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Effects of Voluntary Physical Rehabilitation on Neurogenesis In SVZ And Functional Recovery After Ischemic Stroke

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EFFECTS OF VOLUNTARY PHYSICAL REHABILITATION ON NEUROGENESIS IN SVZ AND FUNCTIONAL RECOVERY AFTER ISCHEMIC STROKE

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fulfillment of the requirements for
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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Anuranjani Balakrishnan ENTITLED Effects of Voluntary Physical Rehabilitation on Neurogenesis in SVZ and Functional Recovery after Ishemic Stroke BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Stroke is the leading cause of long-term disability and 87% of all strokes are due to ischemic strokes. In this current study, we examined whether voluntary physical rehabilitation can influence neurogenesis (measured by Doublecortin) in the subventricular zone and show improved motor functional recovery in 10-12 month female rats after ischemia. We saw a significant increase in the neurogenesis (measured by doublecortin) of all three regions (anterior, middle and posterior) of SVZ in the rehab animals compared to control group when using a two-way variance ANOVA test, although we were unable to see significant differences in paired t-tests of similar regions for control and rehab animals. The control animals showed a significant increase in contralateral functional recovery of 56% with rehab animals displaying a recovery of 23%. These findings suggest that the physical rehabilitation showed increased neurogenesis in the SVZ but did not translate to greater contralateral functional recovery.

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ANOVA	Analysis of variance
AUP	Animal use protocol
AVM	Aeteriovenous malfunction
BDNF	Brain-derived neurotrophic factor
BrdU	5-Bromo-2'-deoxyuridine
CORT	Corticosterone
DCX	Doublecortin
DG	Dentate Gyrus
DPX	Distyrene, aplasticizer and xylene
ELISA	Enzyme-Linked Immunosorbent Assay
ET-1	Endothelin 1
FDA	Food And Drug Administration
GFAP	Glial Fibrillary Acidic Protein
HRT	Hormonal replacement therapy
IACUC	Institutional animal care and use committee
ICH	Intracerebral hemorrhage
L	Left forepaw
MCM2	Mini chromosome maintenance 2
mm	Millimeter
ML	Medial lateral

NEUROD	Neurogenic differentiation
NPC	Neural progenitor cells
NSC	Neural stem cells
NSPC	Neural stem progenitor cells
OCT	Optimal cutting temperature
PAX-6	Paired box gene 6
PBS	Phosphate buffered solution
PCNA	Proliferating cell nuclear antigen
Ph3	Phosphohistone h3
PSA-NCAM	Polysialylated embryonic form of the neural cell adhesion
PSD	Post stroke day
R	Right paw
RMS	Rostral migratory stream
SAH	Subarachnoid hemorrhage
SEM	Standard error mean
SVG	Subventricular zone
SVZ	Subgranular zone
TBR-2	T-box brain gene 2
TIA	Transient Ischemic Attack
tpA	Tissue plasminogen activator

WHI	Women's Health Initiative
μL	Microliters

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1. INTRODUCTION

1.1 STROKE:

Stroke is the leading cause of long-term disability. Nearly 800,000 approximately (795,000) people in the United States are affected by stroke every year and about 31 percent have reported repeating attacks (1). There are nearly 7 million stroke survivors in the U.S and stroke is the fifth leading cause of death in the U.S (2).

About 87% of all strokes are due to ischemic stroke (2), and it is characterized by the occlusion of a brain arterial blood vessel (4). Clinical treatments have advanced in the acute time window but long-term treatment remains limited. However, many of the research studies are focusing on approaches to improve the mechanism of brain repair after a stroke to enhance adult neurogenesis (5).

The main types of strokes are ischemic, hemorrhage and transient ischemic attacks (TIA). Each type of stroke is briefly explained in detail below.

1.2 ISCHEMIC STROKE:

Ischemic stroke occurs when the blood supply to the brain is obstructed which causes brain cells to die, deprived of oxygen supply. This can be caused by the fatty deposits lining the blood vessel, a condition called arthrosclerosis. There are two basic types of ischemic stroke caused by these fatty acid deposits: cerebral thrombosis and cerebral embolism (8).

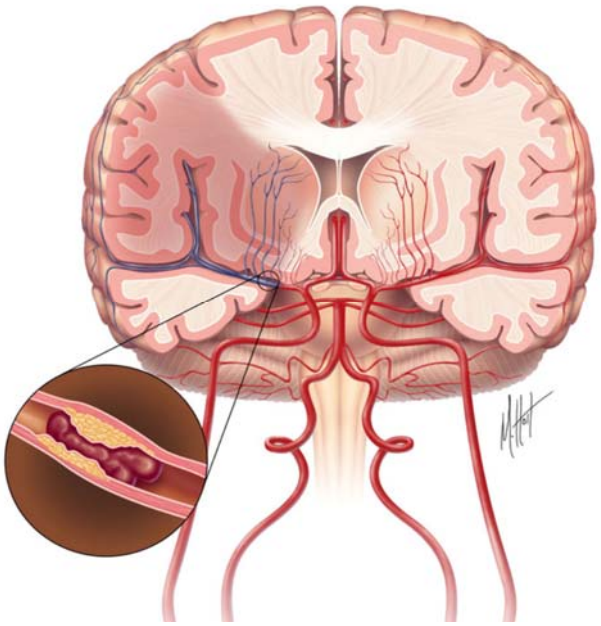


Figure 1: *This image represents an Ischemic stoke showing an obstructed artery to the brain (8).*

Cerebral thrombosis usually occurs when the artery within the brain is blocked by a blood clot (thrombus). This event occurs to 50% of all the stroke victims. Cerebral embolism is also a blood clot but formed somewhere else in the circulatory system, usually the heart which then carries the emboli through the bloodstream and travels to the brain's blood vessels until it becomes lodged. Another risk factor of embolism can be due to irregular heartbeat known as arterial fibrillation (8).

1.3 HEMORRHAGIC STROKE:

Hemorrhagic stroke accounts only for 13% of all strokes. However, 40% of all stroke deaths are due to hemorrhagic stroke (6). The most common causes of hemorrhage stroke include burst aneurysm, arteriovenous malfunction (AVM), arteriolar hypertensive diseases, blood thinners and head injury (7).

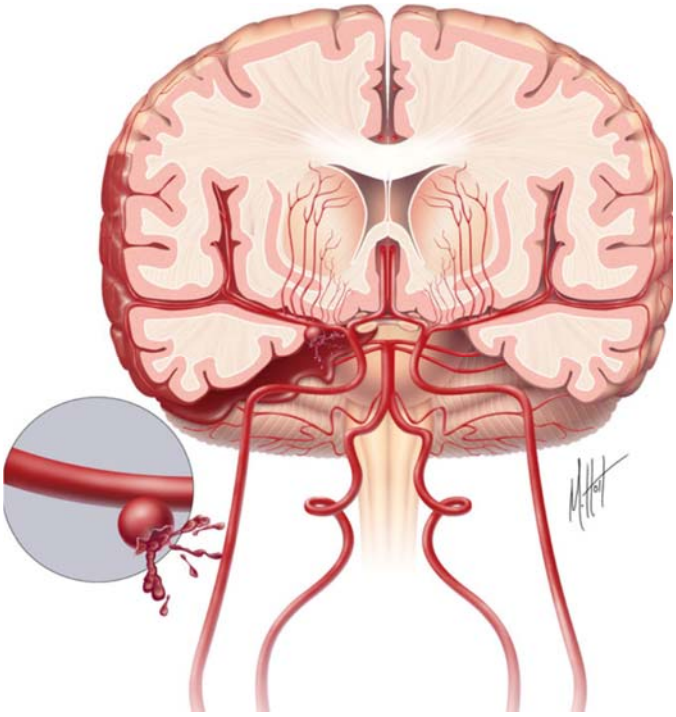


Figure: 2 *The image shows the hemorrhagic stroke of ruptured cerebral aneurysm (8).*

Hemorrhagic stroke occurs when a weakened vessel ruptures and bleeds into the surrounding brain (1). The sudden accumulation of blood increases pressure and compresses the brain tissue. There are two types of hemorrhagic stroke, intracerebral hemorrhage (ICH) and subarachnoid hemorrhage (SAH). ICH occurs when a diseased vessel ruptures and bleeds into the tissue deep inside the brain. The common cause of ICH is due to high blood pressure (hypertension). SAH occurs when a blood vessel ruptures on the surface of the brain and the surrounding brain (the subarachnoid space) are rapidly filled with blood. This can lead to unconsciousness and death due to pressure buildup outside the brain. To reduce the risk of subsequent fatal hemorrhage, endovascular coil embolization is performed as a surgical method. However, there are only few effective options to treat the neurological dysfunction caused by the hemorrhagic stroke (9).

1.4 TRANSIENT ISCHEMIC ATTACKS:

Transient ischemic attacks (TIA) are often known as warning stroke or mini-stroke. They represent a warning of an impending ischemic stroke (10). TIA occurs when an artery is blocked for a short period. The blockage may dissolve on its own by the anticoagulant (blood thinners) in the blood. Approximately 15% of all strokes are led by TIAs. Unlike a stroke, there is no enduring damage to the brain, when a TIA is over.

1.5 CURRENT TREATMENTS FOR STROKE:

The only FDA approved treatment for ischemic stroke is thrombolytic tissue-type plasminogen activator (tPA) (1). It works by dissolving the blood clot and improves blood flow to the brain. Treatment within 3 to 4.5 hours of stroke onset increases the potential benefit. Due to its time constraints and post-stroke complications including intracranial hemorrhage, if the treatment is given after this time period, only 5-8% of ischemic stroke patients are eligible for the treatment (1,64-66).

Mechanical thrombectomy is a surgical treatment, which involves physical removal of blood clot via stent retriever. This procedure is performed within 6 hours after stroke onset, but beneficial if done within 24 hours of onset (1). Furthermore, other current approaches for stroke therapeutics includes magnetic resonance-guided focused ultrasound, neuroprotection (acute), cell replacement therapies, endogenous stem cells, exogenous stem cells, stereotactic radiotherapy, optogenetic stimulation, transcranial magnetic stimulation, PSD-95, hypothermia, induced stem cells, modulation of circuits (recovery) (68).

1.6 POST-STROKE NEUROGENESIS:

Many experimental studies demonstrate the formation of new neurons in the stroke damaged adult brain. The neural stem progenitor cells (NSPC) are localized primarily in two regions: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SVG) of the hippocampus (11). In the SVZ, the neural progenitor cells migrate through the rostral migratory stream and differentiate into neurons in the olfactory bulb, whereas in the SGZ the NSPCs integrate into the neural network as granule cells of the hippocampus (12). A bulk of promising research studies demonstrates an increase in the proliferation of endogenous neural stem cells (NSC) during an ischemic stroke (13). During an incident of brain ischemia, injury or neurodegenerative damages, the migrating new neuroblast escapes from the migratory pathway and are drawn towards the impaired site to repair and regenerate (15-18). Various exogenous, endogenous modulators can alter post-stroke neurogenesis, including growth factors like brain-derived neurotropic factor (BDNF) (14).

1.7 NEUROGENESIS IN THE SVZ:

Most of the newly formed neurons are formed in the subventricular zone of the brain. It is said that SVZ sustains a pool of neural progenitor cells (NPC) that regularly replaces with neurons in the olfactory bulb. While in the case of neurodegenerative disorders, the proliferation of NPCs can be substantially upregulated or downregulated in the SVZ (19). It was reported that the number of proliferating cells significantly increased in the ipsilateral SVZ by 2-14 days with a peak at 7 days after an ischemic attack. In addition, SVZ proliferation showed a similar increase in the olfactory bulb neurons at 14 days and followed by a reduction 28 days after a stroke (5,20,14). Thus understanding the role of endogenous NPCs in

the subventricular zone can aid in analyzing neurogenesis at the site of injury and potentially benefit from the stroke recovery (21).

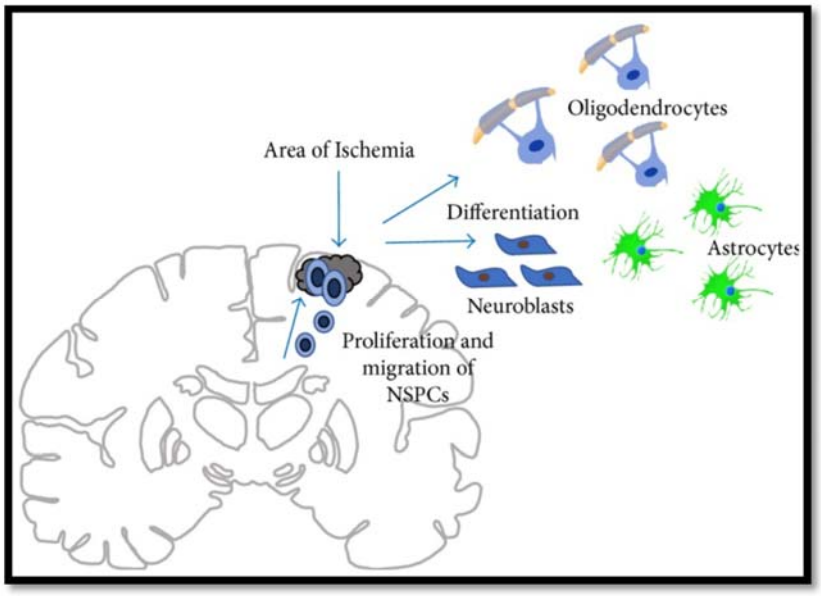


Figure 3: *This figure shows the endogenous NPCs differentiate and migrate from the SVZ to the area of ischemia (21).*

1.8 NEURONAL MARKERS:

In adult neurogenesis, various markers are expressed in the different timeframes of neurogenesis. The markers for proliferating cells are ki-67, phosphohistone h3 (ph3), mini chromosome maintenance 2 (MCM2), proliferating cell nuclear antigen (PCNA). Moreover, markers specific for neuronal lineage are doublecortin (DCX), polysialylated embryonic form of the neural cell adhesion molecule (PSA-NCAM), calretinin, neurogenic differentiation (NEUROD), T-box brain gene 2 (TBR-2). In addition, neuronal markers specific for early stages of neurogenesis are nestin, glial fibrillary acidic protein (GFAP), SOX-2 and paired box gene 6 (PAX-6) (22-25).

1.9 DOUBLECORTIN:

Doublecortin (DCX) is a microtubule-associated protein, which facilitates polymerization of microtubules. DCX is known as a marker for adult neurogenesis, also expressed in newly formed neurons and migrating neuroblasts (25-27). Studies have shown the presence of DCX cells with the morphology of migrating neuroblasts in the SVZ, RMS and olfactory bulbs (23). The morphology of DCX expressing cells in the SVZ was mostly bipolar with short processes. DCX is also used to label neuronal progenitor cells and early immature neurons (28,29). Furthermore, DCX positive cells are specific to newly formed neurons because they express early neuronal antigens but lack antigens specific to undifferentiated cells, glial cells and apoptotic cells (30).

DCX immunoreactive cells are highly expressed over 2-3 week after the ischemic injury but decrease gradually to become NeuN and continue into maturation process (31). Studies have shown that increasing number of DCX cells in the adult brain is predominantly correlated with two factors; enhanced voluntary physical activity and experimentally induced epileptic seizure. However, there is a decrease in the expression of DCX cells and neurogenesis in correspondence to ageing (32-36). Thus, the transient expression of DCX indicates that it could be an applicable marker for measuring the modulation of adult neurogenesis and may provide substitute to BrdU labeling (28), because BrdU can falsely label any cell with injured DNA as a cell that is undergoing mitosis.

