G-CSF GENE THERAPY FOR BRAIN DISEASES AND/OR SICKLE CELL ANEMIA

By

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Howard Prentice, Department of Biomedical Science, and has been approved by all members of the supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Medicine and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

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Ischemic stroke is defined as a blockage or reduced flow of blood to select areas of brain tissue due to either plaque formation or buildup of blood clots in the small blood vessels. A characteristic of sickle cell anemic patients is the potential for them to experience a similar type of blockage due to the sticky nature of the sickled red blood cells as well as defective oxygen delivery to the brain. Because of this similarity, sickle cell anemia may represent a good animal research model for therapeutic intervention based on stroke models. In recent studies, Granulocyte-Colony Stimulating Factor (G-CSF), has been shown to exhibit a robust range of neuroprotective properties against neurological disorders including ischemic stroke through preservation of the endoplasmic reticulum (ER) by modulating various ER stress pathways. Through cognitive deficit analysis in the form of behavioral and locomotor experiments in addition to *in situ* biomarker analysis

by way of western blotting and immunohistochemistry, we found that G-CSF gene therapy exhibited neurogenic and neuroprotective effects in ischemic mouse models and could possibly serve as a good therapy for other diseases that share similar pathology to stroke.

G-CSF GENE THERAPY FOR BRAIN DISEASES AND/OR SICKLE CELL ANEMIA

FIGURES

CHAPTER 1: INTRODUCTION

Stroke is the fifth leading cause of death in the United States affecting approximately 800,000 Americans each year[1]. This brain disease occurs when blood flow stops or is severely reduced to the brain (ischemia) specifically, to neurons. The pathology of other neurodegenerative brain diseases such as sickle cell disease, a severe form of anemia (SCA), have also implicated a stroke-like mechanism involving a blockage or reduction of blood oxygen flow to brain tissue[2]. Ischemic stroke has also been known to encompass hypoxic components regarding its pathology to the neurovascular unit as vascular deficits[2,17]. Sickle cell anemia and/or other brain diseases such as ischemic stroke disease may serve as a good model to conduct research for therapeutic intervention[7]. In previous studies, granulocyte-colony stimulating factor (G-CSF), has been shown to express neuroprotective qualities in ischemic stroke mice models by acting on endoplasmic reticulum stress pathways and can serve as a powerful growth factor[2,19].

Our aims to help us answer these questions will involve performing experiments that will involve analysis of the proposed protective mechanisms and cell signaling pathways underlying the beneficial effects of AAV based G-CSF gene delivery within ischemic stroke and potentially, sickle cell mouse models[2]. In addition, we performed behavioral analysis with Morris Water Maze on mouse model of Sickle cell anemia.

Aim 1: Investigate whether neuronal cell death mechanisms involve ER stress pathways and if G-CSF gene therapy will induce a neuroprotective effect in stroke mouse models.

Aim 2: Discern whether there are measurable cognitive and behavioral deficits within sickle cell anemic mice indicating neuronal cell death and if this be reversed by G-CSF gene therapy.

1.1 Sickle Cell Anemia

Sickle cell anemia is an inheritable blood condition that involves the expression of a defective oxygen-carrying hemoglobin caused by a single amino acid substitution mutation[8]. This disorder distorts the red blood cell into a sickle or crescent shape that is stiff and inflexible resulting in severely impacted oxygen content of red blood cells.[3] Individuals with sickle cell anemia often experience a block of blood flow due to the sticky nature of the sickled cells which allows them to accumulate in the small blood vessels[8]. This can prove to be a fatal consequence to individuals who have this disease as it is pathognomonic to other blood-related diseases such as ischemic stroke. In these cases, sickled cells block vital blood and oxygen flow to important parts of the brain conferring a stroke-like mechanism.

1.2 Stroke

Stroke is often a complication of sickle cell anemia because there is a lack or absence of blood flow whether due to plaque formation or blood clot accumulation. Because of this, various brain tissue areas do not receive oxygen-containing blood and thus become damaged. This damage to brain tissue can prove to be fatal as the areas not obtaining blood cannot operate normal bodily functions and start to fail. During this time, an

inadequate supply of both oxygen and other sugars result in an ischemic cascade of molecular events that can lead to endoplasmic reticulum (ER) stress and dysfunction[2,19]. The ER is especially important because it is the site of protein synthesis, protein folding and post-translational modification. ER stress occurs when there is a buildup or accumulation of unfolded proteins which will trigger the unfolded protein response (UPR) and is known to be a response to ischemia[19].

1.3 ER Stress Pathways

There are three ER stress transmembrane sensors that act as pathways and are associated with the UPR in the ER to help with resolving this dysfunction. These sensors are protein kinase-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1) and activating transcription factor 6 (ATF6).[2] Downstream targets of each of these pathways will generally upregulate UPR genes and ER-associated degradation genes (ERAD).[2] These genes will degrade the unfolded proteins and will allow for homeostasis in the ER. This mechanism is not without its consequences as prolonged ER stress will make these pathways upregulate the expression of the pro-apoptotic transcription factor, DNA damage-inducible transcript 3 or (CHOP).[2] This transcription factor will favor apoptosis by down regulating anti-apoptotic proteins like Bcl-2 as well as upregulating pro-apoptotic proteins such as Bim and Bax.[2]

1.4 G-CSF

Granulocyte-colony stimulating factor (G-CSF) is a glycoprotein that is known to stimulate the production of both granulocytes and stem cells from within the bone marrow and is synthesized by immune cells[6]. Granulocytes are white blood cells that

have wide-ranging functions implicated in initiating immune responses to infection and the repairing of damaged tissue.[2] Currently, G-CSF is FDA approved for the treatment of neutropenia, a condition in which a person has abnormally low levels of neutrophils[16]. In recent studies, G-CSF gene therapy has previously demonstrated to have therapeutic efficacy in ischemic stroke by expressing neuroprotection from cerebral ischemia through preservation of the ER. This results in the attenuation of pro-apoptotic proteins as well as promotion of anti-apoptotic protein expression[2].

1.5 BCAO

Bilateral Carotid Artery Occlusion refers to the partial blockage of one of the arteries to promote a stroke-like mechanism. BCAO is a widely established model for inducing cerebral ischemia in rodents and is performed using standard operating procedures[4]. The animal has one of its arteries occluded or "blocked" for a certain amount of time. After this time period, the occlusion is removed, and the animal is allowed to undergo reperfusion of blood to the brain[14].

1.6 Common Pathogenesis

Sickle cell anemia may represent a good model to better study ischemic stroke and its mechanisms through the use of a sickle cell mouse model. The rationale behind this concept is the defective or dysfunctional blood flow reduction to key areas of the brain being shared by both pathological conditions[3]. One of the big questions during this study was if a therapeutic intervention of G-CSF protein to sickle cell anemic mice will share the same positive outcomes as exhibited by its positive effect seen in ischemic stroke mouse models. If the pathways associated with cell death involving ER stress

mechanisms share parallels to mechanisms of cell death in sickle cell anemia, the purposed work will offer insight into possibly more treatment options[2].

Currently, no research has been done combining behavioral and locomotor experimentation of sickle cell mice with a potential neuroprotective treatment. The rationale behind this notion is that if induced ER stress pathways due to hypoxic conditions (sickle cell mice) can be positively modulated to become neuroprotective by the actions of G-CSF protein treatment. The results for this study will contribute to the field of stroke and sickle cell research and possibly help elucidate the mechanism associated with its pathology. If results from this study are favorable from hG-CSF treatment, it will make an ideal candidate for cerebral ischemia and/or general stroke therapy.

1.7 Specific aims

Our central hypothesis is that ischemic stroke will have the same or similar cell death mechanisms seen in sickle cell anemia will be treatable with G-CSF protein therapy acting as neuroprotection and/or a neurogenesis therapy. To answer this hypothesis, we are pursuing the following specific aims:

Aim 1: Investigate whether neuronal cell death mechanisms involve ER stress pathways and if G-CSF gene therapy will induce a neuroprotective effect in stroke mouse models.

This aim will be addressed in the form of immunological staining and protein detection techniques to look for specific biomarkers implicated by the various ER stress pathways within stroke mice models. We will also explore the potential neuroprotective effects

conferred by G-CSF protein therapy based on treatment time intervals throughout the study.

Aim 2: Discern whether there are measurable cognitive and behavioral deficits within sickle cell anemic mice indicating neuronal cell death and if this be reversed by G-CSF gene therapy.

This aim will be addressed in the form of both behavioral and locomotor experiments using established paradigms on sickle and non-sickle cell anemic mice before treatment. We will also explore the potential neuroprotective effects of G-CSF by repeating the same behavioral and locomotor experiments on treated animals with cohorts receiving treatment at different time intervals.

CHAPTER 2: METHODS

2.1 Mice used for Study

Mice were raised at Florida Atlantic University's Boca Raton campus vivarium under standard pathogen-free conditions. Mice in housing cages will have access to plenty of food and water during the study. Mice in housing cages will be living under the standard 12-hour light/dark cycle provided by FAU's vivarium. Mice will be monitored on a daily basis by the vivarium staff to check for infections, in-fighting, mouse distress and overall well-being of the animals.

The mice that will be used for this study was bred Townes mice containing the appropriate genotype for sickle cell anemia[12]. The mice cohorts for this study will be as follows: a cohort containing the genotype homozygous for sickle cell expression as well as a cohort containing the genotype homozygous for no sickle cell expression which will act as a control. Each cohort will contain ~24 mice for a total of ~48 mice. All mice used for this study will be genotyped by an outside company employed by the lab, Transnetyx.

The mice used in this study were ~8 months old conferring a developing phenotype of sickle cell anemia. After genotyping is performed of all mice, cohort grouping of mice according to their genotype into separate housing cages will be done. Some potential problems or challenges that could be anticipated during cohort grouping could include incorrect genotyping of one or more of the mice which will make the whole study for those mice invalid. Another potential problem or challenge when dealing with rodents is

the potential for in-fighting between other mice within the housing cage prior to cohort grouping. This may lead to serious physical injury to the mouse as well as social isolation which may affect behavioral data.

