BEHAVIORAL ASSAYS TO STUDY SENSORIMOTOR DEFICIT AND RECOVERY IN RATS FOLLOWING MIDDLE CEREBRAL ARTERY OCCLUSION

A Senior Scholars Thesis

by

GLORIAN MARI ROMAN-CRUZ

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2010

Major: Biology

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Approved by:

Co-Research Advisors:	Farida Sohrabji
	Amutha Selvamani
Associate Dean for Undergraduate Research:	Robert C. Webb

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ABSTRACT

Behavioral Assays to Study Sensorimotor Deficit and Recovery in Rats Following Middle Cerebral Artery Occlusion. (April 2010)

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The goal of this study was to identify a sensorimotor behavioral test that predicts infarct volume in animals with ischemic stroke. Stroke is the major cause of behavioral deficits in humans and research on stroke therapies require that neuroprotective compounds should both reduce infarct volume and improve performance. Previous work from our lab shows that estrogen treatment to older acyclic female rats increases infarct volume as compared to animals that have not received estrogen. Here, we used estrogen replaced and estrogen deficient animals and tested their behavior with the corner test, in conjunction with three other behavioral assays (the forelimb placement test, the rotarod test, and the four-point neurological score test). Our goal was to determine whether or not there is a correlation between performance during the corner test and ischemic volume. In this study, 16 acyclic (reproductively senescent) rats were used and assigned to one of two groups: one group received estrogen treatment after being ovariectomized via a gradual release pellet and the other did not receive an estrogen treatment. All the rats then went through a stroke surgery 3 weeks after the ovariectomy surgery. Before

the stroke surgery, all rats also went through training on all behavioral assays and then were administered the assays again after the stroke surgery. Behavioral tests were performed every day after stroke, starting 24hr after stroke. Our results indicate that there is a statistically significant negative correlation between performance on the corner test and infarct volume. These data suggest that the corner test may be a useful test for stroke studies that assess new therapeutic compounds.

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There are so many people who have helped me with this project and whom I would like to thank. First of all, I would like to thank Dr. Farida Sohrabji for the amazing opportunity to do research in her lab as well as for all the guidance she has given me throughout this process. On that note, I would also like to thank everyone at the Sohrabji Lab for all of their patience, support, and all the memorable experiences. Last, but not least, I would like to thank Dr. Amutha Selvamani who took me under her wing and "showed me the ropes" in terms of research. Without all of you, this wouldn't have been possible. Thanks again everyone for making this such an awesome and memorable experience.

NOMENCLATURE

MCAo	Middle Cerebral Artery Occlusion

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CHAPTER I

INTRODUCTION

According to data from the CDC, stroke is the third leading cause of death in the United States for the general population as well as the elderly (Sahyoun et al., 2001). It has also been found that in elderly populations, women are more at risk for a fatal stroke or a stroke resulting in disability than men are and are at an elevated risk between the ages of 45 and 54 which coincides with the ages associated with menopausal onset (Towfighi et al., 2007). Previous studies have shown that acyclic reproductively senescent females (an animal model of the menopause) have a large cortical-striatal infarct after a middle cerebral artery occlusion resulting in stroke, and estrogen treatment to this group increases the severity of brain damage. (Selvamani and Sohrabji 2008). In order to study the affects of a middle cerebral artery occlusion on sensorimotor function, the vibrissae-elicited forelimb placement test is used and has been found to be effective in detecting sensorimotor deficit as well as severe loss of interhemispheric sensorimotor integration (Woodlee et al., 2005, Selvamani and Sohrabji, 2008). However this test is not sensitive enough to detect differences in severity of infarct. In order to study sensorimotor recovery over a prolonged period of time a more sensitive test is needed. In a literature survey we have identified 2 tests that are more appropriate for measuring recovery. Several studies have shown that the rotarod test is a sensitive test for observing and

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This thesis follows the style of the Journal of Neuroscience Methods.

for the assessment of motor impairment induced by ischemia in the rat. In addition, the rotarod test results in objective quantitative data over the rat's balance and motor coordination (Sayeed et al., 2007 and Zhang et al., 2002). This test involves the use of a suspended beam within a semi-enclosed chamber that rotates, thus forcing the rat to maintain balance. Throughout the trial, the rod accelerates gradually, and eventually, the rat loses its balance and falls off the beam. The amount of time the rat remains on the beam is recorded (Kadam et al., 2009). Even though the rotarod test has been widely used in order to establish a correlation between infarct volume and differences in behavior between stroke and control groups after MCAo, these results have only shown a significant correlation up until 7 days post stroke (Lim et al., 2008, Hunter et al., 2000 and Rogers et al., 1997).

