CORRELATING INNATE FUNCTIONAL RECOVERY FROM STROKE EITHER WITH STEM CELL PROLIFERATION AND/OR LIMB REHABILITATION

A thesis submitted in partial fulfillment of the requirements

for the degree of

Master of Science

By

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2016

Wright State University

WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL

July 28, 2016

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Devipriyanka Nagarajan ENTITLED Correlating Innate Functional Recovery From Stroke Either With Stem Cell Proliferation and/or Limb Rehabilitation. BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Nagarajan, Devipriyanka. M.S., Microbiology and Immunology, Wright State University, 2016. Correlating Innate Functional Recovery from Stroke either With Stem Cell Proliferation and/or Limb Rehabilitation.

In the present study 10-12 month female rats were examined for functional recovery from stroke and this recovery was compared with the stem cell/progenitor cell proliferation in the brain (which was measured by Ki67). The cell proliferation indicated by Ki67 showed a 6 fold increase in control animals compared to the rehabilitation animals. The contralateral functional recovery in control animals were 46.6% and in the rehabilitation animals were 24.5%.

The physical rehabilitation was carried out to determine if limb rehabilitation can promote greater functional recovery. The results showed that when the animals were made to over use their impaired limb compared to non-impaired limb, they experienced physical stress that decreased the stem cell/progenitor cell proliferation in the subventricular zone. This is the first study which shows that increased physical stress (due to voluntary exercise) will decrease the Ki67 levels in the subventricular zone in animals after ischemic stroke.

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AF	Atrial Fibrillation
ANOVA	Analysis of variance
Ant	Anterior
AP	Anterior Posterior
AUP	Animal use protocol
BDNF	Brain derived neurotrophic factor
BrdU	5-Bromo-2'-deoxyuridine
DAB	3,3' – diaminobenzidine
DCX	Doublecortin
DPX	Distyrene, aplasticizer and xylene
FDA	Food and Drug Administration
IACUC	Institutional animal care and use committee
IgG	Immunoglobulin G
ip	Intraperitoneal
L	Left
Mid	Middle
ML	Medial Lateral
mm	Millimeter
OCT	Optimal cutting temperature
PB	Peanut butter
Post	Posterior
PSD	Post stroke day
R	Right
R^2	R square
rec	Recovery
SEM	Standard error of mean
SGZ	Subgranular zone
SVZ	Subventricular zone
TIA	Transient Ischemic stroke
Tot	Total
tPA	Tissue plasminogen activator
µL	Microliters

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Adrian Corbett for being such a wonderful mentor and who has supported me throughout my thesis with her patience, motivation and immense knowledge. She has been the best adviser I ever had and always responded to my questions so promptly. I am so grateful to her, she helped me fulfill my dream of earning a Master's degree in the United States.

I would like to express deepest gratitude to my program adviser Dr. Barbara Hull for her full support, expert guidance, relevant assistance and help to complete this study and research. I also thank Dr. Debra Mayes for being a part of my thesis committee, and also for her encouragement and intellectual guidance. I am also grateful to my lab members and my friends who have also been there for me through thick and thin.

Finally, I would like to thank my family to whom I owe a great deal. My mother Nirmala and brother Sridhar for their unconditional love, support, encouragement and have made an untold number of sacrifices for me.

This thesis is dedicated to my father R. Nagarajan, who had always been a great source of inspiration in my studies and life.

I. INTRODUCTION

1.1 STROKE

Stroke is considered the fourth leading cause of death in the world (1), with one out of every nineteen individuals dying because of stroke (2). Stroke is a primary cause of long term disability (2) and is said to affect/damage the arteries leading to the brain and the arteries within the brain (3).

Stroke is the fifth leading cause of death in the United States of America. Nearly 795,000 Americans suffer from new or recurrent stroke every year (4) . Stroke occurs every 40 seconds in the United States (6) and it kills about 129,000 a year (approximately 60% females). It is also the leading cause of adult disability (4).

Stroke risk factors can be high blood pressure , Diabetes Mellitus, disorders of heart rhythm, high blood cholesterol and other lipids , smoking / tobacco use, physical inactivity, nutrition and family history and genetics, as well as chronic kidney disease (4) .

1.2 TYPES OF STROKE

There are two major types of stroke: ischemic and hemorrhagic. Ischemic stroke occurs when the blood supply leading to the brain gets blocked, due to the blood in the vessel clotting. When this happens, the brain is deprived of blood and oxygen, therefore cells in the brain die (3).

Stroke-enabling clot formation occurs following the deposition of fatty acids (cholesterol), calcium and cellular waste products inside arteries. These settling substance are termed as plaque and results in the hardening and narrowing of the blood vessel (arteries) which is called Atherosclerosis. The blockage by a clot of the brain's blood vessels which are atherosclerotic is called cerebral thrombosis (3).

When a portion of clot breaks from the blood clots that have formed at other locations (arteries of upper chest, neck or heart), it travels through the bloodstream and reaches the brain blood vessels and blocks the blood supply. This is called as cerebral embolism. Embolism can also be caused due to atrial fibrillation (irregular heartbeat). Cerebral embolism and thrombosis are two types of ischemic stroke (3).

Thirteen percent of stroke cases are hemorrhagic stroke which includes intracerebral hemorrhagic stroke (10%) and sub arachnoid hemorrhagic stroke (3%) (5). The weakening of the artery's walls and bulging of the arteries is called an aneurysm. When these weakened arteries rupture, they burst and bleed in the tissue deep within the brain surface, then they are said to be intracerebral hemorrhagic stroke. The blood accumulation in the surrounding brain causes compression of the surrounding tissues there (6). Sudden increases in the blood within the brain cause pressure to be built up, which causes unconsciousness and death in severe cases (7). Subarachnoid hemorrhage is when the bleeding occurs from rupture of a surface artery, so the blood fills the space between the skull and the brain (3)

The Transient Ischemic Attack (TIA) is caused due to a temporary clot and it causes minor damage to the brain. This might lead to full stroke, if it is left untreated (3). People suffering from TIA are at higher risk of having a stroke (8).

1.3 RISK FACTORS SPECIFIC TO WOMEN

Women are more prone to suffer a stroke than men, and they seem to be approximately four years older at the time of their first stroke compared to men (9). In US approximately 55,000 more women have a stroke than number of men who are affected by stroke (10). Also women with Atrial Fibrillation (AF) are at higher risk of stroke occurrence than men (11-15). Women who use replacement estrogen and progestin during menopause, also seem to be at higher risk of getting a stroke (16). Data from Framingham Heart Study say that women with early menopause (before 42 years) have twice the risk of having a stroke (17). Females who use low-estrogen-dose oral contraceptives have a 93% increased chances of getting an ischemic stroke compared to women who do not take oral contraceptives (18, 19).

1.4 ISCHEMIC STROKE TREATMENT

Nearly two million brain cells die each minute, in a untreated stroke (6). About 87% of stroke cases are of ischemic stroke. So far the only successful treatment for ischemic stroke has been the tPA (Tissue plasminogen activator). It is the only drug approved by Food and Drug Administration (FDA) and it is administered intravenously into arm. tPA is an enzyme and it functions in the breakdown of fibrin in blood clots (20), tPA was approved in the year 1996 for stroke treatment (21). But, tPA can be only given to the patients within the first three to four hours after stroke onset, otherwise it would cause a bleeding in the brain (22). For that reason, many patients do not get to the hospital soon enough for this treatment.

Mechanical thrombectomy is another alternative method to the tPA treatment, where a stent retriever is inserted into the groin arteries via catheter to the blocked up

brain clot, the stent will engulf the clot and stent is removed from the body with trapped clot (6). This technique must be performed within six hours of stroke occurance and the mechanical thrombectomy can be performed only after performing the tPA treatment (23).

1.5 NEUROGENESIS

Aging occurs concomitantly with alterations in the physiology and plasticity of neurons in brain (i.e. decrease in dendritic synapses or loss of synaptic plasticity) (24), increase in oxidative stress (25) and increase in pro-inflammatory cytokines (26). Such factors influence majority of neurodegenerative diseases (27). Neurogenesis refers to the production of new neurons from stem/progenitor cells in the subventricular zone (SVZ) of the lateral brain ventricles and the sub granular zone (SGZ) of the hippocampus (28).

Neurogenesis can be altered by factors like drugs (29), exercise (30-32), environmental enrichment (30, 33), learning and stress (psychosocial and physical stress) (34-36). Stress and aging downregulated neurogenesis. Subventricular zone and subgranular zone are considered as two crucial stem cell locations or neurogenic niches in brain. A decrease in adult neurogenesis may cause disorders like Alzhemier's disease, Parkinson's disease and dementia. Researchers suggest aging could be a stem cell disorder, as a decrease in the proliferation of stem cell was observed during aging (37, 38)

Stroke can trigger the striatal neurogenesis (measured by Ki67 expression in SVZ and DCX (striatum)) in older rats (39). Stroke has the ability to trigger neurogenesis not only in the neurogenic niches but allow these migrating neuroblasts to reach the injury site due to increased growth factors in the injured region of the brain (39).

1.6 Ki67

Ki67 a nuclear protein which is used as marker for cell proliferation. It has also been called Antigen Ki67 in humans: at interphase it is seen within the nucleus and at the mitosis stage it would be seen at the surface of the chromosomes (40). Ki67 is present in cell division phases of G1, S, G2 and mitosis. Cells in G0 phase do not express it (41). For staining purpose, Ki67 antibody reacts with nuclear structure of the proliferating cells alone (40), so it is a very good marker for stem/progenitor cell proliferation in the brain.

1.7 REHABILITATION POST STROKE

Functional deficits occurs when there is ischemic injury within the motor cortex, which greatly impairs routine life of patients (42). Patients with ischemic stroke show varied neurological deficits, which primarily depend on the location and size of the brain injury (42). Injuries in the motor cortex would result in functional deficits in the limbs (motor impairments) and it could potentially be shattering as they affect routine tasks like dressing, eating, drinking, using the bathroom, showering, shopping, transportation, and writing (42-45). Rehabilitation is important as it helps in the restoration of movement, stability and its co-ordination (46) Stroke physicians generally recommend rehabilitation therapies to maintain physical independence post stroke (47). A study also shows that rehabilitation therapy at an early stage post stroke (24-72 hours) have greater effects (48). It also might enhance self-esteem as the patient relearns basic skills impaired due to stroke (49). Rehabilitation post stroke has been seen as an effective therapy for stroke patients with varying degrees of severity(50).

The enlargement of ischemic injury may be also prevented by exercise (51, 52). Exercise at the early stage also can suppress pro inflammatory response (53) or increase angiogenesis in ischemic injury site (54). Exercises are also said to regulate BDNF (brain derived neurotrophic factor) expression (55). BDNF is a growth factor which supports growth and survival of neurons (56), stimulation of neurogenesis (57), learning and memory (58-60) promotion of neuroplasticity (61).

1.8 HYPOTHESES

1. Functional recovery from stroke will be correlated with stem cell/ progenitor cell proliferation (measured by Ki67) in the Subventricular zone.

2. Rehabilitation of impaired limb will lead to greater functional recovery.

1.8.1 Specific Aims:

1. Analyze the effect of limb rehabilitation on stem cell/ progenitor cell proliferation (Ki67 staining) and overall functional recovery, measured with Montoya Staircase.

A larger population of women (aged) than men suffer from stroke and these studies are focused on older female rats who have undergone stroke. This study will examine the functional recovery from stroke and compare it with the stem cell/ progenitor cell proliferation in the brain, which will be measured using the Ki67 as a marker. These animals are not treated with any sort of drugs in the pre stroke and post stroke period. We measured stem/progenitor cell proliferation in the Subventricular zone as it is closest to the actual infarct produced in the forelimb motor cortex. An ischemic

stroke has the ability to trigger neurogenesis, which we hypothesized will promote functional recovery. From numerous stroke studies in the literature (22, 62-64), we also hypothesize the greater functional recovery can be achieved when there is physical rehabilitation treatment post stroke.

II.MATERIALS AND METHODS

All the protocols mentioned here were done with the guidelines provided by Wright State University Institutional Animal care and use committee (IACUC).

Twenty four Sprague Dawley female retired breeder rats of 10-12 months of age were used for the study. The weight of the rats was recorded before the start of the protocols and each week thereafter.

2.1 PRE-STROKE TRAINING

Montoya staircase was used for Pre-stroke training. It is used to evaluate the independent use of forelimbs in skilled reaching and grasping tasks(65). Sucrose pellets are placed on the staircase and presented bilaterally at seven graded steps of reaching difficulty to provide objective measures of side bias, grasping skill and maximum forelimb extension (65). Three sucrose pellets were placed on each step in the staircase. Thus the staircase on each side of the platform which supports the rat's body had 21 pellets. Some of the pellets were coated with maple extract. Each rat was trained for 15 minutes daily for one and half weeks during the dark cycle pre-stroke (66). Training data (number of pellets retrieved by each paw) were recorded to determine the pre-stroke function. The sucrose pellets collected by each forepaw were recorded and the total highest number of pellets achieved during the last three trials was considered as the pre-

stroke function. The animals should at least have the ability to pick up nine pellets to be considered for the post stroke analysis (22)

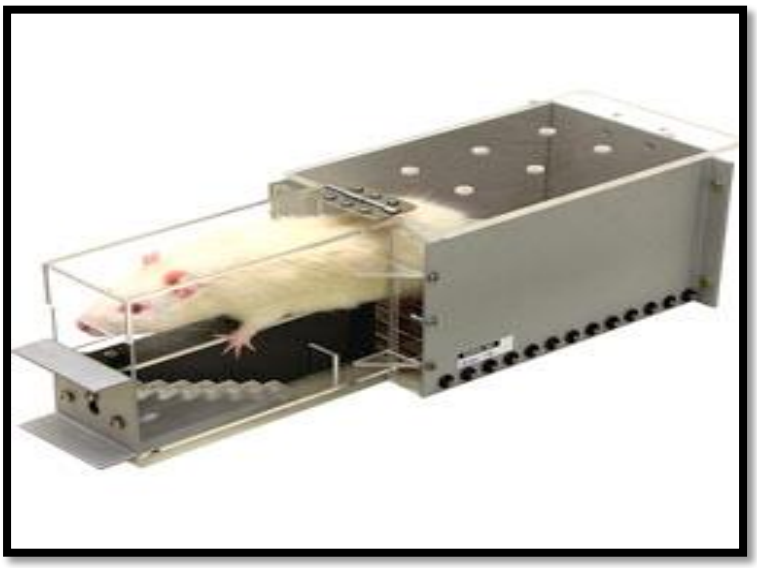


Figure 1: Image showing Montoya Staircase Setup

2.2 DIET RESTRICTION

The rats were placed on restricted diet to optimize the Montoya staircase results during the pre-stroke Training and post stroke testing. The diet was restricted to 15% of their normal intake. In general rats only lost about five to ten percent of their body weight (65) during the pre-stroke training and suffered no significant loss of weight during the post stroke testing.

2.3 INTIAL RESPONSE TO REHABILITATION SHELVES

Shelves which contained about 10-12 grams of peanut butter were hung outside the rat cages, to the left side of an opening at the front of the cage as one faces the cage, or to the right side of the opening as the rat faces the opening. The shelf was positioned in such a way that the rat could use only the left forelimb (which would be impaired after

stroke surgery) to consume the peanut butter. Animals which consumed peanut butter were considered for the rehabilitation study.

2.4 STROKE INDUCTION

All rats in the study had a stroke surgery (Animal approval protocol: AUP 1015). The surgery was carried out with the help of the stereotactic apparatus using non-traumatic ear bars (Stoelting Co., USA) (22, 67). The stereotactic apparatus helps to align the microdrill tip with bregma position on the skull, which is necessary to determine the co-ordinates of the forelimb motor cortex (68). Initially the rats were placed in a closed plexi-glass chamber which we supplied with five percent of isoflurane to induce anesthesia. The animal was removed from the glass chamber and its head was shaven, then was quickly placed on the stereotactic apparatus, its head was stabilized using the non-traumatic ear bars and tooth hold and was supplied with approximately 2.5% of isoflurane during the surgery through an anesthesia mask. To determine if animal had reached a surgical plane of anesthesia its foot or tail was pinched (in a surgical plane of anesthesia there is no response to pain). Puralube® was applied on the animal's eyes to keep them hydrated/moist throughout the surgical procedure. Povidone Iodine was used to clean the incision site, it was followed by 70% ethanol and once again by povidone iodine to maintain sterility at incision site. An incision was made on the scrubbed region of the shaven head and few drops of bupivacaine (analgesic) was applied on the incision edges. Bupivacaine will numb the region up to twelve hours. Blood and fascia were removed from the incision site until the skull was fully exposed. Bregma was located on the skull and marked using a fine point marker (22). Stereotactic apparatus was used to determine the bregma co-ordinates. The forelimb motor cortex location on the right

hemisphere of the brain was calculated using bregma co-ordinates (68). The two locations are shown in the table, where the first hole is located at the edge of the forelimb motor cortex and the second hold is directly in the center of the forelimb motor cortex.