2.2 Morris Water Maze

The Morris water maze is a well-established behavioral paradigm for testing learning and spatial memory in rodents. In the test, mice will be placed in a large tub of room temperature water deep enough so that they cannot stand and must swim to stay afloat. Within the tub will be placed a transparent platform roughly the same size as the mouse at ~2 cm below the water surface so the mouse cannot see it[10]. The platform will be placed in specific "quadrants" of the tub which will allow the mouse to swim and try to find the platform to rest and dry on.

Spatial orientation is very important from the mouse's perspective and could possibly help with learning and memory in finding the platform. Stationary visual cues will be placed at each quadrant above the water maze tub and will consist of cut-out styrofoam shapes made of a circle, triangle, square and X[10]. Black curtains will be draped around the tub as not allow other environmental cues other than the stationary shapes placed on the curtain. As mouse tries to find the hidden platform, its movement data such as speed, direction, latency to platform and platform area duration will be recorded by a mouse sensor camera placed at the top of the water maze tub apparatus. 4 adjustable light bulbs will be placed around the tub to help with lighting for camera recordings. Recordings

from the camera will be available to view from a computer interface within the water maze room[10].

All mice will undergo water maze training every day, once a day, for a one-week period that will slowly acclimate the animal to the water. This will include introducing the mouse to a specific quadrant of the tub and let loose to swim and try to find the hidden platform placed at an opposite quadrant of the tub. No data collection will be performed at this time as this is just the training/pre-handling period. The mouse will have an allotted time of 60 seconds to try and find the platform within the maze. If the mouse cannot find the platform target within the allotted time, the mouse will be physically placed or "guided" to the platform for an independent 15 seconds. This will theoretically teach the mouse that there is a platform in the tub for future training/probe trial runs. After each run of the water maze, every mouse will be transferred to an adjacent room containing warming stations so that the mice can be dried before being placed back into housing cages.

After the one-week training period, each sickle cell and non-sickle cell mouse will be challenged in the water maze for 60 seconds and will be recorded by the camera at the top of the maze. During this time, the hidden platform will be placed at another quadrant of the tub as well as dropping the mouse at a different start site (mouse facing wall when dropped into water) and will be considered a probe trial. If the mouse finds the platform or is circling the platform, calibrated software of the camera will stop the timer and record the appropriate maze data. Like training runs, if the mouse does not find the platform within the given time, the mouse will be place

d onto the platform for 15 seconds and returned to warming room. The probe trail will last for approximately 1 week. We expect to see sickle cell mice finding the hidden platform less often and non-sickle cell mice due to problems in spatial learning or spatial memory.

Some potential problems or challenges that can come up at this point of the study includes both animal error as well as possible human error regarding software recording calibration. Concerning the animal side, the mouse may be exposed to harmful skin bacteria within the water maze tub due to urine and fecal matter accumulation. Proper cleaning of the tub must be done after each water maze run or ever other run that will involve draining the water and scrubbing of the tub with appropriate cleaning products. Another possible challenge the researcher might encounter is the sensitivity of camera calibration to the water's reflection of the tub. This may be counteracted by the use of adjustable light bulbs facing the water surface.

These mice will again be challenged to the water maze under the same probe trial parameters at 7-days, 14-days and 21-days after treatment respectively before being euthanized. After treatment we expect to see a positive trend in decreased time and increased speed to the hidden platform in sickle cell mice.

2.3 Rotarod

The rotarod performance test is often used on rodents to test for endurance, motor coordination, grip strength and balance. This type of performance test is especially useful in challenging the rodent to access the effects of experimental drugs and procedures[11]. In this test, the animal is placed on a mechanically driven rotating cylindrical rod that

slowly builds up speed until the animal cannot keep up and subsequently falls. The rotational speed of rotarod spindle is controlled by software from a computer interface. This fall is not enough to injure the rodent but large enough to trigger avoidance in the animal. When the animal does fall, an infrared beam sensor below the rods and above the collection area records the appropriate data such as current speed at which the rodent fell at and total time the rodent spent on the rotarod.

For this study, each mouse cohort will be placed on the Rotamex-5 system in groups of 4. When all mice are ready, the machine will drive the cylindrical rods to rotate first at a slow speed that will gradually increase in velocity. As the rods increase in speed, mice will progressively start to fall into their collecting chamber which will activate the infrared laser and record the appropriate data. There will be no training period for this test so each mouse will have ~2 trials on the rotarod. We expect to find sickle cell mice performing poorly compared to non-sickle cell mice on the rotarod test.

For this type of test, minimal to no problems or challenges faced by both the mice and researcher since the mice being tested face no injury as well as an easy-to-use apparatus for the researcher. After G-CSF protein treatment of selected animals, these mice will again be challenged to the rotarod performance test under the same probe trial parameters at 7-days, 14-days, 21-days, and 30-days after treatment respectively before being euthanized. After treatment we expect to find better motor coordination in sickle cell mice compared to pre-treatment sickle cell mice.

2.4 Locomotion (Open Field Maze)

For further analysis of animal behavior mechanisms can include the use of an open field maze or locomotion machine. The open field maze has been commonly used to measure both behavior in rodents as well as emotionality of the animal based on their overall movement during the test. This involves putting the rodent into a sealed chamber (allowing oxygen to enter) that is internally supported by four corner pegs that are extremely sensitive to any type of movement[13]. The animal will then be left alone in the chamber for ~30 minutes so the computer software interface can record any locomotor activity produced by the rodent.

For this study, we will be implementing this type of behavioral and locomotor test to further examine general ambulatory functionality of both cohorts of sickle cell and non-sickle cell mice. There will be no training period for this type of experiment so each mouse used in the study will only perform the test once. We expect to find that sickle cell mice will not move as much distance when compared to non-sickle cell mice due to a reduced energy supply. There should be no anticipated problems for the mouse when conducting this test as the mouse will face no challenges or harmful stimuli. After G-CSF protein treatment of selected mice, these mice will again be placed into the open field maze apparatus at 7-days, 14-days, 21-days, and 30-days after treatment respectively before being euthanized. After treatment we expect to see sickle cell mice moving more distance over a longer period of time when compared to pre-treatment sickle cell mice.

2.5 Vectors used for BCAO mice

BCAO mice will be treated with the hG-CSF gene therapy while control mice will be treated with green fluorescent protein (GFP). We chose random mice from each cohort to

be treated or not treated. Administration of the hG-CSF treatment group will be 50µg/kg body weight dissolved in 0.3 ml of 5% dextrose.[2] After administration of the treatment, mice will be left alone in their housing. Per the studies intervals, the mice will be removed from the housing cage euthanized.

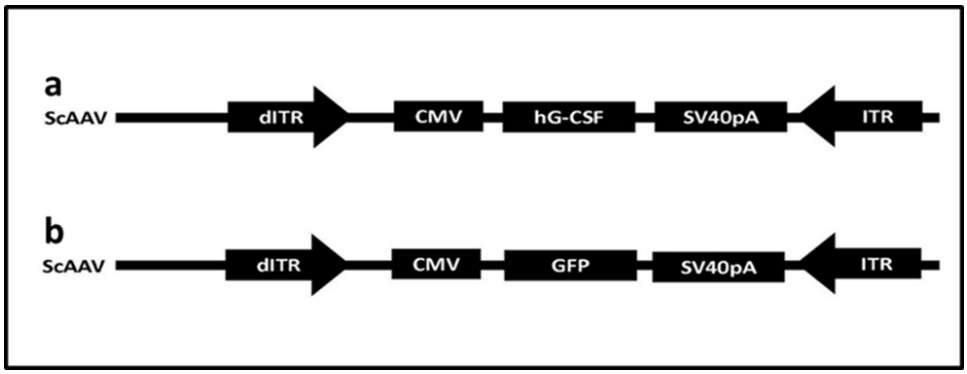

Figure 1. *AAV vectors used for hG-CSF gene therapy. Mice were given AAV-CMV-hG-CSF or AAV-CMV-GFP (1.5ul) by eye-drop in the left eye following BCAO procedure. Sham non-ischemic mice received no virus. All analysis was carried out on the frontal and middle brain sections.*

2.6 Western Blotting

Western blotting is an immunoassay technique that allows for the detection and quantification of specific proteins within biological samples or tissues. This technique for protein detection has been and still is widely used in almost every molecular science/ life science laboratory since first invented in 1975. At its foundation western blotting takes advantage of the antibody-protein complex formed through specific binding of antibodies to those proteins.[17] For western blotting to work correctly, a series of steps on the sample must be performed.

First, an electrical current separates protein in a sample by its size in a thin gel membrane which is known as polyacrylamide gel electrophoresis (PAGE). The proteins from the gel

are then transferred to a nitrocellulose support membrane. Proteins on the membrane are exposed to a primary antibody specific to the protein of interest (antibody probing). Then the membrane is soaked in random proteins to prevent nonspecific binding of the secondary antibody. Next, a secondary antibody coupled with a fluorescent enzyme that binds to the primary antibody to subsequent analysis. Finally, the membrane is imaged with charge-coupled camera-based imager. What makes this possible is the use of specific wavelengths to excite fluorophores that are conjugated to either the primary or secondary antibody. Analysis involves using an imager to detect bands on the membrane where both molecular weight of the protein (comparison from marker proteins) and the amount of protein (band intensity) can be quantified.

For this study, only a select number of mice from both sickle and non-sickle cohorts, will be used for western blotting. This is because the other half of mice left that are not being used for western blotting will be used for immunohistochemistry and must be euthanized by perfusion. We anticipate using ~24 mice for western blotting which will include extracting the whole mouse brain and sectioning it into 1/6s. In this study, we will be looking for proteins that are expressed in the different endoplasmic reticulum or mitochondrial stress pathways due to hypoxic conditions faced by the sickle cell anemic mice. The pathways in question are the PERK, IRE1, and ATF6 pathways.

There are certainly potential challenges and problems the researcher could face during western blotting protocols. Some of these include having unknown protein blotting, loss of a protein during membrane transfer and general transfer difficulties. Most of these

potential problems would occur from the human side where the researcher can start off with a bad gel, make transfer mistakes and/or use poor primary or secondary antibodies.