The corner test has demonstrated sensitivity as well as objectivity when applied to assess the long term functional outcome after stroke (Zhang et al., 2002). The corner test integrates vibrissae stimulation, postural motor function, and the use of the forelimbs and hindlimbs in order to observe changes in behavior due to ischemia. The rat is placed between two boards that are placed at a 30˚ angle to one another. The room and corner are then darkened in order to entice rat to the corner. Once in the corner, the rat's vibrissae will be stimulated, causing the rat to rear and then turn either left or right facing away from the corner.

In this study, a series of behavioral tests including the corner test and the rotarod test, were performed routinely after MCAo while the vibrissae-elicited forelimb placement

test was performed before MCAo as well as 7 days post MCAo. With a battery of behavioral tests such as the rotarod test, the corner test, and the vibrissae-elicited forelimb placement test, as well as the information already gained from past experiments concerning the effect of estrogen on stroke, we expect to correlate performance on the task with infarct volume (severity of brain damage) as well as evaluate long term functional outcomes after stroke (Zhang et al., 2002).

CHAPTER II

MATERIALS AND METHODS

Animals and housing

Female retired breeder Sprague Dawley rats were purchased from Harlan Laboratories and housed two to a cage and allowed free access to both food and water. All the animals were kept under a 12 hour dark and 12 hour light cycle. Vaginal smears were obtained daily from all animals in order to verify the length of their estrous cycle or if they were acyclic.

Determination of estrus cycle

Vaginal smears were acquired daily with a cotton swab and then put on a slide. The cells on the slide were then observed under a microscope (Olympus Leeds Instruments, TX; 20x objective). The cells were then categorized as being either in the proestrus, estrus, metestrus, or diestrus stage of the estrus cycle. Cells in the proestrus stage are usually round epithelial cells, those in estrus are usually enucleated cornified cells, metestrus cells appear to be cornified and in proportional numbers to leuckocytes, and the diestrus stage is usually characterized by the presence of a few cells and a thick mucus. The Smears were acquired over a 21 day period and any rat that stayed in one stage for 7 days were considered acyclic. Since reproductively senescent rats were the main focus of this study, those that were in constant diestrus were included in the present study.

Surgical procedures

Ovariectomy and hormone treatments

The animals were anesthesized with a solution of xylazine (200mg/kg) and ketamine (10mg/kg). A bilateral ovariectomy was completed by means of a dorsal midline incision and the established procedure by Jezierski and Sohrabji (2000). The ovaries were then removed and then 60-day time-release 17β-estradiol pellets or control pellets were positioned subcutaneously before the incision was closed. These time-release pellets have been used in previous studies and were designed to keep the plasma hormone level between 60 pg/ml and 80 pg/ml. It has been shown that these levels are maintained for 3, 4, and 6 weeks, although an initial supraphysiological increase of hormone levels has been reported immediately after placement of the pellet (Singh et al., 2008). Instead of receiving an estradiol pellet, the control animals received a pellet made up of the same material but that contained no hormone. Once the animals were sacrificed, the uterus was removed, cleaned of fat and weighed.