1.10 POST-STROKE REHABILITATION:

Due to functional disability and limitations of post-stroke treatment, stroke

rehabilitation program is only the potential treatment for the majority of stroke patients (37,38). The objective of the post-stroke rehabilitation program is to improve activities of daily living and to regain independence (37,39). Many research studies have shown voluntary exercise and enriched environment can predominantly increase neurogenesis after cerebral ischemia, although various studies show neurogenesis on different regions of the brain (40-45). Some studies show that post-ischemic exercise can enhance neurogenesis in the SVZ, but not in dentate gyrus (DG) (46). In contrast, there are studies that show increased levels of neurogenesis in the DG after voluntary exercise, but not in the SVZ (41).

Furthermore, voluntary exercise can influence all phases of neurogenesis such as cell proliferation, differentiation and survival (47-50). It has been evident that the time-limited window is crucial for rehabilitation and neuroplasticity (58-61). Voluntary exercise delivered within first few weeks after stroke onset can potentially aid in the sensorimotor recovery by reducing infarct volume and stimulating brain organization (51-52,56-57). In addition, voluntary exercise and environment enrichment has proven to increase the levels of BDNF (63). The elevated levels of BDNF following exercise enhance recovery and reduce depression like behavior in chronic and sub acute patients (52-55,62). Finally, physical exercise has not only proven to attenuate motor dysfunction but also showed enhanced neuroplasticity.

1.11 EFFECTS OF FEMALE SEX HORMONE ON ISCHEMIC STROKE:

Studies have shown that, approximately 40,000 more women are affected by stroke every year (68). The chances of cardiovascular diseases and stroke are higher in women after menopause (69-73). This gender difference can be related to neuroprotective roles of estrogen, a female sex hormone responsible in reproductive

function and sex characteristics in females (74). In addition, researchers have shown strong evidence suggesting that estrogen may have a neuroprotective and therapeutic function in stroke (75). Experimental studies using MCAO models of stroke has demonstrated that female rats have less brain damage compared to the male rats. To overcome this problem in animal models, overiectomy was performed to equalize the results (76-77). In contrast to numerous studies that showed beneficial role of estrogen in preventing stroke in early aged women, the Women's Health Initiative (WHI) trial demonstrated that hormonal replacement therapy (HRT) containing estrogen and progestin increased the risk of stroke and heart disease in women (78-85). Researchers argue that the discrepancies between studies may have resulted due to critical parameters like dosage, timing, route of administration (86-87). In addition, it might have resulted from the alterations in the timing of estrogen administration in relation to perimenopausal (75,87).

1.12 HYPOTHESES:

- Increased neurogenesis in the subventricular zone (measured by doublecortin) will significantly lead to functional recovery following ischemic stroke.
- We also hypothesized that rehabilitation after stroke induction will increase functional recovery.

II. MATERIALS AND METHODS

2.1 ETHICS STATEMENT:

All experimental procedures were carried out in accordance with the Wright State University Institutional Care and Use Committee (IACUC). Female Sprague Dawley outbred retired rats obtained from Harlan Laboratories, Indianapolis, Indiana were used in the study.

2.2 ANIMALS & HOUSING CONDITIONS:

According to stroke studies, more women are affected by stroke each year than men (68). Female rats ranging 10-12 months of age were chosen in this study to mimic the older women suffering from stroke attacks. In addition, adult rats mirror the neurological properties of human stroke patients to make precise comparison (88). There were twenty-four rats each grouped into (N=12 control) and (N=12 rehabilitation). The animals were housed in individual cages and placed in 952cm^2 hanging shoeboxes with wire lids. They were on a 12-h light/dark cycle and maintained at a room temperature of 22 ± 2°C. All rats were fed Harlan Teklad rodent chow throughout the experiment.

2.3 MONTOYA STAIRCASE:

Montoya staircase was used as a pre-stroke training to evaluate the forepaw ability (to reach with the left or right forepaw independently). Montoya staircase can assess reaching and grasping motor function and provides a sensitive

and quantitative measurement of skilled reaching in rats for each limb separately (89). The staircase apparatus has been widely used in animal models to evaluate unilateral forelimb deficits in ischemic and hemorrhage stroke models (90). The staircase apparatus is made of the Plexiglas and consist of a raised central platform with seven bilateral graded steps on each side. The rats can climb onto the central platform and reach for the small food pellets which is placed on every step. The apparatus is designed in such a way that rats can retrieve pellets from the right steps using the right forelimb and the left steps using the left forelimb. The rats cannot turn around in the platform or retrieve the dropped pellets from the floor (91). In this study, a mixture of plain and maple extract coated sucrose pellets were used to lure rats to pick up pellets, with the scent of maple extract attracting the rats. In each staircase three pellets were placed in the well, a total of 21 pellets placed on both sides of the staircase.

2.4 TRAINING:

The rats must be trained properly in the Montoya staircase apparatus before surgery for dependable post-stroke data. The rats were trained on a daily basis during the dark-phase for 1.5 weeks prior to the surgery. During the training, the rats were placed in the box for 15 min and then returned to their home cages. The amount of pellets retrieved by each rat from the wells was noted to determine the pre-stroke function. The highest number from the last three trials was recorded as the pre-stroke function. The minimum reaching performance for the animals to be considered for the post-stroke analysis is to retrieve at least 9 pellets out of 21 from the staircase. The rats that did not retrieve 9 pellets by the end of the training were excluded from the post-stroke training because their forelimb deficit could not be reliably measured (rats

can pick up pellets from the first well and possibly the second well with their tongues; they must use their forelimb for the third well). We prefer to get a training level of 15 pellets, with rats retrieving from 5 wells, as ensures the forelimbs are being used and their loss will show up after the stroke. The rats were tested on the staircase to record their initial functional deficit at 3-5 days of post-stroke and every 30 days for functional recovery.

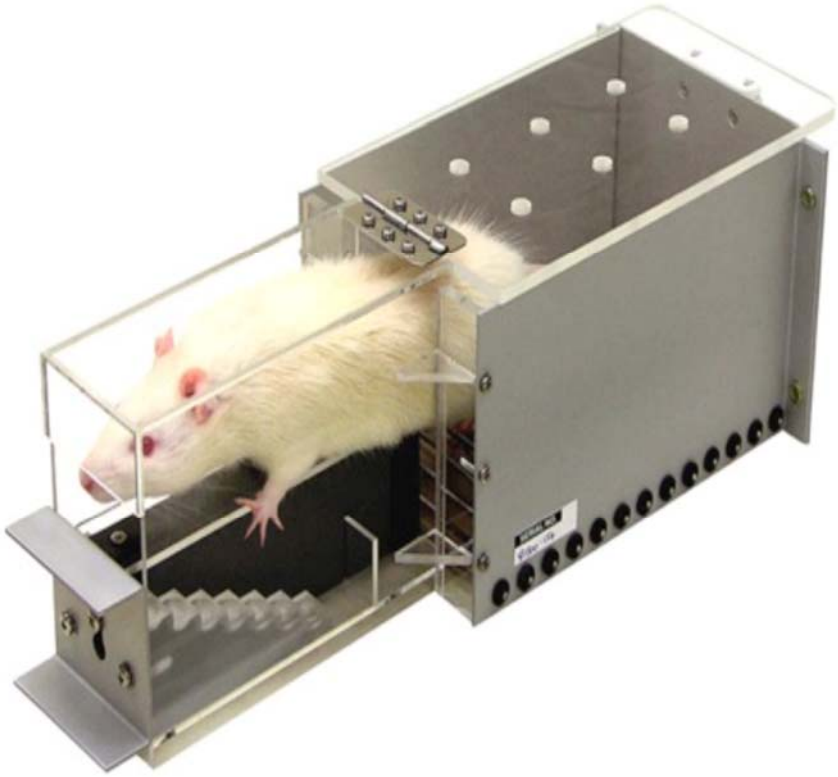


Figure 4: *Image showing Montoya staircase apparatus during the training*

2.5 DIET RESTRICTION:

The rats were placed on a diet restriction to ameliorate the results of pre-stroke and post-stroke training. The amount of ad lib feed was restricted to 15% of their

daily intake. This was calculated by the amount of initial ad lib chow given to each rat and then subtracted from the amount of remaining food that was left in the feeder for each day. The rats were carefully observed and were weighed every three days until the end of the training. The rats were fasted overnight before the day of the training and were fed restricted diet until the training was completed. The rats only lost about 10% of their body weight during the week & ½ training period and did not suffer from significant weight loss during any of the post-surgical testing periods (3 days).

2.6 INDUCTION OF STROKE:

The stroke was induced to the right hemisphere of all rats in this study (Animal approval protocol: AUP 1015), which caused impairment to the left forelimb. The surgery was performed using a stereotactic apparatus (Stoelting Co., USA) (92,93). The stroke was stimulated via stereotactic injection of a vasoconstrictive peptide, endothelin -1.

Prior to the surgery, each animal was brought individually into the surgical room and was placed into a glass chamber, with 5% of isoflurane for 4 minutes to induce anesthesia. Once the animal was sedated, the top of the head was shaven and Puralube ointment was applied to their eyes to keep them from drying out and hydrated during the surgery. The animal was then placed onto the stereotactic apparatus using non-traumatic ear bars and was administered with 2.5% of isoflurane throughout the surgery with an anesthesia mask. The animal's foot or tail was pinched as a retraction reflex method and the respiration rate and the color of the skin was observed to check their effectiveness of anesthesia during the surgery. The surgical site was sterilized with povidone-iodine, then with 70% ethanol and again with povidone-iodine. A small incision was made using the scissors on the scrubbed area

of the head and bupivicane (analgesic) was applied on the incision area. The blood from the incision area was cleaned and bregma was positioned on the skull using the fine point marker. The bregma was aligned with the burr bit in a micro drill and bregma co-ordinates were recorded using the stereotactic apparatus.

	First hole	Second hole
Anterior posterior position from bregma	0mm	1.5mm
Medial lateral position from bregma	2.5mm	2.5mm

Table: 1 *Co-ordinates of stroke induction*

The forelimb cortex on the right hemisphere was marked using bregma co-ordinates, the identified location are shown in the table 1. Small burr holes were drilled at two positions. Next, the micro drill was removed and the Hamilton syringe was attached to the stereotactic apparatus. The syringe was filled with a total of 3µl of endothelin-1 (Human/porcine, EMD chemicals). At each hole, the endothelin (1.5 micro liters) was slowly injected at the pace of 0.1 microliter every 10 seconds at a depth of 2 milliliter. After the endothelin administration at both sites, the Hamilton syringe was removed from the apparatus and the surgical site was sutured. Then the sutured site was applied with povidone iodine and the animal was injected with 2 ml of saline subcutaneously. Once the animal regained consciousness, they were taken back to their cages with heating pad to keep them warm. The animal was closely monitored until it regained consciousness and locomotion was observed. The animal was taken back to its housing area and the next animal was prepared for the surgery.

All the rats were given moist chow for three days post-stroke and normal diet was given after that.

2.7 POST-STROKE TREATMENT:

The rats that were in the vehicle group received 4 grams of Pillsbury cookie dough for 60 days post-stroke, beginning 24 hours after stroke surgery. The rat chow diet was restricted only during staircase training, rest of the days they were fed with ad lib rat chow. The weight of the rats was regularly monitored each week.

2.8 VOLUNTARY PHYSICAL REHABILITATION:

The shelves were hung outside the cages with about 10-12 grams of peanut butter. The rehab shelves were hung in such a way that the rats were only able to use their left forelimb (contralateral limb) to grab the peanut butter. Prior to the surgery, the shelves with peanut butter were hung and the rats that consumed the peanut butter were chosen for the rehab study.

At the beginning of 8 days post-ischemia, the rats that were selected for the rehab study started their voluntary physical rehabilitation by consuming peanut butter from the hanging shelves. The functional recovery was measured and analyzed upon the daily movement of the rat's contralateral limb. Every morning, the amount of peanut butter consumed by the rats were weighed and recorded and this routine was followed for a total of 52 days.

Figure: 5 *This image is showing the rehab hanging shelves with peanut butter and the rat using its contralateral limb (left forelimb) to grab the peanut butter.*

2.9 MEASURING ARM SWIPES:

The amount of peanut butter a rat takes up in a single swipe using its forelimb was calculated to determine the number of arm swipes a rat achieved everyday during the rehab study. This was calculated by weighing the peanut butter shelve before hanging them into the cage and reweighed it again when the rat had attained 5,10,15,20 arm swipes and the average of amount peanut butter a rat would attain in a single swipe was calculated. For example, if a rat had attained 0.30 grams of peanut butter with 15 arm swipes, then the mean value of a single swipe would be 0.02 grams for that rat. Overall we attained values from at least 15 rats to determine the mean value of peanut butter picked up per arm swipe.

2.10 POST-STROKE STAIRCASE FUNCTIONAL TESTING:

Montoya staircase functional testing began at 3-5, 28-30, 58-69 days post-stroke. The baseline deficit was determined on the 3-5 days post-stroke and the baseline function pre-stroke was calculated from the last three days of pre-stroke training. For example, if the rat retrieved 15L (left forepaw) and 16R (right forepaw) on the first day, 12L and 13R the next day, 14L and 17R on the final day, then the pre-stroke baseline function for the left forepaw is 12L and for the right forepaw is 17R. The contralateral and the ipsilateral functional deficit were calculated as shown below.

Contralateral function = $\frac{\textbf{\textit{Number of pellets PSD3}}}{\textbf{\textit{Baseline function (left)}}}$	**Ipsilateral function =** $\frac{\textbf{\textit{Number of pellets PSD3}}}{\textbf{\textit{Baseline function (right)}}}$
Contralateral deficit = 1− contralateral function	**Ipsilateral deficit =** 1− ipsilateral function
Total deficit = Contralateral function + ipsilateral function	

Table: 2 *Calculations to determine contralateral and ipsilateral functional deficit.*

2.11 FUNCTIONAL RECOVERY CALCULATION FOR POST-STROKE

DAY- 30:

Contralateral recovery = Contralateral function (PSD30) – Contralateral function (PSD3)
Ipsilateral recovery = Ipsilateral function (PSD30) – Ipsilateral function (PSD3)
Total recovery = Ipsilateral recovery + Contralateral recovery

Table 3: *Calculation of Contralateral and ipsilateral recovery on post-stroke day 30.*

2.12 FUNCTIONAL RECOVERY CALCULATION FOR POST-STROKE

DAY-60:

Contralateral recovery = Contralateral function (PSD 60) – Contralateral function (PSD3)
Ipsilateral recovery = Ipsilateral function (PSD 60) – Ipsilateral function (PSD3)
Total recovery = Ipsilateral recovery + Contralateral recovery

Table 4: *Calculation of Contralateral and ipsilateral recovery on post-stroke day 60.*

2.13 EUTHANIZATION AND CARDIOPERFUSION:

After 60 days of post-stroke study, the animals were euthanized. Each animal was taken separately into the surgery room and euthanization was performed. The animal was administered with euthasol (100mg/kg pentobarbital) via intraperitoneal injection. Once the animal has reached the surgical plane of anesthesia, 5-6 cm lateral incision was made just beneath the rig cage (94). Then the perfusion was initiated by inserting catheter into the left ventricle of the heart while flowing phosphate buffered saline (PBS) and the right atria was cut to drain the blood from the body. Once the blood was sufficiently removed from the body, then the solution was switched to 4% formaldehyde in PBS to fix the tissues and it was kept until 150ml of solution flushed through the heart. Next, the rat's head was decapitated and dissected and the brain was stored in the 4% formaldehyde solution overnight. After 24 hours, the brain was transferred to 30% sucrose solution for three days.

2.14 CRYOSTAT SECTIONING OF THE BRAIN:

The brain tissue was removed from the sucrose solution and was prepared for the cryostat by placing them into the block using optimal cutting temperature (OCT) compound in combination with a freezing peltier device. We waited until 30 minutes for the tissue and compound to reach the same temperature of the chamber temperature (−25°C) so that the tissue can be sliced without damage. Next, the brain was sectioned into coronal slices of 50μm in thickness. The tissues were sliced until the infarct region was seen in the right hemisphere. The collected slices were stored in the four vials containing phosphate buffer solution, each labeling them as DCX, ki67, infarct and control respectively.