Table 1: Stroke induction co-ordinates. AP refers to Anterior-Posterior and ML refers to Medial Lateral

	AP Position from Bregma	ML Position from Bregma
First Hole	0 mm	2.5 mm
Second Hole	1.5 mm	2.5 mm

A micro drill (0.7 mm diameter, Fine Science Tools. Foster City, CA) was used to drill holes at the two positions. The site was cleared of the bone dust, and a Hamilton syringe with three micro liters of endothelin was positioned on the stereotactic apparatus. The syringe was positioned in the hole, a depth of two millimeter (69). Each of the holes were injected with about 1.5 micro liters of the endothelin. The endothelin-1 (Human/Porcine, EMD Chemicals; 400 pmoles/microliter) was injected very slowly, about 0.1 microliter (69) for every ten exhalations. After endothelin administration, the surgical site was sutured (Vicryl absorbable sutures) and povidone iodine was applied on the incision site (to avoid microbial contamination). The animal was injected subcutaneously with 2 ml of saline and the anesthesia supply was cut off but oxygen flow was left on until the animal regained consciousness. The animal's tail was pinched at intervals to determine if the animal was leaving the surgical plane of anesthesia. When the animal woke, the anesthesia mask was taken off and the animal was put back in its cage, on a heating pad, until full locomotion was observed, at which time it was returned

to its normal housing area. The animal was given moist chow for three post stroke days and normal chow after that (22).

2.5 POST STROKE TREATMENT

The rats were given 4 grams of Pillisbury sugar cookie dough daily for sixty days (vehicle control) (62). The rat weights were recorded once a week. They were fed with rat chow daily, restricted only during the Montoya Staircase testing intervals (3 day periods) at various times post stroke.

2.6 REHABILITATION SHELVES

The animals that were selected for rehabilitation (see page 9) were given about 12-14 grams of peanut butter on a hanging shelf, every other night beginning 8 days post stroke. The shelves were hung in such a way that the animals could use only their contralateral forelimb (left limb) to consume the peanut butter. Next morning, the amount of peanut butter consumed by the animal was measured and recorded. Rehabilitation continued over a total period of about fifty two days. The number of arm swipes the rat performed to get the peanut butter was also calculated to determine number of times the animal used its forelimb to reach the peanut butter. For this measurement, we weighed the peanut butter on the shelf before hanging it, and then waited until the rat had performed 5, 10, 15 or 20 arm swipes across the peanut butter in the shelf, and then reweighed the shelf with peanut butter to determine how much peanut butter an animal would obtain in a single swipe (average calculation). For example, if an animal ate 0.20 grams of peanut butter with 20 arm swipes, then each arm swipe obtained a mean of 0.01

grams of peanut butter for that animal. We obtained values from at least ten rats to determine the mean amount of peanut butter obtained per arm swipe.

2.7 POST STROKE FUNCTIONAL TESTING

Montoya staircase testing was performed on post stroke days 3-5, 28-30 and 58-60. The functional test on post stroke day 3-5 is carried out to determine the baseline deficit following the stroke and the rest of the days to determine the functional recovery post stroke. The animal's baseline deficit can be calculated as below

The Montoya staircase was performed on post stroke day 3, 4, 5 and to identify if the animals that underwent the stroke surgery had a motor deficit or not. The Baseline Function pre stroke for the left and right forepaw was obtained from the highest amount of total pellets obtained in the last three days of pre-stroke training. For example, if the animal retrieved 13L, 15R one day, 14L, 15R the next day and 12L, 18R the final day, then the pre-stroke baseline function for the left would be 12 pellets and the baseline function for the right would be 18 pellets. The contralateral and ipsilateral function were recorded and the functional deficit was calculated accordingly.

The contralateral and ipsilateral function were calculated by the following way:

$$\text{contralateral function} = \frac{Number\ of\ pellets\ \ PSD3}{\text{Baseline Function (Left)}}$$

$$\text{ipsilateral function} = \frac{Number\ of\ pellets\ \ PSD3}{\text{Baseline Function (Right)}}$$

$$\text{Contralateral Deficit} = 1 - \text{contralateral function}$$

$$\text{Ipsilateral Deficit} = 1 - \text{ipsilateral function}$$

$$\text{Total Deficit} = \text{contralateral function} + \text{Ipsilateral function}$$

2.7.1 Functional Recovery Calculation for Post stroke days 30 and 60:

For Post stroke day 30

$$\text{Contralateral Recovery} = \text{Contralateral function (PSD 30)} - \text{Contralateral function (PSD 3)}$$

$$\text{Ipsilateral Recovery} = \text{Ipsilateral function (PSD 30)} - \text{Ipsilateral function (PSD 3)}$$

$$\text{Total Recovery} = \text{Contralateral Recovery} + \text{Ipsilateral Recovery}$$

For Post stroke day 60

$$\text{Contralateral Recovery} = \text{Contralateral function (PSD 60)} - \text{Contralateral function (PSD 3)}$$

$$\text{Ipsilateral Recovery} = \text{Ipsilateral function (PSD 60)} - \text{Contralateral function (PSD 3)}$$

$$\text{Total Recovery} = \text{Contralateral Recovery} + \text{Ipsilateral Recovery}$$

Contralateral refers to the side affected by stroke (Left side) or the opposite side from where the stroke was induced and ipsilateral refers to the paw on the same side that the stroke was induced. The right forepaw would be controlled by the left hemisphere, which did not have a stroke, so we do not normally see any functional deficit on this side.

2.8 EUTHANIZATION

Sixty days post stroke the animals were euthanized. The animals were injected ip with euthasol (100mg/kg i.p pentobarbital) and the cardio-perfusion was carried out when the animal reached the surgical plane of anesthesia. The Cardio-perfusion was carried out to get rid of the blood from the animal's whole body by flushing it with phosphate buffered solution through a catheter in the left ventricle, allowing the blood to leave the body through a cut in the right atria. It was followed by 4% paraformaldehyde in phosphate buffered saline to fix the tissues. The rat's head was decapitated and brain dissected, and the brain coronally blocked and stored in the 4% paraformaldehyde solution overnight. Twenty four hours later the brain was removed from paraformaldehyde and stored in 30% sucrose solution for three days (67) to prepare it for cutting on a cryostat.

2.9 CRYOSECTIONING OF BRAIN TISSUE

The brain tissues were removed from sucrose solution and they were prepared for cryo-sectioning using OCT (Optimal cutting temperature) compound on a stage in combination with a freezing peltier device. Thirty minutes were given for the tissue to reach a frozen state and also to let the tissue temperature match with that of the chamber temperature (-25 degrees Centrigrade) of the cryostat. The tissue was then placed in the pedestal support and sliced coronally (50 μm thickness). The sliced sections were collected starting before the infarct appeared and stopped when the infarct disappeared. The slices were collected in four vials and stored in Phosphate buffer solution. The vials

were numbered 1, 2, 3 and 4 and they were labelled as infarct, Ki67, Doublecortin (DCX) and control antibody staining (minus primary antibody) respectively.

2.10 Ki67 ANTIBODY STAINING

For the Ki67 antibody staining, the phosphate buffered saline solution (PBS) in the vials was removed and 1.5 milliliters of blocking solution (PBS with 0.3% tween and 3% goat serum) were added to the vials and incubated for one hour. Then about of 1:750 dilution (2µL) of AbCam anti-Ki67 ab15580 GR181198-1 (rabbit anti-Ki67 primary antibody) was added to the Ki67 vial and incubated overnight in cold (5°C) and dark room, nothing was added to the control vial and it was just left with the blocking solution and incubated overnight in cold dark room. Sixteen hours later the vials were brought back to room temperature, the blocking solution and unbound antibody was removed from the Ki67 vial and washed thrice with PBS-Tween. To the Ki67 vial, 1.5 mL of blocking solution was added, followed by addition of 7.5 µL of biotinylated secondary antibody (goat-anti-rabbit IgG) from Vector ABC kit to both the vials (Ki67 and Ki67 control). The vials were incubated for one hour and then washed thrice with phosphate buffer solution and replaced with 1.5mL of ABC reagent (Avidin, and biotinylated goat anti rabbit IgG labelled with horse-radish peroxidase) was incubated for forty five minutes and then removed, and washed twice with PBS-Tween. Finally, the DAB substrate was added and the reaction was stopped with water after 10 minutes of development. We then replaced this solution with phosphate buffered saline solution.

2.11 TISSUE MOUNTING

The vials containing stained tissue slices were emptied into a petri-dish containing PBS. A thin tip paint brush was used to mount the coronal brain slices onto gel coated slides. About 4-5 slices were mounted in a slide. We tried to make sure that the slices were not folded or damaged. After the tissues were dry, DPX mountant was put over the slices and a coverslip was placed on top to cover and pushed down until all sections were covered with the mountant (Sigma- Aldrich).

2.12 IMAGE ANALYSIS

The slides were observed under the bright field microscope (40X total; 4X objective and 10X ocular) to spot Ki67 antibody staining along the sub ventricular zone, which was a dark staining. On identification of the Ki67 antibody staining in the slices, digital pictures were taken and saved with respect to rat's identification number, position on a particular numbered slide, and condition for staining (Ki67 staining or control)

The Ki67 antibody staining area was estimated by using image J software and a calibration scale for each microscope used. We first set the calibration scale for the microscope picture (number of pixles per mm), then adjusted the threshold until only the Ki67 staining was highlighted. We then saved the mask of Ki67 staining and measured it with a free-hand tool, enabling us to remove any extraneous dots.

2.13 STATISTICAL ANALYSIS

Graph pad prism 6 software was used to determine the statistical difference between the groups. Statistical methods used include T test (Welch’s correction for

unequal variances), linear regression, correlation analysis, two way ANOVA and contingency tests.

III. RESULTS

There were twenty four initial animals in the following study and they were grouped as control with rehabilitation and control without rehabilitation. Table 2 shows number of animals in each of the groups.

All twenty four animals were given ischemic stroke which was induced by endothelin-1 (a powerful vasoconstrictor). The surgery was successful and all the animals survived post stroke except for one animal in the control with rehabilitation group. Control without Rehabilitation animals are represented as Control and the control with Rehabilitation animals are represented as Rehab or Rehabilitation.

Table 2: Showing number of animals in each groups

	Control without Rehabilitation **(CONTROL)**	Control with Rehabilitation (**REHAB)**
Number of animals	12	12
Number of animals after surgery	12	11

3.1 RAT WEIGHTS

3.1.1 Pre Stroke Rat Weight Analysis:

We recorded the animals' weight from the beginning of the study (5/31) until the rats were sacrificed (7/20). The two group weights were compared statistically using a T test (Welch's correction for unequal variance). We started recording the rats' weight from pre stroke (5/31) and until post stroke day 60. The rats' initial weight can be seen below, which shows the rat weight comparison between two groups at pre stroke period.

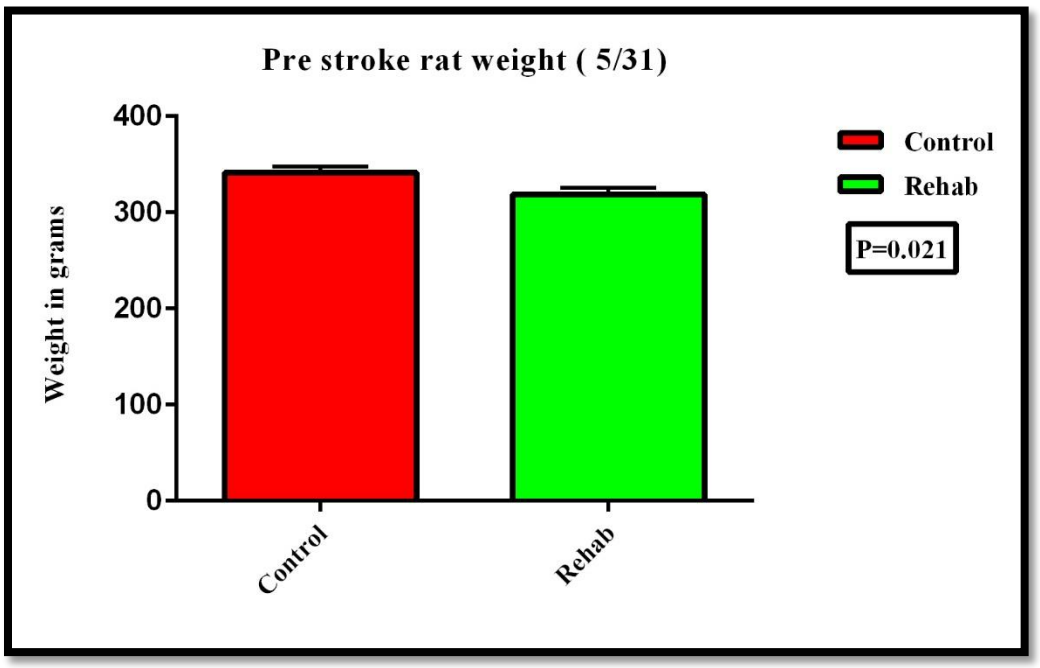


Figure 2: Comparison of rat weights at pre stroke period. The x axis denotes two groups: control animals (red bar, n=12) and rehabilitation animals (green bar; n=12). The y axis denotes the rat weights in grams. The statistical comparison was performed using T test (with Welch's correction for unequal variance) and was significantly different (P=0.021). Columns show mean weights for each group and error bars represent standard error of mean (SEM)

From the figure 2 we can observe that there is a significant difference (P=0.021) between two groups. The mean ±SEM for control was 341.6 ± 6.277 grams and rehab was 318.9 ± 6.577 grams. The animal grouping was chosen upon their ability to eat the peanut butter in a test pre-stroke. Animals which consumed large quantities of peanut butter were considered for rehabilitation procedure. It was just unfortunate that in the initial weight recordings, rehabilitation animals weighed lesser than the control animals. It might have also been due to animal's appetite. The animals that weighed less were not eating enough rat chow, hence they were hungry all the time and ate more of peanut butter. The grouping was actually made only after the initial weight recordings, but were not based on the animal's weight.

3.1.2 Post Stroke Rat Weight Analysis:

Figure 3 shows the final weight recording (7/20) of the animals before they were sacrificed. It was recorded to determine, if there were any weight gain or significant differences between two groups due to peanut butter consumption in the rehabilitation group.

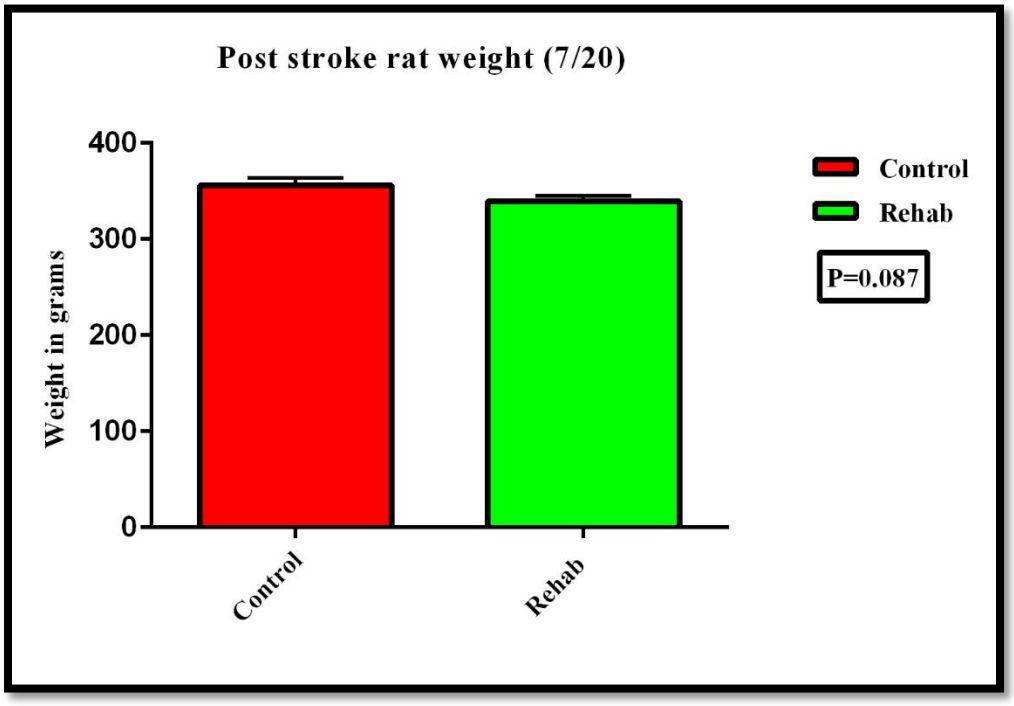


Figure 3: Comparison of rat weights in both groups at post stroke day 60. The x axis denotes two groups: control (red bar; n=12) and rehabilitation animals (green bar; n=11). The y axis denotes the rat weights in grams. The statistical comparison was performed using T test (Welch's correction) and showed no statistical difference. Column show mean weights for each group and error bars represent SEM.

There was no significant difference (P=0.087) between two groups at the end of this study (Figure 3). The mean ±SEM for control was 356.3 ± 7.354 grams and rehab was 339.6 ± 5.578 grams. The mean weights of the animals in the two groups at post

stroke day 60 did not differ significantly. Thus, initial significant weight difference seen in Figure 2 was abolished over time.