Intracerebral transient MCAo

Animals were anesthetized and then subjected to a stereotaxic surgery in order to occlude the middle cerebral artery (MCAo). During the surgery, oxygen saturation levels and respiratory rate were monitored constantly using the Mouse Oximeter (STARR Life Sciences Corp., PA). In addition, the animals were maintained at 37˚C throughout the surgery. In order to achieve a MCAo, after being anesthetized and positioned in a Kopf

stereotaxic device, a midline incision was made in the scalp. A small hole was then made on the left side of the skull, using specific coordinates (stereotaxic coordinates +0.9 mm anterior, +3.4 mm relative to Bregma and a depth of -8.5 mm from the dural surface), by using a small drill. 3 µl of a 0.5 µg/µl solution of endothelin-1 (American Peptide Company INC, CA) was injected through the hole using the procedure described in Selvamani and Sohrabji (2008) in order to cause a MCA occlusion. The rate at which the endothelin-1 was injected was 1.0 µl over 2 minutes. Once the solution was injected, the 10 µl Hamilton syringe was removed slowly 3 minutes after the solution was administered. The needle used in conjunction with the Hamilton syringe was a 26s/2 in./2S needle. The animals used as controls were not injected with the endothelin-1, instead they were injected with saline. After the occlusion, animals were put under heat lamps while recovering and then later transported to the animal holding room to continue recovering from both the surgery and anesthesia. After being monitored daily, the animals were sacrificed at 7 days post-MCA occlusion (Selvamani and Sohrabji, 2008).

TTC staining

Brains are immediately removed from the skull after the animals are terminated and then placed in a brain mold (BrainTree, MA) and manually sectioned into 2 mm slices. Slices between the rostral measurement of -2.00 mm and the caudal measurement of +4.00 mm from Bregma were analyzed. The slices were then placed on ice and later incubated in 2% TTC solution for 20 minutes at 37˚C. The slices are then fixed in 4% paraformaldehyde for 5 minutes at room temperature, observed under a microscope, and photographed with a Nikon E950. The Quantity One software program (Bio-Rad, CA)

was used to quantify infarct volume. Typically 3 slices are analyzed using the algorithm described in Selvamani and Sohrabji (2008). Cortical and striatal infarct was measured separately and normalized to the cortical or striatal volume of non-occluded hemisphere.

Behavioral testing

Corner test

The Corner test was performed as established in Zhang et al. (2002) with a couple of modifications. The rat was placed between two wooden boards 60 in. in length, 1 in. in width, and 12 in. in height. The boards were at a 30˚ angle to one another and the rat placed facing the corner halfway between the corner and the open ends of the boards. The room lights were then turned off and a cover placed on the corner in order to entice the rat to go to the corner. Once in the corner, the rat usually rears and then turns either left or right. Ten trials were performed and the experimenter recorded the side that the rat turned. If the rat did not go towards the corner or turn within one minute, then the trial was started over again. The rat was also not handled for the 30 seconds immediately after a turn.

Rotarod test

Each rat was exposed to the Rotarod two days before MCAo in order to acclimatize the animal to the apparatus. Each animal is placed on the suspended beam of the Rotarod, facing away from the viewer, for 1 minute. Animals were taken off the Rotarod once they fell off of the beam, and placed back on the Rotarod for 3 trials that were 5 minutes in

length. If they fell off the beam before the allotted time, they would be placed back on the beam. The length of time spent on the beam was recorded. The animals were also tested post-MCAo daily for seven days. During this time, animals were placed on the accelerating beam and the time at which they fell off the beam was recorded unless it was less than 5 seconds, in which case the trial was started over again. If the animal fell off the beam before 30 seconds, then the animal would be given 5 minutes off the beam and then placed back on the beam for an additional 2 trials. If the animal fell off at or after 30 seconds, then the animal would only go through 1 trial. All times were recorded for trials over 5 seconds.

Vibrissae-elicited forelimb placement test

This test was performed 2 days before MCAo as well as 7 days post MCAo as described in Woodlee et al., (2005) and Selvamani and Sohrabji, (2008). This was done by same-side forelimb placing trials as well as cross-midline placing trials. A detailed description of the procedure is reported in Selvamani and Sohrabji, (2008).

Statistics

The infarct data was analyzed using a 2-way ANOVA coded for region (cortex or striatum) and treatment (estrogen or no estrogen). The vibrissae-elicited forelimb placement test was analyzed using a paired t-test. For the rotarod test, the recorded times were correlated with infarct size. In addition, for the corner test, the number of right turns was correlated to infarct size.