2.15 DOUBLECORTIN STAINING:

The following protocol was followed for the staining of DCX. The brain slices for DCX were kept in the vials marked 1 and the control was kept in the vials marked 4.

- The PBS was removed from the vials and was replaced with 1.5 ml of blocking solution (PBS 0.3% with tween and 3% goat serum). The vials were incubated for one hour.
- Next was the primary antibody incubation step. In this step, vials 1 were added with 1:400 dilution (3.75μL) of doublecortin antibody (DC #4604 cell signaling) into the existing blocking solution. The primary antibodies were not added in the control vials 4. The vials were then taken to the cold room and were kept in shaker overnight at 5°C.
- The next morning the vials were brought back to the room temperature and unbound antibodies were removed by washing the vials 1 twice with PBS-Tween.
- Subsequently, the second wash solution was replaced with 1.5 ml of the block solution in the vials 1. Then the vials 1 and 4 were added with 7.5μL of biotinylated secondary antibody (goat-anti-rabbit-IgG) from vector ABC kit and were incubated for an hour.
- At the end of the secondary antibody incubation, the vials were washed twice with PBS-Tween. Then 1.5 ml of ABC solution (avidin and goat-anti-rabbit-IgG labeled with horse-radish peroxidase) were added to the vials and washed off with PBS-Tween twice after 30 minutes of incubation.
- Finally 1.5 ml of DAB substrate were added to the vials 1 and washed off with

distilled water after five minutes. Then the vials were replaced with phosphate buffer saline solution.

- After all the substrate was developed, tissues from vials 1 and 4 were ready to mount onto slides.

2.16 TISSUE MOUNTING:

After the immunostaining protocol the vials 1 and 4 were emptied onto to a petri dish with PBS. The sections were carefully mounted onto the gel-coated slides using a thin paintbrush. Each slide was mounted with five sections and we thoroughly checked for any folding or tearing. Once the slides were dry, DPX mountant was put over the sections to preserve the staining and finally coverslip was placed on top to cover all the sections in the slide.

2.17 MICROSCOPY AND IMAGE ANALYSIS:

The slides were observed via bright field microscopy using a 4X objective lens to capture the DCX staining along the regions of anterior, middle and posterior subventricular zone. The DCX staining were captured via digital microscope and were montaged using Adobe Photoshop. All the images were stored and categorized by animal number and were later keyed to either DCX control or DCX rehab.

A software program called imageJ was used to quantify the amount of neurogenesis in the subventricular zone. First, the program calibration was set as the microscope picture (area in mm^2), DCX staining was outlined and threshold was adjusted. This was saved as mask of the original image and by using free hand tool redundant spots were removed. Simultaneously, the values for the area of the mask were stored in the excel sheet. The values from image J were then added up together and averaged to get an average area of neurogenesis for one rat.

2.18 STATISTICAL ANALYSIS:

Statistical analysis was done using the Graphpad prism 7 software and Sigma plot. To analyze the statistical difference between the groups unpaired t-test with Welch's Correction and contingency test was performed using the Graph pad prism. Two-way ANOVA was plotted using the sigma plot.

III. RESULTS

In this study, there were 24 rats initially and we grouped them into two groups: control with rehabilitation and control without rehabilitation. Ischemic stroke surgery was performed in all the 24 rats using the vasoconstrictor, endothelin-1. The surgery was effective in both the groups and all the animals survived except one animal in the control with rehabilitation group.

Number of animals	**Control without Rehabilitation (Control)**	**Control with rehabilitation (Rehab)**
Before the Surgery	12	12
After the surgery	12	11

Table: 5 This table summarizes the number of rats in each group.

3.1 MONTOYA STAIRCASE TRAINING ANALYSIS:

3.1.1 Pre-stroke rat weight analysis

The rat's weight was measured for both the control and rehab groups from the beginning of the study until post-stroke day 60. The initial rat weights were compared statistically using the t-test (Welch's correction for an unequal variance) between the two groups and the graph is shown below.

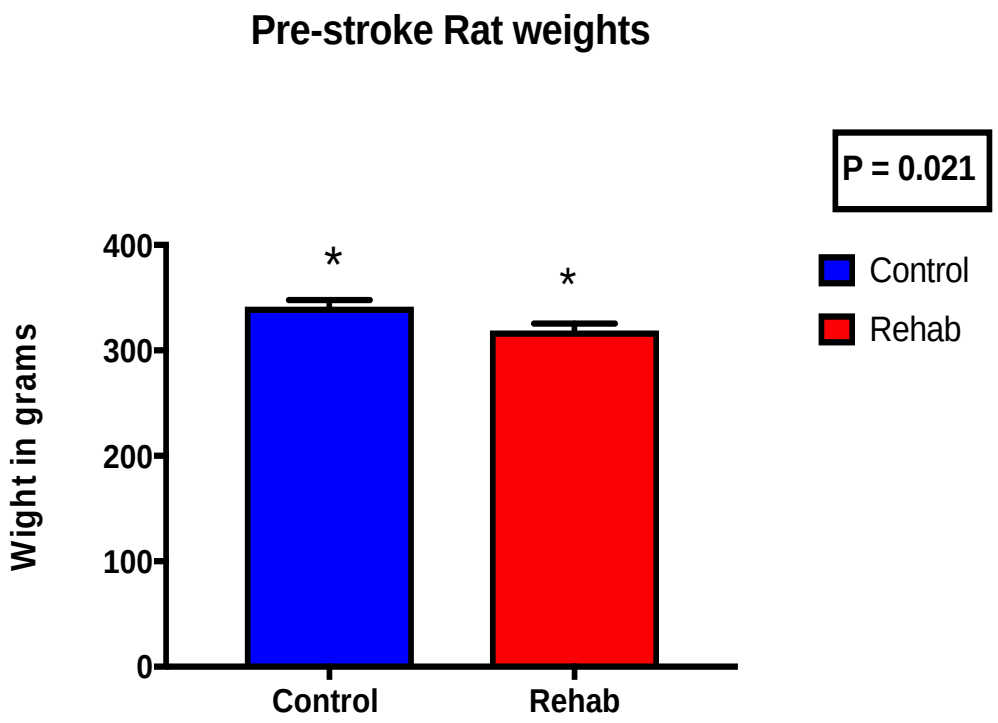


Figure 6: *Graph shows the comparison of pre-stroke rat weights. The blue bar in the X-axis denotes the control group and the red bar denotes the rehabilitation group. The Y-axis denotes mean weight in grams. t-test using Welch's correction for an unequal variance was performed and the error bar indicates the standard error of the mean (SEM).*

From the above figure 5, we can observe that there is a significant difference between the two groups (P value = 0.021). The mean and SEM for the control group is 341.6 ± 6.277, n=12 and the mean and SEM for the rehab group is 318.9 ± 6.577, n=11. The rats that consumed more amount of peanut butter were chosen for the rehab study and were grouped accordingly. But it happened by chance that the control group

weighed more than the rehab animals. This might be because of the rats in the rehab group did not eat rat chow and consumed more peanut butter when they were hungry. The initial grouping of the rats was based upon the consumption of the peanut butter but was not based on the animal's weight.

3.1.2 Post-stroke rat weight analysis

The rat's weight was finally recorded at the post-stroke day 60, to analyze if there was any significant difference in the weights of the two groups over time.

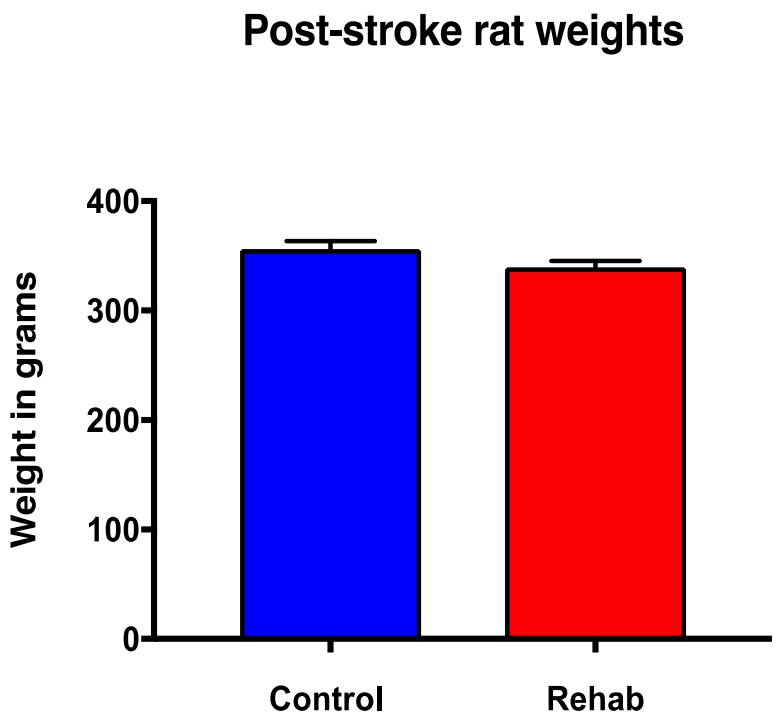


Figure 7: *Comparison of final rat weights at post-stroke day 60. The X-axis denotes two groups: control and rehab. The blue bar in the X-axis denotes the control group and the red bar indicates the rehab group. t-test using Welch's correction was performed statistically to compare between the both groups. Y-axis represents the mean weight in grams. The error bar represents the standard error of the mean (SEM).*

The figure 6 shows that there was no significant difference between the control and the rehab group (P-value =0.0870). The mean and SEM for the control is 356.3 ± 7.354, n=12 and the mean and SEM for the rehab is 339.6 ± 5.578, n=11. Here, we can note that significant difference seen in the pre-stroke rat weights in figure 5 went away over time, which may have had something to do with the rehab animals eating the fat peanut butter.

3.1.3 Control Animals Weight Over Time:

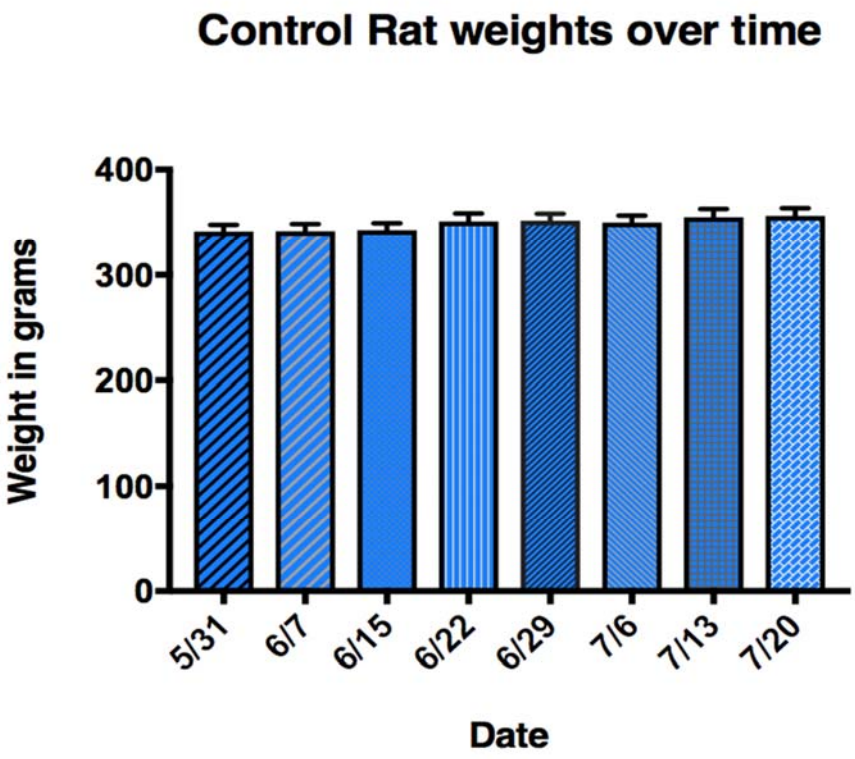


Figure 8: *This graph is showing the control rat weights from the beginning of the study until the post-stroke day 60. Patterned blue bars in the X-axis denote different time points in the control study. Y column indicated the mean weight in grams and the error bars represent standard error of mean. One-way ANOVA was done to analyze statistical difference of the weights over time.*

The rat weights were measured every week throughout the study. From the above figure, we can see that there was no significant difference over time in the

control group as the P-value = 0.6441. This explains that there was no weight loss after the stroke induction (6/1, 6/2, 6/3) in the control group and the weights remained stable over time.

3.1.4 Rehabilitation animals weight over time:

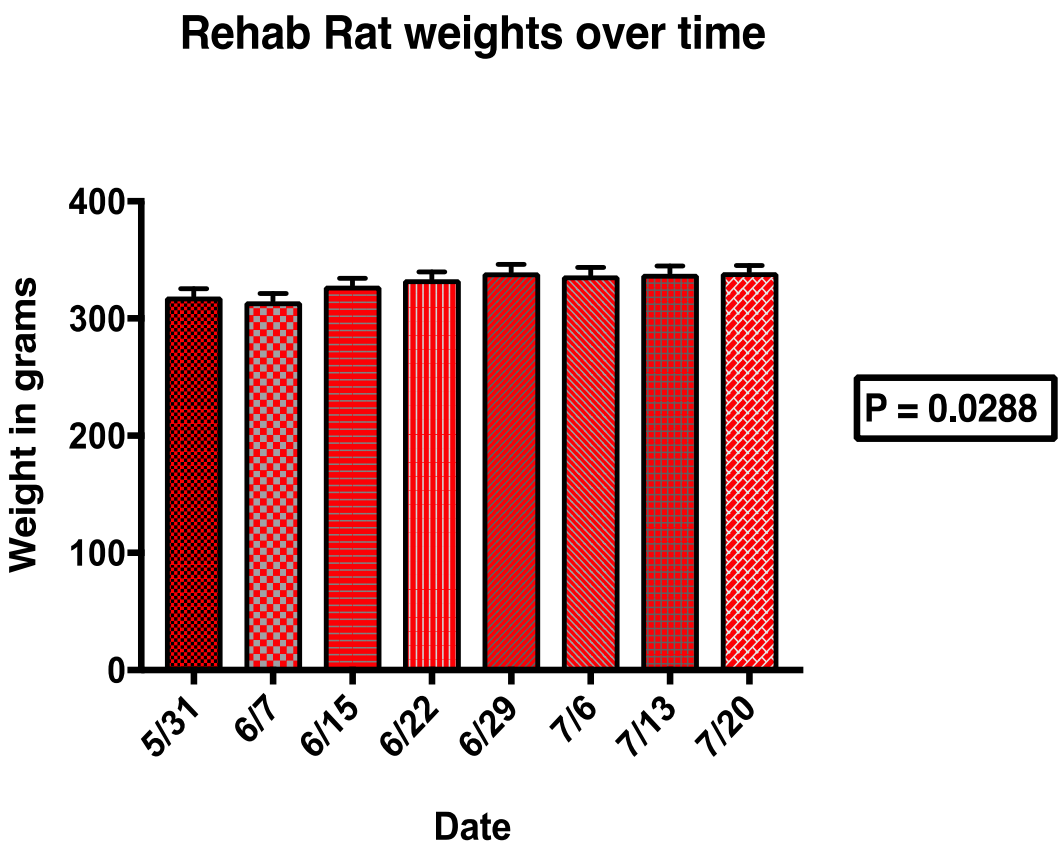


Figure 9: *This graph is showing the One-way ANOVA analysis of the rehab rat weights from the beginning of the study until the post-stroke day 60. Patterned red bars in the X-axis denote different time points in the rehab study. Y column indicates the mean weight in grams and the error bars represent standard error of mean.*

In figure 8, shows the rehabilitation weight measured every week from the beginning of the study until the post-stroke day 60. One-way ANOVA was performed with the data and the P-value = 0.0288, which shows a significant difference over time. At the beginning of the study, rehab rats weighed significantly lesser than the

control rats. This figure explains that the rehab rats have increased their weight over time by eating a sufficient amount of peanut butter and rat chow until they got stable at the end of the study. This also shows that the restricted diet during the Montoya staircase did not affect the weight of the rats. There is a slight decrease on the weight of the rats between 5/31 to 6/7, which might be due to the stroke surgery, which was performed on 6/1, 6/2, 6/3 and their weights increased during the rehabilitation study which started on post-stroke day 8 for all groups. Rehabilitation study began after the completion of the baseline Montoya staircase deficit following the stroke (post stroke days 3-5).