3.1.3 Comparison of Initial Rat Weight and Final Rat Weight:

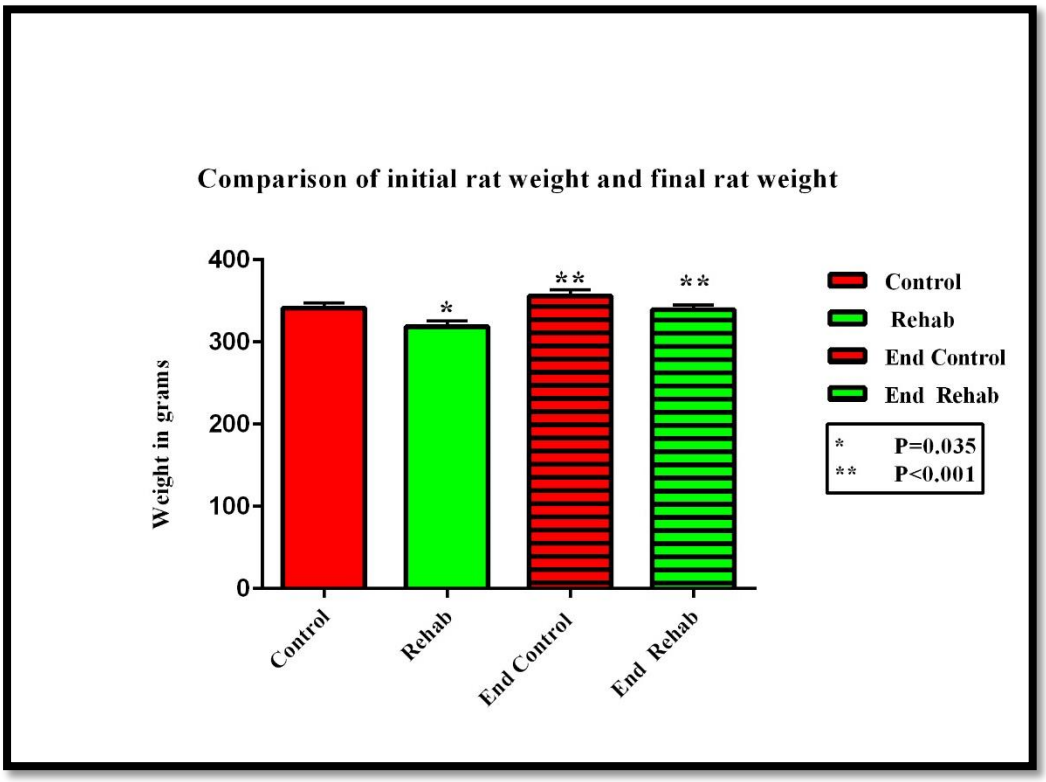


Figure 4: Comparison of initial rat weight and final rat weight. The x axis denotes two groups change over time: control animals pre stroke weight (red bar; n=12) , rehabilitation animals pre stroke weight (green bar; n=12) , control animals post stroke weight (red striped bar; n=12) and rehabilitation animals post stroke weight (green striped bar; n=11). The y axis denotes the rat weight in grams. A repeated measures 2-way ANOVA was performed to determine the statistical differences. Columns show mean weights for each group and error bars represent standard error of mean (SEM). An asterix (*) denote P=0.035 and double asterix (**) denote P<0.001.

In Figure 4 the initial rat weight (5/31) and the final rat weight (7/20) were compared for the two groups. The figure shows that there is a high statistical difference (P<0.001) between weights with respect to time periods, and also a statistically

significant difference between the groups at the initial weighing period (P=0.035). Thus all of animals gained significant weight over the period of study.

3.1.4 Control Animals Weight History:

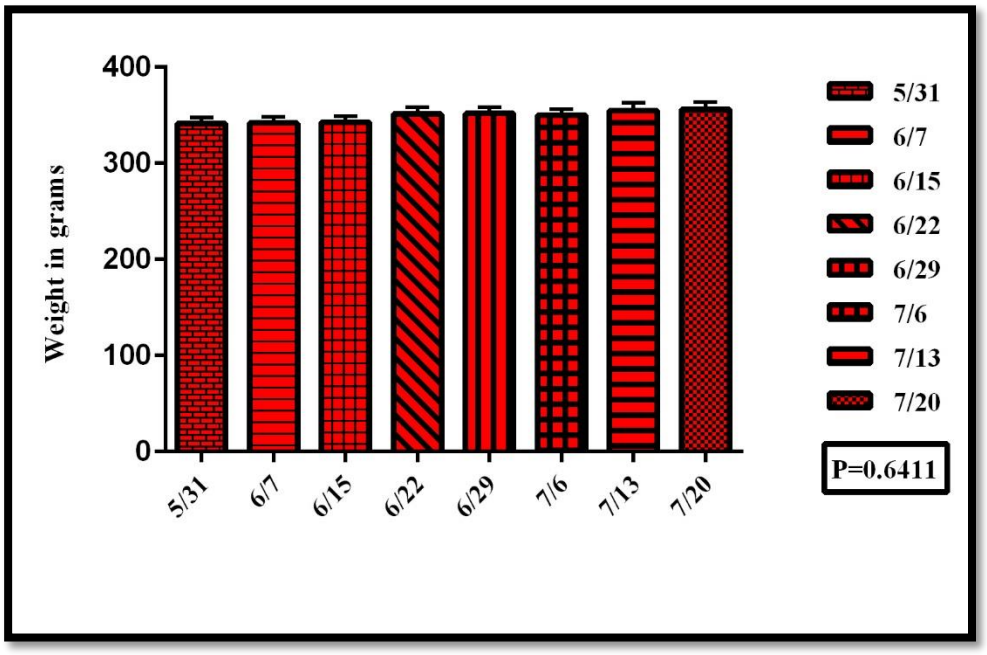


Figure 5: Weight changes in control animals over time in x axis. Each of the patterned red bar (n=12) denote different time points. The y axis denote rat weights in grams. A one way ANOVA was done. Column show mean weights for each group and error bars represent standard error of mean.

As shown in Figure 5 the weights measured weekly over a period of 60 days. The animals showed no significant difference (P=0.6411) in their weights over the time period. Thus the control animals' weights were stable and there was no major weight loss following the stroke surgery. It also shows that the animal was not over eating and the diet restriction during the Montoya staircase testing had little effect on the animals' weight.

3.1.5 Rehabilitation Animals Weight History:

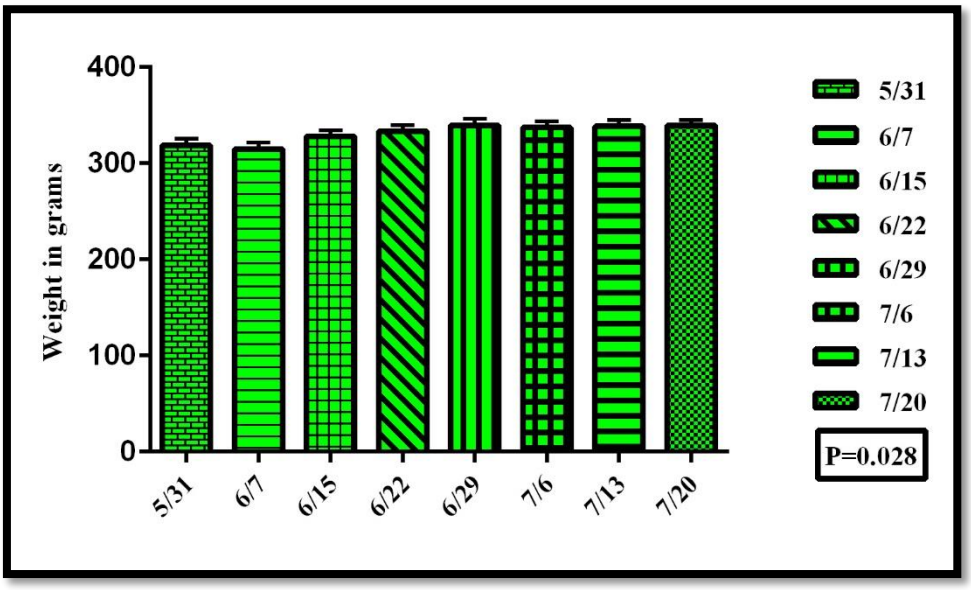


Figure 6: Rehabilitation animals weight history from pre stroke period until the rats were sacrificed. The x axis denote the rehabilitation animal weight change over time. Each of the patterned green bar (5/31, n=12; 6/7 through 7/20, n=11) denote different time points. The y axis denotes rat weights in grams. A one way ANOVA was done. Column show mean weights for each group and error bars represent standard error of mean.

In the Figure 6 shows the rehabilitation animal weight history from the beginning of the study until the animals were sacrificed sixty days later. We can see a significant difference (P=0.028) in animal weights in this group over a period of time. Initially the animals weighed less and they started increasing over time period until they stabilized. This shows that the animals which weighed less initially compared to the control group, gained weight by eating sufficient amount of rat chow and peanut butter. This has improved their diet. Alternatively the animals did not lose any weight post stroke, showing that diet restriction during the Montoya staircase has not affected the animals' health. These rehabilitation animals had stroke surgery on 6/1, 6/2 and 6/3. A little weight loss can be observed between 5/31 and 6/7, which was presumably due to the stroke

surgery. And a gain in weight is seen from 6/22, which is after we started rehabilitation procedures.

3.2 FUNCTIONAL RECOVERY ANALYSIS

3.2.1 Pre Stroke Training:

Twenty four animals were used for the pre-stroke functional training. The Montoya Staircase was performed to obtain pre-stroke function data for right and left forelimb. Each animal was trained for seven days for about fifteen minutes. The Number of pellets picked by each paw was recorded to determine the pre-stroke function of the animal and their best performance in the last three day of training was set as their baseline pre-stoke performance. The baseline pre-stroke performance was required to find out the animal's functional deficit and functional recovery in the post stroke period (see Post-stroke Functional Testing in Methods).

3.2.2 Contralateral Function Analysis:

Figure 7 includes data from four different time periods and the functional recovery of two groups have been statistically analyzed. A statistically difference is not seen between the groups, whereas a high significant difference is seen with respect to time ($P < 0.0001$). The statistically significant difference is seen between the pre stroke period and post stroke day 3-5.

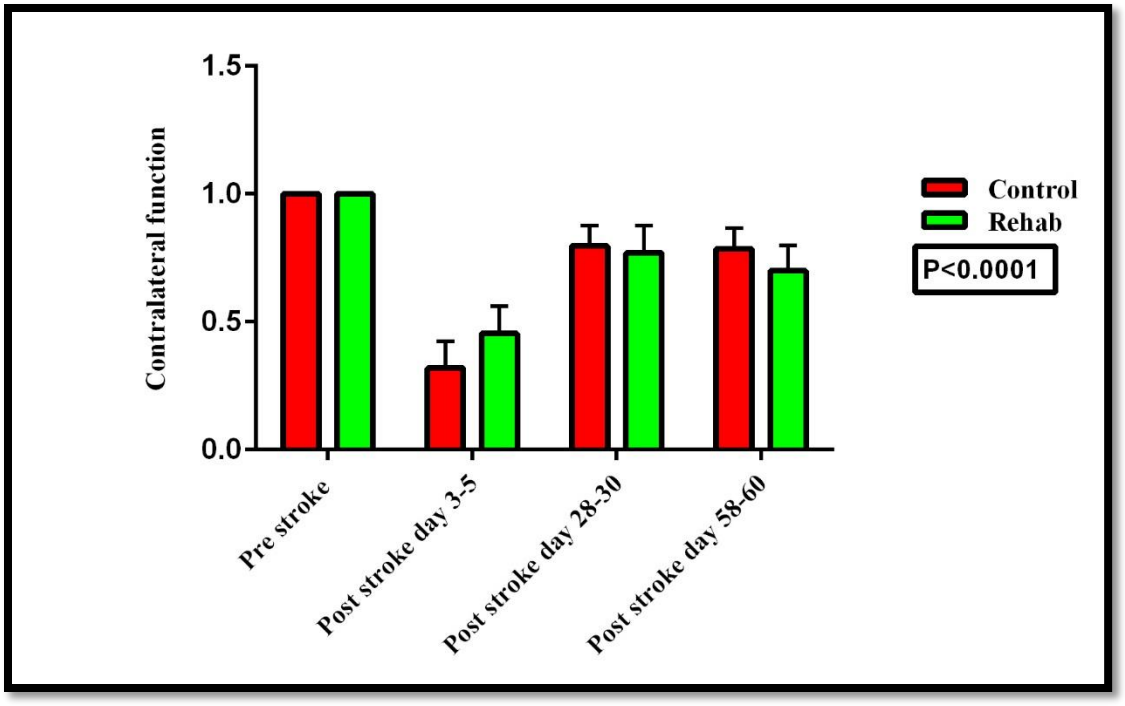


Figure 7: Comparison of contralateral function over time between control animals and rehabilitation animals. The x axis denotes different time periods: pre stroke, post stroke days 3, 30 and 60. The y axis denotes contralateral function; 1.0 = normalized pre stroke contralateral function. Two way ANOVA was done.

In Figure 7 the pre stroke function is normalized to 1 for the contralateral side for both the groups. Pre stroke function serves as the baseline function. For post stroke day 3-5 function, the contralateral function for control group was only 32% percent of the pre-stroke function and for the rehabilitation group, it was only about 45.45% percent. Thus animals now had at least a functional deficit of about 68% (control) to 54.55% (rehabilitation) following stroke induction, thus the endothelin injection has impaired the forelimb movement in the animals. This also indicates that hit the right co-ordinates (forelimb motor cortex) during the surgical stroke induction. At post stroke day 28-30, the animals have regained more than fifty percent of their contralateral function, which is an unusual result for our laboratory. We generally only see about an 11% recovery at this

stage, which leads us to question whether some animals in the control group might have been pre-menopausal (still have estrogen release). At post stroke day 58-60 the control animals had a contralateral function of about 78.6% and the rehabilitation animals had a contralateral function of about 70%. Therefore the functional recovery for the control animals were about 46.6% and for the rehabilitation animals the recovery was 24.5%.

3.2.3 Ipsilateral Function Analysis:

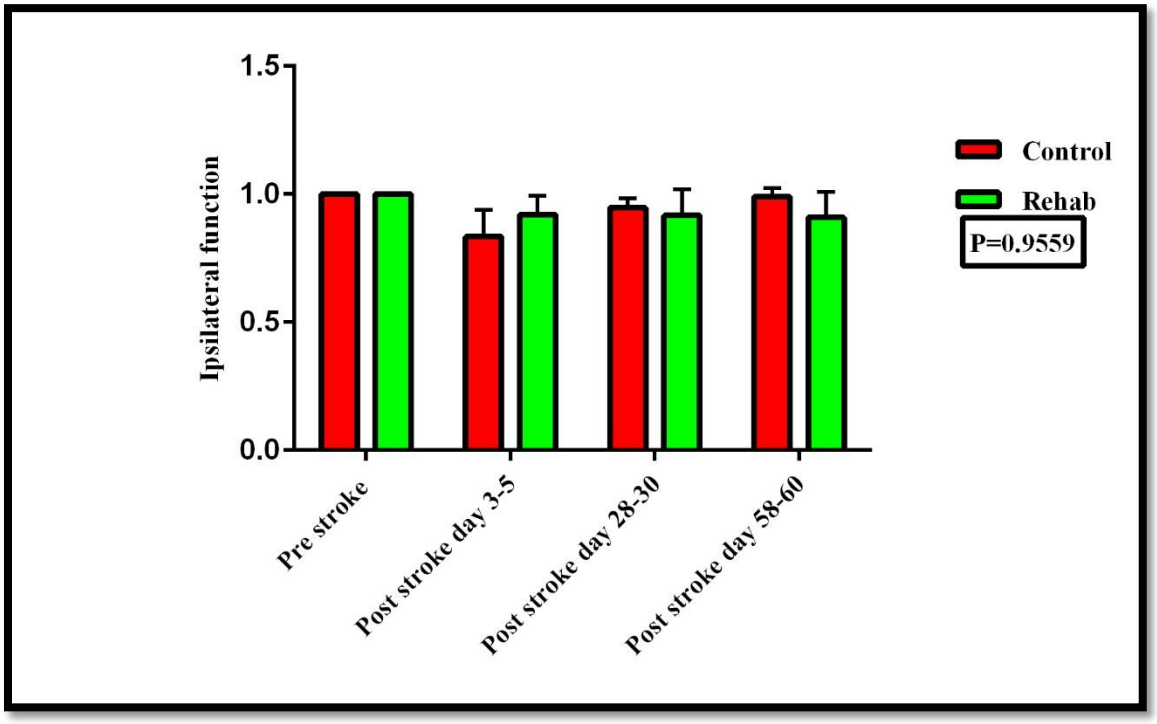


Figure 8: Comparison of ipsilateral function over time between control animals and rehabilitation animals. The x axis denotes different time periods: pre stroke, post stroke days 3-5, 28-30 and 58-60. The y displays ipsilateral function; 1 = normalized pre stroke baseline. Two way ANOVA yielded a P value of 0.0559. The error bars represent standard error of mean.