CHAPTER III

RESULTS

Infarct size

Three weeks after ovariectomy surgery (with and without estrogen treatment), animals were then subjected to a MCAo. Seven days after the occlusion, the animals were sacrificed and the brains extracted in order to analyze infarct volume. For both sets of animals, the infarct volume spanned both the cortex and the striatum. As seen in figure 3.1, reproductively senescent animals that received estrogen had significantly larger infarct volume than those animals that received the placebo ($F_{(1,4)}$ 57.82, $p<0.05$.).

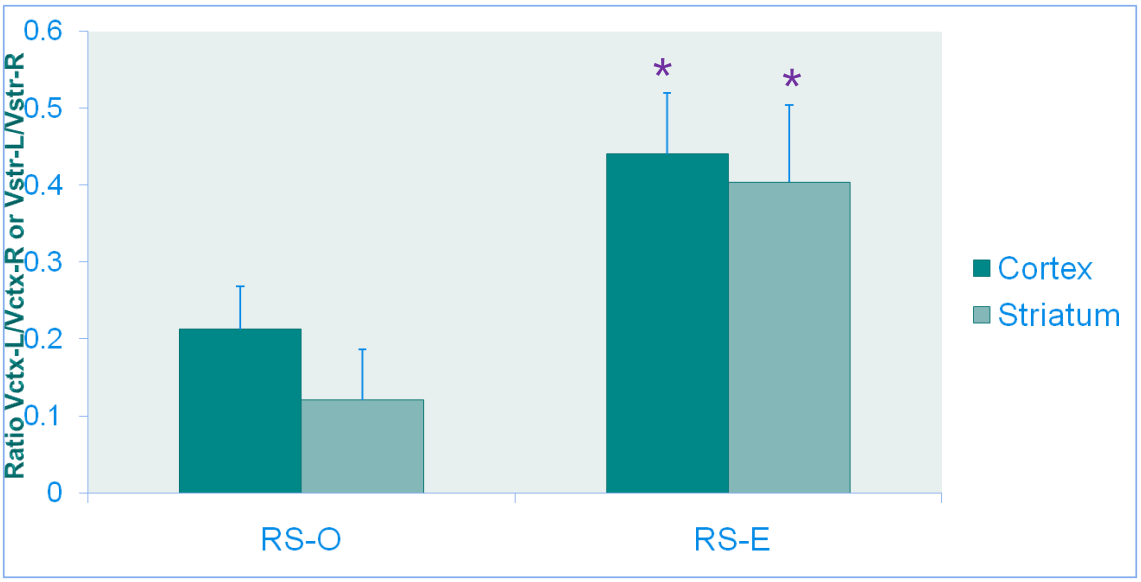

Fig. 3.1 Effect of estrogen on infarct volume in reproductively senescent females. In both the cortex as well as the striatum, there was a significantly larger infarct size in the estrogen treated (E) group of females as compared to the placebo (O) treated group.

Vibrissae-elicited forelimb placement test

The animals were subject to the vibrissae-elicited forelimb placement test before MCAo. All the animals scored either a 3 or a 2 which were considered successful trials. Post surgery, trials were performed on days 1, 3, 5, and 7 days after MCAo. On these trials, both groups revealed a statistically significant behavioral deficit as compared to pre-MCAo. For the same-side test, there was a significant difference in performance between pre and post-MCAo in that the animals performed worse on the right side post-MCAo than pre-MCAo as seen in figure 3.2a. For the cross-midline test, there was a behavioral deficit both on the left and right sides with there being a significant difference on the right side post-MCAo when compared to pre-MCAo as seen in figure 3.2b. There was no statistically significant difference between reproductively senescent animals that were given an estrogen treatment and those that were given a placebo.