3.2 FUNCTIONAL RECOVERY ANALYSIS:

The rats were trained daily for 1.5 weeks for pre-stroke function analysis. The number of pellets retrieved by each rat from the wells were recorded to determine the baseline pre-stroke function. The best performance from the last three days of training was recorded as the baseline pre-stroke function. This number was then used as a gauge level to determine functional deficits and functional recovery in the post-stroke animals. The rats were tested on the staircase to record their initial functional deficit at 3-5 days of post-stroke and every 30 days (post stroke days 28, 29 and 30; post stroke day 58, 59 and 60) post-stroke for functional recovery.

3.2.1 Unilateral and Bilateral deficits

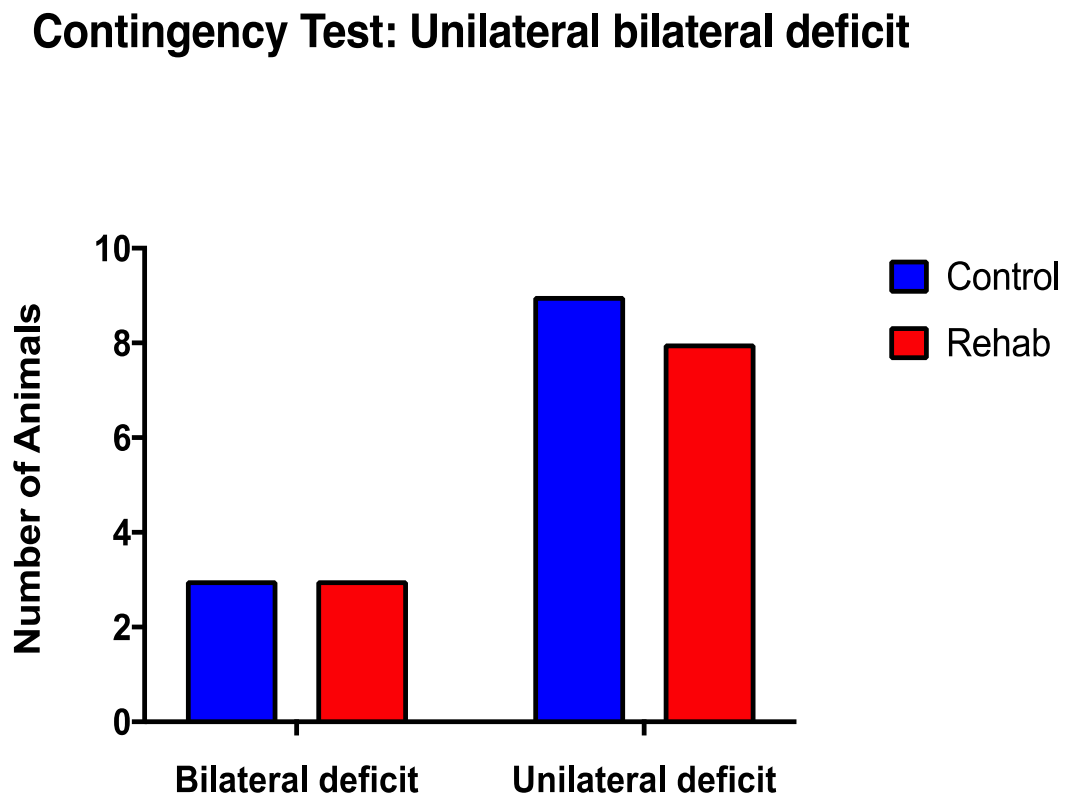


Figure 10: *Contingency test was statistically performed to compare the animals in each group with unilateral or bilateral deficits following the stroke induction. The X-axis denotes two groups: bilateral or unilateral deficit; blue bar indicates the control group and the red bar indicates the rehab group. The Y-axis represents the number of animals. The graph shows no statistical difference between the groups, P=1(Fisher's exact test).*

At post-stroke day 3, the animals were tested for unilateral or bilateral deficits. If the number of pellets retrieved by the rats after the surgery decreased from the baseline value, then it is said to have a functional deficit. If they showed a decrease in both contralateral and ipsilateral side, then deficits were bilateral. The above graph shows the contingency test comparing two groups for the unilateral or bilateral deficits, where P=1 (Fisher's exact test) showing no statistical difference between the

groups. There were 23 animals after the surgery and 3 animals in the control and rehab group were affected with the bilateral deficit, 9 animals in the control group and 8 in the rehab group had a unilateral deficit. The bilateral deficit may have occurred due to an error in the surgical procedures, endothelin might have damaged the corpus callosum.

3.2.2 Contralateral Functional Analysis

To calculate the contralateral function, the number of pellets retrieved on a post-stroke day were divided by the pre-stroke baseline number of pellets to give the normalized function for that limb. The below graph shows the contralateral function between control and rehab groups at post-stroke day 3, 30 and 60.

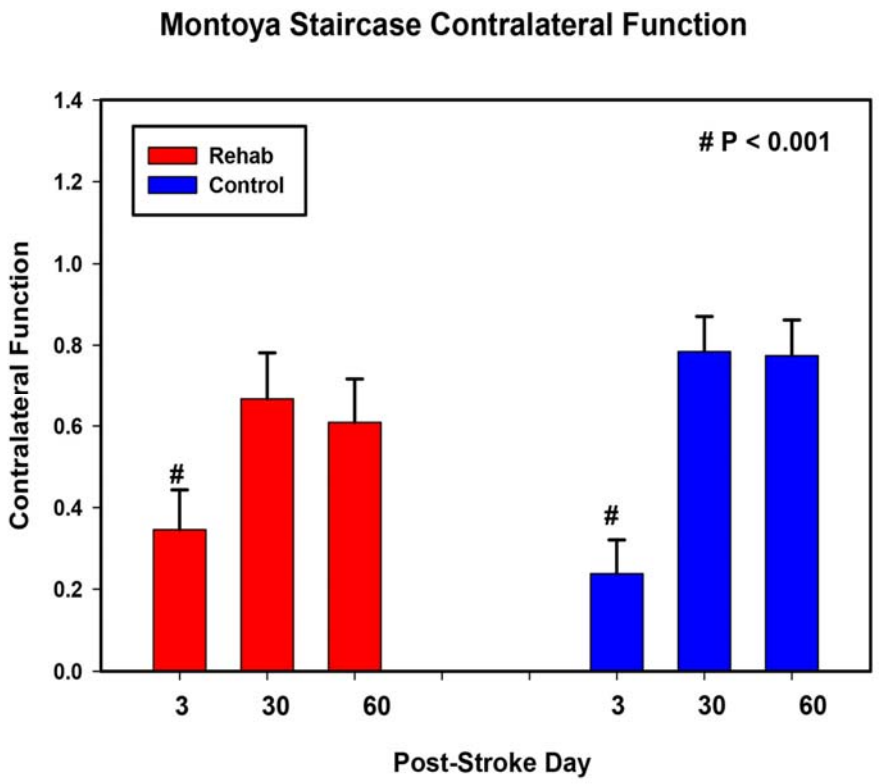


Figure 11: *Comparison of contralateral function between control and rehab over time. The X-axis denotes post-stroke day 3, 30 and 60. The red bar in the X-axis indicates the rehab group and blue bar represents the control group. The Y-axis denotes the contralateral function. Two way ANOVA was statistically done.*

In figure 10, two way repeated measures ANOVA was performed and the statistically significant difference were seen between the post-stroke day 3 and 30, post-stroke day 3 and 60 for both control and rehab groups, where P value = <0.001. On post-stroke day 3-5, the contralateral function mean value for control was 0.237 (23.7%) and rehab was 0.346 (34.6%). This signifies that the functional deficit following the stroke induction for the control group was about 76.3% and 65.4% for the rehab group. On post-stroke day 28-30, the animals recovered more than 50% of their contralateral function. The mean value for the control group was 0.784 and the rehab group was 0.667. On post-stroke day 58-60, the control animals had the contralateral function mean value of 0.774 (77.4%) and rehab animals mean value was 0.609 (60.9%). However, there was no overall significant difference seen between the control and rehab groups for the contralateral function.

3.2.3 Ipsilateral Functional Analysis

To analyze the ipsilateral function, the number of pellets taken on a given post-stroke day were divided by the pre-stroke number of pellets to give the normalized value for ipsilateral function. The below graph shows the ipsilateral function between control and rehab groups at post-stroke day 3, 30 and 60.

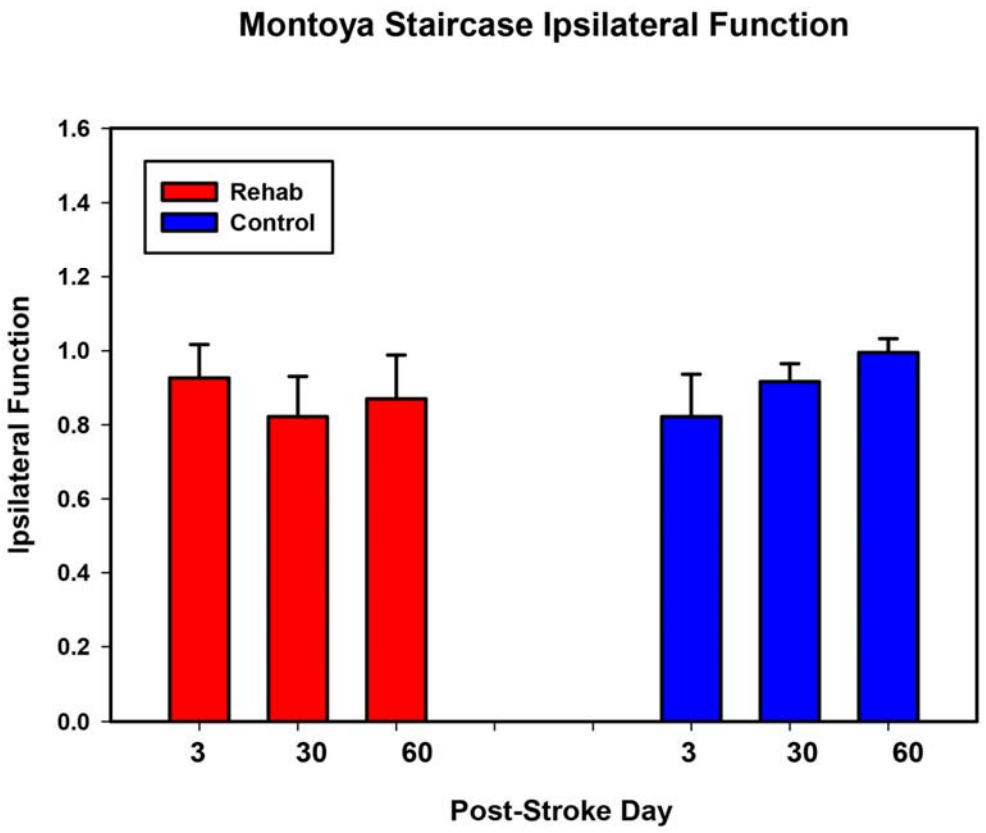


Figure 12: *Results from the Montoya staircase training for the ipsilateral function between control and rehab over time. The X-axis denotes post-stroke day 3-5, 28-30 and 58-60. The red bar in the X-axis indicates the rehab group and blue bar represents the control group. The Y-axis denotes the ipsilateral function. Two way ANOVA was statistically done and the error bar represents SEM.*

Two way repeated measures ANOVA was performed to compare the ipsilateral function between control and rehab over time. The data for the ipsilateral function failed the normality test (Shapiro-Wilk). There was no statistically significant difference seen between the groups or with respect to time. At post-stroke day 3-5, the mean value for the control group was 0.822 (82.2%) and the rehab group was 0.927 (92.7%). This implies that the ipsilateral function was not affected much with the surgery, animals had less than 20% deficit for both control and rehab groups.

On post-stroke day 28-30, there was a slight decrease in the rehab group, which showed a mean value of 0.822 and an increase in the control group with the

mean of 0.917. Finally, at post-stroke day 58-60, the control animals almost recovered with the mean value of 0.996 (99.6%) and the rehab group displayed an increase with the mean of 0.870 (87%).

3.2.4 Contralateral Recovery

To analyze and calculate the contralateral final recovery, the normalized contralateral function on the current day of testing (indicated on the X-axis) subtracts the normalized contralateral function on post-stroke day 3. The below figure shows the results from the Montoya staircase contralateral recovery.

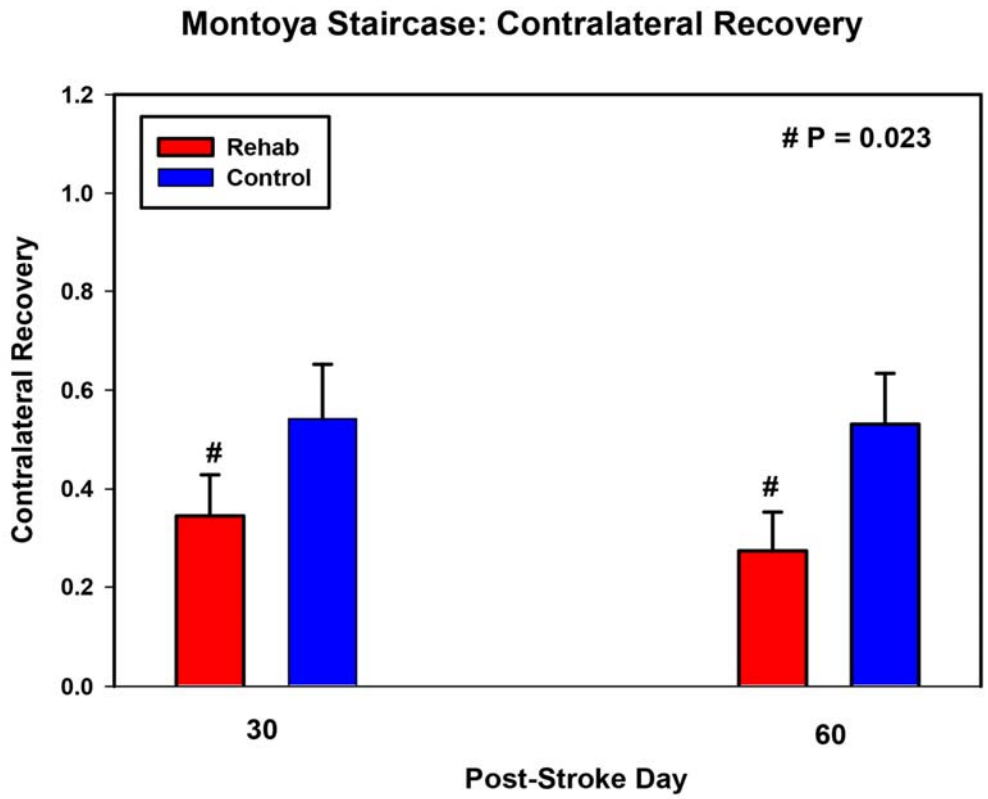


Figure 13: *This graph compares the contralateral functional recovery on the post-stroke day 30 and 60 based on the deficit 3-5 post-stroke days. Two-way repeated measures ANOVA was done. The X-axis denotes the post-stroke day 30 and 60. Blue bar indicates the control group; red bar denotes the rehab group. The Y-axis represents the contralateral recovery and the error bars displays the SEM.*

From the figure 12, we can observe that there is a statistically significant difference between the control and the rehab group at both post-stroke day 30 and 60 as the P value = 0.023. The contralateral recovery for the control group was significantly higher than the rehab group, where the mean value of control at post-stroke day 30 was 0.542 (54.2%) and rehab was 0.346 (34.6%). This implies that the animals in the control group regained their contralateral function more effectively than the rehab group. At post-stroke day 60, the control group regained about 53% of the contralateral function and the rehab group regained only 27% of their contralateral function.