The ipsilateral function analysis of control and rehabilitation animals was preformed over a period of 60 days. Figure 8 has been plotted with the data from four different time periods and the ipsilateral functional recovery of two groups has been

statistically analyzed. No statistical difference is seen between the groups or respect to time. Pre stroke function is 1 which means 100% ipsilateral function normalized at pre-stroke. At post stroke day 3-5, ipsilateral function is about 83.5% for control animals and 92% for rehabilitation animals, which means that the animal's ipsilateral side was not affected much with the surgery. The animals seem to have an ipsilateral deficit less than 20% for both the groups.

At post stroke day 28-30, we see a slight increase (94.7%) in the ipsilateral function of the control animals from the post stroke day 3-5. By post stroke day 58-60, we see ipsilateral function has almost reached pre stroke function (99.1%). With the rehabilitation animals the ipsilateral function remained the same (91%) from post stroke day 3-5 through post stroke day 58-60.

3.2.4 Total Functional Recovery Analysis:

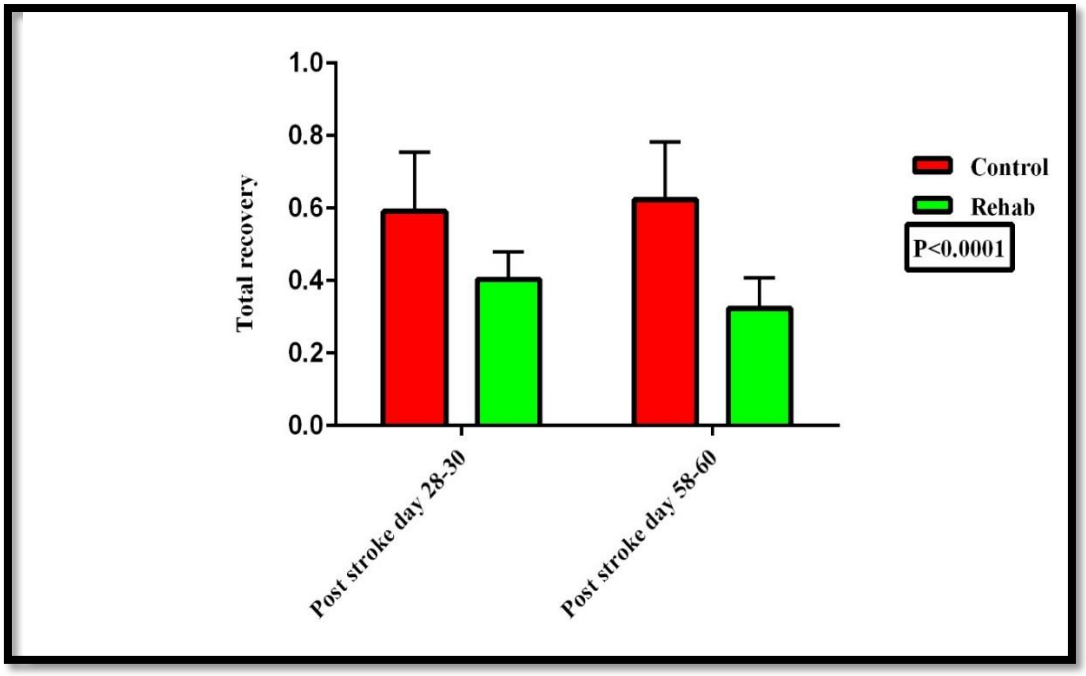


Figure 9: Comparison of total functional recovery over time between control animals and rehabilitation animals. The x axis denotes different time periods: post stroke days 30 and 60. The y axis displays total functional recovery; 1 = 100% function on one side so that 2 would equal 100% functional recovery on both sides. Two way ANOVA was done.

The total functional recovery for control and rehabilitation animals is shown in figure 9. We can see Figure 9 the total functional recovery from post stroke days 28-30 and post stroke days 58-60 for control and rehabilitation animals. Here we see a significant difference ($P < 0.0001$) between the groups in their total functional recovery. In Figure 7 and 8, we have seen that the contralateral function and ipsilateral function did not show any sort of significant difference with respect to groups. By contrast, here we can see that the total functional recovery for the control animals is higher compared to the rehabilitation animals. The total functional recovery for the control animals has a slight increase from post stroke days 28-30 (59.1%) through post stroke days 58-60 (62.3%).

In the rehabilitation animals, the recovery has diminished slightly (not significant) by post-stroke days 58-60. The total functional recovery on post stroke day 58-60 is much lower (32.27%) for rehab group compared to the control, which probably led to the statistical difference of P<0.001 in the 2-way ANOVA.

3.2.5 Bilateral and Unilateral Deficit:

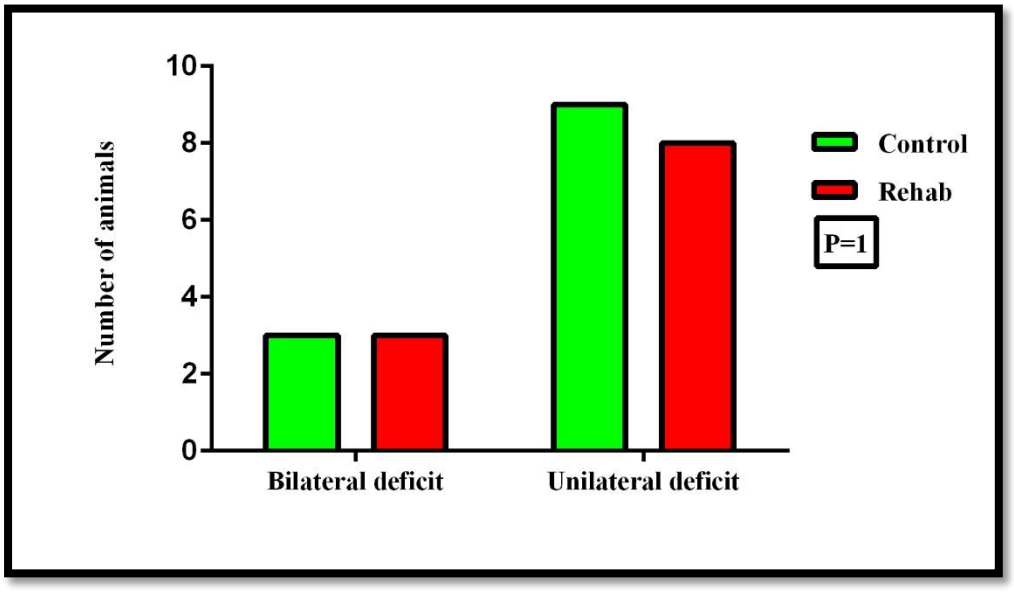


Figure 10: A contingency test is done to statistically compare the proportion of animals in each group with a bilateral deficit following stroke surgery. The x axis denotes the different types of deficits: bilateral and unilateral deficit. The y axis displays the number of animals. Contingency table analyses P=1 (Fischer's exact test), shows no statistical difference between groups.

The animals that had either Bilateral or unilateral deficit at post stroke day 3 are shown in Figure 10. From the graph, we can see that three animals in each of the groups had bilateral deficit. Therefore out of twenty three animals, six animals suffered bilateral deficit at post stroke day 3.The rest of the animals (seventeen total with 9 in the control group and 8 in the Rehabilitation group) had a unilateral deficit. A contingency test with Fischer's exact test was done to produce the graph showing a P=1(Figure 20). P value

signifies that bilateral deficit probably happened due to errors in the surgical procedures (endothelin injection probably damaged the corpus callosum).

3.3 VOLUNTARY PHYSICAL REHABILITATION

Figure 11: This Figure shows a rat swiping at the peanut butter in the hanging shelf using the left paw (contralateral forelimb)

3.3.1 Peanut Butter Consumption:

The amount of peanut butter (PB) eaten by each animals was recorded and averaged for each day. The peanut butter shelves were hung every other night, beginning on post stroke day 8, until post stroke day 52, when the rats were euthanized. Therefore the rehabilitation days totaled 23 days (the shelves were not hung during Montoya staircase post-stroke testing due to the restricted diets required during testing)

A linear regression analysis was done to determine the relationship between the amount of peanut butter eaten per day (average amount of peanut butter consumed per day by all the animals) and rehabilitation days. No significant difference (P = 0.1531; deviation from zero) was seen (Figure 12) and the r square value (R^2 = 0.007436) was very low, which indicates the linear regression fit is not very good. The slope obtained was negative and the b intercept was 4.36, which implies as the number of rehab days increased the amount of peanut butter consumption decreased (Figure 12).

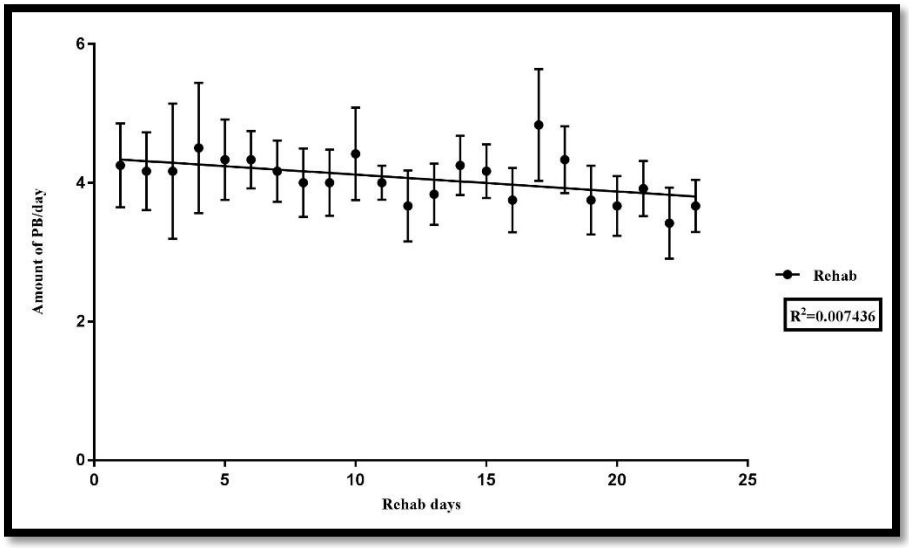


Figure 12: Graph showing amount of peanut butter consumed by all the rehabilitation animals throughout the rehabilitation period. The x axis denotes the rehabilitation days and the y axis displays the amount of peanut butter in grams. Error bars represent standard error of mean.

3.3.2 Forelimb Swipes Analysis:

We watched several animals to count the number of arm movements (up to 20 swipes) in order, to determine the average amount of peanut butter eaten per arm swipe. We converted the total amount of peanut butter consumed by the animal into the number of arm swipes by each animal. Figure 13 shows a better correlation compared to the

previous result (Figure 12). The R square value was 0.2861, which indicates a 40 fold better fit than the previous figure, and the P value (0.0086) was highly significant, which indicated a significant deviation from zero. A linear regression analysis was performed, to determine the relationship between the number of swipes and rehab days. The slope obtained was negative and b intercept was 93.67, which shows that the arm swipes decreased as the number of rehab days increased (Figure 13).

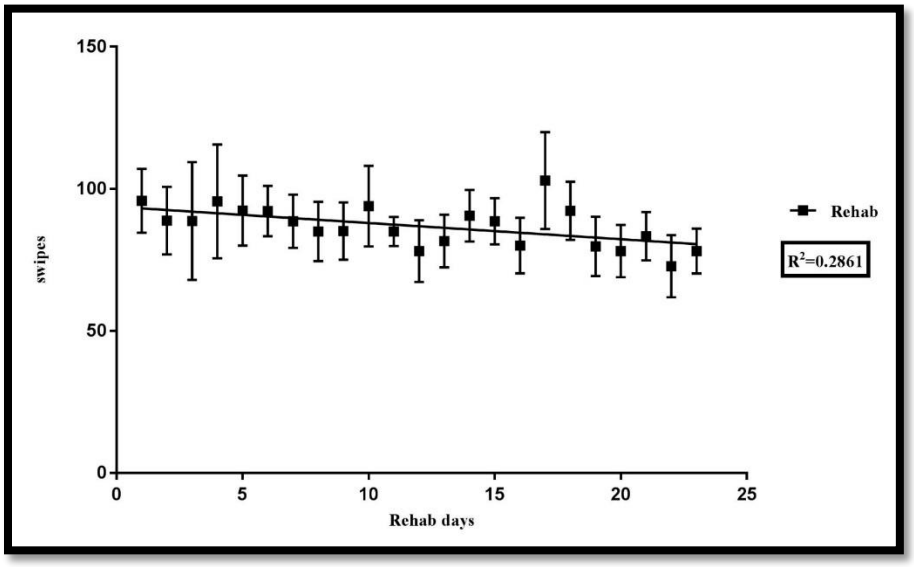


Figure 13: Graph showing linear regression of number of arm swipes made by all the rehabilitation animals per day throughout the procedure. The x axis denotes the rehabilitation days and the y axis displays the number of arm swipes per day. Error bars represent standard error of mean.

3.4 KI67 STAINING

Around 2000 digital images were taken using the brightfield optical microscope at 40X (4X objective and 10X ocular) magnification to identify the Ki67 staining along the sub ventricular zone of the lateral ventricles. Images from both the groups were taken and analyzed using the NIH image J software before the group identifiers were assigned, to prevent any bias in image analysis.

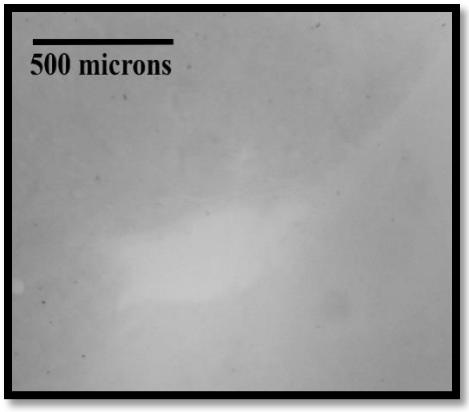


Figure 14: Image showing control Ki67 antibody staining in the control animal (minus primary antibody, plus secondary antibody). Image shows very little secondary antibody nonspecific binding

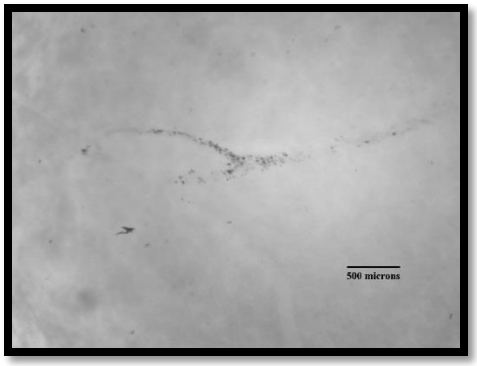


Figure 15: Image showing positive Ki67 antibody staining in the control animal(plus primary antibody and plus secondary antibody)

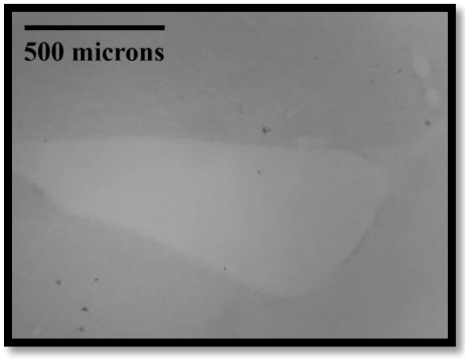


Figure 16: Image showing control Ki67 antibody (no primary antibody, plus secondary antibody) staining in rehab animals

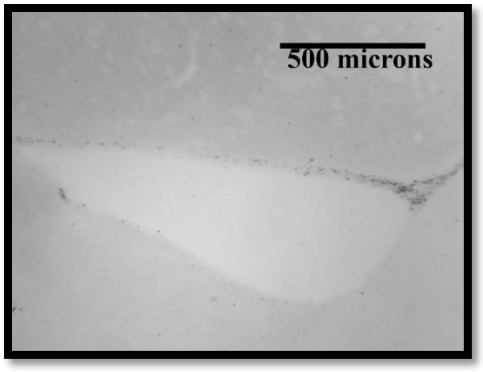
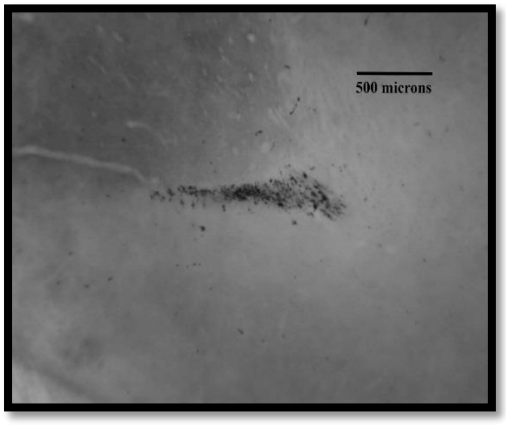


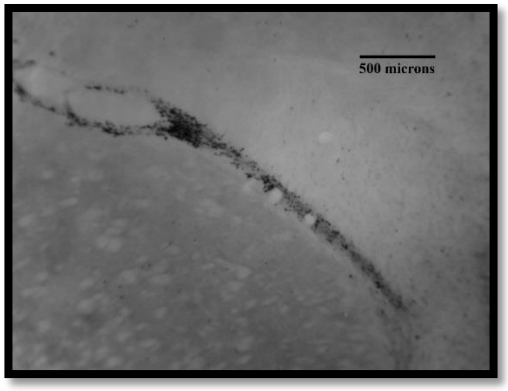
Figure 17: Image showing positive Ki67 antibody (plus primary antibody, plus secondary antibody) staining in rehab animals.