2.7 Immunohistochemistry

Immunohistochemistry is commonly used staining technique used for the purpose of detecting antigens within a biological tissue sample using antibodies that takes advantage of antigen-antibody binding.[13] Immunohistochemistry is often used in biomedical research to detect specific antigens in tissue samples and is often implemented by healthcare professionals to diagnose diseases like breast cancers regarding cellular events such as increased cell death. What separates this technique from other antigen-antibody reaction techniques like western blotting is the ability to gather *in situ* data regarding relative distribution and/or localization of specific antigen(s) or cellular components within a tissue sample. This type of immunostaining is quite an involved process requiring a delicate touch and patience of the tissue.

Immunohistochemistry first starts with the tissue sample. In this study we will be using whole mice brains from both cohorts of sickle cell and non-sickle cell. The process first starts after adequately anesthetizing the animal with isoflurane prior to the opening of the body cavity to have access to the heart and is necessary for perfusion. This type of perfusion involves replacing the blood in the rodent with a saline solution by injecting a small saline pump through the animal's left ventricle. This done to optimize the health of the overall brain and to reduce excitotoxicity. After making an incision in the ascending aorta, ice-cold saline will be pumped throughout the body (in through the left ventricle and out the ascending aorta) until fluid exiting the body cavity is clear with no blood

residue. The ending stage of this procedure will have the researcher physically extract the whole mouse brain from the mouse skull and placed into an ice-cold saline or paraformaldehyde solution so the brain can be stored for future use.

After acquisition of the mouse brain, preparation of brain section will be performed next. This will be done with the use of a cryostat microtome. This is an apparatus made specifically to cut desired sizes of tissue with a fine blade.[12] For this study we will be cutting our mouse brains at 40μm sections. The cut sections will be organized into 12-well plates such so that each well will have a representation of the whole brain from the anterior to posterior stopping at cerebellum structures. The sections will be placed into either Tris-buffered saline, TBS, (for staining the same day) or an ethylene glycol/ glycerol antifreeze mix (for future use). If the section will not be used that day or the next, the sections in the antifreeze solution will be placed into a -20℃ freezer.

For immunostaining the tissue, a series of stepwise reactions must be performed to ensure proper tissue staining coverage. The process starts with ordering the tissue in a sperate petri dish containing TBS where the sections of one well is organized in an anterior to posterior orientation. This step is important because it will allow the researcher to add or subtract tissue based upon what structures are of importance in staining (i.e. lateral ventricles, hippocampus, dentate gyrus, etc.). Next, the tissues are washed three times in TBS on a shaker at 25 rpm to remove any residue if the tissue was stored in the antifreeze solution. The next step involves the removal of excess aldehydes by incubating tissues in a 1% sodium borohydride solution for 30 minutes on 25 rpm shaker. After incubation,

excess bubbles formed by the reaction will appear and must be hand washed with TBS until there are no more bubbles in the well.

Afterwards, quenching of the endogenous peroxidases will need to be performed. The tissue will again be incubated in a solution containing 10% methanol and 3% hydrogen peroxide for 5 minutes on 25 rpm shaker. After incubation, excess bubbles may appear and must be washed by handheld TBS. Blocking is the next important step that will consist of incubating the tissues in TBS, 10% goat serum, 0.3% Triton-X100, 0.3M Glycine and a fractional antibody solution on shaker at 25 rpm for 1 hour. This blocking step is done so as to limit non-specific binding.

After blocking, the next step is applying primary antibody(s). The primary antibody solution will contain TBS, 0.3% Triton-X100, 2% goat serum and the primary antibody(s). Once the solution is made up, it is important to open as many sections of tissue flaps as possible in a large well to allow for complete surface area coating of the primary antibody solution to the tissue. After this step, the tissues containing the primary antibody(s) solution must be placed into 20℃ fridge and left to sit overnight or 24 hours. After the primary antibody has been set, the secondary antibody(s) can be applied.

The secondary antibody(s) solution will be made up of the secondary, 0.3% TritonX-100 and 2% goat serum in TBS. Application of the secondary antibody(s) solution will take place in the same well as the primary-stained sections and placed under cover on a shaker at 25 rpm for 2 hours. It is very important to note that at the beginning of creating the secondary antibody solution, the laboratory's lighting must be turned down or turned off. This is because the secondary antibody(s) contain the fluorescence antibodies and will

fade rather quickly if within the presence of light. After setting for 2 hours on the shaker, washing of the tissue with a mixture of tris-buffered saline and Tween 20, TBST, must be performed four times with 10 minutes for the first wash and all subsequent washes at 5 minutes.

After last wash of TBST the tissue sections will be ready for mounting on confocal microscope slides. We will analyze the sections under a Zeiss LSM 700 confocal microscope[20]. Some potential problems or challenges that could come about during the entire immunohistochemistry process can vary. Complications in tissue extraction can lead to adverse histological data further down the study as well as difficulties in staining the tissues at the appropriate times while following the appropriate protocol. Immunohistochemistry will be one of the more important experiments in this study because it will give a visual representation of specific antigen localization, distribution, and quantification within our affected mice brains.

In this study, immunohistochemistry was used for the purpose of detecting biomarkers within the brain that modulate cellular growth or cellular regeneration by the actions of G-CSF. Specifically, we will be staining for biomarkers related to endoplasmic reticulum or mitochondrial stress pathways such as the PERK, IRE1, and ATF6 pathways[2,13]. We also quantified number of cell biomarkers using quantification techniques related to confocal microscope analysis.

CHAPTER 3: RESULTS

3.1 Morris water maze results of Sickle cell and non-Sickle cell cohorts

The first part of the study focused on the spatial learning and memory aspect of sickle cell phenotype mice compared to non-sickle celled cohorts. This was done by way of the Morris water maze test. We first observed distance moved by the mice cohorts in regard to distance moved in the water tub. Across three separate probe trials, non-sickle celled mice traveled more mean distance in the tub in trying to find the hidden platform compared to the sickle celled mice (**Figure 1**). This could be due to the sickle cell mice having insufficient oxygen transferred to muscle groups to facilitate more swimming. This also could be correlated with reduced brain function due to the phenotype.

We next observed the velocity of the mice cohorts were swimming at during the probe trials in trying to find the hidden platform. We found that across all three probe trials, non-sickle celled mice traveled at a higher mean velocity than that compared to the sickle celled mice in trying to find the hidden platform (**Figure 2**). This could also be due to the sickle cell phenotype affecting travel speed in regard to locomotor capability.

We then measured the cumulative duration of the sickle cell and non-sickle cell mice cohorts were in the relative zone of the hidden platform across three separate probe trials. We found that across all three probe trials, non-sickle cell mice exhibited better spatial memory of where the hidden platform was compared to the sickle cell phenotype who spent less time in the zone where the hidden platform was located (**Figure 3**). Sickle cell

mice seemed to have a harder time in locating the hidden platform after first being exposed to it compared to non-sickle mice.

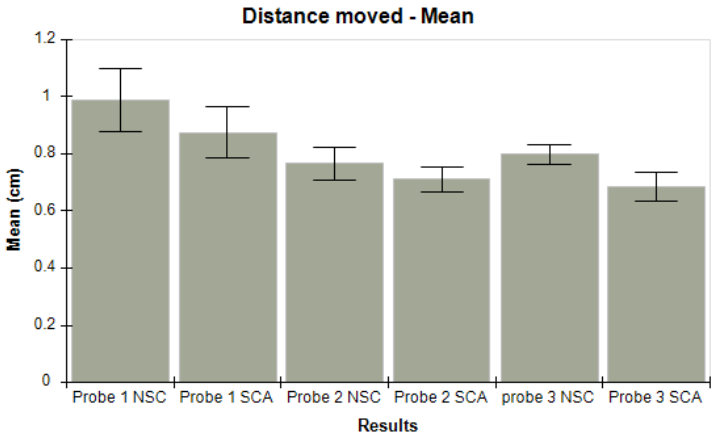

Figure 2. *Across 3 probe trials, Townes non-sickle cell mice (NSC) groups distance compared to the same-aged sickle cell mice (SCA) groups. These mice were breaded in-house and genotyped using Transnetyx. (n =4 (NSC); n =3 SCA)*

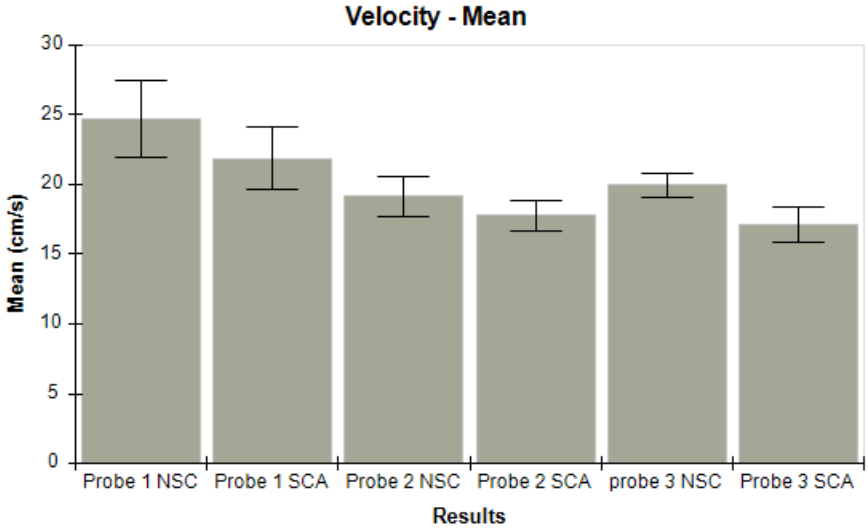

Figure 3. *Across 3 probe trials, Townes non-sickle cell mice (NSC) groups compared to same-aged sickle cell mice (SCA) groups. These mice were breaded in-house and genotyped using Transnetyx. (n =4 (NSC); n =3 SCA)*