3.2.5 Ipsilateral Recovery

To calculate the ipsilateral final recovery, the normalized ipsilateral function on the current day of testing (indicated on X-axis) subtracted the normalized ipsilateral function post-stroke day 3. The below figure shows the results from the Montoya staircase ipsilateral recovery.

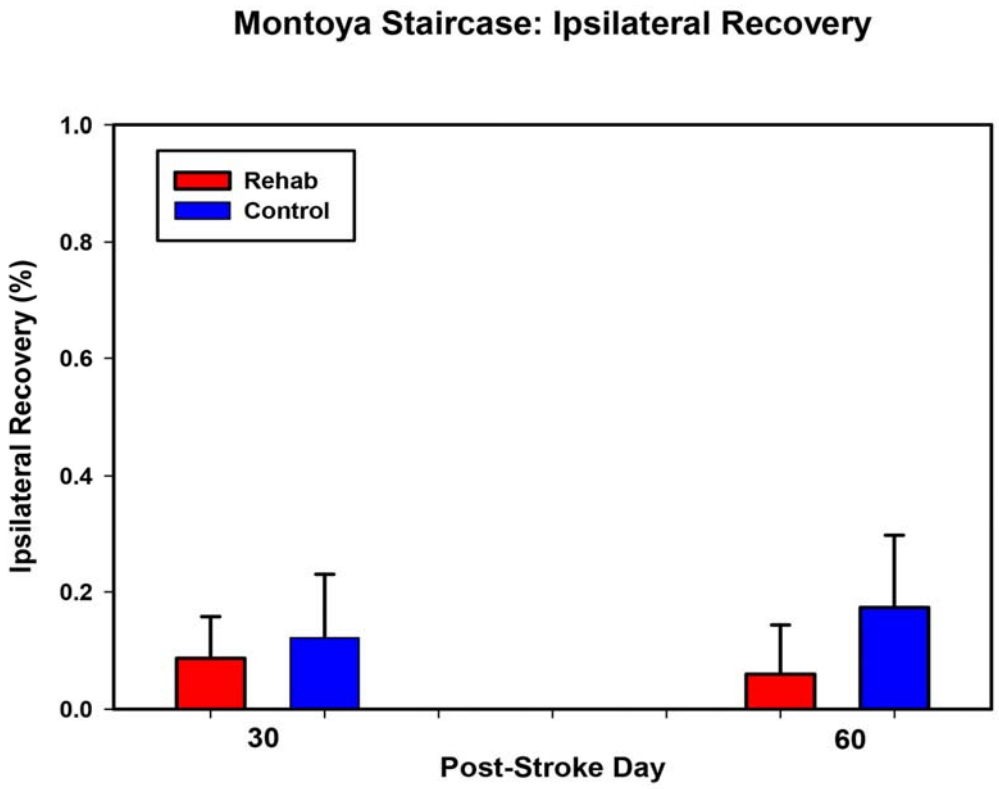


Figure 14: *Comparison of the ipsilateral functional recovery on the post-stroke day 30 and 60 based on the deficit 3-5 post-stroke days. Two-way repeated measures ANOVA was done. The X-axis denotes the post-stroke day 30 and 60. Blue bar indicates the control group; red bar denotes the rehab group. The Y-axis represents the ipsilateral recovery and the error bars displays the SEM.*

In figure 12, two-way ANOVA was statistically done to compare ipsilateral functional recovery on the post day 30 and 60. The data failed the normality test (Shapiro-Wilk), no significant difference was seen between the two groups for the ipsilateral recovery (P value = 0.702). At post-stroke day 30, the mean value for the control group was 0.121 and the rehab was 0.0863. At post-stroke day 60, the control animals mean value was 0.173 and rehab was 0.060.

3.2.6 Total Post-stroke Recovery

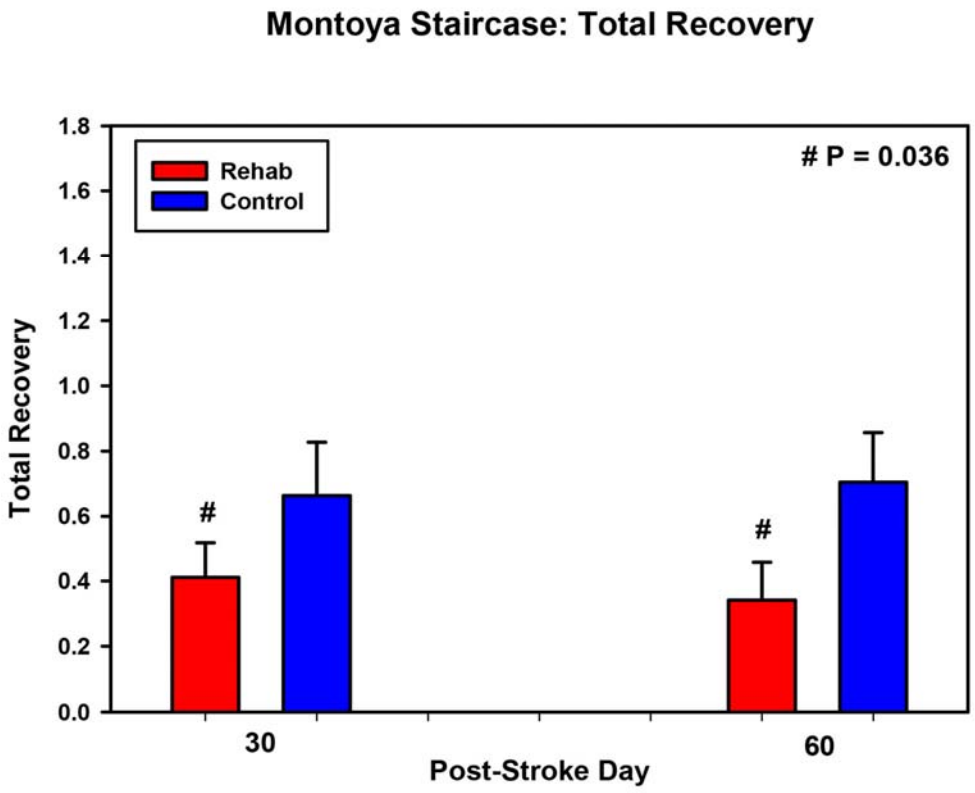


Figure 15: *Total recovery analysis between control and rehab groups on the contralateral and ipsilateral sides at post-stroke day 30 and 60. Two way ANOVA was done. The X-axis represents the post-stroke day 30 and 60; the blue bar indicates the control and red bar indicates the rehab group. The Y-axis represents the total recovery. The error bar indicates SEM.*

The total recovery was calculated by adding the contralateral and the ipsilateral recovery from the post-stroke days 30 and 60. The total recovery results were shown in figure 14, we can observe that there was a significant difference between the control and rehab groups (P-value = 0.036). The total recovery for the control group showed an increase from the post-stroke day 30 (mean = 0.663; 66.3%) through the post-stroke day 60 (mean = 0.704 70.4%). Whereas the total recovery for the rehab group showed a slight decrease from the post-stroke day 30 (mean = 0.411 41.1%) through the post-stroke day 60 (mean = 0.341 34.1%).

3.3 DOUBLECORTIN STAINING ANALYSIS:

The stained images were captured via bright field microscopy using a 4X objective lens. The DCX staining along the regions of anterior, middle and posterior subventricular zone was analyzed and measured using the quantitative tool called Image J software.

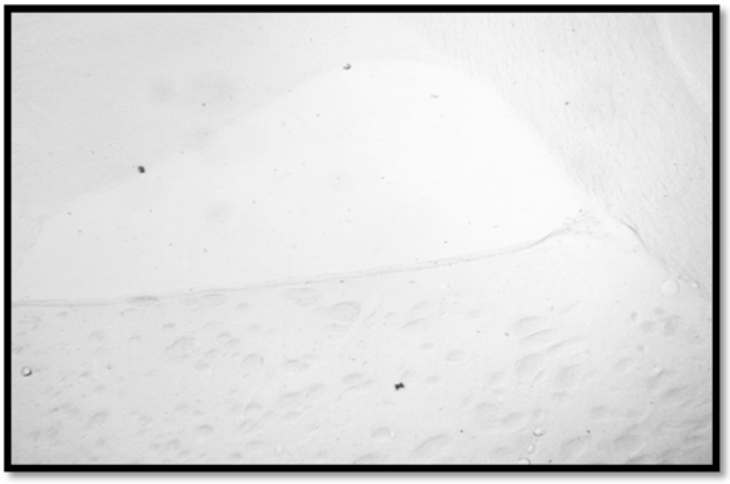


Figure 16: *Image showing control DCX staining (no primary antibody, plus secondary antibody) in a control animal. Scale bar:500μm*

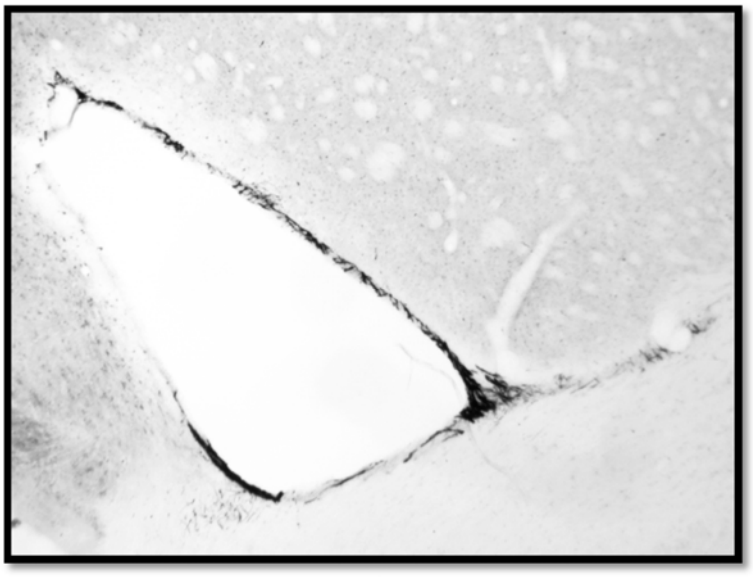


Figure 17*: Image showing DCX positive cells in the subventricular region (plus primary antibody, plus secondary antibody) of a control animal. Scale bar: 500μm*

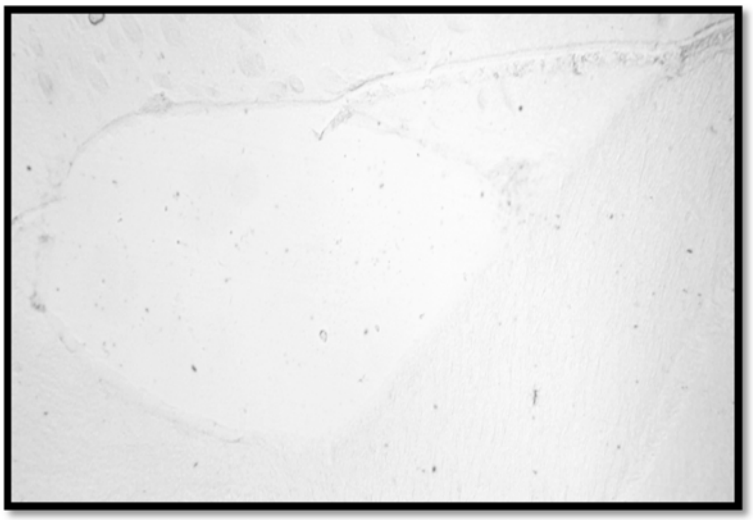


Figure 18: *Image showing the control DCX staining (no primary antibody, plus secondary antibody) in a rehab animal. Scale bar:500µm*

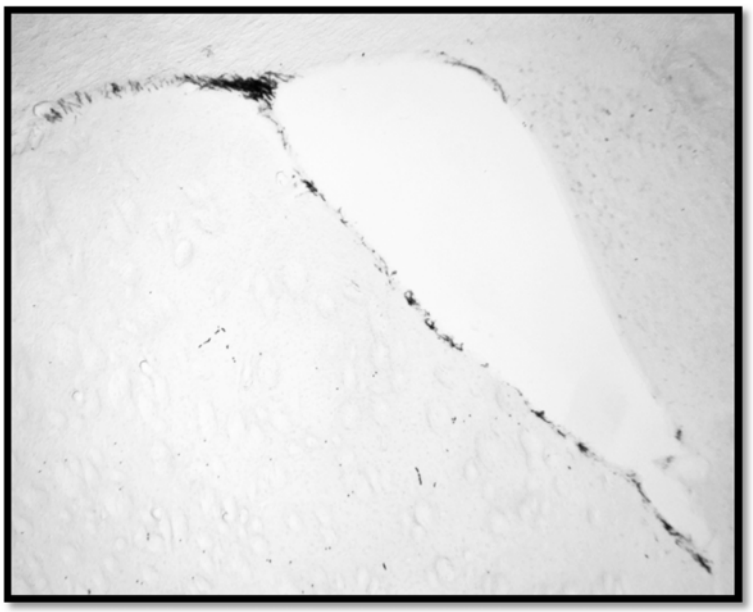


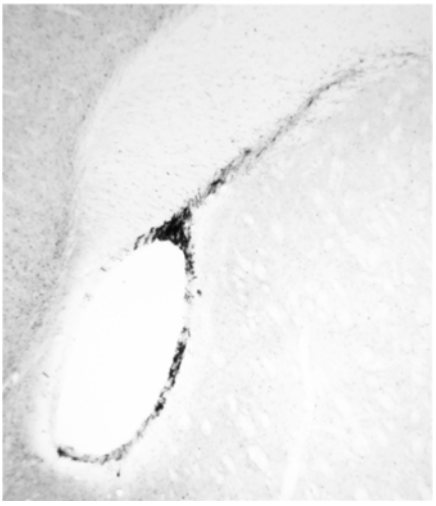
Figure 19: *Image showing DCX positive cells (plus primary antibody, plus secondary antibody) in the subventricular zone of a rehab animal. Scale bar:500µm*

3.3.1 Doublecortin levels in the Anterior SVZ region

To estimate the DCX levels in the anterior subventricular zone, coronal slices were captured with the digital camera and the area of DCX was calculated using Image J. The staining was often seen on both sides of the ventricle. In the coronal slices (Figure 19) below, we can see a tear-shaped opening in the anterior SVZ.

