 s in tap water. Slides were submerged in 0.1% Meyers Hematoxylin (Sigma-Aldrich, St. Louis, MO) for 8 min before going into running tap water for 10 min. The tissue was then immersed and the rats trained to lever-press on a fixed ratio 1 (FR1) schedule of reinforcement, whereby a lever-press on either lever delivered a reinforcer. The FR1 training sessions completed once 20 reinforcers were earned or an hour had elapsed, whichever occurred first. After learning to respond on a FR1 schedule, the rats were placed on a FR5 schedule of reinforcement, which required 5 consecutive lever presses on either lever to earn a reinforcer. Sessions continued until 20 reinforcers were earned or 1 h had elapsed, whichever occurred first. Training sessions took place throughout week 5 at approximately 1000 h each day (Fig. 1).
in a 0.05% ethanol eosin (Sigma-Aldrich, St. Louis, MO) for 2 min. Directly following the H&E staining the slides went into a 50% ethanol solution for 30 s (all alcohols from Deacon Laboratories, Inc., Allentown, PA), a 70% ethanol solution for 30 s, a 90% ethanol solution for 30 s, and a 100% ethanol solution for 1 min. Tissue was then submerged in xylene (Sigma-Aldrich, St. Louis, MO). Hematoxylin and eosin images were scanned using a Nikon Coolscan IV scanner (Nikon, Melville, NY) and analyzed with Image J software (NIH, Rockville, MD). As described previously [12], tissue was assessed for damage and reported as a percentage of the contralateral hemisphere.

2.6. Statistical analysis

All statistics were calculated in SPSS (IBM, Armonk, NY) with an alpha level of $p < 0.05$. Behavioral baselines and infarct size between groups were analyzed using independent samples t-tests while behavioral tasks following MCAo were analyzed using repeated-measures ANOVA. If Mauchly’s test of sphericity was found significant, a Greenhouse-Geisser correction was applied. Infarct size in the MCAo group was correlated with the last session of each behavioral measure using Pearson correlation.

3. Results

3.1. Infarct size

An independent samples t-test showed an increase in damaged area in the 60-minute MCAo group as compared to the sham group, $t(18) = -3.21, p = 0.005$ (Fig. 3). Infarct size in the 60-minute MCAo group did not significantly correlate with any behavioral measure except for the limb-placement task (Fig. 4A) which was negatively correlated to infarct size ($r = -0.727, n = 10, p = 0.017$; Fig. 4B).

3.2. Cylinder task

The laterality index was calculated and averaged by group for each session (Fig. 5). No significant difference was found during baseline, $t(18) = 0.039, p = 0.970$. The laterality index was compared between groups across sessions using a 2 x 7 mixed ANOVA. Mauchly’s test of sphericity was not significant ($p = 0.214$) so no correction was applied. There was a significant difference in laterality index between groups ($F(1,16) = 34.077; p < 0.001$) although no significant difference was found over time ($F(6,96) = 0.836; p = 0.545$) and there was no significant group x session interaction effect ($F(6,96) = 1.713; p = 0.126$).

3.3. Limb-placement task

The total score obtained for the left side (side of deficit) for each rat was used for statistical analysis (Fig. 6). Baseline data showed no significant difference between groups, $t(18) = 0.849, p = 0.407$. Limb-placement scores were compared between groups across sessions using a 2 x 7 mixed ANOVA. Mauchly’s test of sphericity was not found to be significant ($p = 0.075$) and no correction was applied. There was no significant difference across sessions ($F(6,108) = 0.705; p = 0.647$) although there was a significant difference between groups ($F(1,18) = 7.802; p = 0.012$), and a significant group x session interaction ($F(6,108) = 2.365; p = 0.035$).

3.4. Rotorod task

The average of all three trials within each rotorod session was used for statistical analysis (Fig. 7). Baseline data revealed no significant difference between groups, $t(18) = 0.983, p = 0.339$. A 2 x 7 mixed ANOVA revealed Mauchly’s test of sphericity not significant ($p = 0.053$) so no correction was used. There was a significant difference between groups ($F(1,18) = 9.219; p = 0.007$) but no difference was found in latency to fall across sessions ($F(6,108) = 1.909; p = 0.086$) and no group x session interaction effect was observed ($F(6,108) = 0.717; p = 0.637$).

3.5. Operant measures

There were two sham rats that never contacted the alternating lever contingency (i.e. never earned more than a single reinforcer in a session) which were excluded from the analyses. With the exclusion criteria in place, the number of reinforcers earned, the total number of lever presses, and the percentage of incorrect lever presses was compared across all 7 operant sessions between the sham ($n = 8$) and 60-minute ($n = 10$) MCAo group.

The total number of lever presses was examined between groups across sessions using a 2 x 7 mixed ANOVA (Fig. 8A). Mauchly’s test of sphericity was significant ($p < 0.001$) and the Greenhouse-Geisser correction was used. Total number of lever presses significantly increased across sessions ($F(2.500,39.993) = 9.100; p < 0.001$), and was significantly different between groups ($F(1,16) = 9.978; p = 0.006$). No significant group x session interaction was observed ($F(2.500,39.993) = 0.681; p = 0.543$).