(a)

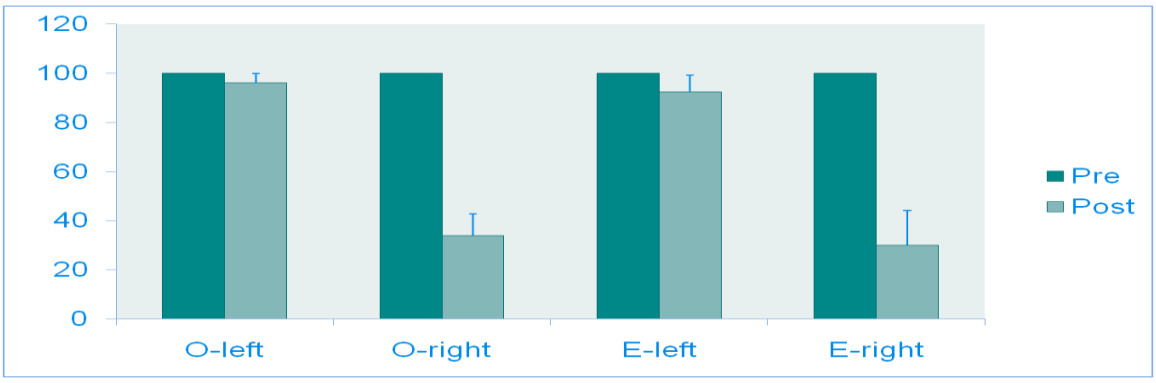

Fig. 3.2 Effects of estrogen on the vibrissae-elicited forelimb placement test. (a) In the same-side test, there was no significant difference in behavior on the left side when comparing the results of the trials pre and post stereotaxic surgery. There was, however, a significant difference detected on the right side when comparing pre and post stereotaxic surgery. There was no significant difference found between the estrogen treated (E) group and the placebo treated (O) group.

(b)

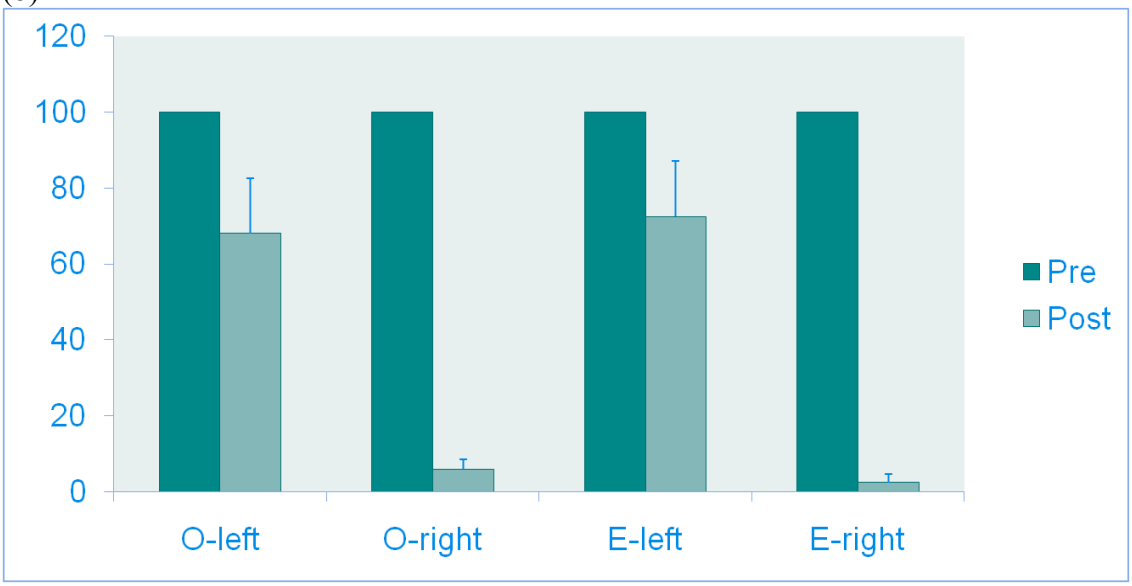

Fig 3.2 Continued. (b) In the cross-midline test, both sides were visibly affected. The right side was more significantly different when comparing pre and post stereotaxic surgery. There was no significant difference in behavior between the estrogen treated (E) group and the placebo (O) treated group.