A)

B)



C)

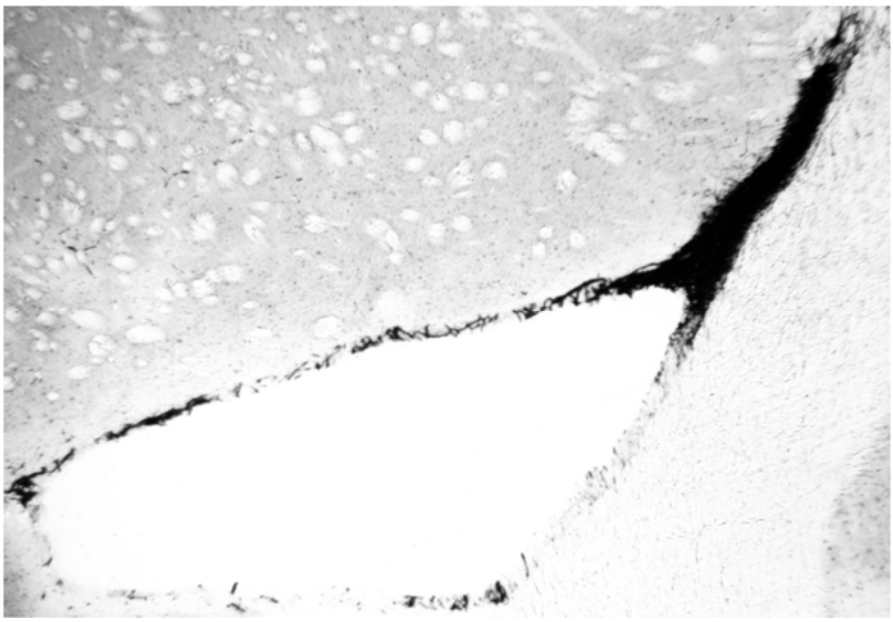


Figure 20 (A-C): *Representing images showing DCX positive immunostaining of the anterior SVZ. Scale bar:500µm*

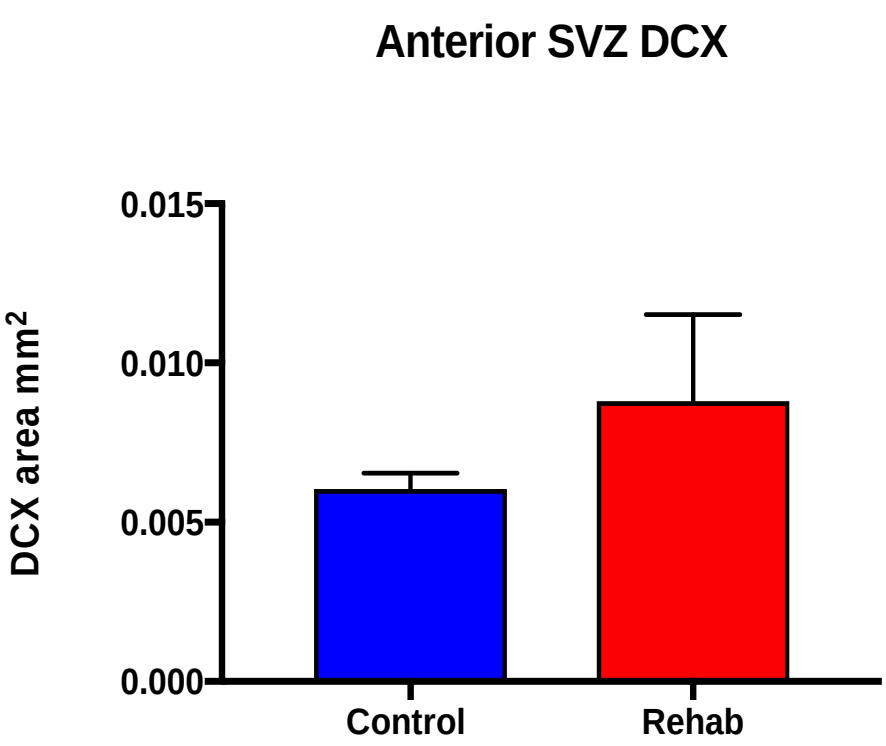


Figure 21: *Graph showing the analysis of DCX levels in the anterior SVZ for control and rehab animals. The blue bar (control; N=12) and the red bar (rehabilitation; N=11). The Y-axis indicates DCX area in mm^2. T-test with Welch's correction for unequal variance was done (P=0.3376). The error bar indicates SEM.*

Figure 20 shows the average area of DCX positive cells in the anterior portion of the subventricular zone for the control and the rehab groups. There was no significant difference seen between the groups (P=0.3376). The mean and SEM of the control group was 0.006038 ± 0.0005053 and the mean and SEM of the rehab group was 0.008805 ± 0.00271.

From the figure 20, there was an outlier identified using ROUT method (Q =1%). The cleaned data removed one value from the rehab group and the graph was performed using T-test with Welch's correction for unequal variance. There was no statistically significant difference after the outlier was removed (P=0.7839). The mean and SEM value for the control was 0.006038 ± 0.0005053, n=12 and the rehab was 0.006505 ± 0.001585, n=10.

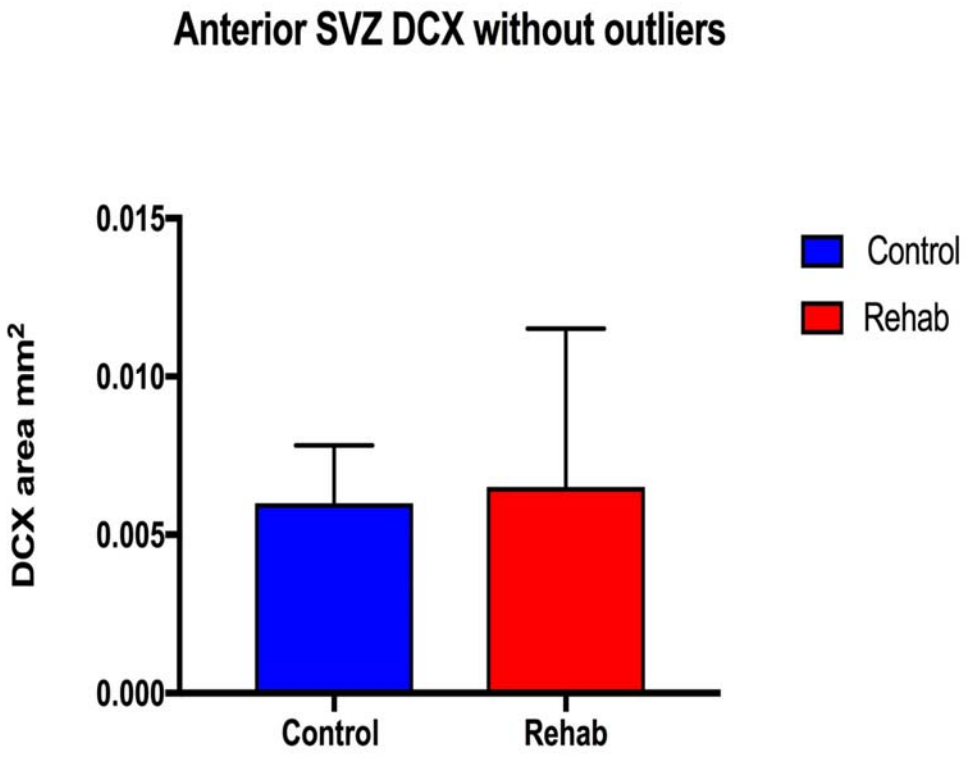
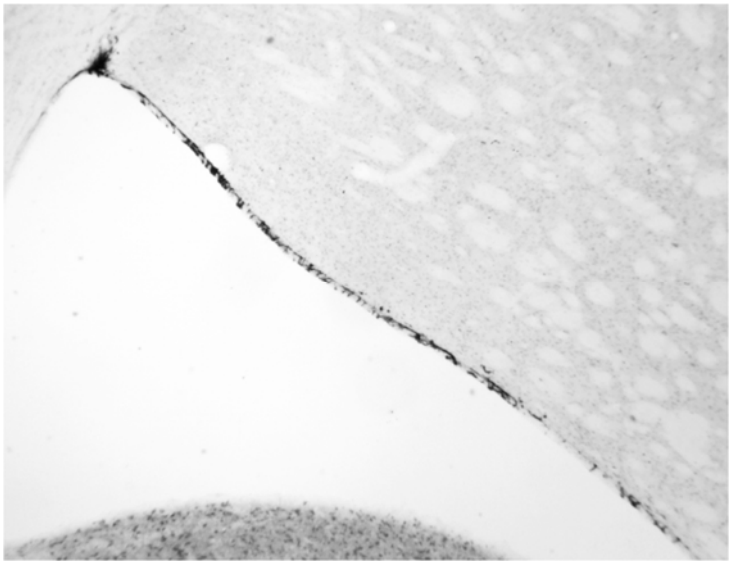


Figure 22 : *Graph showing the analysis of DCX levels in the anterior SVZ after removing outliers from the data. T-test with Welch's correction for unequal variance was performed (P=0.7839)*

3.3.2 Doublecortin levels in the Middle SVZ region

A)



B)

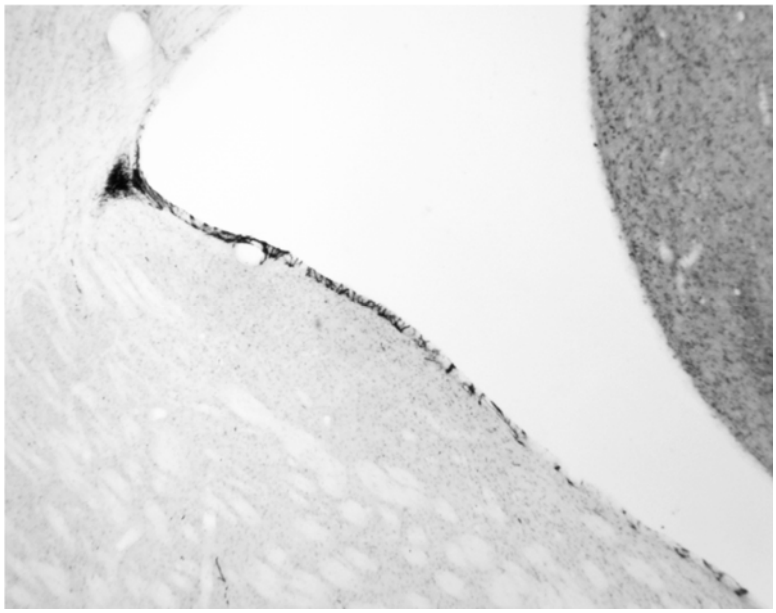


Figure 23(A-B): *Representing images showing DCX positive immunostaining of the middle SVZ. Scale bar:500µm*

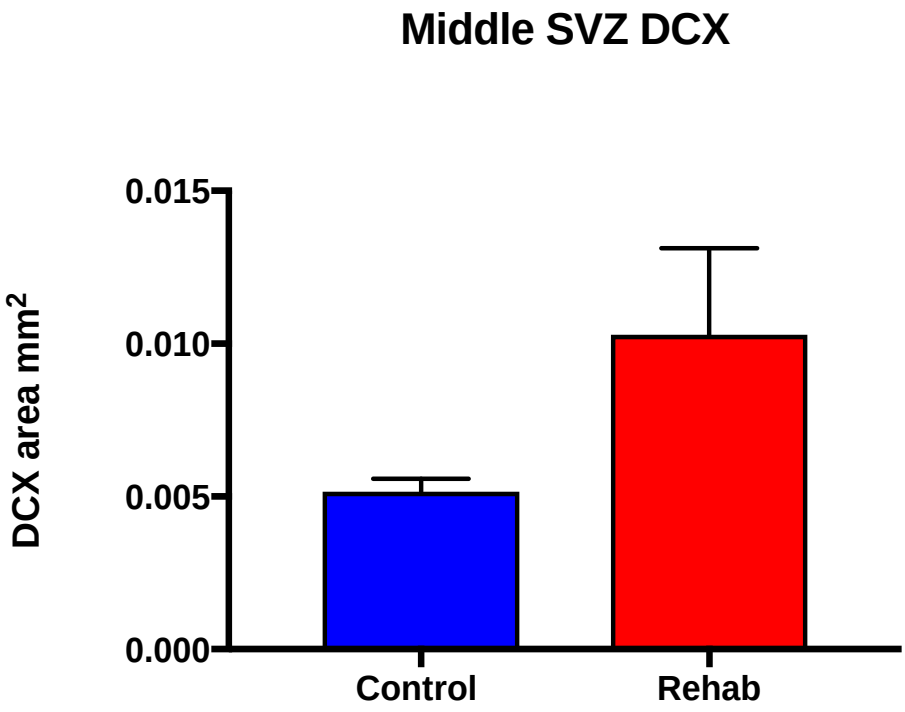
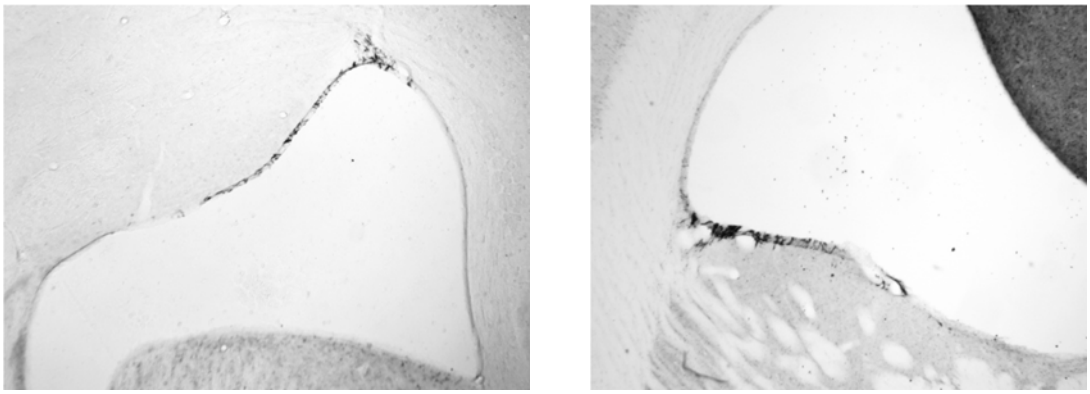


Figure 24: *Graph showing the analysis of DCX levels in the middle SVZ for control and rehab animals. The blue bar (control; N=12) and the red bar (rehabilitation; N=10). The Y-axis indicates DCX area in mm^2. T-test with Welch's correction for unequal variance was done (P value = 0.1039). The error bar indicates the SEM.*

From the figure 22, we can observe that the ventricles in the middle region of the SVZ have opened up and the DCX staining was mostly seen on the side of the ventricle away from the midline. T-test with Welch's correction was performed statistically (figure 23) to compare neurogenesis (DCX) levels on the middle region of SVZ between control and rehab groups. There was no significant difference (P value = 0.1039) between the groups. The mean and SEM for the control group was 0.005159 ± 0.0004208, n=12 and the rehab group was 0.01029 ± 0.00282, n=10

3.3.3 Doublecortin Levels In The Posterior SVZ Region

A) **B)**



C)

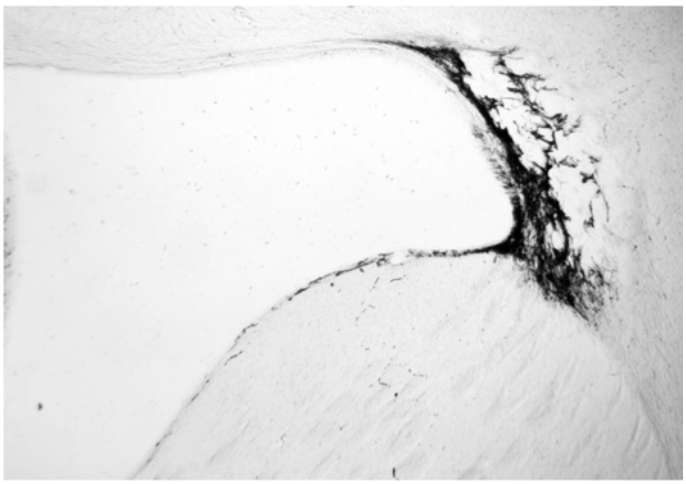


Figure 25: A-C): *Representing images showing DCX positive immunostaining of the posterior SVZ. Scale bar:500µm*