3.4.1 Estimation of Ki67 Antibody Staining in the Anterior Sub Ventricular Region

Pictures of about 6-8 anterior coronal slices per animal were taken with the digital camera on the brightfield scope to obtain the Ki67 antibody staining in the anterior portion of the sub ventricular zone. In the anterior Subventricular zone, the ventricles have a tear-drop shaped if they are open and we often see staining on both sides of the ventricle. In the full coronal sections below, we see a line of Ki67 staining in the left panel and tear- drop shaped openings in the right panel

A) 

B)



C)

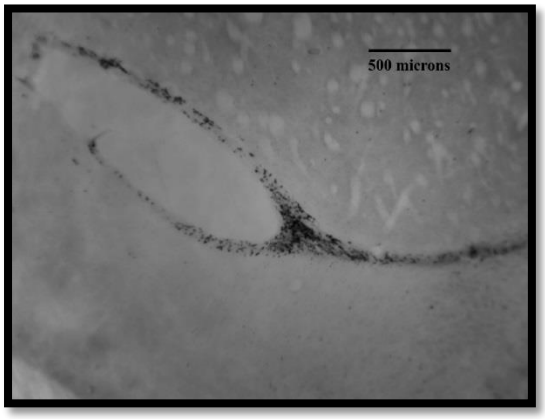
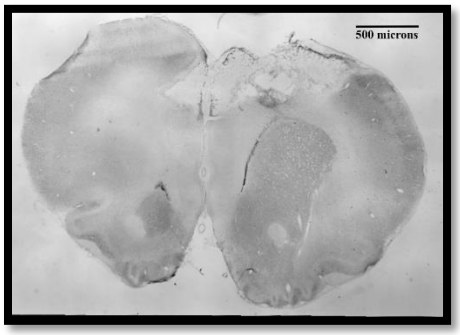
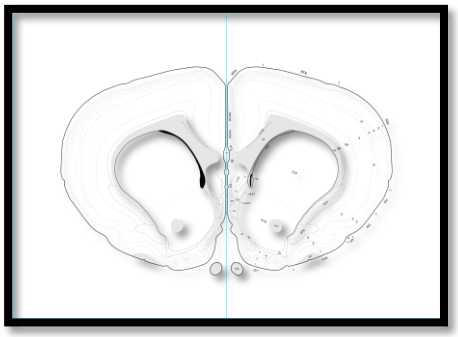


Figure 18 (A-C): Images show Ki67 antibody staining in the anterior portion of the subventricular zone.

A) **B)**





C) **D)**

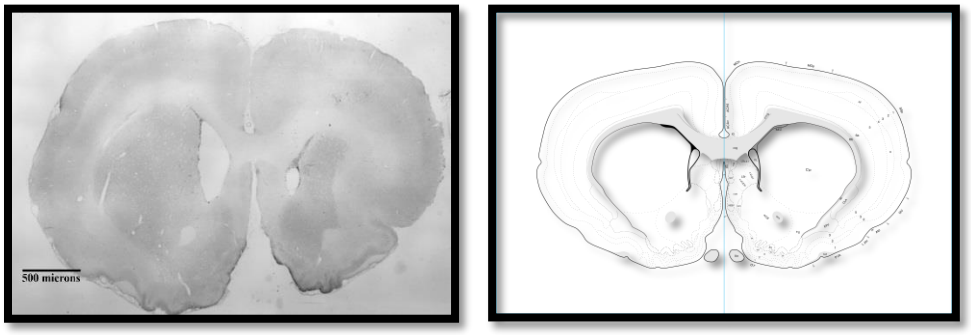


Figure 19: Images A and C show full coronal section of Ki67 antibody staining in the anterior portion of the subventricular zone. Images B and D show the corresponding image from rat brain atlas.

The Ki67 area staining in the anterior sub ventricular zone for control animals and rehabilitation animals were compared statistically using T test (Welch's correction for unequal variance). The Ki67 area for the control animals seems to be higher compared to the rehabilitation animals (Figure 20). The two values are significantly different (P = 0.0114). The mean ±SEM for control was 0.03262 ± 0.008474 mm^2 and rehab was 0.006830 ± 0.001522 mm^2 which indicates about 5 fold increase in stem cell proliferation.

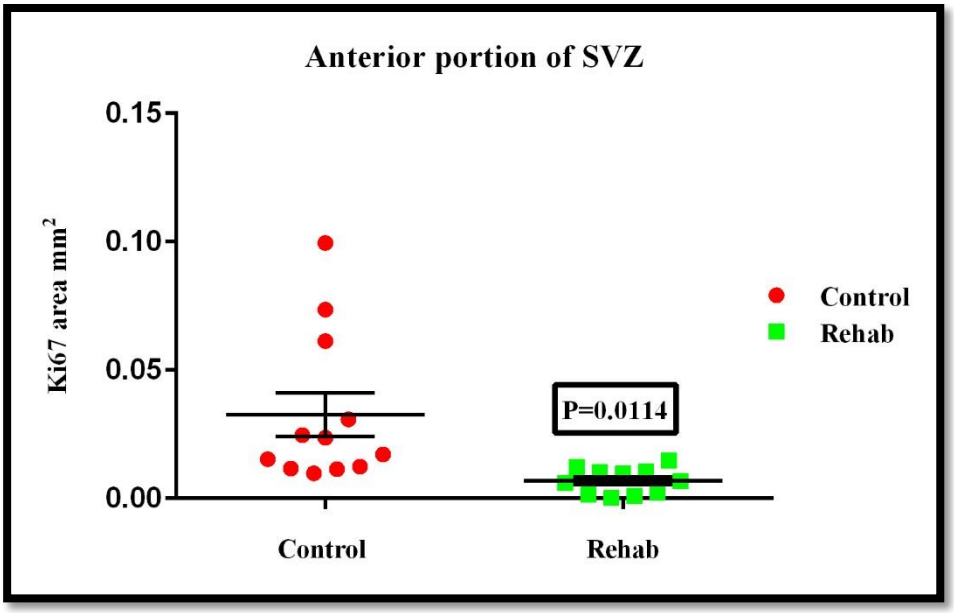


Figure 20: Graph showing average Ki67 antibody staining in the anterior portion of the sub ventricular zone in the control and rehabilitation animals. Each of the red (control; n=12) and green (rehabilitation; n=11) dots represent a single animal. The x axis denotes two groups and the y axis displays the Ki67 area in mm^2. The broad horizontal bar indicates the group mean and the error bars represent SEM. P=0.0114 using T-test with Welch's correction for unequal variance.

ROUT method when Q=1% was used to identify outliers for the data in the Figure 20, the cleaned data removed two values from the control group. A graph was created after removing the outliers and t-test with Welch's correction for unequal variance was performed (Figure 21). The data still remained significantly different (P=0.0142). The mean ±SEM for control was 0.0218 ± 0.004898 mm^2 and rehab was 0.006830 ± 0.001522 mm^2 which indicates about 3 fold difference in stem cell proliferation.

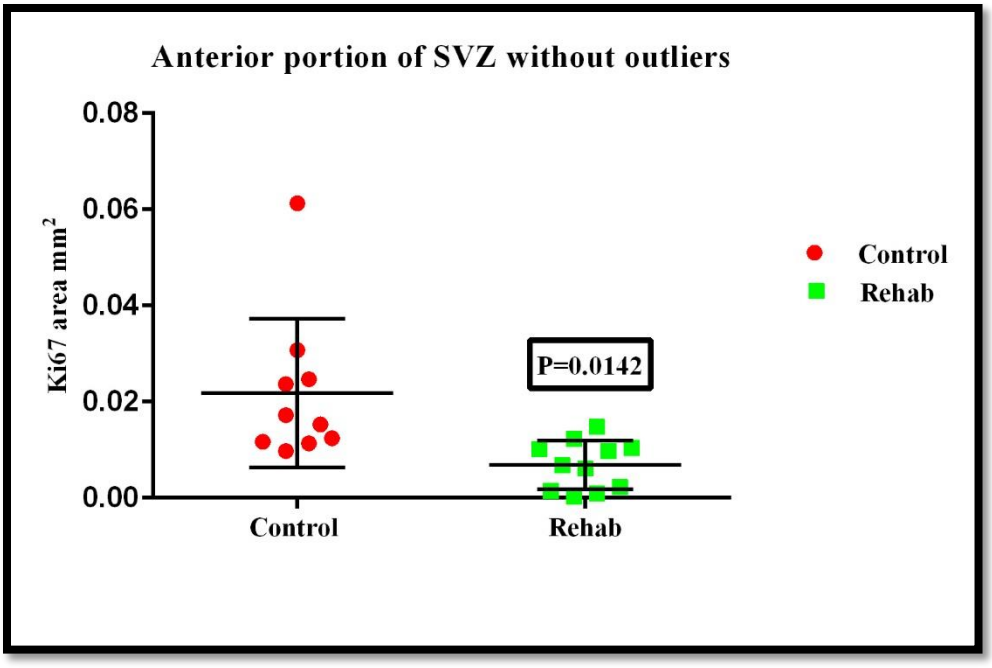
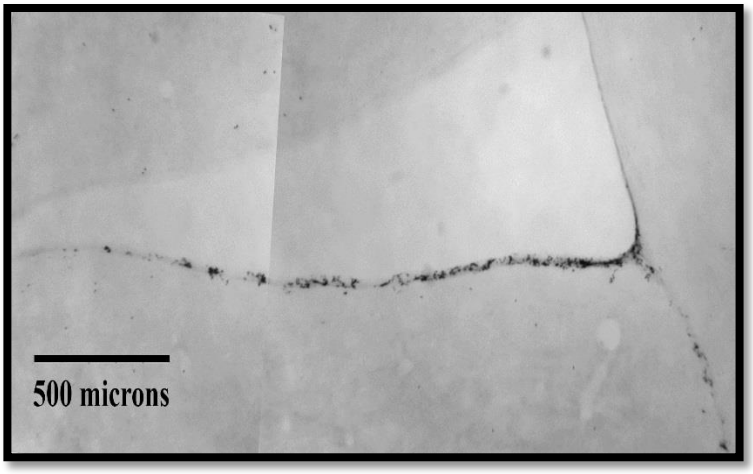


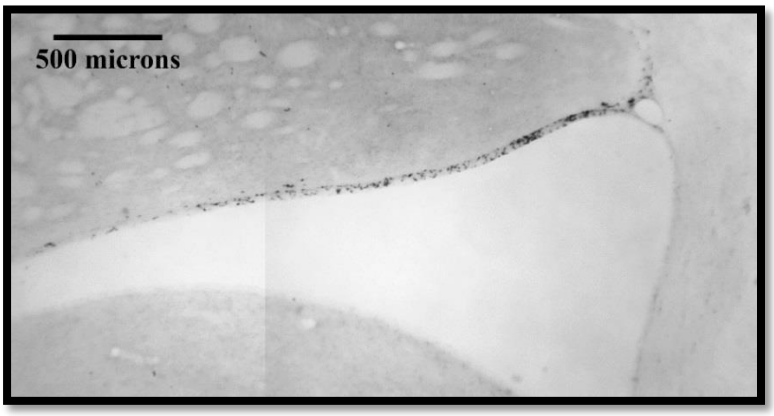
Figure 21: Graph showing average Ki67 antibody staining in the anterior portion of the sub ventricular zone after removal of outliers in the control and rehabilitation animals. Each of the red (control; n=10) and green (rehabilitation; n=11) dots represent a single animal. The x axis denotes two groups and the y axis displays the Ki67 area in mm^2. The broad horizontal bar indicates the group mean and the error bars represent SEM. P=0.0142 using T-test with Welch's correction for unequal variance.

3.4.2 Estimation of Ki67 Antibody Staining In the Middle Sub Ventricular Region

Pictures of about 5-8 coronal brain slices per animal were taken using the digital camera on the bright field scope, to obtain the Ki67 antibody staining in the middle sub ventricular zone. In this region, we see that the ventricles have completely opened up and the Ki67 antibody staining was seen predominantly seen on the side of the ventricle away from the midline (sub ventricular zone). Visibly the staining looked denser in the control animals compared to the rehabilitation animals.



A)



B)

Figure 22: Images A and B show Ki67 antibody staining in the middle portion of the subventricular zone of the lateral ventricles

A) **B)**

Figure 23: Image A shows full coronal section of Ki67 antibody staining in the middle portion of the subventricular zone. Image B shows the corresponding image from rat brain atlas.

T test (Welch's correction) was performed on the data to compare the average Ki67 antibody staining area in the middle Subventriular zone for the two treatment groups (control vs rehab). The total Ki67 staining area was statistically higher (P = 0.0066) in the control animals compared to the rehabilitation animals (Figure 24). The mean ±SEM for control was 0.02671 ± 0.006445 mm^2 and rehab was 0.004104 ± 0.001058 $mm^{2,}$ which indicates about a 6 fold difference in stem cell proliferation.

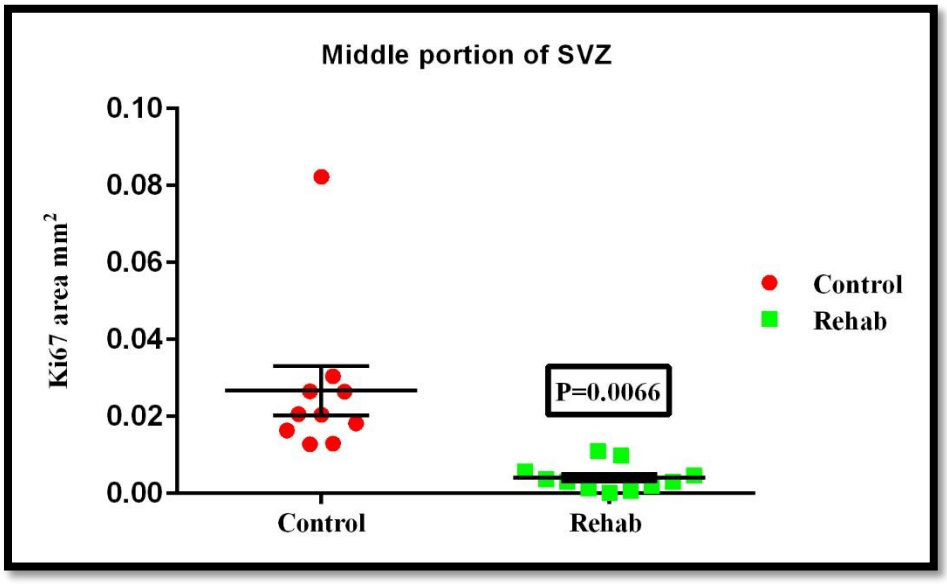


Figure 24: Graph showing average Ki67 antibody staining in the middle portion of the sub ventricular zone. Each of the red (control; n=10) and green (rehabilitation; n=11) dots represent a single animal. The x axis denotes the two groups and the y axis displays the Ki67 area in mm^2. The broad horizontal bar indicates the group mean and the error bars represent SEM. P=0.0066 using t-test with Welch's correction for unequal variance.

ROUT method when Q=1% was used to identify outliers for the data in the Figure 24, the cleaned data removed one animal from the control group. A graph was created after removing the outlier and t-test with Welch's correction for unequal variance was performed (Figure 25). The data highly significant (P<0.0001). The mean ±SEM for control was 0.02054 ± 0.002072 mm^2 and rehab was 0.004104 ± 0.001058 mm^2, which shows about a fivefold difference in stem cell proliferation.