Corner test

The corner test was performed 2 days before stereotaxic surgery as a training and then performed 24 hours, 3 days, 5 days, and 7 days after stereotaxic surgery. As seen in figure 3.3, before the surgery, animals usually made a minimum of 5 right turns. Post-MCAo, however, there was a significant difference in performance between the estrogen treated group and the placebo treated group in that the placebo treated group made a significantly larger amount of right turns as compared to left turns. Also, there was a significant negative correlation between the number of right turns and the cortex and striatum infarct volume (-0.855 and -0.947, respectively).

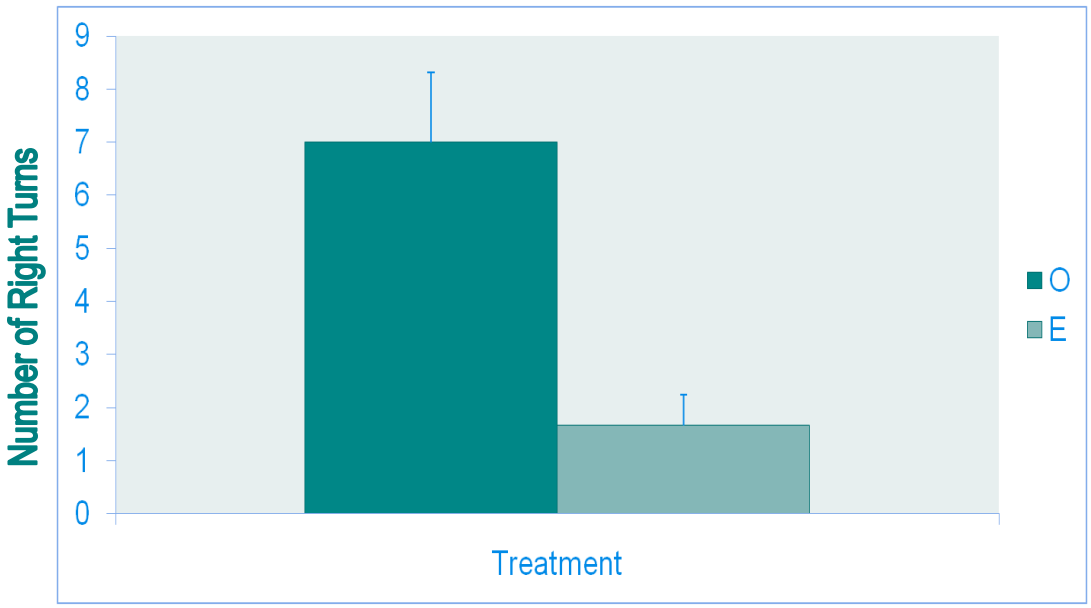

Fig. 3.3 Effects of estrogen on the number of right turns on the corner test. The estrogen treated (E) group made a significantly lower number of right turns when compared to the placebo treated (O) group.

Rotarod test

Like the corner test, the rotarod test was performed 2 days before MCAo as well as on day 1, 3, 5, and day 7 after MCAo. As seen in figure 3.4, there was no significant difference between the estrogen-treated group of reproductively senescent female animals and the placebo-treated group. In addition to this, there was no significant correlation between the infarct size and time spent on the rotarod.

