SELECTIVE INHIBITION OF INSULIN GROWTH-LIKE FACTOR RECEPTOR (IGFR) ON BRAIN ENDOTHELIAL CELLS (BMECS) USING ADENO-ASSOCIATED VIRUS (AAV) SERTOTYPE 9

An Undergraduate Research Scholars Thesis

by

JESSICA LEE

Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Farida Sohrabji
Dr. Shameena Bake

May 2018

Major: Biomedical Sciences

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ABSTRACT

Selective Inhibition of Insulin Growth-like Factor Receptor (IGFR) on Brain Endothelial Cells (BMECs) using Adeno-associated Virus (AAV) Serotype 9

Jessica Lee
Department of Veterinary Medicine and Biomedical Sciences
Texas A&M University

Research Advisor: Dr. Farida Sohrabji
Department of Neuroscience and Experimental Therapeutics
Texas A&M University

Research Advisor: Dr. Shameena Bake
Department of Neuroscience and Experimental Therapeutics
Texas A&M University

Ischemic stroke is the 5th leading cause of death within the U.S. and a major cause of long-term disability, especially in older females in comparison to men and younger women. This phenomenon is associated with a decrease in several steroid hormones including estrogen and the peptide hormone insulin growth-like factor (IGF)-1.[1] In animal models, age-related loss of IGF-1 is associated with an increase in BBB permeability and worse stroke outcomes, including larger infarct volumes, greater sensory motor impairment, and increased neuroinflammation. Exogenous treatments of IGF-1 is shown to decrease BBB permeability and reduce brain infarct volume in middle-age female rats.[8] Two main components of the BBB are astrocytes and brain microvessel endothelial cells (BMECs). Our recent studies demonstrated that targeted increases in IGF-1 expression in astrocytes through AAV-mediated gene transfer improves stroke outcomes in middle-age rats. [10] These findings suggest that secreted astrocytic IGF-1 may act on endothelial cells to maintain the integrity of the blood brain barrier. To test this hypothesis, we

propose to block IGF-1 receptors (IGFR) in BMECs of young female rats that still produce normal amounts of IGF-1. By successfully doing so, we would then be able to understand the significance of IGF-1 signaling on stroke outcomes as it interacts with BMECs. Adeno-associated virus (AAV) serotype 9, packaged with IGF1R shRNA and a mCherry reporter gene, was used to suppress the receptor. Mature adult female rats (5mo old) were injected with the control and siRNA containing AAV-9 vector. Integration of viral contents was assessed by immunohistochemistry and qPCR for IGFR on brain microvessels.

DEDICATION

This is dedicated to my mother, Feng Ying Guo, father, Chik Man Lee, and brother, Tommy Lee.

ACKNOWLEDGEMENTS

I would like to thank Dr. Farida Sohrabji for giving me the opportunity to work and learn in your lab and for mentoring me as I participate in the Undergraduate Research Scholars Program. I would also like to thank Dr. Shameena Bake, for teaching me valuable research skills and challenging me to be a better student and critical thinker. I thank you both for all your guidance through out this project and helping me prepare the poster presentation and thesis.

Lastly, I would like to thank Texas A&M University for awarding me the Reagents' Scholarship to help achieve my educational goals.

These studies were supported by an NIH grant (NS074895) and a gift from the Warren Brain Tumor fund to Dr. Farida Sohrabji. I was supported by a stipend from the Warren Brain Tumor fund.

NOMENCLATURE

BBB	Blood Brain Barrier
IGF-1	Insulin Growth-like Factor 1
IGFR	Insulin Growth-like Factor Receptor
AAV9	Adeno-associated Virus Serotype 9
BMECs	Brain Microvessel Endothelial Cells

CHAPTER I

INTRODUCTION

Age and Stroke Severity

Stroke is more prevalent and has more severe impairment in older females after menopause. Initially, this was attributed to the decrease in ovarian steroids such as estrogen, which is seen at menopause, and supported by preclinical studies showing that estrogen replacement decrease stroke infarct volume in younger females. However, in middle aged females, estrogen replacement has the opposite effect as evidenced by increased infarct volume and behavioral impairment in animal studies and in increased stroke occurrence in postmenopausal women.[1] These results led our laboratory to investigate other molecules that may affect stroke outcomes.