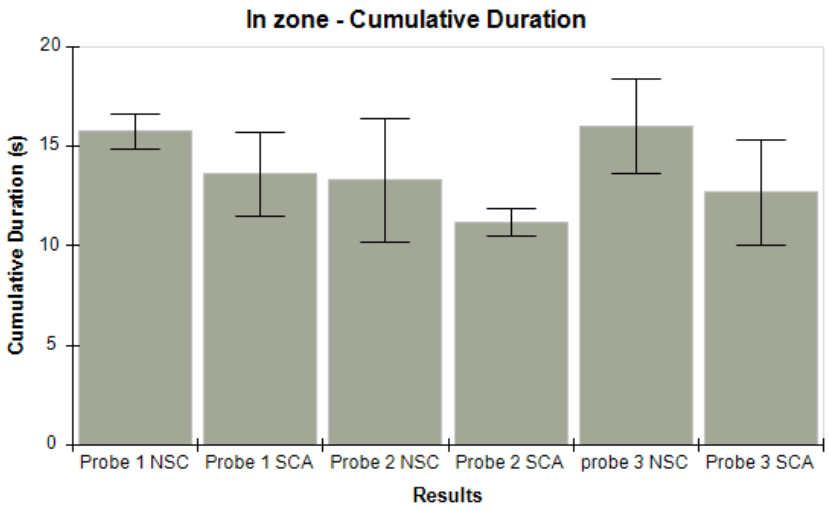

Figure 4. *Across 3 probe trials, Townes non-sickle cell mice (NSC) groups compared to the same-aged sickle cell (SCA) mice group time on the platform area. These mice were bread in-house and genotyped using Transnetyx. (n =4 (NSC); n =3 SCA)*

3.2 Rotarod test results of Sickle cell and non-Sickle cell cohorts

We then examined the locomotor activity of the sickle cell and non-sickle cell cohorts using a Rotarod test to measure motor coordination/skill and access if any neurodegeneration was implicated by way of motor impairment. We found that non-sickle cell mice were able to last longer on the rotating rod which increased in speed the longer the mouse was on it compared to the sickle cell mice (**Figure 5**). A similar result was also observed which non-sickle cell mice fell off the rod at a higher rod speed than compared to sickle cell mice (**Figure 5**). This could be due to neurodegeneration of the affected phenotype and it's subsequent motor skill impairment.

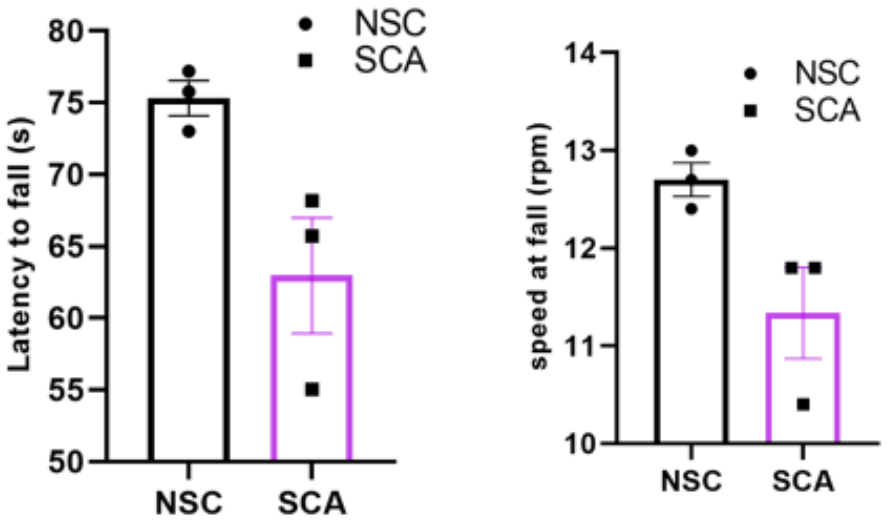

Figure 5. *Rotarod motor endurance and coordination of non-sickle cell compared to sickle cell phenotype Townes mice of the same age. These mice were bread in-house and genotyped using Transnetyx. (n =4 (NSC); n =3 (SCA)*

3.3 Locomotion results of Sickle cell and non-Sickle cohorts

We next looked at the relative behavior of sickle cell and non-sickle cell cohorts by way of the Force Plate Actimeter. This measured the overall locomotor activity in millimeters and would assess whether the animal's overall behavior in an enclosed and quiet environment over a period of time. We observed that the sickle cell mice cohort displayed slightly more locomotor activity than the non-sickle cell mice (**Figure 6**). Sickle cell mice might have been in distress and/or agitated during the test.

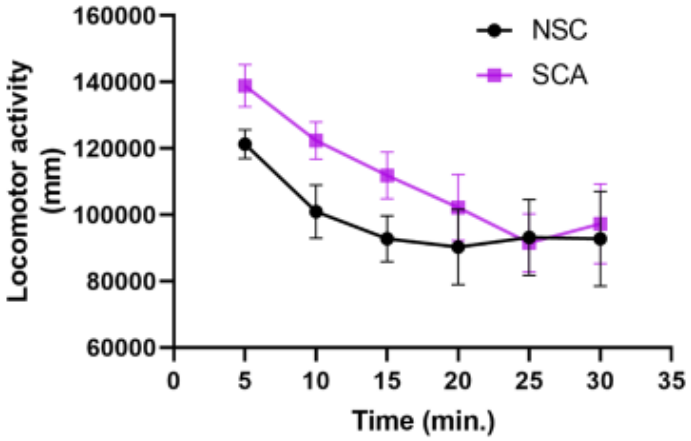

Figure 6. *Mean (+/- SEM) behavior score on Force Plate Actimeter (FPA) for Townes mice of Sickle cell anemia phenotype (SCA) compared to non-sickle cell phenotype (NSC) at 8 months of age. Mice were bread in-house and genotyped using Transnetyx. (n =4 per group)*

3.4 Immunohistochemistry results

The second part of the experiment focused on immunohistochemistry imaging of 4-day stroke mouse brain post-BCAO by GAD (green) stain of G-CSF gene therapy on the cortex, CA1 region and CA3 region of the hippocampus. Image J software was used to quantify cell numbers. We first found that more GAD neurons were present in the treated hG-CSF mice when compared to the GFP control mice in areas of the cortex, CA1 and CA3 regions (**Figure 7a**). This can also be seen within the percentage of GAD neurons present in those regions of the brain indicated by bar graphs (**Figure 7b-d**).

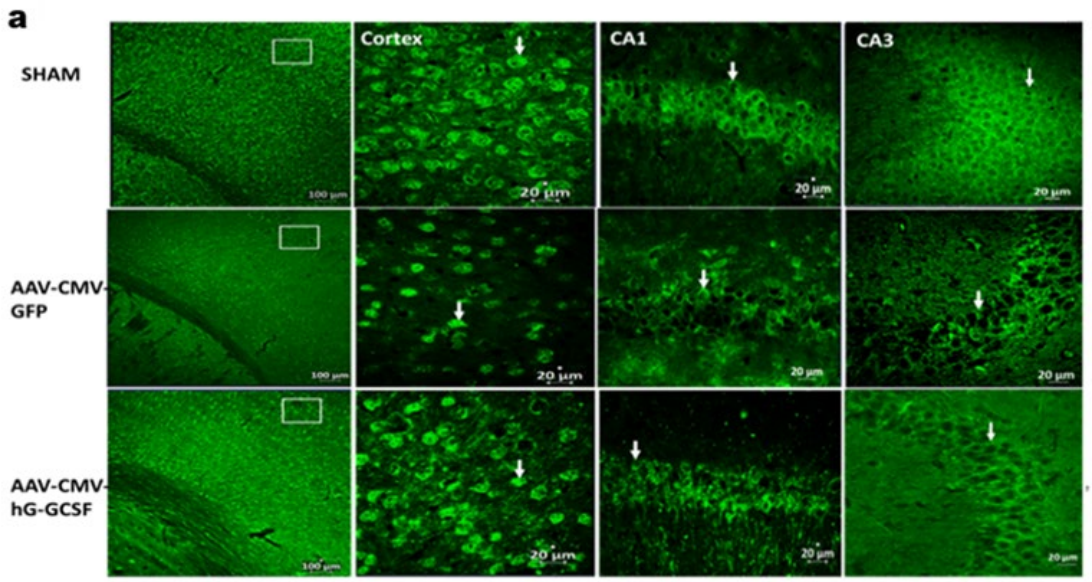

Figure 7a*. GAD stain of hG-CSF-treated and non-treated 4-day stroke mouse brain of cerebral cortex, CA1 region and CA3 region.*

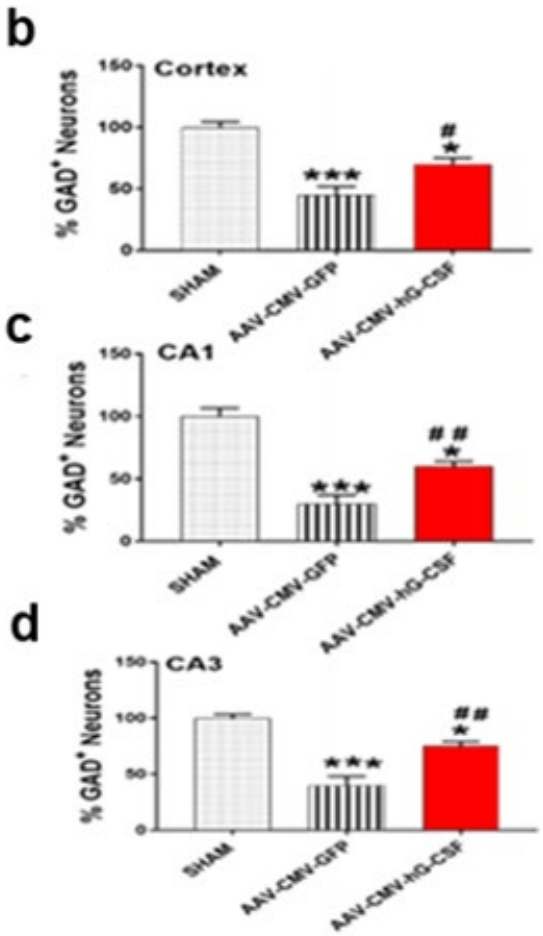

Figure 7b-d. *Percentage of GAD neurons present in SHAM, untreated-GCSF and treated GCSF 4-day stroke mice of cortex, CA1 and CA3 regions, respectively. Cells quantified by Image J.*

Imaging of the basal forebrain of 4-day post-BCAO treated and untreated mice was done using specific biomarkers for neuronal maturation (NeuN) and neurotransmission (chAT). At 4-days post-BCAO within the basal forebrain, treated hG-CSF mice exhibited much more chAT neurons than compared to untreated GFP mice as seen in both the image and bar graph (**Figure 8**). NeuN expression of treated BCAO mice was similar to that of GFP untreated mice (**Figure 8**).