The number of reinforcers earned was examined using a 2 x 7 mixed ANOVA to indicate the ability of rats to learn the alternating active lever pattern (Fig. 8B). Mauchly’s test of sphericity was found to be significant ($p < 0.001$) so the Greenhouse-Geisser correction was used. The number of reinforcers earned significantly increased across sessions ($F(1.428,22.849) = 27.509; p < 0.001$) and differed significantly between groups ($F(1,16) = 12.264; p = 0.003$). There was also a significant group x session interaction effect ($F(1.482,22.849) = 4.222; p = 0.039$).

Since a significant difference was found between groups in the total number of lever presses, the number of incorrect lever presses (presses made on the inactive lever) were represented as a percentage of overall lever presses to diminish any confounding effects of overall motoric differences (Fig. 8C). The percentage of incorrect lever presses was
analyzed using a 2 × 7 mixed ANOVA, and Mauchley’s test of sphericity was found to be significant (p < 0.001) so the Greenhouse-Geisser correction was used. The percentage of incorrect lever presses was shown to significantly decrease over time (F(2.125,33.997) = 23.034; p < 0.001), and was shown to be significantly different between groups (F(1,16) = 10.607; p = 0.005). No group x session interaction effect was observed (F(2.125,33.997) = 1.353; p = 0.273).
4. Discussion

All behavioral tasks conducted within this study (cylinder, limb-placement, rotorod, and operant testing), revealed significant differences across groups over seven weeks of testing. The infarct size, as shown by H&E staining, also revealed a significant difference at 45 days after surgery. The infarct size was also negatively correlated with the limb-placement task. Taken together, it appears that when using this MCAo model in rats, these specific tasks are sufficient to identify deficits up to seven weeks after stroke. The behavioral flexibility operant task was used in our study to determine the ability of each rat to adapt to changing environmental contingencies. We found that it provided a long-term, sensitive, cognitive measure that successfully detected long-term deficits in a rodent stroke model. While behavioral flexibility can be tested in a variety of ways, such as the Wisconsin Card Sorting task in humans [22], our use of a simple alternating contingency operant task that required rats to learn to alternate back and forth on two levers to earn reinforcers proved successful in distinguishing rats with strokes from those given sham surgery. This task was a modified version of the one used by Linden et al. [13] whereby mice given MCAo were tested 30 days after surgery and showed deficits. Linden et al. [13] concluded that the disruption of behavioral flexibility is due to damage that occurs explicitly in the dorsomedial striatum. This conclusion is supported by others who have shown that damage to the dorsomedial striatum produces deficits in other measures of behavioral flexibility as well [23,24].

Behavioral flexibility can be measured in a variety of ways, like the operant task we used here where the percentage of incorrect lever presses is the representative measure of flexibility. However, there are other constructs that this specific operant task may be measuring, including motor deficits, differences in food motivation, or even a deficit in general learning of the lever pressing contingency.

While the lower number of total lever presses in the MCAo group compared to the sham group could support the argument that deficits in the operant task are due to motor deficits rather than deficits in behavioral flexibility, it would be unlikely given that lever presses can be made using multiple topographies (the rat can use the contralateral limb, the ipsilateral limb, their teeth, etc.) and does not require the use of the impaired limb. The number of incorrect lever presses was also shown as a percentage of overall lever presses, further suggesting that motor deficits did not confound the results obtained on this task. It is also unlikely that these results are due to a difference in food motivation, as all rats were similarly motivated during training to earn food pellets. During the same training period, all rats learned to lever press for food pellets within the time allotted and consumed all food pellets that were delivered. The successful training of all rats also suggest that our results are a product of deficits in behavioral flexibility deficit rather than a general learning impairment. Also, the data obtained from any rat that did not contact the alternating lever contingency (i.e., never earned two reinforcers within a single session) was not used in the analyses of the operant task. This was done to ensure all rats being analyzed were being assessed for their inability to adapt to changing environmental contingencies (i.e., the alternating active lever).

To our knowledge, this is the first time that the behavioral flexibility operant task was used to assess long-term (6-weeks post-MCAo) cognitive deficits in this rat model of stroke. The findings confirmed those of Linden et al. [13] who found deficits at 3-weeks post-MCAo. In both of these studies, the sham-operated rats earned more reinforcers and made less incorrect lever presses than those given MCAo. These findings support the contention that the behavioral flexibility operant task provides a promising protocol for detecting stroke deficits, since it is objective, only takes a week of testing, can detect deficits 30 + days following stroke, has numerous measurable parameters, and is comparable to human tasks (such as the Wisconsin card sorting task). While the behavioral flexibility operant task in this situation was sufficiently sensitive to detect deficits in MCAo rats, it also proved to be so challenging for a few rats that never learned the task. Though the absence of learning is still valuable information, changing how the task is completed (making it easier, running it longer than seven days, or conducting more rigorous training) may provide a means of refining the task.

4.1. Conclusions

In conclusion, we found that the behavioral flexibility operant task was sufficiently sensitive to detect deficits in rats for up to seven weeks following stroke and that a 60-minute MCAo was adequate to produce clear deficits. Pairing the behavioral flexibility task with rotorod, limb-placement, and cylinder, gave a well-rounded battery of behavioral tasks that revealed significant chronic deficits in the rat MCAo stroke model, which should translate well to the human condition and provide a useful model to test potential therapies.

Declaration of interest

None.

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