Functions of IGF-1 in the Brain

Insulin growth-like factor (IGF)-1 is a polypeptide composed of 70 amino acids, which play many roles in growth and development. A majority of IGF-1 is produced in the liver and circulates the body. Some amount of IGF-1 eventually gets transported across the BBB; however, brain cells such as astrocytes, neurons and microglia may also synthesize IGF-1.[2] IGF-1 has similar insulin-like functions within the brain by promoting anabolic activity, which results in an overall increase of neuronal survival. IGF-1 promotes glucose utilization with the help of phosphatidylinositol 3- kinase (PI3K).[3] Studies have also shown that IGF-1 contributes to neuroprotection from oxidative stress by activating signals that promote neuron protection.[4] IGF-1 contributes to maintaining overall brain integrity after stroke, such that a decrease in IGF-1 is

associated with worse stroke outcomes, while exogenous treatments of IGF-1 improves stroke recovery in males and middle aged females [1].

Blood Brain Barrier

Recent studies from this laboratory have investigated the effects of IGF-1 on the blood brain barrier (BBB) and overall stroke outcomes. The principal cell type of the BBB is BMECs, and this structure creates an entryway for the transport of oxygen and nutrients from the blood circulation. Physicially, the BBB also serves as a protective barrier that maintains brain homeostasis by preventing pathogens and cytotoxic proteins from entering the brain.[5] After stroke the BBB becomes more permeable and results in greater brain exposure to toxic products. Limiting permeability of the BBB is thought to help protect the brain during the occurrence of a stroke.[6] Studies from our lab showed that post-stroke IGF-1 treatment targets proteins related to the PI3K-Akt pathway and extracellular matrix proteins, both of which suggested that IGF-1 may target BBB function. [6]

Blood Brain Barrier Composition

Two major components of that make up the BBB are astrocytes and endothelial cells.[7] Astrocytes play a major role in repair after ischemic injury. With age, the neuroprotective capacity of astrocytes decreases.[8,9] Specifically, aging astrocytes produce less IGF-1, which is associated with a poor stroke recovery. Our previous studies using adenovirus mediated delivery of the open reading frame of the human IGF-1gene (packaged in rAAV serotype 5), under a glial specific promoter (GFAP), increased hrIGF-1 in astrocytes in the middle-aged brain and resulted in better post-stroke outcomes in this age group. [10]. In parallel, our tissue culture studies showed that IGF-1 treatment to BMEC increases barrier properties in these cells. Thus we propose that IGF-1 secreted by astrocytes, in vivo, may act on IGF-1 receptors (IGFR) on

endothelial cell of the BBB to reduce stroke injury. Astrocytes grown in contact with BMECs result with a tighter endothelium, which leads to an increased electrical resistance and overall protection.[11] Furthermore, the IGF-1 receptor is reported to maintain normal brain function by interacting with glucose transporter 1 (GLUT1), regulating transport of glucose in the brain.[12].

Adeno-associated Virus Construct

Adeno-associated virus (AAV) vectors are a commonly-used gene delivery tool for animal models. These replication-deficient vectors are used for their ability to localize in specific tissues, depending on the serotype, without eliciting a harmful immune response.[11] The most recent study from this laboratory used AAV-5 vector to successfully transfect the IGF-1 gene in astrocytes.[10] In this study, we will use AAV serotype 9 to target endothelial cells and silence the IGF-1 receptor in these cells.

CHAPTER II

METHODS

Animals

Mature adult (5-6 months old) Sprague-Dawley female rats were purchased from Envigo Laboratories, Indiana and maintained at 12 hour light-dark cycles in the animal care facility at the MREB. All procedures were conducted in accordance with National Institute of Health guidelines on animal welfare and approved by the institutional committee on animal care.

AAV Mediated Suppression of IGFR-1

Adeno-associated viral vector (serotype,-9) was used to silence IGFR-1 expression in brain microvessel endothelial cells in the cortex and striatum. This viral vector can establish latent infection and recombine genetic components into the DNA of specific cells of the body. Viral contents take at least 6 weeks to fully integrate. As shown in Fig 1, the viral vector was packaged with or without the IGF-1 receptor siRNA downstream of an endothelial specific promoter (PgP), and a mCherry fluorescent reporter gene. Once transfected, the virus integrate in brain cells and the siRNA results in silencing of IGF-1 receptor gene only in BMECs. The mCherry marker is used to detect integration of the viral contents using fluorescent microscopy.