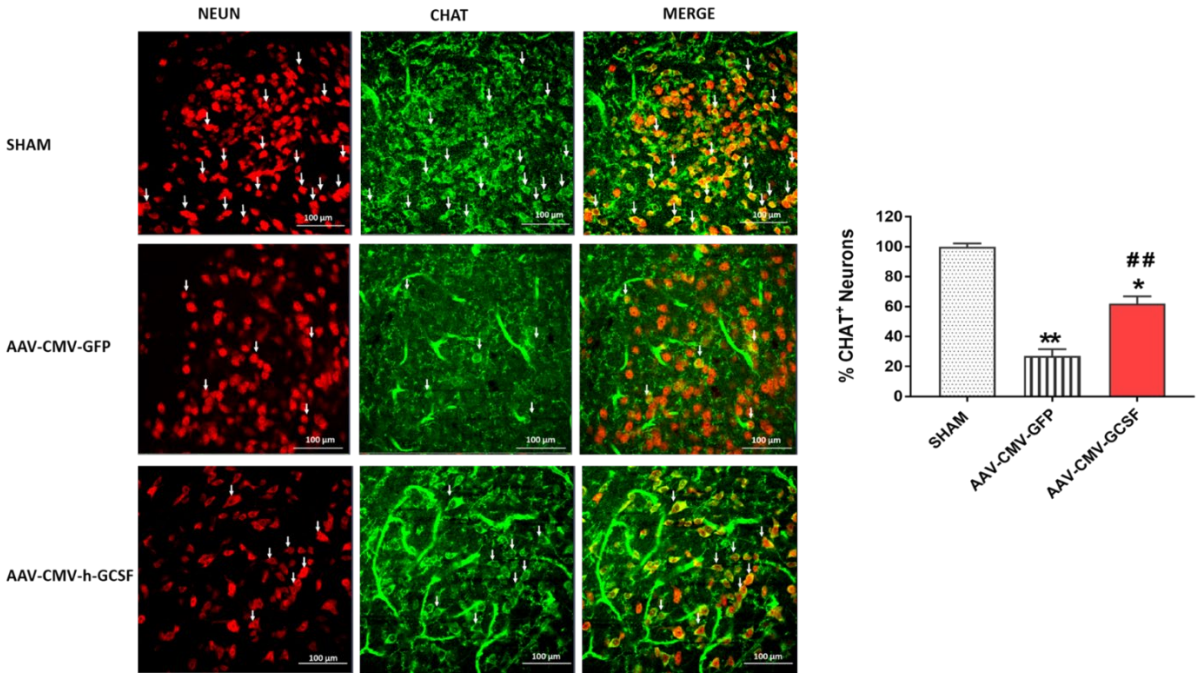

Figure 8. *NeuN and chAT neuron immunohistochemistry images and quantification of chAT positive neurons for BCAO mice at 4 days. Images are of sections are of the basal forebrain.*

Next, we measured hG-CSF gene expression of 4-day post-BCAO mice using western blot analysis. Sections were of the frontal and middle part of the mouse brain. Mice were given AAV-CMV-hG-CSF or AAV-CMV-GFP (1.5ul) by eye-drop in the left eye following 30 mins of BCAO. Sham non-ischemic mice received no virus. All analysis was carried out on the frontal and middle brain sections. We saw significant mRNA gene expression as well as protein expression in hG-CSF treated mice compared to the untreated GFP mice (**Figure 9a-b**). Visual representation of the brain sections also indicated h-GCSF treated mice had less infarcted brain tissue (**Figure 9c**).

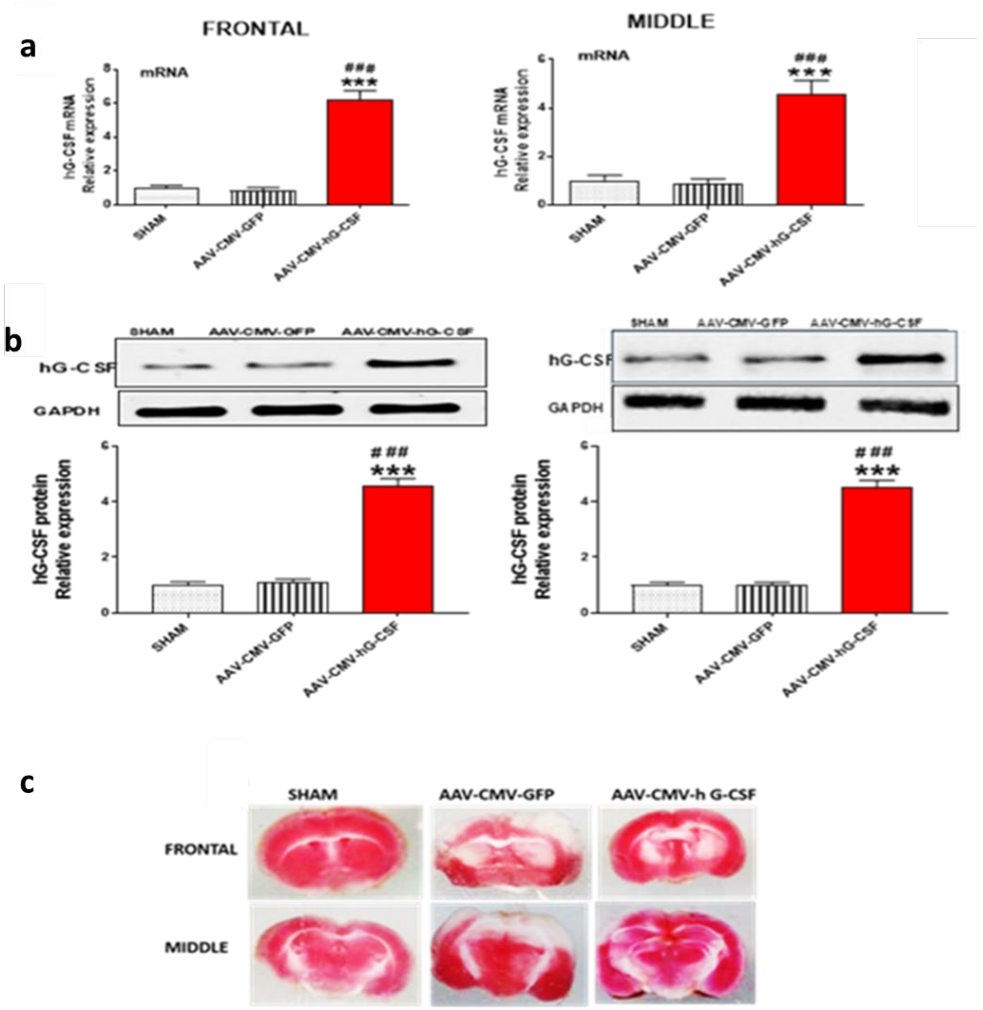

Figure 9a-c. *Demonstration of hG-CSF gene expression via four days post BCAO. (a) qRT-PCR expression of mRNA hG-CSF levels in the frontal and middle brain sections. (b) Western blot of mRNA hG-CSF to the hG-CSF protein. (c) TTC representation of infarcted regions.*

We then measured the levels of pro- and anti-apoptotic proteins present in treated and untreated 4-day post-BCAO mice. This was done by way of western blotting of Bcl2 and Bax. In the frontal section of the brain, Bcl2 was expressed more in the treated mice than in untreated mice (**Figure 10a**) while Bax was expressed more in the middle part of the brain (**Figure 10a**). Relative expression levels of Bcl2 were much higher in the treated hG-CSF mice than in the untreated GFP mice (**Figure 10b**). Relative expression levels of Bax were also measured in these mice indicating a similar level when compared to the

GFP mice (**Figure 10c**). We also saw the relative ratio between the two proteins in both cohorts indicating that treated hG-CSF mice had significantly higher ratio than in the non-treated GFP mice (**Figure 10d**).

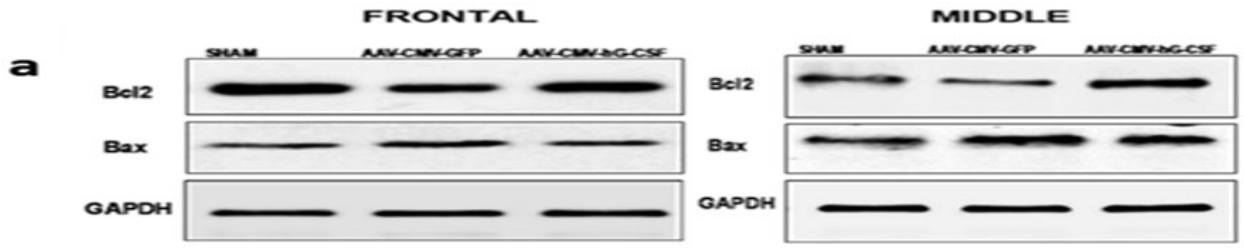

Figure 10a. *Effect of hG-CSF gene therapy on Bcl2 and Bax 4 days post-BCAO. (a) Western blot analysis using antibody against Bcl2 and Bax. Statistical analysis was performed by One Way ANOVA, with Tukey as post hoc test and expressed as means ± S.E.M; * p < 0.05 /** p < 0.01/***p <0.001 versus Sham group while ## p <0.01/ ### p <0.001 versus AAV-CMV-GFP-treated group. (n =6 animals/group).*