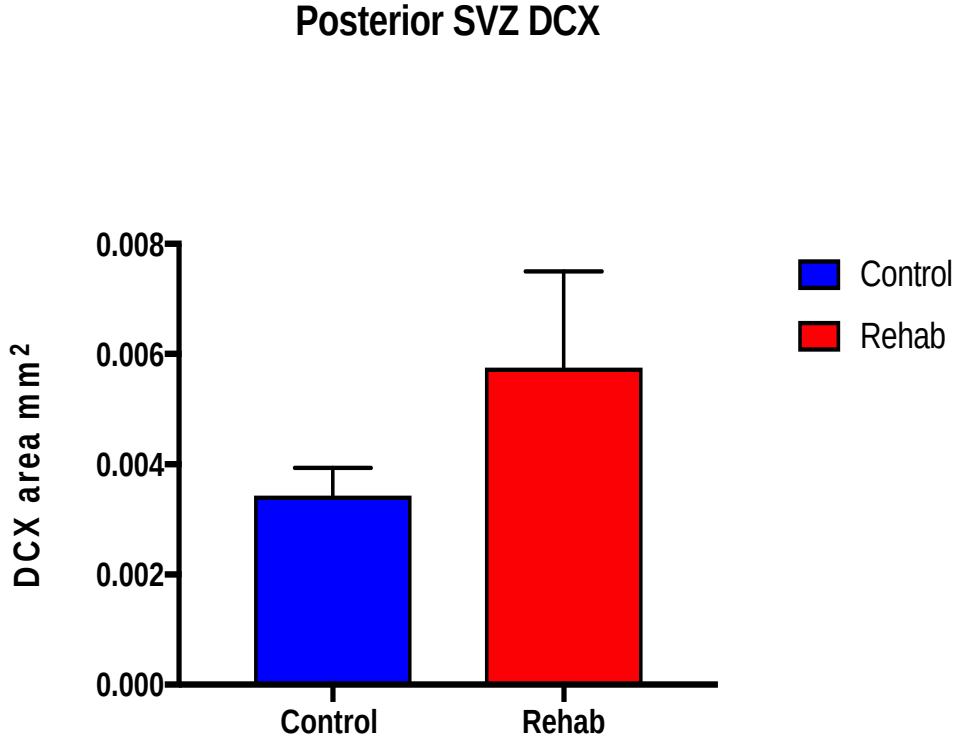


Figure 26: *Graph showing the analysis of DCX levels in the posterior SVZ for control and rehab animals. The blue bar (control; N=12) and the red bar (rehabilitation; N=11). The Y-axis indicates DCX area in mm^2. T-test with Welch's correction for unequal variance was done (P = 0.2263). The error bar indicates the SEM.*

To analyze the DCX staining in the posterior region of the SVZ, t-test with Welch's correction was statistically performed to compare the average area of the posterior SVZ in both the control and rehab animals. No statistical significant difference (P = 0.2263) was found between the groups. The mean and SEM value for the control group was 0.003437 ± 0.0004994, n=12 and the rehab was 0.005757 ± 0.001746, n=11.

3.3.4 Total mean area (DCX) of Subventricular zone

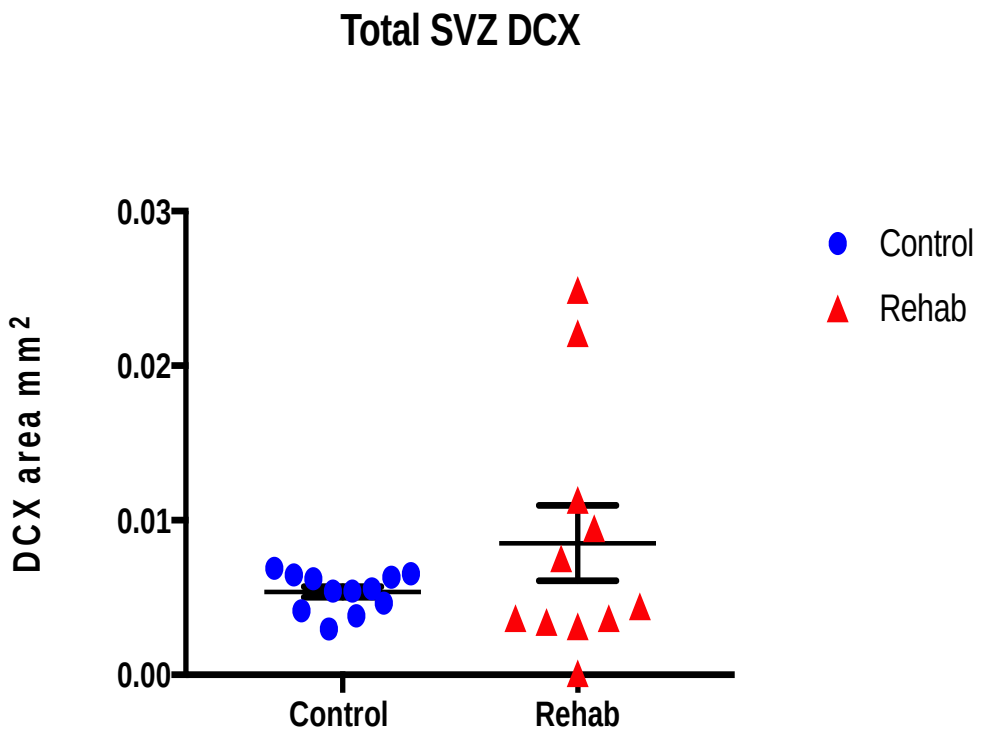


Figure 27: *Analysis of total anterior, middle, posterior regions of SVZ for control and rehab groups. Each of the blue dots (control; N=12) and the red dots (rehab N=11) represents a single animal. T-test with Welch's correction was statistically done (P-value = 0.2272). Y-axis indicates the DCX area mm^2 and the error bar represents SEM.*

The total mean of anterior, middle and posterior regions of SVZ for both the control and rehab groups were analyzed and compared using t-test with Welch's correction (Figure 26). No statistically significant difference (P value = 0.2272) was found between the groups although the rehab group showed a higher mean value compared to the control group. The mean and SEM for the control group was 0.005358 ± 0.0003567, n=12 and the rehab was 0.008517 ± 0.002436, n=11.

3.3.5 Comparing Different Regions of Subventricular Zone

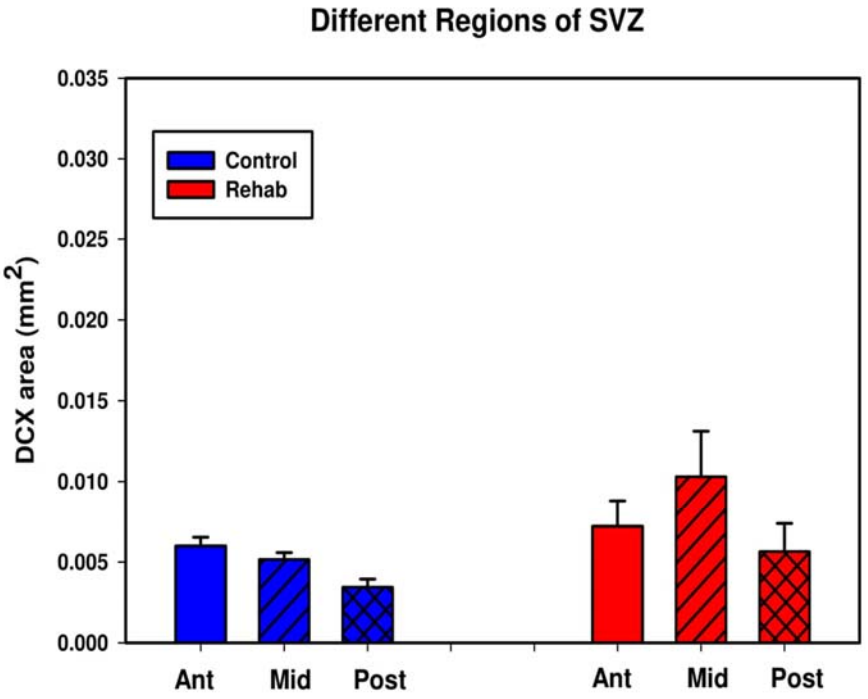


Figure 28: *Comparison of different regions (anterior, middle, posterior) of the subventricular zone for the control and rehab group. X-axis denotes the different regions of SVZ for the control (blue) and the rehab (red) group. Two-way ANOVA was statistically performed, P=0.018. The error bars indicates the SEM.*

The different regions (anterior, middle, posterior) of the subventricular zone were statistically compared using the two-way ANOVA. There was a significant difference between the control and the rehab groups (P-value = 0.018) but not with respect to the different regions. This signifies that the rehab group has a significant increase in the stem cell proliferation compared to the rehab group overall.

IV. DISCUSSION

4.1 OVERALL RAT WEIGHT SUMMARY:

Initially, in the pre-stroke rat weight analysis, we saw a significant difference between the control and the rehab group (Figure 5). The rat's weight were measured every week throughout the study to monitor their health conditions. The rats that consumed more peanut butter were considered for the rehabilitation study. But unfortunately, the control animals weighed more than the rehab group, when this initial assignment was made. This might be because the animals in the rehab group did not eat as much rat chow and consumed more peanut butter when they were hungry. The initial animal grouping was purely based on the consumption of peanut butter but not based upon the animal's weight.

The post-stroke results showed no significant difference between the control and the rehab groups as regards weight. This indicates that the animals in the rehab group gained their weight after the stroke surgery, which was performed on the days 6/1, 6/2, 6/3. Moreover, this also signifies that the rats used their forelimb to consume more peanut butter (beginning post-stroke day 8) and due to the high-fat diet, we saw an increase in the weights in the post-stroke rat analysis (Figure 6). The animals did not lose any weights during the Montoya staircase testing and remained consistent over time.

Overall, the control animals weight history from day 5/31 (pre-stroke) through 7/20 (at the end of the study) showed no significant difference (Figure 7). This indicates that the there was no weight loss after stroke induction and the weight

remained stable over time. Furthermore, the rehab animals weight history from day 5/31 through 7/20 showed a significant increase in the animal weights (Figure 8). The significant difference may have resulted due to rehab animals weighing less initially (pre-stroke) and the statistical difference was seen with respect to the time (between 6/7- 6/15) after stroke induction. The fat content in the peanut butter may have increased the weight in the rehab animals, showing this statistical difference over the time period (Figure 8). Studies have shown that rats fed with high-fat diet increases weight and fat tissue mass gain in rats (104).

4.2 VOLUNTARY PHYSICAL REHABILITATION:

The rehabilitation for the animals started at post-stroke day 8 and continued for a total of 23 days. The rehab shelves were filled with peanut butter and were hung outside the cages so that the rats can use their contralateral forelimb to grab the peanut butter voluntarily. The rehab was held every-day except during the functional testing days. The average amount of peanut butter consumed by the rats and the average number of arm swipes a rat achieved every-day was also measured. The below graphs (Figure 30 and Figure 31) was acquired from the same study by Ms. Devipriyanka Nagarajan (97), illustrates the linear regression analysis to determine the amount of peanut butter consumed by animals per day and the average amount of peanut butter consumed per arm swipe.

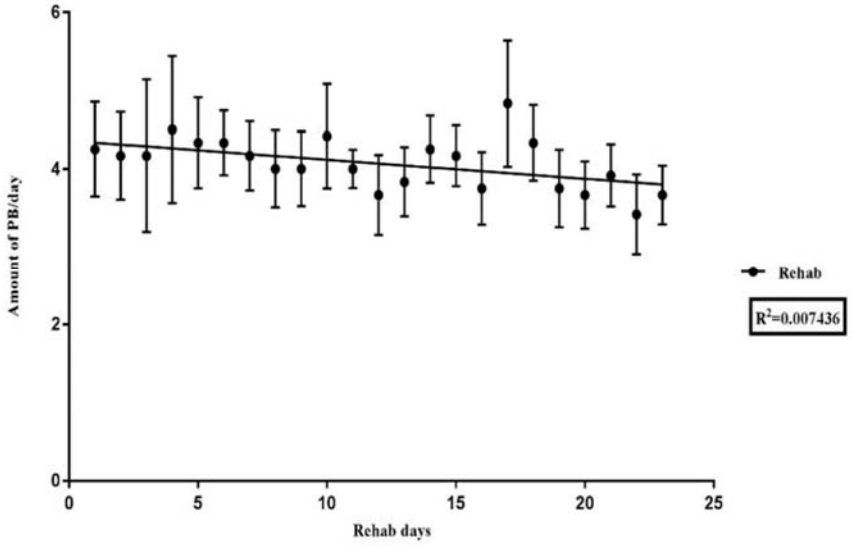


Figure 29: *The linear regression analysis shows the amount of peanut butter consumed by rehab animals throughout the study (97).*

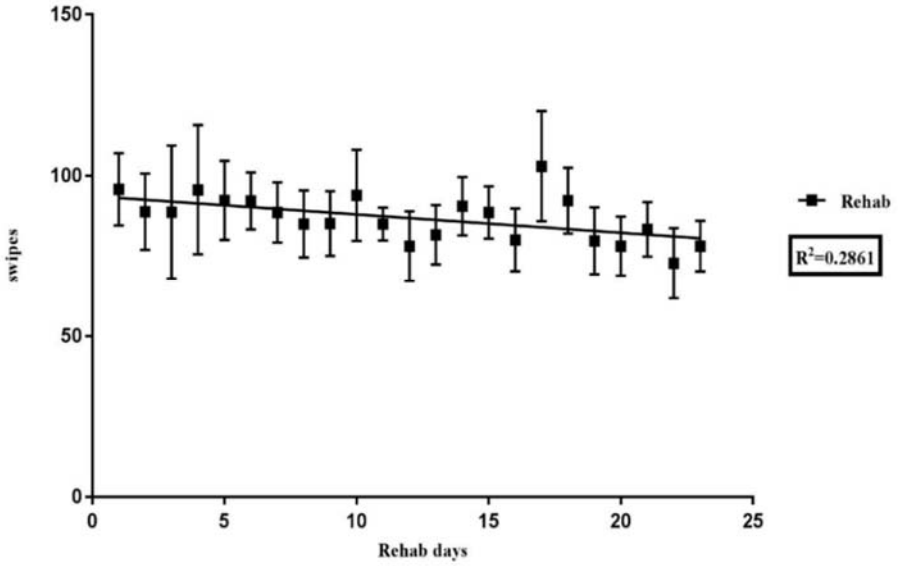


Figure 30: *The linear regression analysis shows the average amount of peanut butter per forelimb swipe throughout the rehabilitation days (97).*

From the above Figure 28 and Figure 29, we can clearly see that the amount of peanut butter consumption and forelimb swipes per day decreased as the rehab days increased in the study. The rehabilitation procedure began 8 days after the onset of stroke, the animals were given a sufficient amount of time to reconcile from the stroke injury. So the best possible explanation for the decrease in the consumption of peanut butter and forelimb movement might be due to physical stress. Post-stroke physical stress can lead to depression which in turn affects the functional recovery and cognitive function (95). The previous stroke study from our laboratory showed a better recovery in the rehabilitation animals compared to the control group. The previous rehab study was carried for 90 days with a rest period of 30 days before euthanization and also only about one-fourth of the peanut butter was hung when compared to our study (96). Whereas in the current study, the animals were consuming an excessive amount of peanut butter by using their forelimb which might have caused physical stress and fatigue in the animals.