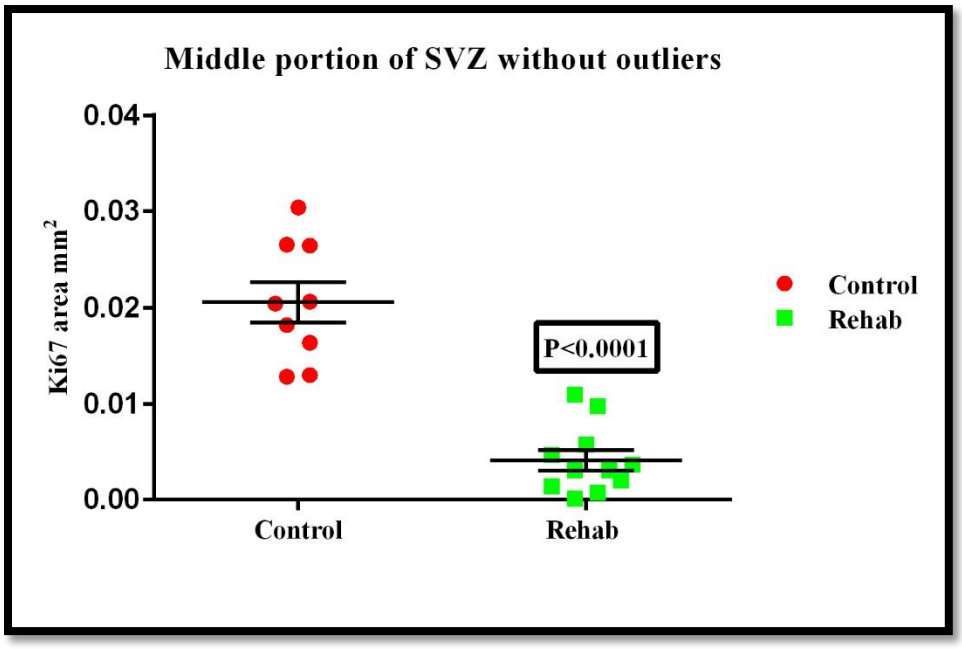


Figure 25: Graph showing average Ki67 antibody staining in the middle portion of the sub ventricular zone after removal of outliers in the control group. Each of the red (control; n=9) and green (rehabilitation; n=11) dots represent a single animal. The x axis denotes the two groups and the y axis displays the Ki67 area in mm^2. The broad horizontal bar indicates the group mean and the error bars represent SEM. P<0.0001 using t-test with Welch's correction for unequal variance.

3.4.3 Estimation of Ki67 Antibody Staining In the Posterior Sub Ventricular Zone

Pictures of around 3-5 coronal brain slices per animal were taken using the digital camera on the bright field scope, to obtain the Ki67 antibody staining in the posterior sub ventricular zone. In the posterior Subventricular zone, the Ki67 antibody staining is seen

only at one corner of the ventricle, away from the midline. Generally the Ki67 present in the posterior portion of the ventricles will be much reduced compared to that in the anterior and middle portion of the ventricles.

A) **B)**

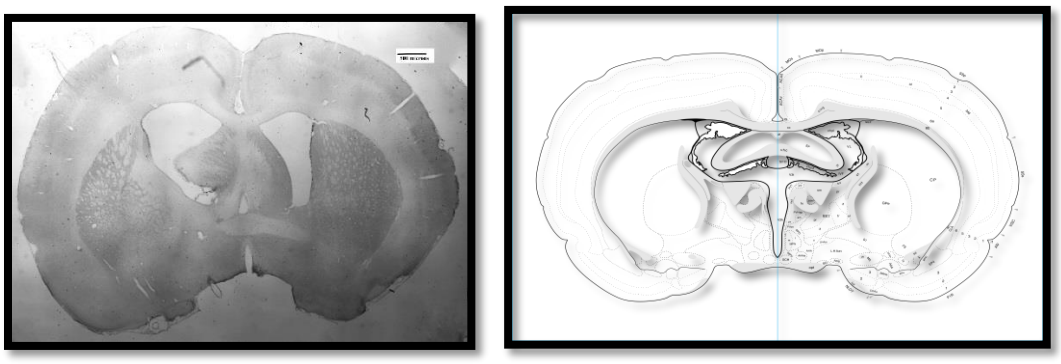


Figure 26: Image A shows full coronal section of Ki67 antibody staining in the posterior region of the Subventricular zone. Image B shows the corresponding image from rat brain atlas.

The Ki67 area in both the groups are compared using T test (Welch's correction). Significant difference (P = 0.0189) is seen between the groups in the presence of statistical outliers. The mean ±SEM for control was 0.01832 ± 0.005499 mm^2 and rehab was 0.002583 ± 0.0007364 mm^2. The controls animals have denser Ki67 antibody staining than the rehabilitation animals (Figure 27).

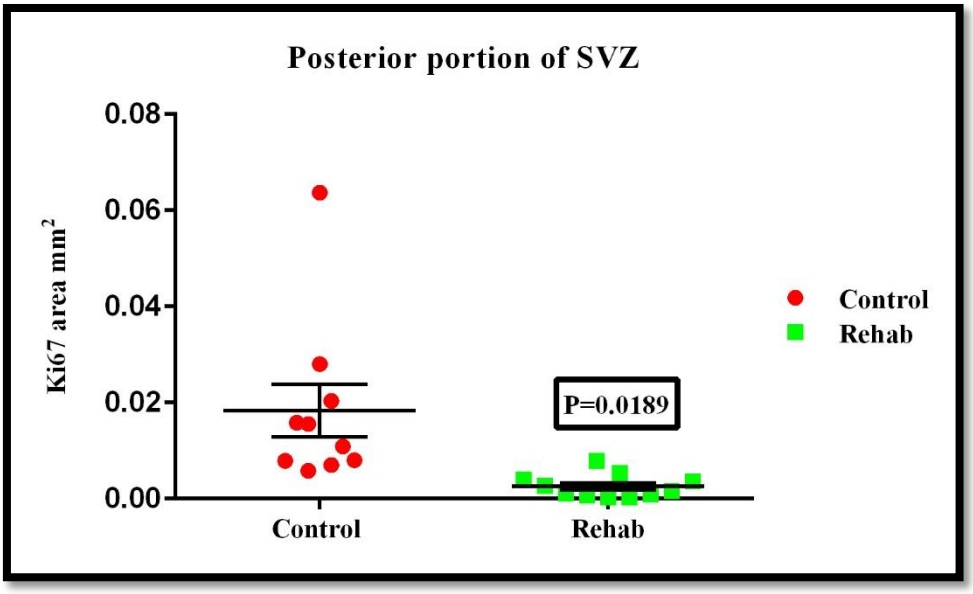


Figure 27: Graph showing the average Ki67 Antibody Staining in the posterior portion of the Sub Ventricular Zone. Each of the red (control; n=10) and green (Rehabilitation; n=11) dots represent a single animal. The X axis denotes two groups and the Y axis denotes Ki67 Area In mm^2.The broad horizontal bar indicates the group mean and the error bars represent SEM. P=0.0189 using T-test with Welch's correction for unequal variance.

ROUT method when Q=1% was used to identify the outliers for the data in the Figure 27, one animal was removed from the control group. A graph was created after removing the outlier and t-test with Welch's correction for unequal variance was performed (Figure 28). The data were highly significantly different (P=0.0022). The mean ±SEM for control was 0.01328 ± 0.002459 mm^2 and rehab was 0.002583 ± 0.0007364 mm^2, reflecting approximately a 4 fold change in stem cell proliferation.

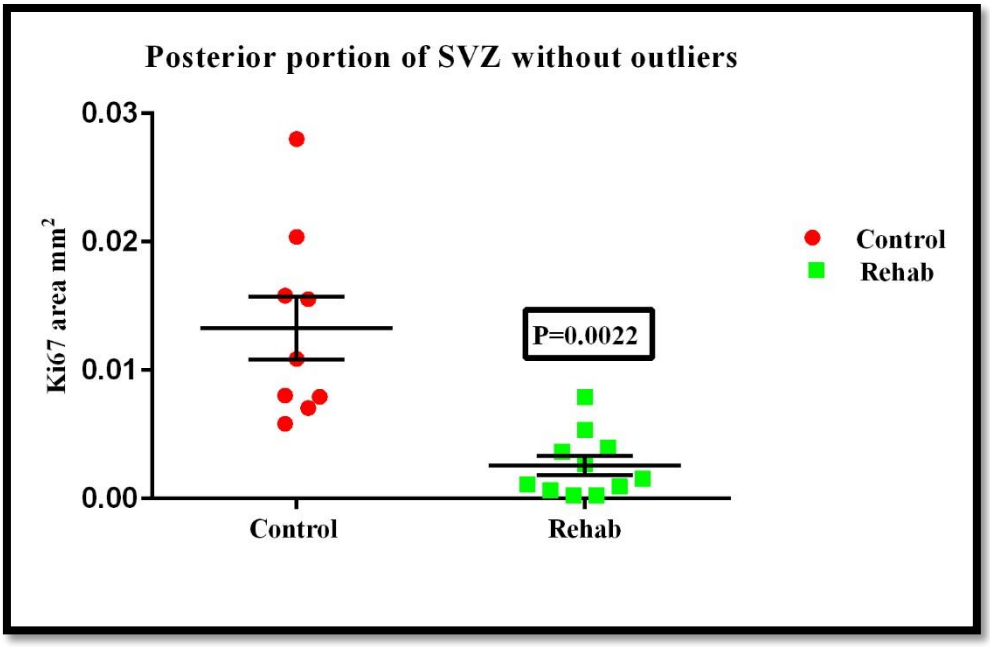


Figure 28: Graph showing the average Ki67 Antibody Staining in the posterior portion of the Sub Ventricular Zone after removal of the outlier from the control group. Each of the red (control; n=9) and green (Rehabilitation; n=11) dots represent a single animal. The X axis denotes two groups and the Y axis denotes Ki67 area in mm^2.The broad horizontal bar indicates the group mean and the error bars represent SEM. P=0.0022 using T-test with Welch's correction for unequal variance.

3.4.4 Estimation of Overall Ki67 Antibody Staining

In total there were about 18-21 coronal slices per animal to analyze. Overall average Ki67 staining area from anterior through posterior subventricular zone for two groups were compared statistically. T test (Welch's correction) was used to compare the staining in the groups. The control animals had higher mean Ki67 area staining compared to the rehabilitation animals. They were highly significant having a P value of 0.0093 (Figure 29). The mean ±SEM for control was 0.03031 ± 0.008021 mm^2 and rehab was 0.005035 ± 0.001161 mm^2, indicating about a six-fold difference in stem cell proliferation.

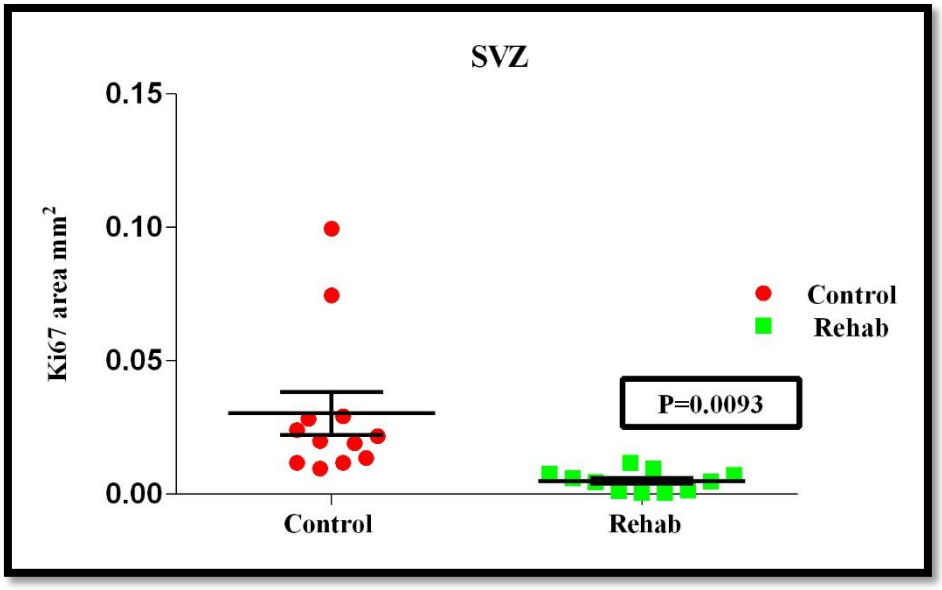


Figure 29: Graph showing overall mean Ki67 antibody staining throughout the whole Sub-ventricular zone. Each of the red (control; n=12) and green (rehabilitation; n=11) dots represent the mean Ki67 staining from a single animal. The x axis denotes the groups and the y axis displays the Ki67 area in mm^2. The broad horizontal bar indicates the group mean of the average Ki67 staining throughout the Subventricular zone and the error bars represent SEM. P=0.0093 using T-test with Welch's correction for unequal variance.

ROUT method when Q=1% was used to identify the outliers for the data in the Figure 29, the cleaned data removed two animals from the control group. A graph was created after removing the outlier and t-test with Welch's correction for unequal variance was performed (Figure 30). The data was highly significant (P<0.0001). The mean ±SEM for control was 0.01896 ± 0.002228 mm^2 and rehab was 0.005035 ± 0.001161 mm^2,

indicating at least a 3 fold difference in stem cell proliferation.

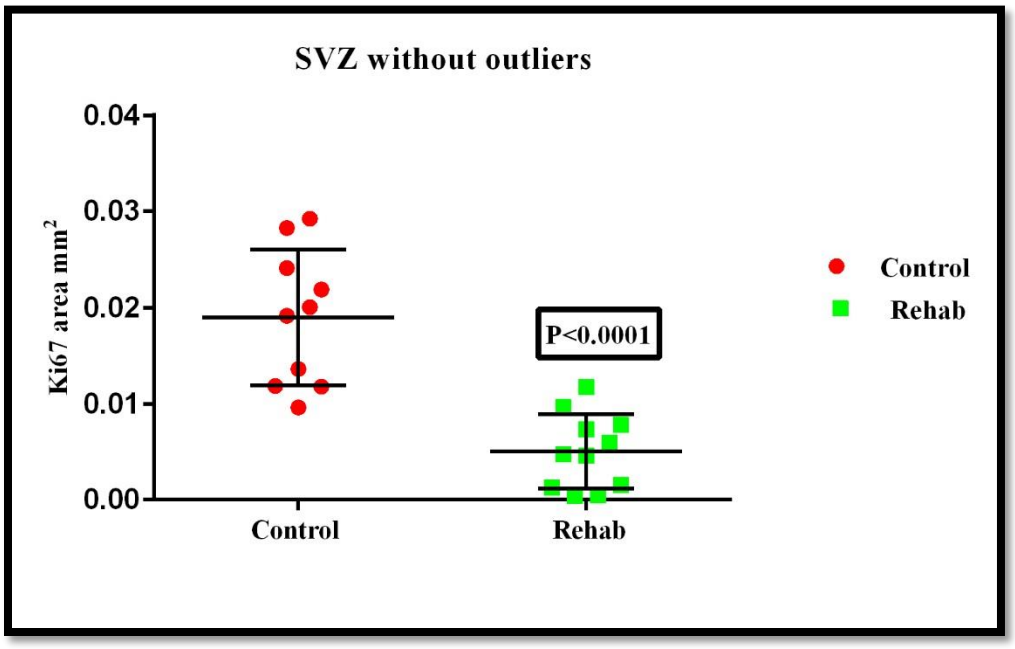


Figure 30: Graph showing overall mean Ki67 antibody staining throughout the whole Sub-ventricular zone after removal of outlier from the control group. Each of the red (control; n=10) and green (rehabilitation; n=11) dots represent the mean Ki67 staining from a single animal. The x axis denotes the groups and the y axis displays the Ki67 area in mm^2. The broad horizontal bar indicates the group mean of the average Ki67 staining throughout the Subventricular zone and the error bars represent SEM. P<0.0001 using T-test with Welch's correction for unequal variance.

3.4.5 Anterior Versus Middle versus Posterior Sub-ventricular Zones.

The average Ki67 antibody staining varies with each of the regions. This graph was analyzed using two way ANOVA. We statistically compare the mean Ki67 area in the three different Sub-ventricular zone regions (anterior, middle and posterior) for control and rehabilitation animals. Control animals have higher Ki67 area staining in all the three regions of the Subventricular zone. A two way ANOVA was performed on the data to produce the statistical differences. There is a statistical difference between the treatment groups but not with respect to the three different regions (Figure 31) within

each treatment group. The P value shows that a highly significant ($P < 0.0001$) difference between treatment groups.

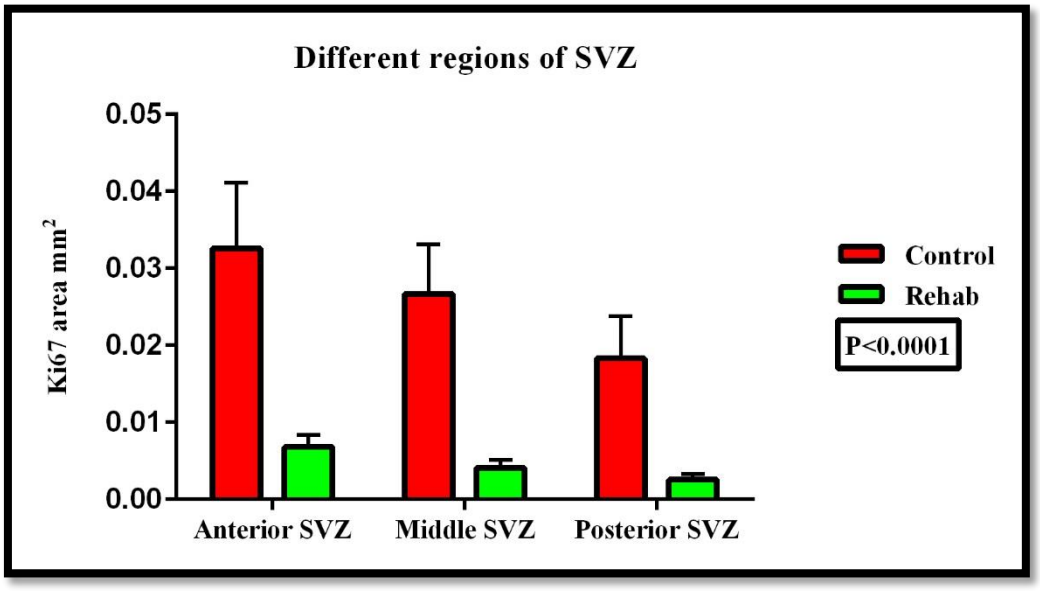


Figure 31: Graph showing the comparison between different regions of the sub ventricular zones for mean Ki67 staining. The x axis denotes different regions of the sub ventricular zones for control and rehabilitation groups. The y axis displays the mean Ki67 staining area in mm^2. A two way ANOVA found a significant difference between treatment groups (control versus rehab) with $P<0.0001$. Error bars represent Standard error of mean (SEM).