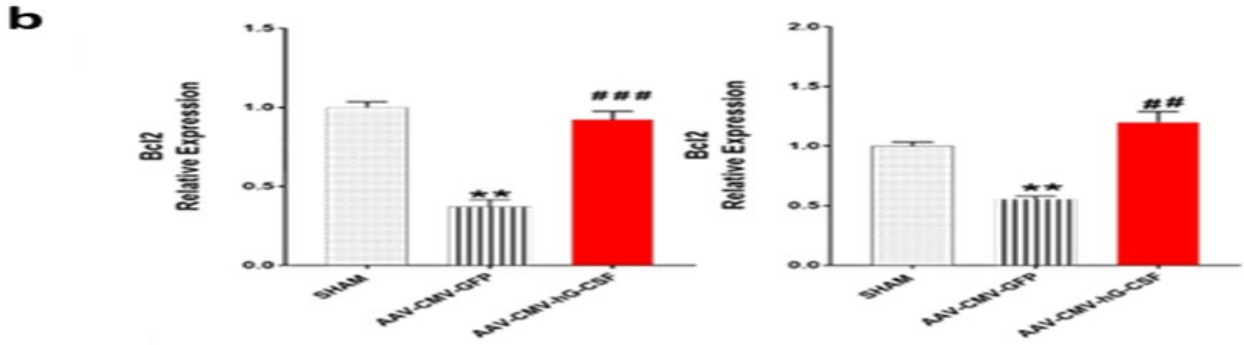

Figure 10b. *Effect of hG-CSF gene therapy on Bcl2 and Bax 4 days post-BCAO. (b) Densitometry analysis of Bcl2. Statistical analysis was performed by One Way ANOVA, with Tukey as post hoc test and expressed as means ± S.E.M; * p < 0.05 /** p < 0.01/***p <0.001 versus Sham group while ## p <0.01/ ### p <0.001 versus AAV-CMV-GFP- treated group. (n =6 animals/group).*

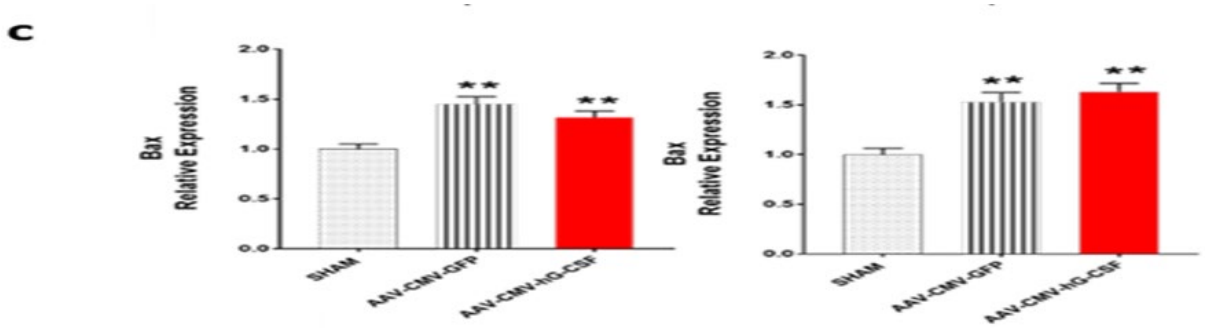

Figure 10c. *Effect of hG-CSF gene therapy on Bcl2 and Bax 4 days post-BCAO. (c) Densitometry analysis of Bax. Statistical analysis was performed by One Way ANOVA, with Tukey as post hoc test and expressed as means ± S.E.M; * p < 0.05 /** p < 0.01/***p <0.001 versus Sham group while ## p <0.01/ ### p <0.001 versus AAV-CMV-GFP- treated group. (n =6 animals/group).*

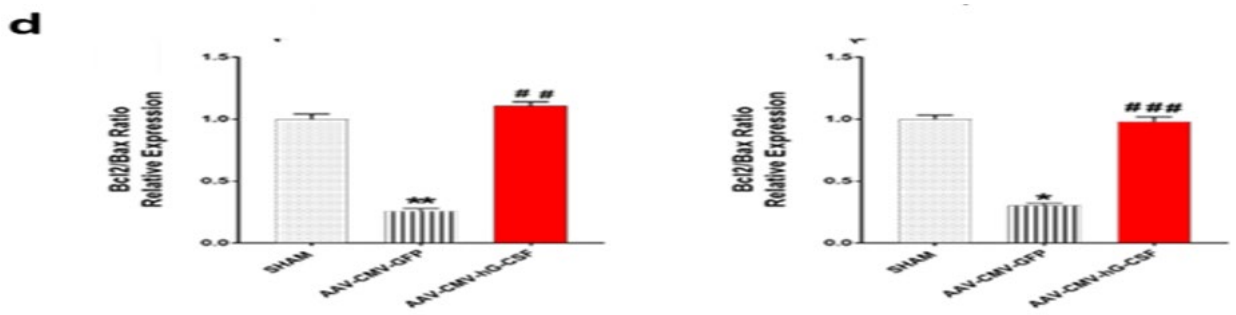

Figure 10d. *Effect of hG-CSF gene therapy on Bcl2 and Bax 4 days post-BCAO (c) Ratio of Bcl2:Bax protein expression. Statistical analysis was performed by One Way ANOVA, with Tukey as post hoc test and expressed as means ± S.E.M; * p < 0.05 /** p < 0.01/***p <0.001 versus Sham group while ## p <0.01/ ### p <0.001 versus AAV-CMV-GFP- treated group. (n =6 animals/group).*

We then measured GAD65 mRNA relative expression in both hG-CSF treated and non-treated 4-day post-BCAO mice. This was done by qRT-PCR analysis of GAD65 mRNA expression in both the frontal and middle sections of the brain. We found that the relative expression of GABAergic neurons was significantly more expressed in treated hG-CSF mice than in the GFP untreated mice in both sections of the brain (**Figure 11a**). We used western blotting to measure the protein level expression of GAD65 in these cohorts and found that hG-CSF mice had more protein expression when compared to that of untreated GFP mice in both sections of the brain (**Figure 11b-c**).

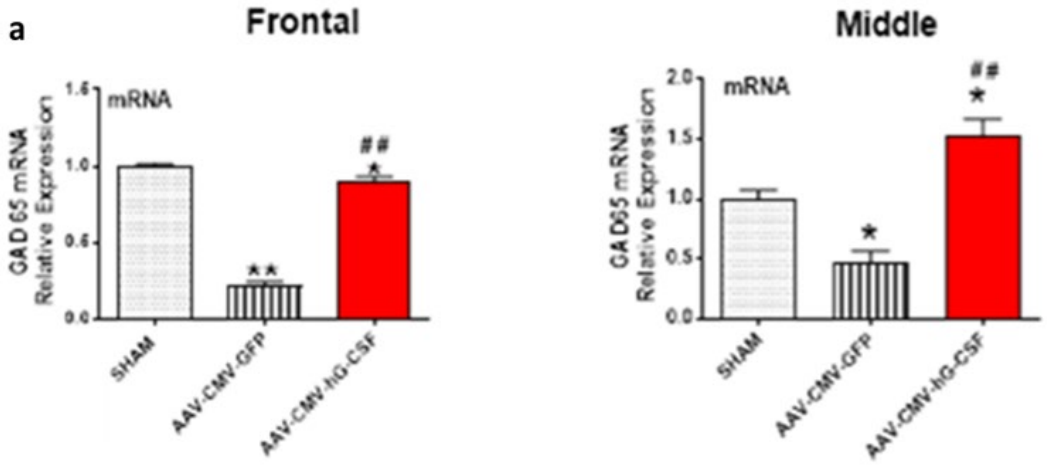

Figure 11a. *Effect of hG-CSF gene therapy on GABAergic neurons. (a) qRT-PCR analysis of GAD65 mRNA in the frontal and middle sections of the brain, four (4) days post-BCAO. Statistical analysis was performed by One Way ANOVA, with Tukey as post hoc test and expressed as means ± S.E.M; * p < 0.05 /** p < 0.01/***p <0.001 versus Sham group while ## p <0.01/ ### p <0.001 versus AAV-CMV-GFP- treated group. (n =6 animals/group).*

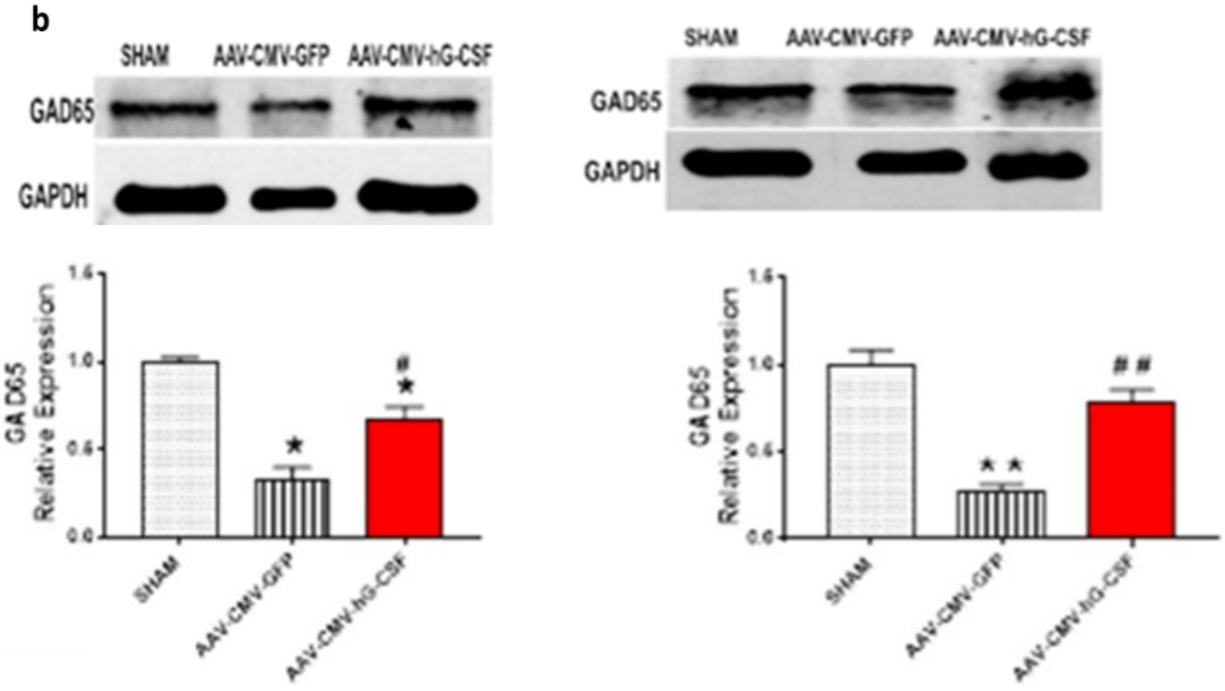

Figure 11b. *Effect of hG-CSF gene therapy on GABAergic neurons. (b) Western blot analysis of GAD65 protein expression and bar graph of GAD65 densitometry. Statistical analysis was performed by One Way ANOVA, with Tukey as post hoc test and expressed as means ± S.E.M; * p < 0.05 /** p < 0.01/***p <0.001 versus Sham group while ## p <0.01/ ### p <0.001 versus AAV-CMV-GFP- treated group. (n =6 animals/group).*