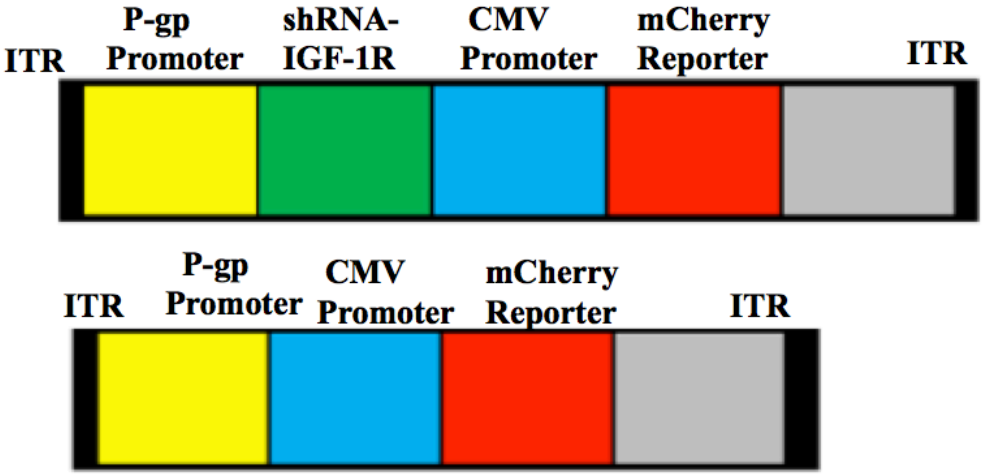

Fig. 1. Schematic representation of AAV9-shRNA-IGF1R construct (top) and control construct with mCherry reporter but no shRNA (bottom).

Surgeries

Stereotaxic Injections

Rats were anesthetized using a combination of Xylazine and Ketamine based on their body weight (13 mg Xylazine/kg, 87 mg Ketamine/kg). Rats were then placed on a heating pad to maintain normal body temperature of 37^{o}C. In preparation for surgery, the animals head was shaved and cleaned with alcohol wipes. The rats were placed in a stereotaxic instrument, and two small holes were drilled into their skulls at specific coordinates relative to the bregma, corresponding to the striatum and the cortex respectively. The coordinates for the striatum were +0.9mm anterior posterior, -3.6 mm medial lateral, and -6.5 mm depth. For the cortex, the coordinates were +0.9 mm anterior posterior, -5.5 mm medial lateral, and -6.0 depth. The control or siRNA containing AAV9 construct was injected injected at a flow rate of 0.5 ul every 30 seconds for 3 min. The concentration of AAV9 injected was 2.5 x 10^{12} viral particles (VP)/mL (Signagen, MD). After injection, the scalp was sutured and the rats were returned to their cages and placed under a warm lamp. Amimals were carefully monitored until they regained

consciousness. The rats were then returned to the vivarium and maintained for 6 weeks allowing for recovery and integration of the viral contents.

Brain Extractions for Sectioning

Rats were anesthetized using a combination of Xylazine and Ketamine (13 mg Xylazine/kg, 87 mg Ketamine/kg), and perfused transcardially with sterile phosphate buffered saline (dPBS) followed by 4% paraformaldehyde. Brain were then removed from the skull and placed in PBS at 4 ^{o}C overnight. The brains were then transferred to a 15% sucrose solution overnight and later embedded in cryomedia and cryosectioned. Sections (30 um thickness) were placed on glass slides in preparation for histological analysis.