3.4.6 Correlating Total Functional Recovery and Ki67 Staining

The total functional recovery data from the Montoya staircase test were correlated with the aveage Ki67 staining area, to determine if there is any correlation between the two quantities and also to see if the Ki67 staining (proliferation of stem/progenitor cells in the Subventricular zone) contributed in functional recovery. In the Figure 32, the total functional recovery (post-stroke days 58-60) and the Ki67 antibody staining for the control group were analyzed using the linear regression analysis. The R square value for the Anterior SVZ was 0.1054, Middle SVZ was 0.09311 and Posterior SVZ was 0.04211,

which indicates little correlation. The highest correlation was seen in the anterior SVZ with a positive slope.

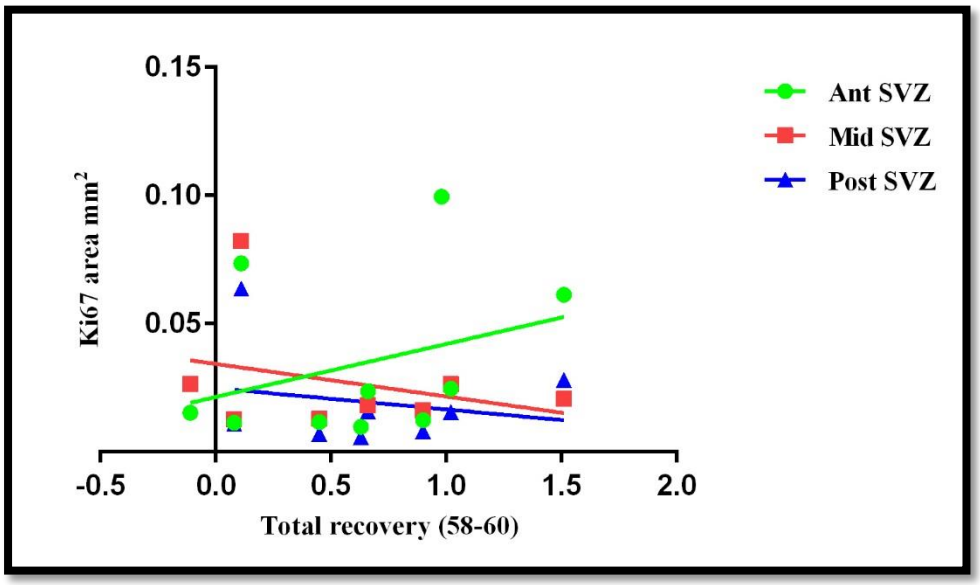


Figure 32: Graph comparing total functional recovery and the mean Ki67 staining from anterior SVZ, middle SVZ and posterior SVZ in control group. The x axis denote the total functional recovery from post stroke day 60. The y axis displays the mean Ki67 area in mm^2. The dots represent each region of the sub ventricular zone: green circles (anterior SVZ), red squares (middle SVZ) and blue triangles (posterior SVZ)

In the Figure 33, the total functional recovery from post stroke day 60 and the Ki67 antibody staining for the rehabilitation group has been correlated. Linear regression analysis was performed to obtain the graph (Figure 33). The R square value for the rehabilitation group were anterior SVZ 0.03638, middle SVZ 0.002853 and posterior SVZ 0.1397. The highest correlation was seen in the posterior SVZ. All of the linear regression had a negative slope for this treatment group. No correlation was seen between total functional recovery and Ki67 staining (Anterior, middle and posterior SVZ).

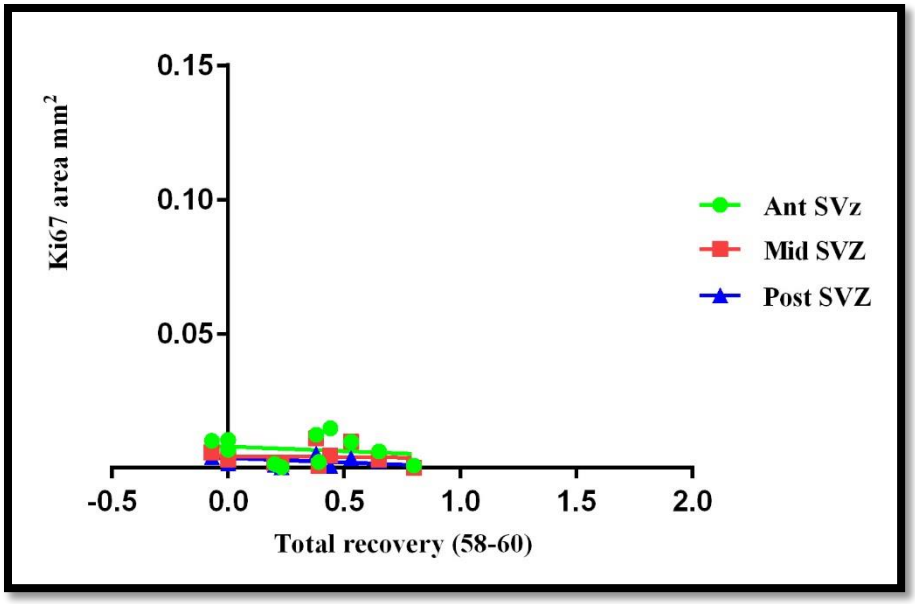


Figure 33: Graph comparing total functional recovery and mean Ki67 staining from anterior SVZ, middle SVZ and posterior SVZ for the rehabilitation group. The x axis denote the total functional recovery from post stroke day 60. The y axis displays the mean Ki67 area in mm^2. The dots represent each region of the sub ventricular zone: green circles (Anterior SVZ), red squares (Middle SVZ) and blue triangles (Posterior SVZ).

In the Figure 34, the overall mean Ki67 staining was compared with the total functional recovery from post stroke days 30 and 60. A linear regression analysis was performed to correlate the quantities. The R square values for control animals on post stroke day 30 was 0.4135 and post stroke day 60 was 0.3842, showing some correlation with functional recovery. The slopes obtained were positive.

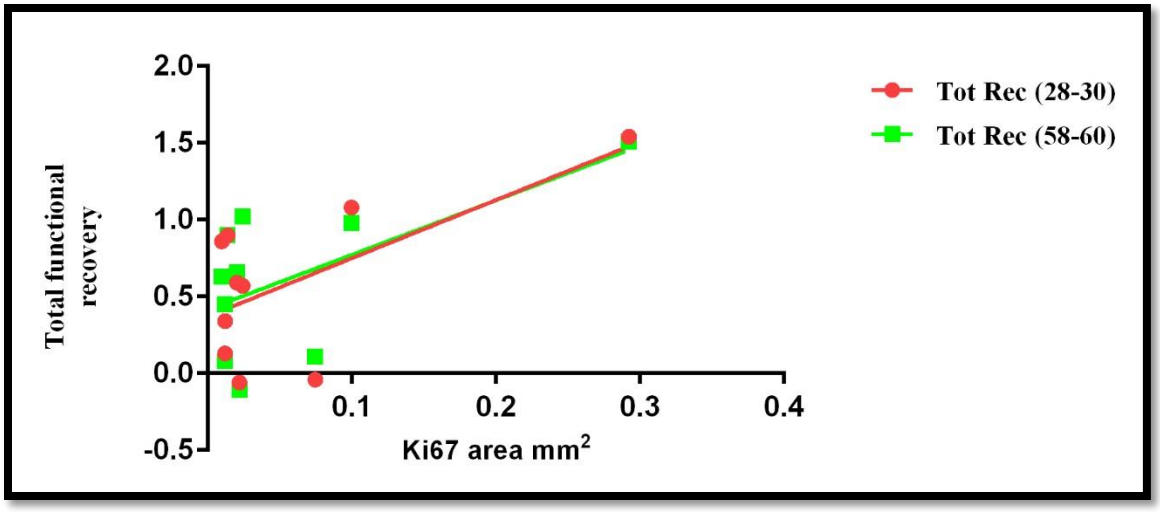


Figure 34: Graph comparing the mean SVZ Ki67 antibody staining and total functional recovery from post stroke days 30 and 60 for control animals. The x axis denotes overall mean Ki67 antibody staining area in mm^2. The y axis displays the total functional recovery. The dots represent total function recovery at post stroke days 30 (red circles) and 60 (green squares)

In the Figure 35, the overall Ki67 staining and the total function recovery from post stroke days 30 and 60 for rehabilitation animals are correlated using the Linear regression analysis. The R square values for the rehabilitation animals on post stroke day 30 was 0.008895 and post stroke day 60 was 0.01024, which shows no correlation with functional recovery. This would seem to indicate that in this group, any functional recovery was due to the rehabilitation, rather than the stem cell proliferation, which was almost non-existant.

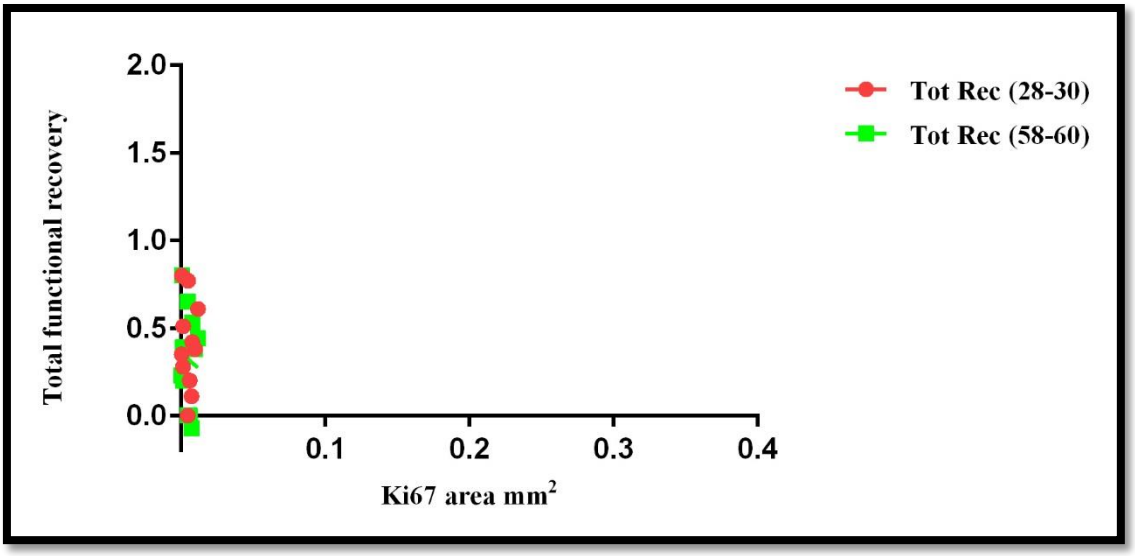


Figure 35: Graph comparing the mean SVZ Ki67 antibody staining and total functional recovery from post stroke days 30 and 60 for rehabilitation animals. The x axis denotes overall mean Ki67 antibody staining area in mm^2. And the y axis displays the total functional recovery. The dots represent total function recovery at post stroke days 30 (red circles) and 60 (green squares)

3.4.7 Correlating Arm Swipes with Total Functional Recovery and Ki67 Staining

The amount of peanut butter consumed by the animals in the rehabilitation group was converted into number of arm movements (swipes) in the entire rehabilitation period. A correlation analysis was performed between total functional recovery and swipes. The R square value obtained was 0.03151and P value was 0.6016 (Figure 36), which shows little correlation and non-significant deviation from zero.

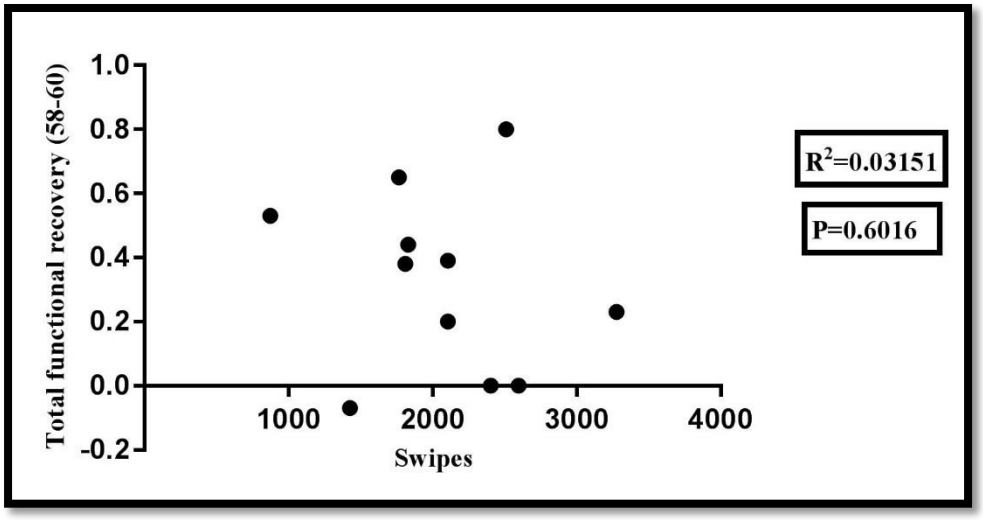


Figure 36: Graph showing correlation between swipes and total functional recovery from post stroke day 60. The x axis denotes swipes and the y axis displays total functional recovery. Each of the dots represent a single animal.

In the Figure 37, mean Ki67 antibody staining in the SVZ was plotted against the total swipes in the whole rehabilitation period. A correlation analysis was performed to statistically analyze the data. The R square value was 0.384, which is a good correlation, and P value was 0.0420. This figure shows that increases in the overall arm movements above 2000 had a negative impact on the stem/progenitor cell proliferation in the Subventricular zone. This was likely due to increased stress from the physical rehabilitation, as the animals used their impaired arm too much.

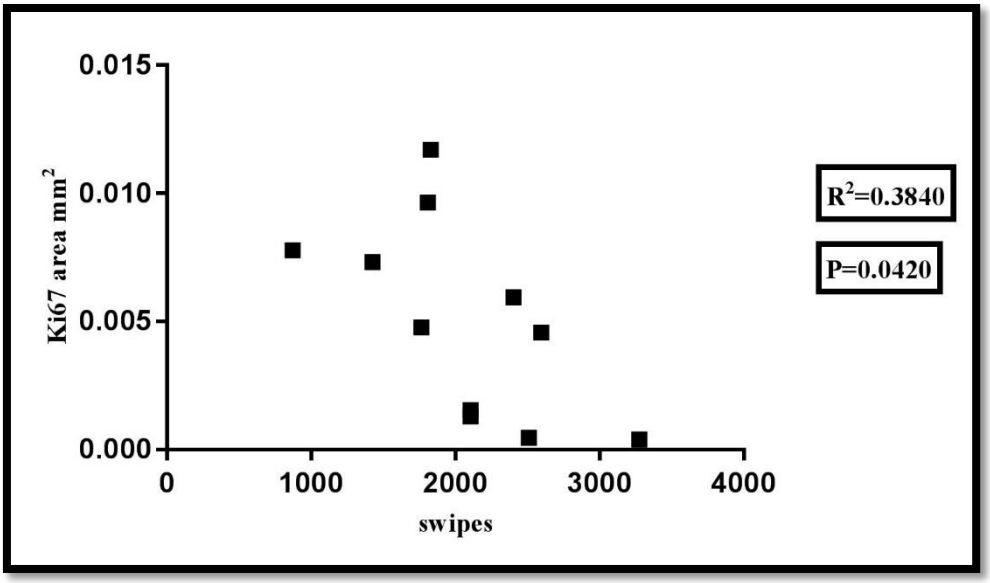


Figure 37: Graph showing correlation between total number of arm swipes during rehabilitation and Ki67 area in mm^2. The x axis denotes swipes and y axis displays mean Ki67 area in mm^2 .Each of the dots represent a single animal in the group.

3.4.8 Correlating Ipsilateral functional Recovery and mean Ki67 Staining

In the Figure 38, we see the correlation of the total ipsilateral functional recovery with the mean Ki67 staining in the SVZ for the control animals. A correlation analysis was performed. The R square value and P value for control animals 0.7827 and 0.0007 respectively, which shows the highest correlation and significance for any of our correlation analysis. We separated the ipsilateral functional recovery since this would involve injury to the corpus callosum, which is physically closer to the SVZ region, and more likely to feel the impact of increased growth factors in this region.