Imaging of the effect of hG-CSF gene therapy on GABAergic neurons in the cortex and CA1 region of the hippocampus of 4-day post-BCAO treated and untreated mice was also performed. We found that hG-CSF treated mice exhibited high GAD expression in areas of the cortex and CA1 region of the hippocampus compared to the untreated GFP mice with white arrowheads indicating immuno-positive expression of GAD (**Figure 12a**). Quantification of the percentage of GAD positive neurons was also measured and showed that treated hG-CSF mice had a higher number of them compared to the GFP mice (**Figure 12b**).

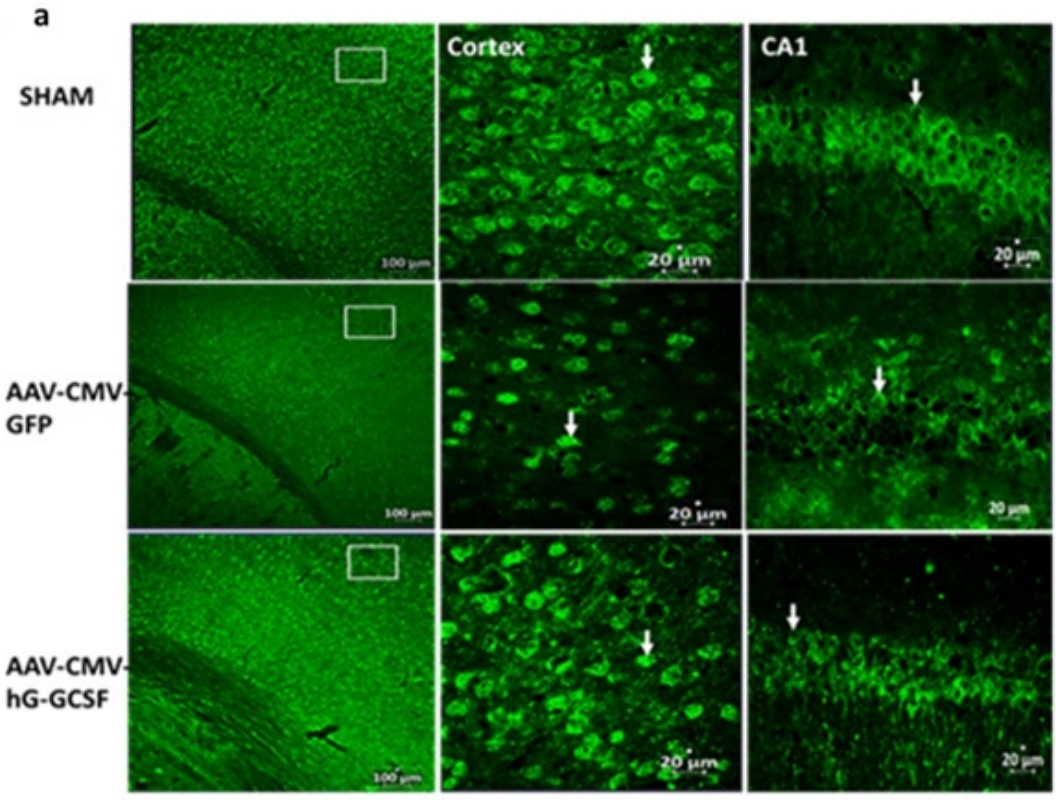

Figure 12a. *Effect of hG-CSF gene therapy on GABAergic neurons in the cortex and CA1 region of the hippocampus. (a)Photomicrograph of GAD expression in the cortex, and CA1regions of the hippocampus at 4 -days post-BCAO. White arrowhead indicates immuno-positive expression of GAD.*

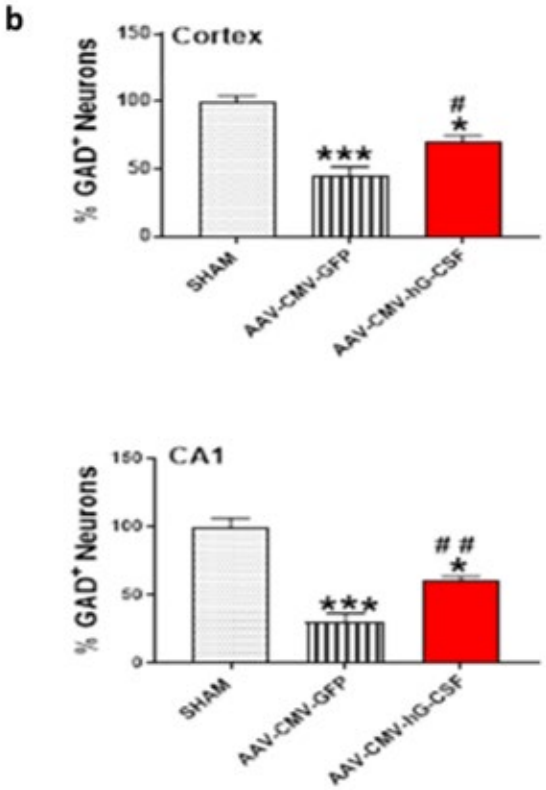

Figure 12b. *Effect of hG-CSF gene therapy on GABAergic neurons in the cortex and CA1 region of the hippocampus. (b) Quantitative analysis of GAD expression in cortex, and hippocampal CA1. Statistical analysis was performed with One Way ANOVA, with Tukey as post hoc test means ± S.E.M; *p < 0.05/***p < 0.001 versus Sham group while $^{\#}p<0.05/\ ^{\#\#}p < 0.01$ versus AAV-CMV-GFP- treated group (n = 6 per group).*

Lastly, we observed colocalization of both BrdU and DCX biomarkers present in 14-day post-BCAO hG-CSF treated mice (**Figure 13a**). We also saw a rather large difference in the amount of migrating neuronal cells in both blades of the dentate gyrus. BrdU+/DCX+ cells located along the subgranular zone (SGZ) of the DG, with a few migrating into the granular layer (**Figure 13b**). Quantification of BrdU+/DCX+ cells in both blades of the dentate gyrus showed hG-CSF treated mice having significantly more cells compared to the untreated GFP mice (**Figure 13c**).

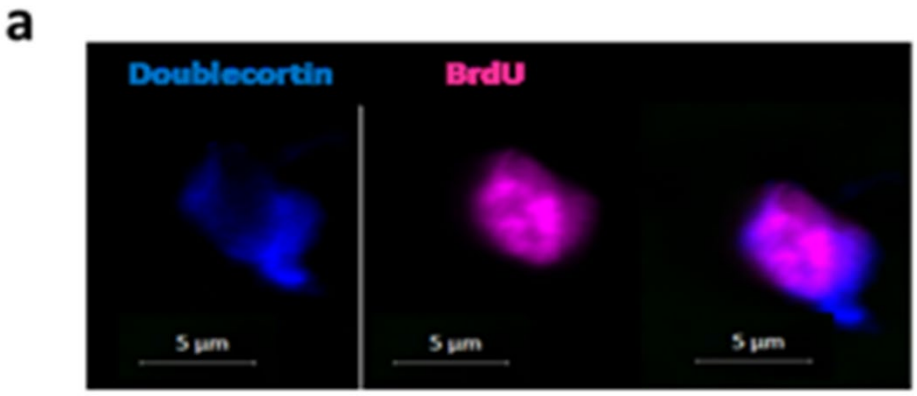

Figure 13a. *Representation of colocalized BrdU (violet) and DCX (blue) in a single cell. 14-day post-BCAO hG-CSF treated mice. BrdU is located in the nucleus of the cell, while DCX is located in the cell's cytoplasm.*

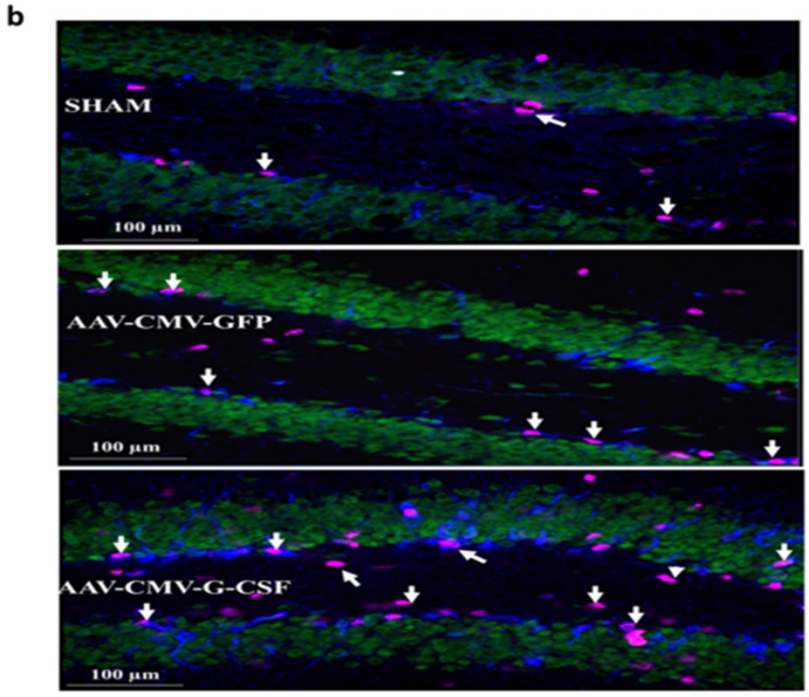

Figure 13b. *14-day post-BCAO Triple immunofluorescence of anti-BrdU, anti-DCX (immature neuronal marker) and anti-NeuN (mature neuronal marker). The expression level of DXC (blue), BrdU (violet) in both blades of the dentate gyrus. $BrdU^{+}/DCX^{+}$ cells located along the subgranular zone (SGZ) of the DG, with a few migrating into the granular layer.*