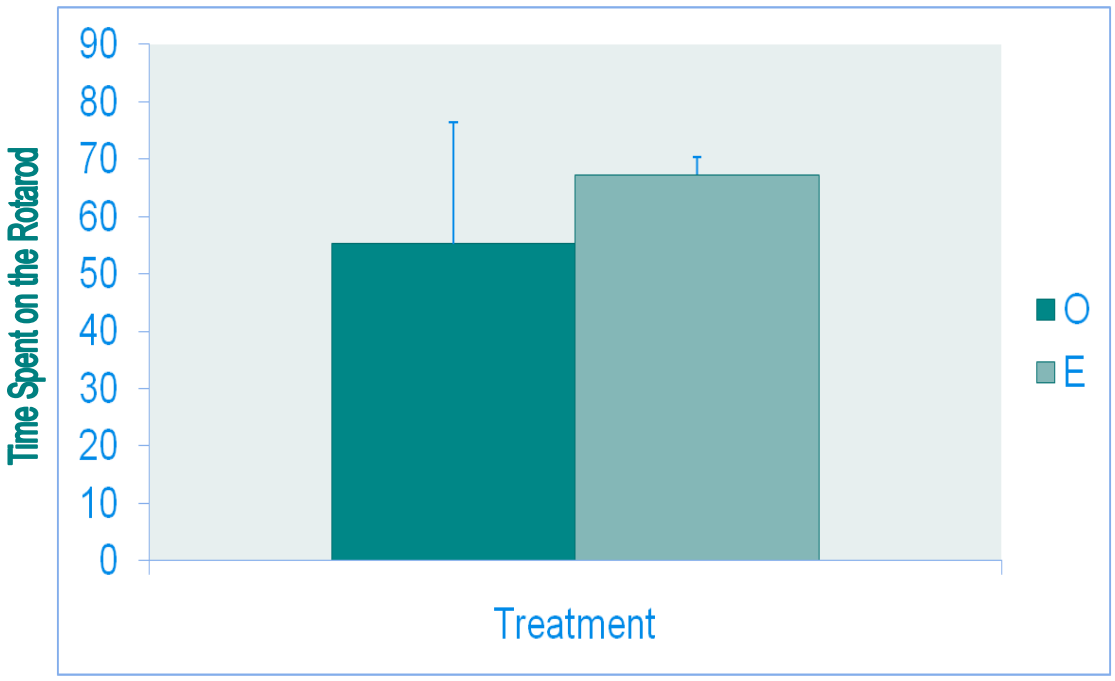

Fig. 3.4 The effect of estrogen on behavior on the rotarod. There was no statistically significant difference on time spent on the rod between the estrogen treated (E) group and the placebo treated (O) group.

CHAPTER IV

SUMMARY AND CONCLUSION

Discussion

In the present study, we compared the effectiveness of three different behavioral assays to detect a sensorimotor deficit and to discriminate the severity of tissue damage after MCAo. Consistent with previous studies, reproductively senescent females who received estrogen treatment incurred a larger striatal and cortical infarct than those reproductively senescent females who were given the placebo treatment. The three tests that were compared were the vibrissae-elicited forelimb placement test, the rotarod test, and the corner test. The vibrissae-elicited forelimb placement test was found to detect damage due to stroke on both the same side as well as the cross midline tests. However, in the same side test, there was only a significant difference in behavior when comparing pre and post-MCAo on the right side. This is to be expected as the stroke was induced on the left side of the brain and thus the right side of the animal would be affected. In the cross-midline test, a significant difference was found between post and pre-MCAo in both the right and left sides. In the rotarod test, there was no significant difference in behavior between the estrogen treated females and the placebo treated females. This is surprising in that the rotarod in past studies has been found to be a reliable behavioral assay to detect sensorimotor deficit. However, the results obtained from this study have shown that there is no correlation between infarct volume and performance on the rotarod and that the rotarod is not sensitive enough to distinguish between more and less severe tissue damage. The corner test, on the other hand, was found to be more effective and more reliable at these than the rotarod test. Specifically, estrogen treated animals (which had

larger infarcts) actually completed fewer right turns than the placebo treated group (which had smaller infarcts). This was underscored by a statistically significant negative correlation between infarct volume and the number of right turns performed. Since the stroke was induced on the left side, the right side would be affected. In theory, an intact animal would make an unbiased number of right turns, which in this case would be 5, whereas an animal that has had a MCAo would make more left turns than right turns. This proved to be the case in the present study. Our results therefore show that the corner test may be a good predictive assay for the extent of tissue damage in stroke and especially useful for studies where novel therapies are being tested. Although both tests measure sensorimotor integration, the corner test is likely a low stress test compared to the rotarod test, since it involves a behavior that is natural for the animal (seeking a corner) as opposed to a balancing behavior which is less natural and more stressful.

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