Figure 31: *Image showing trough filled with peanut butter from our previous lab study (96).*

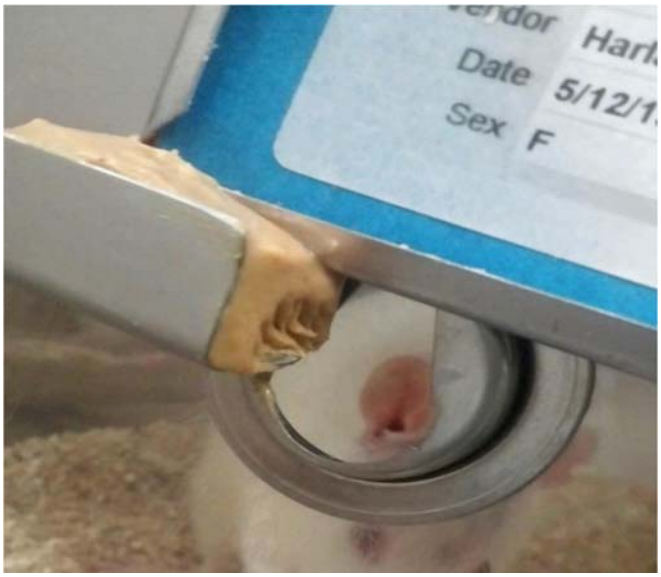


Figure 32: *Image showing trough filled with peanut butter from our current lab study.*

This demonstrates that the repetitive use of impaired forelimb have caused physical stress in the animals. Higher BDNF concentration in the hippocampus region is related to motor and functional recovery in animal models. Research studies have shown that excessive amount of exercise can lead to down-regulation of BDNF. In addition, corticosterone (CORT) a biomarker used for evaluating stress in animal models, have shown to down-regulate BDNF and increased stress levels (100). Thus by analyzing brain BDNF and plasma CORT in our study, we can compare the relationship between motor recovery and stress in animals. The concentration of BDNF and CORT can be evaluated using an enzyme-linked immunosorbent assay (ELISA) (101,102), but we would have done this in the subventricular zone. Overall, we can say that the intensity of a training plays a key role in the rehabilitation and thus the training should be gradually increased to benefit from the rehabilitation outcome.

4.3 FUNCTIONAL RECOVERY

4.3.1 Bilateral deficits and ipsilateral functional recovery

After the stroke induction, the animals were tested for bilateral and unilateral deficits on post-stroke day 3. There are some animals that showed functional deficit on both contralateral and ipsilateral sides (Figure 9). This might have occurred due to the surgical errors, hitting the wrong coordinates or ET-1 reaching the corpus callosum which connects to the right and left hemisphere of the brain, damaging both contralateral and ipsilateral sides leading to the bilateral deficits.

The ipsilateral function was not affected much with the surgery, animals had less than 20% deficit for both control and rehab groups. At post-stroke day 58-60, the ipsilateral function for the control group showed a recovery of about 99.6% and the rehab group displayed an increase with 87%. Although there was no significant difference seen with respect to time or groups, we can see that the animals with the bilateral deficits showed a substantial increase in ipsilateral function. The ipsilateral function almost recovered in control group and bilateral deficits essentially faded at post-stroke day 60. This implies the impromptu recovery of white matter tracts in the control and the rehab groups (98,99)

Furthermore, a correlation between Ki67 and ipsilateral functional recovery from the same study (97) illustrated a strong correlation and significance with the P value of 0.0007 in the control groups. This correlation shows an enhanced recovery in the white matter (Figure 32). In contrast, Figure 33 illustrated a correlation between Ki67 (neurogenesis) and ipsilateral functional recovery in rehab animals and showed no correlation between them. This might be due to the reduction of stem/progenitor cells which in turn have caused due to the stress in rehab animals (97).

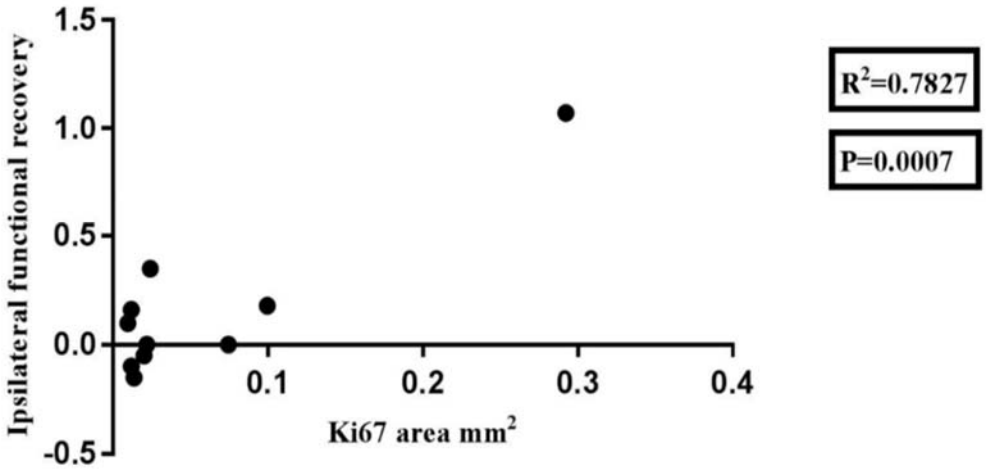


Figure 33: *Image showing correlation between Ki67 total area mm^2 and ipsilateral functional recovery in the control group (97).*

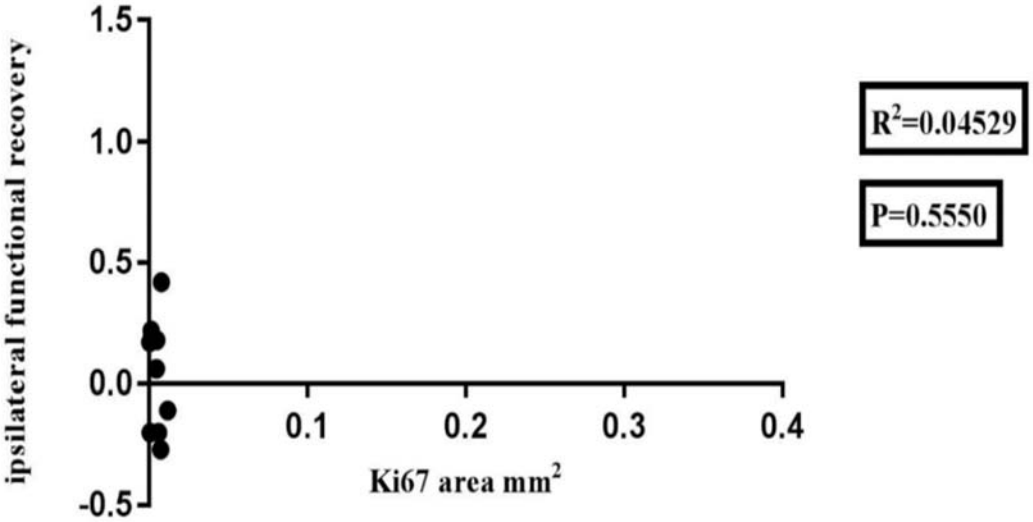


Figure 34: *Image showing correlation between Ki67 total area mm^2 and ipsilateral functional recovery in the rehab group (97).*

In contrast, my results showed a better correlation between DCX staining and ipsilateral functional recovery in rehab and control animals. This is because DCX

immunoreactive cells are highly expressed over 2-3 week after the ischemic injury but Ki67 are expressed at a particular period. Thus Ki67 total area mm^2 showed no correlation with the ipsilateral functional recovery while DCX staining should have shown a correlation.

4.3.2 Contralateral functional recovery:

The results of this study demonstrated that the functional recovery was significantly higher in the control group (contralateral side) compared to the rehab group (Figure12). At post-stroke day 30 and 60, the contralateral function for the rehab group and the control group showed a significant increase with respect to time. The contralateral function on post-stroke day 58-60 in the control group displayed an increase of 77.4% while the rehab group only showed an increase of 60.9%.

This study demonstrated a significant difference in the contralateral functional recovery between the control and rehab groups. The control group displayed a higher contralateral functional recovery of about 53% and the rehab group regained only about 27% at post-stroke day 60. We saw a 10% decrease in the contralateral functional recovery in the rehab animals with respect to post-stroke day 30. Whereas the control group contralateral recovery remained stable after post-stroke day 30.

Furthermore, we also saw a significant difference in the total recovery in the control and rehab groups. The control group recovered better with an increase of 70.4% and the rehab group regained only about 34.1% of their total recovery. These results explain that the control animals displayed a better functional recovery compared to the rehab animals. In our previous study, rehab animals recovered better compared to the control animals. The contralateral function for the rehab group was 26% and the control group was 8.6% on post-stroke day 90. Although physical

rehabilitation did not show any correlation with the functional recovery, there was a steady statistical trend (96).

From the graph (Figure 34) we can explain that there is no strong correlation between the physical rehabilitation (measured by arm swipes) and the total functional recovery (97). As the arm swipes increase the total functional recovery decreased in some of the animals. This might be the result of physical stress (increased CORT levels) in the rehabilitation animals. We hypothesized to see increased functional recovery correlated with the voluntary physical rehabilitation. However, we saw no correlation between them. Physical rehabilitation did not result in increased functional recovery.

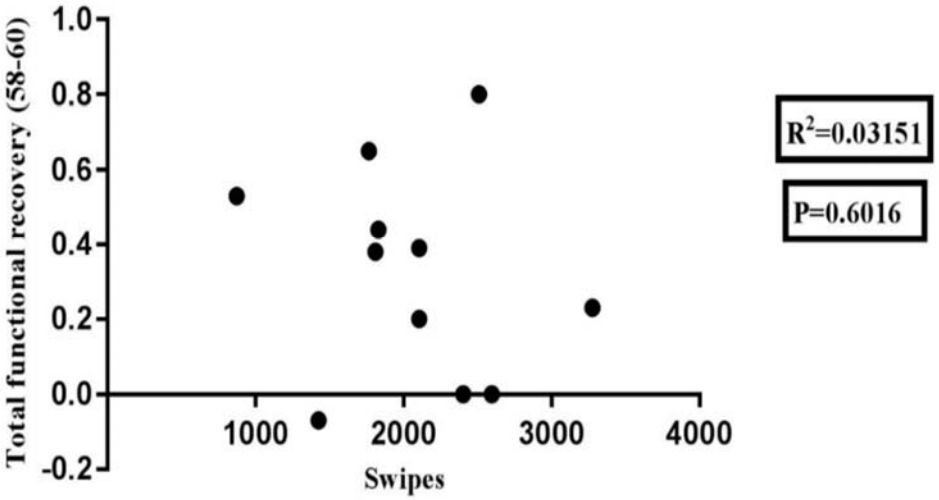


Figure 35: *Graph showing correlation between the arm swipes and the total functional recovery. Each dots represents a single animal (97).*

4.4 NEUROGENESIS:

In our study, doublecortin (DCX) was used as a neuronal marker for measuring neurogenesis after ischemia. We studied all the three regions (anterior,

middle, posterior) of the subventricular zone in the rat brain. Although we did not see a significant difference between the control and the rehab groups, the rehab group emphatically showed a higher mean value in all the three SVZ regions compared to the control groups (Figure 21, Figure 23 and Figure 25). Likewise, there was no significance seen between the control and the rehab groups for the total mean of the SVZ doublecortin, but certainly the rehab group demonstrated a higher mean value compared to the control group (Figure 26).

Also, we compared all the three regions of the SVZ using the two-way ANOVA (Figure 27). We saw a significant difference between the rehab and the control group, where the rehab group showed a higher production of neurogenesis (DCX) compared to the control group.

4.4.1 The Effects Of Stress On Adult Neurogenesis:

Some studies have shown that there is a reciprocal relationship between adult neurogenesis and stress in rodents. The stress modulators can affect adult hippocampal neurogenesis in the sub-granular zone (SGZ) which is the hippocampal neurogenesis niche (103). Although there are several modulators that induce stress including pro-inflammatory cytokines, neurotropic factor (BDNF) and morphogen signaling pathways, glucocorticoid is one of the key modulators for stress. Both acute and chronic stress in rats can predominately reduce neurogenesis, cell proliferation in the hippocampal region (103).

In our study, the potential reason for not displaying a statistically significant difference in the total SVZ region (DCX neurogenesis) between control and rehab might be due to the overuse of impaired forelimb during rehabilitation procedure. The overuse of forelimb has resulted in the stress which in turn affected the production of

neurogenesis in rats. We did not anticipate that the animals would voluntarily stress with overuse of impaired forelimb.

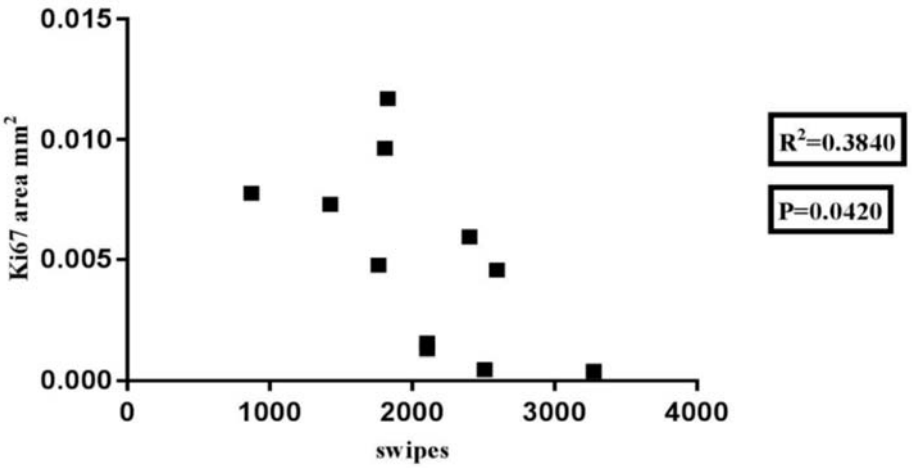


Figure 36: *Graph showing correlation between total arm swipes during rehabilitation and Ki67 area in mm^2 (97).*

Figure 35 was acquired from the same study (97), the graph shows the correlation between the total arm swipes during rehabilitation and Ki67 (stem cell proliferation). This demonstrated that the increase in the forelimb swipes above 2000 had triggered a negative impact on the stem cell proliferation (Ki67) in the subventricular zone (97). This is possibly due to the effect of stress from overdoing of physical rehabilitation.

4.4.2 Effects of Estrogen on Neurogenesis and Functional Recovery After Ischemia:

Many studies have shown that estrogen can increase the number of newborn neurons in the SVZ of an animal model and also undergo brain repair after ischemia. Estrogen expression is prominent in the pre-menopausal rats, which has shown

neuroprotective role and increase in the stem cell proliferation in some of the studies (75,104).

In our study, we assume that some of the rats in the control group may still have estrogen expression (pre-menopausal) after ischemia. This can be one of the possible explanations for an increase in neurogenesis seen in the control group. Estrogen levels have to be tested to validate the results. Estrogen can be quantified using ESTRADIOL ELISA kit.

4.5 FUTURE WORKS:

In the future study, some alterations are required to further investigate and validate the results. The levels of corticosterone need to be quantified in order to understand the stress response in animals. High levels of corticosterone can down-regulate BDNF which decreases neurogenesis in rats. The concentration of BDNF and CORT can be evaluated using an enzyme-linked immunosorbent assay (ELISA) (100-101). Since estrogen plays an important role in neuroprotection and increase in the stem cell proliferation, estrogen levels have to be evaluated to determine if the animals are post-menopausal.

Since rehabilitation was a major part of this study, rehab time and intensity of the exercise should be reduced to improve stress endurance and show better recovery in animals. The intensity of the exercise should be gradually increased in order to see better recovery without stress in animals. Furthermore, trough height and distance can be modified to strengthen recovery of impaired forelimb by making them use different postures. Also the amount of peanut butter given to the animals during rehabilitation should be reduced to avoid abrupt physical stress in animals. A gradual program of rehabilitation, with slow buildup of use of the arm would avoid physical

stress in the arm and would likely improve functional recovery.

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