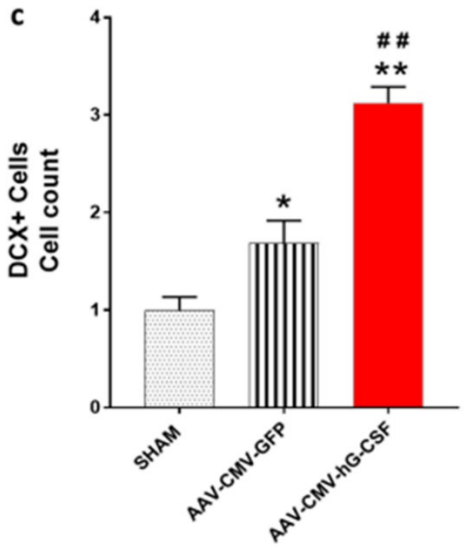

Figure 13c. *Quantification of BrdU+/DCX+ cells in both blades of the Dentate Gyrus. Statistical analysis was performed by One Way ANOVA, with Tukey as post hoc test and expressed as means ± S.E.M; * p < 0.05 /** p < 0.01/***p <0.001 versus Sham group while ## p <0.01/ ### p <0.001 versus AAV-CMV-GFP- treated group. (n =6 animals/group).*

CHAPTER 4: DISCUSSION

For the water maze results, we challenged both sickle and non-sickle mice in three separate probe trials that consisted of placing the hidden platform in three distinct quadrants of the water maze. The mice were dropped at quadrants located opposite of the placed platform and left to swim for 1 minute. Mice that did not find the platform were placed on it for 15 seconds after the allotted 1 minute of swimming. We found across all probe trials that non-sickle cell mice exhibited better spatial memory and locomotor abilities than compared to sickle cell mice.

We first saw this in the amount of distance traveled, measured in cm, with non-sickle cell mice traveling more distance to find the platform. This might indicate that they have more energy to put into finding the platform compared to their diseased counterparts (**Figure 2**).

Across three probe trials, the mean velocity of non-sickle cell mice was also higher than that of sickle cell mice. In each trial, the control group displayed a higher velocity of swimming to find the hidden platform compared to the diseased mice. This could indicate that non-sickle cell mice had more available energy to use in finding the platform than the sickle cell mice who did not travel as fast and used their energy sparingly (**Figure 3**).

Across three probe trials, non-sickle cell mice spent more time in the area of the hidden platform when compared to the sickle cell mice. This could indicate that the non-diseased control group exhibited better spatial memory and/or learning of where the hidden

platform was located based on previous runs. The sickle cell mice groups did not spend as much time in the relative area of the platform which might imply cognitive deficits (**Figure 4**). Due to the low *n* number of the preliminary studies, further testing on the water maze with a larger mice population would be necessary to better interpret this locomotor test. We hypothesized that G-CSF treatment at the various time intervals would improve performance on this test in the sickle cell mice groups.

In regard to latency to fall and the speed at which the mice fell, sickle cell anemic mice demonstrated a decreased motor performance on an accelerating rotarod than compared to the control of non-sickle cell anemic mice. When compared to non-sickle cell mice, sickle cell mice had a much lower latency to fall meaning that it took less time (measured in seconds) for the mouse to fall from the rod than compared to their healthier counterparts. This could be interpreted as the diseased mice not having adequate muscle strength and/or motor coordination due to our hypothesized concept of insufficient blood oxygen content delivered throughout the brain and body. This theory can also be observed in at which sickle cell mice fell at a much slower rod speed than non-sickle mice indicating a possible motor coordination dysfunction. We hypothesize that G-CSF intervention at various time intervals will improve performance on this test in sickle cell mice (**Figure 5**).

The preliminary locomotor activity demonstrated by both types of mice showed a rather interesting and unexpected result. In this test, we observed more locomotor activity in the sickle cell anemic mice when compared to non-sickle cell anemic mice. Both cohorts also showed a similar trend in distance traveled as time went on with both groups moving

more distance early in the experiment and progressively meeting at the same amount of locomotor activity at the 25-minute mark. This observation was a surprise for our group as we hypothesized that a diseased mouse would not move as much distance when compared to a healthy mouse since it would prove more taxing on the animal. This preliminary data was from a relatively low *n* number so further studies with additional mice is needed to obtain more accurate results. We hypothesize that G-CSF intervention in sickle cell anemic mice will show even more distance traveled when compared to the control (**Figure 6**).

Neuroprotective effects were seen in G-CSF-treated cerebral cortex of brain compared to untreated-G-CSF (GFP). Percentage of GAD neurons across all regions are increased in G-CSF-treated mouse compared to untreated G-CSF mouse. CA2 junctions are very resistant and not affected by ischemia (**Figure 7a-d**).

NeuN neuron immunofluorescent images and quantification of chAT positive neurons for BCAO mice at 4 days showed sections of the basal forebrain exhibiting increased chAT in hG-CSF treated BCAO mice compared to untreated GFP-BCAO mice (**Figure 8**). This could be due to the enzyme catalyzing the biosynthesis pf acetylcholine; an important neurotransmitter that regulates signal transduction. NueN staining for neuron maturation was also observed in the hG-CSF treated BCAO mice with imaging showing roughly the same amount of NeuN in the untreated GFP BCAO mice (**Figure 8**).

qRT-PCR expression confirmed the presence of mRNA hG-CSF levels in the frontal and middle brain sections (**Figure 9a**). Western blot validated the significant translation of mRNA hG-CSF to the hG-CSF protein (**Figure 9b**). 2,3,5-Triphenyltetrazolium chloride

(TTC) staining is a commonly used immunohistochemistry staining method to determine the volume of the cerebral infarction in experimental stroke models, and for this study, BCAO affected mice. TTC was used in this study for the purpose of physical representation of infarcted regions showed by white areas of brain tissue (**Figure 9c**).

The effect of hG-CSF gene therapy on Bcl2 and Bax 4 days post-BCAO was also tested by way of western blot analysis. Western blot analysis using antibody against Bcl2 and Bax (Figure 10a). Densitometry analysis of Bcl2 showed more expression than that of the untreated BCAO-GFP mice (**Figure 10b**). The densitometry of Bax western blot was also measured showing the same if not less than the untreated BCAO mice which could be due to the sequestering of Bax by Bcl2 in those areas of the brain to a more anti-apoptotic response (**Figure 10c**). Ratio of Bcl2:Bax protein expression (**Figure 10d**). Our data demonstrated that treatment with hG-CSF gene increases the ratio of Bcl2:Bax mRNA and the ratio of Bcl2:Bax protein expression. This observed increase of Bcl2:Bax ratio increases cell survival and could be due to the hG-CSF-induced increase of pAKT.

Effect of hG-CSF gene therapy on GABAergic neurons was measured using qRT-PCR analysis of GAD65 mRNA in the frontal and middle sections of the brain, 4 days post-BCAO. Western blot analysis of GAD65 protein expression and bar graph of GAD65 densitometry was also measured. We observed that hG-CSF gene expression rescued GABAergic neurons in vulnerable brain regions such as the cortex and CA1 and CA3 of the hippocampus (**Figure 11a**). The increased number of GABAergic neurons with hG-CSF gene expression is evident by the increase of both GAD65 mRNA and protein expression (**Figure 11b**). The underlying mechanism of the increase in GABAergic

neurons could be due to both neuroprotection and neurogenesis.

Effect of hG-CSF gene therapy in the cortex and CA1 region of the hippocampus was also measured using GABAergic neurons. Photomicrograph of GAD expression in the cortex, and CA1 regions of the hippocampus at 4 days post BCAO. (White arrowhead indicating immuno-positive expression of GAD). We observed that hG-CSF gene expression rescued GABAergic neurons in vulnerable brain regions such as the cortex and CA1 and CA3 of the hippocampus (**Figure 12a**). Quantitative analysis of GAD expression in cortex, and hippocampal CA1 was done by western blot analysis. The increased number of GABAergic neurons with hG-CSF gene expression is evident by the increase of both GAD65 mRNA and protein expression (**Figure 12b**). The underlying mechanism of the increase in GABAergic neurons could be due to both neuroprotection and neurogenesis.

The potential neurogenic effect of hG-CSF gene therapy in the Subgranular Zone of the hippocampus 14 days post-BCAO was measured using BrdU for cell proliferation and doublecortin (DCX) for immature neurons as biomarkers. BrdU is located in the nucleus of the cell, while DCX is located in the cell's cytoplasm. Representation of colocalized BrdU (violet) and DCX (blue) in a single cell (**Figure 13a**). Triple immunofluorescence of anti-BrdU, anti-DCX (immature neuronal marker) and anti-NeuN (mature neuronal marker), showing most of the $BrdU^{+}/DCX^{+}$ cells located along the subgranular zone (SGZ) of the DG, with a few migrating into the granular layer. The expression level of DXC (blue), BrdU (violet) in both blades of the dentate gyrus. Treated hG-CSF BCAO mice showed significantly more expression of immature and proliferating neurons

compared to the non-treated GFP mice (**Figure 13b**). Quantification of $BrdU^+/DCX^+$ cells in both blades of the dentate gyrus of treated hG-CSF mice exhibited significantly more DCX+ cells than untreated GFP mice (**Figure 13c**).

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