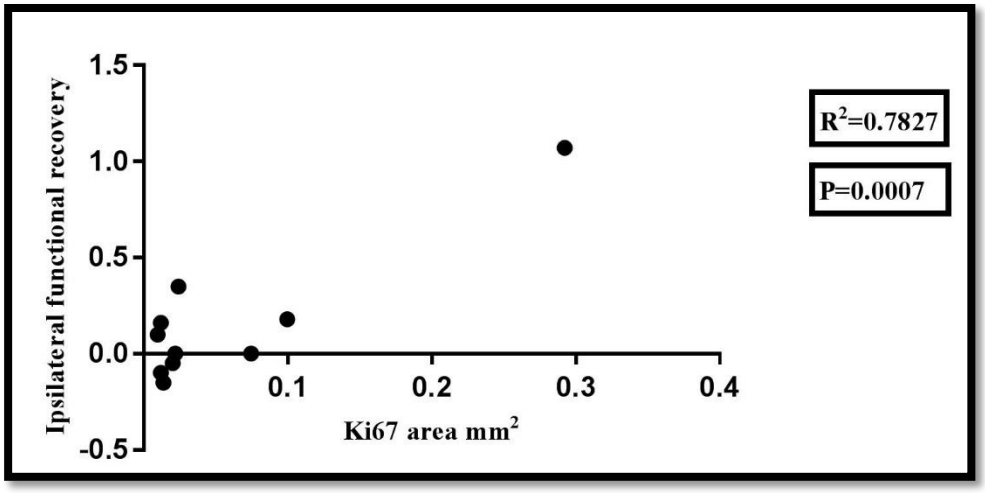


Figure 38: The x axis denotes mean Ki67 staining area in mm^2 for the whole SVZ and the y axis denotes total ipsilateral functional recovery for control animals. Each of the dots represent a single animal.

In Figure 39, we see ipsilateral functional recovery correlated with the mean Ki67 staining in the SVZ for the rehabilitation animals. The R square value and the P value were 0.04529 and 0.5550 respectively. This indicates virtually no correlation between ipsilateral recovery and the mean Ki67 staining area in the rehabilitation group, probably because the stem/progenitor cell proliferation was so reduced in this group.

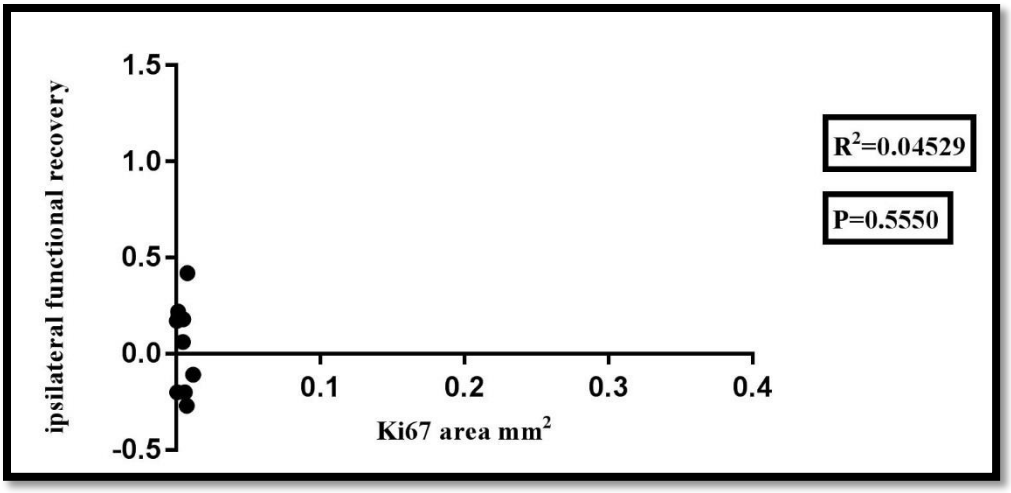


Figure 39: The x axis denotes mean Ki67 staining area in mm^2 for the whole SVZ and the y axis denotes total ipsilateral functional recovery for rehabilitation animals. Each of the dots represent a single animal.

IV DISCUSSION

4.1 Rat Weight Analysis:

We recorded weekly the rat weights from the beginning of the study until the rats were euthanized. Rat weights were recorded to determine their health conditions and ability to use their forelimb, and for animals receiving a drug treatment, to determine their fixed dosage of drugs. The initial rat weight recording was done at pre stroke period. From Figure 2, we see that there was a significant difference in the weights among the two groups (control and rehabilitation) analyzed here. We normally randomly assign animals to their groups, but in this case, we first tested whether they would eat the peanut butter on the hanging shelf before separating the animals into groups. Those that had readily eaten the peanut butter were put into the rehabilitation group: this may have been due to animal's greater appetite at a lower weight. The animals that weighed less may not have been eating enough rat chow, hence they might have been hungry all the time and ate more of peanut butter when it was offered to them (thereby being chosen for rehabilitation group).

All the rats in this study underwent an ischemic stroke in the right hemisphere of their brain in the first week of June. From Figures 3 and 4, we see that the animals have gained weight from their pre stroke period and this gain remained consistent over time (from 6/29 through 7/20). This shows that the animals have used their forelimb for carrying out their routine work. This also indicates that the diet restriction during the Montoya staircase three day test periods did not affect the animal's weight loss (there is

no any major loss). There is no significant difference in weight from post stroke day 30 until post stroke day 60, showing that any weight loss after stroke surgery was rapidly recovered.

The weight history of control animals does not show any statistical difference (Figure 5) over time whereas the rehabilitation animals' weight history does show a good statistical difference over time (Figure 6). Because the animals in the rehabilitation groups weighed less initially (Figure 2), the statistical difference is seen with respect to time in post stroke period (between early dates of 6/7 and 6/15 and later dates). The additional fat content in the peanut butter (Jif brand) may have helped them recover this weight difference quickly (in about a week after starting rehabilitation on post stroke day 8).

4.2 Bilateral Deficit

Bilateral deficits were seen in the some animals in each group at post stroke days 3-5 functional testing of their right and left forepaw grasping, may have been due to either a surgical error (hitting wrong co-ordinates, wise Bregma positioned incorrectly), or the endothelin injections reached the corpus callosum. The corpus callosum connects the right and left hemisphere of the brain, so any damage in the corpus callosum can lead to a bilateral deficit in the animals. The bilateral deficit was analyzed from the functional test – Montoya Staircase. Animals with a functional deficit of more than 20% on both the sides (contralateral and ipsilateral), were considered to have suffered a bilateral deficit.

The Bilateral deficit observed in the animals at post stroke day 3, seems to have vanished by either post stroke days 30 or post stroke day 60, which indicates there was

recovery in the white matter tracts either spontaneously (control group) or with rehabilitation. Since the control group recovered more (46.6%) than our previous female control groups (about an 11% recovery) we have to consider the possibility that this group may still have estrogen expression (pre-menopausal) (70), which has been shown to increase stem/progenitor cell proliferation, whereas most of our previously used control female rats were post-menopausal. Estrogen levels in animals have to be tested to determine if they have an influence in the increase in stem/progenitor cell proliferation and also white matter recovery. Estrogen can be tested by using ESTRADIOL ELISA kit, it can quantitatively determine the estradiol concentration in rat serum and plasma.

4.3 Rehabilitation:

As the rehabilitation days increase the animal's ability to perform, the number of swipes started declining. In the beginning stage of the procedure, the animals were averaging about 90 swipes per night but later it decreased. Apart from eating the peanut butter, the animals were also eating 4 grams of sugar cookie dough and *ad lib* rat chow every day except for the functional testing days. We summarized only the average number of swipes performed to consume the peanut butter per night in the graph. Any animal could have either higher or lower number of arm swipes to get this amount peanut butter due to normal variation, if some arm swipe were ineffective in obtaining peanut butter. We obtained the swipes by converting the amount of peanut butter consumed by the animal into forearm movements. Previous rehabilitation studies from our laboratory showed that the rehabilitation animals recovered better than control animals, which is opposite what we see in this study (63). The previous study was carried out for about 19 total rehabilitation days whereas this study was done for 23 total rehabilitation days.

Although the amount of peanut butter consumed by the animals and the arm swipes were not quantified in previous trial in our laboratory (Figure 40), the amount hung seems to be about one-fourth (63) the amount hung for this study (Figure 41). Animals in the previous study were also given a rest period before euthanasia (30 days) where they had no rehabilitation. This combination of factors made a big difference in the amount of recovery seen, which seems to indicate that too much physical stress in rehabilitation can work against recovery in that limb.

Figure 40: Image showing rehab shelves with little amount of peanut from our previous laboratory study(63)

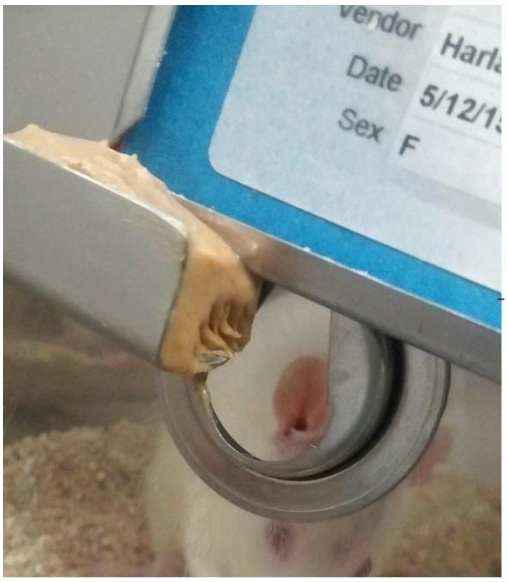


Figure 41: Image showing rehab shelves filled with trough full of peanut butter

Figure 40 shows that the shelves had in our previous rehabilitation study had less peanut butter and animals would not have been physically stressed by consuming this quantity. In the previous study these animals were kept for 30 days post rehabilitation period, for a total of 90 days (63).

The rehabilitation shelves were hung for almost 16 hours overnight, Although the procedure was voluntary, the animals may have been craving more peanut butter. Repeated movement of the impaired forelimb may have caused a physical stress in the animal. Hence it would have been increasingly tiring for the animals as the days passed. So, they started retrieving less peanut butter. The other reason might have been due to increased levels of BDNF (Brain derived neurotrophic factor), moderate exercise increase levels of BDNF in the brain (71-77) which in turn reduces the appetite (78-82). BDNF levels can be tested using immunohistochemistry technique, BDNF antibody to develop BDNF expression.

4.4 Functional Analysis:

In the rehabilitation animals both the contralateral and ipsilateral function seem to be affected post stroke. The contralateral function for the rehabilitation group decreased by 6% from post stroke day 30 to post stroke day 60 (Figure 7). Rehabilitation animals did not show any improvement in the contralateral side, initially they showed increase in function at post stroke day 30, but by post stroke day 60 their recovery decreased. The ipsilateral function for the rehabilitation group remained the same from post stroke day 3 until post stroke day 60, showing no recovery over time.

The control animals with a bilateral deficit showed a steady increase in ipsilateral function from post stroke day 3 through post stroke day 60 (Figure 8), although a two ANOVA did not reveal a statistical difference with respect to time or groups. This may reflect that only about three animals in each group had a bilateral deficit, so averaging across the group of 12 diluted the impact.

This is the first time ever, that we have seen a very high recovery of contralateral function post stroke in control animals. This study shows contralateral function recovery of about 46.6 % with the control animals and 24.5 % with the rehabilitation animals. We see almost 2 fold increase in the contralateral function recovery with the control animals when compared to the rehabilitation animals.

If we compare the total functional recovery, control animals had higher functional recovery compared to the rehabilitation animals. The control animals showed a nearly 2 fold higher total recovery compared to the rehabilitation animals.

From our previous laboratory studies, we have seen contralateral function of 8.6 % with control animals and 26% with rehabilitation animals on post stroke day 90. Previously the rehabilitation animals had a better functional recovery the control animals. The physical rehabilitation did not significantly alter the functional recovery in those animals, although there was a strong statistical trend. For these animals physical rehabilitation procedure was carried out for 19 days (63). In case of Female Long Evans rat, previous studies from our laboratory show recovery of only about 10.86% contralateral function with the control animals on post stroke day 30 (22).

These results were unexpected, since we hypothesized that the rehabilitation procedure could to speed up the recovery in the animals, as we have previously seen a 3 fold increase in the rehabilitation animals recovery post stroke (63). In this study, control animals showed the best functional recovery. The rehabilitation procedure seem to have suppressed the functional recovery in the rehabilitation animals.

4.5 Ki67 antibody staining:

The control animals showed higher mean Ki67 staining area in the SVZ compared to the rehabilitation animals. Average Ki67 staining area in the control group is almost 6 fold higher than seen in the rehabilitation group. These animals did not received any drugs to alter the staining (stem/progenitor cell proliferation) either way. Possible reason for this decrease in stem/progenitor cell proliferation would be the overuse of the impaired limb to reach the peanut butter and to consume it, which resulted in a stress and induced decrease in stem/progenitor cell proliferation. The rehabilitation procedure we designed was completely voluntary and we did not predict that the animals would

voluntarily stress their impaired limb with over-use, but it appears that we were wrong in that assumption.

Elevated levels of Ki67 in the control stroked animal show stem cell/ progenitor cell proliferation (neurogenesis) taking place although the animals received no drugs to stimulate neurogenesis. This may have been due to elevated estrogen or prolactin levels in this group (pre-menopausal), both of which would result in increased neurogenesis. An Elisa technique can be used to test estrogen levels.

Mean Ki67 staining (stem/progenitor cell proliferation) was very low in the rehabilitation animals compared to control animals. We also saw that the total functional recovery with rehabilitation animals was poor. Stress is known to decrease stem cell proliferation(83). This can be tested by giving the control animals small quantities of peanut butter inside cage, expecting them to show non-elevated levels of corticosterone.

We decided to correlate the total functional recovery with the Ki67 staining, to see if they follow the same trend. Increase in functional recovery was associated with an increase in Ki67 staining in the control animals.

For the rehabilitation animals mean Ki67 staining was so low, we saw very little correlation with functional recovery with a very low R^2 value (0.01024). To determine what was actually affecting the functional recovery in the rehabilitation groups, we tried to correlate the number of arm swipes with the Ki67 staining. The animals that performed arm swipes that totaled more than 2000 in the whole rehabilitation period, had lower mean Ki67 staining. As the physical effort increased, the mean Ki67 staining in the

Subventricular zone decreased. This suggests that physical stress in this limb was reducing the stem/progenitor cell proliferation in this region.

Previous studies from our laboratory on Ki67 staining, showed a mean Ki67 staining of about 0.0034 for control female retired breeder Sprague Dawley rats (10-12 months old). These animals did not have a stroke. Based on the stem/progenitor cell proliferation level, we concluded that these animals were all post-menopausal (84). The animals from the present study show a mean Ki67 staining of 0.030, which is almost 8.8 fold higher Ki67 staining than the previous studies. The stroke may have triggered increased neurogenesis (39) but this increase usually only lasts a week or two, or estrogen might still be around (if the animals are pre-menopausal). Estrogen can increase neurogenesis (85, 86) and lead to functional recovery (87). When animals are made to over use their impaired limb compared to the non-impaired limb, they were shown to increase the volume of the injury (88).

There are numerous research studies showing , that stress can elevate levels of glucocorticoids in brain, which in turn can cause death of cells in hippocampus (89), which is the other neurogenesis niche in the brain (Subgranular zone). These elevated glucocorticoids can inhibit the genesis of new neurons in this region and this study shows that the same seems to happen for the Subventricular zone of the lateral ventricles. To definitely prove this we would need to show elevated corticosterone in the Subventricular zone of the rehabilitation rats when their exercise is too intense compared to non-elevated controls.

Most studies show corticosterone decrease stem/progenitor cell proliferation (90) and the voluntary exercise has the ability to increase stem/progenitor cell proliferation

(30), the combination of both the effects were discussed by Lee *et.al*, 2016. So far his study has been one of them to demonstrate that elevated levels of corticosterone with voluntary exercise will reduce neurogenesis in the subventricular zone in similar way to the hippocampus. The neurogenesis was measured by BrdU positive cells for their study(64) We could not use BrdU as a marker for stem/proliferation because we are using an injury model, and BrdU gives false positives when DNA is undergoing repair.

The subventricular zone is a region with continuous neurogenesis. Researchers have found that neurogenesis in the SVZ have contributed in neuroprotective mechanisms, especially these stem/progenitor cells turn into neuroblast which migrate to cortex or striatum post stroke to modify prevailing synaptic connections inside the brain regions (91, 92)

This research used Ki67 to measure the neurogenesis. This is the first study to show that increased physical stress (due to voluntary exercise) decrease the Ki67 levels in the animals after ischemic stroke. Further investigation in this study is required, to quantify circulating levels of corticosterone and quantify estrogen levels and lactate levels (muscle stress) and to limit the rehabilitation procedure to 30 minutes to an hour a day from 16 hours. Amount of peanut butter given to the animals can be reduced to 3-4 grams from 10-12 grams a day.

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