Microvessel Extraction

A parallel set of animals were deeply anesthetized (13 mg Xylazine/kg, 87 mg Ketamine/kg) and their brains were rapidly removed from the skull. The overlying meninges were removed and the cortex and striatum were dissected and harvested for microvessels. Briefly, tissues were homogenized in sterile PBS in a Dounce homogenizer with loosely fitting pestle of 6-7 gentle strokes. The homogenates were spun briefly at 720 g for 5 min at 4^{o}C. The resulting pellet was suspended in PBS and layered over 18% dextran, spun at 2500 rpm for 30 min. The pellet was suspended again in PBS and then filtered through a 150 um filter. The filtrate were passed through a 70 um filter. The vessels were recovered from the filter by squirting PBS. The suspension of vessels were spun again at 1000 rpm for 5 minutes and the final pellet suspended in PBS, and dropped onto coated/charged slides and air dried. For RNA studies, vessels were frozen and kept at -80 deg. C until

RNA extraction.

Immunohistochemistry

Histochemical staining and immunohistochemistry was performed on brain sections and microvessels. (1) Lectin (1:500, vector laboratories,CA) conjugated to a green fluorescent marker was used to detect endothelial cells. (2) Antibodies to IGFR were used to detect IGFR by immunostaining (1:200 primary; 1:500 secondary), (3) Antibodies to Glial Fibrillary Acidic Protein (GFAP) (1:200 primary; 1:500 secondary) was used to identify astrocytes. For IGFR and GFAP immunohistochemistry, a negative control was also prepared that received the secondary antibody (1:500) only. For all staining protocols a nuclear counterstain was applied prior to coverslipping the slides. Images were taken with a Q-colors 3 microscope.

RNA Preparation

RNA Extractions

Brain microvessels previously stored in -80 deg. C were allowed to thaw on ice. A Qiagen miRNAeasy Mini Kit (stored at room temperature) was used to extract RNA from microvessels. QIAzol Lysis reagent was added to 1.5 mL tubes containing microvessels, sonicated for ~20 seconds, and incubated at room temperature for 5 minutes. Chloroform was added to the homogenized cells, shaken, and centrifuged at 12,000 x g for 15 minutes. The upper aqueous phase was transferred to another collection tube and mixed with 100% ethanol. This solution was then added to a column-collection tube and centrifuged at 8000 x g for 15 sec. (room temperature). This step was repeated after addition of RWT and RPE buffer respectively. RPE buffer was added again and centrifuged for 2 minutes (8000 x g). Lastly, RNAase-free water was added to the column and centrifuged for 1 minute (8000 x g); the flow through contained extracted RNA and was stored at -20 deg. C. Nucleic acid concentration was quantified using the Nanodrop 2000 spectrophotometer.

Complementary (c)DNA Synthesis

cDNA synthesis from microvessel RNA was performed using the qScript kit following manufacture's instructions. Briefly, equal volume of RNA template (100ng) was used in the reaction mix along with RNAase free water. Tubes with reaction mix and samples were placed in a thermocycler programmed for incubation for 5 minutes at 25 deg. C, 30 minutes at 42 deg. C, 5 minutes at 85 deg. C,. The synthesized cDNA was stored in -20 deg. C until further use.

Quantitative PCR (qPCR)

qPCR was performed on the cDNA to confirm the suppression of the IGF-1 receptor gene expression in BMECs. Synthesized cDNA was diluted (1:3) with RNAase-free water. Samples were mixed with PerfeCTa SYBR Green Fastmix (1x), and primers for 18 S (housekeeping gene) or rat IGF1R primer (Table 1). Each primer mix contained the forward and reverse primer. cDNA and reagents were pipetted carefully into a PCR plate, sealed and spun, and amplified in a ViiA7 Real-time PCR system. Cycle threshold (C_T) values were calculated, and group differences were tested using student's t-test (significance was considered at $p<0.05$)

Table 1: Forward and Reverse Primer Sequences for qPCR

Gene Name	Forward Primer	Reverse Primer
Rat IGF-1R	CAATATCACAGACCCGGAAG	ATGTCATCTGCTCCTTCTGC
18S	ATGGCCGTTCTTAGTTGGTG	CGCTGAGCCAGTCAGTGTAG

CHAPTER III

RESULTS

Immunohistochemistry Analysis

Brain Sections

Brain microvessels were clearly detected by lectin staining in whole brain sections (Fig 2). In Figure 2, green fluorescence indicated lectin stained microvessels, while red fluorescence (due to the mCherry reporter) indicates the integration of the AAV-9 viral contents within these microvessels. These images provide evidence for co-localization of AAV9-mcherry in these brain sections and confirm the integration of the injected viral contents.

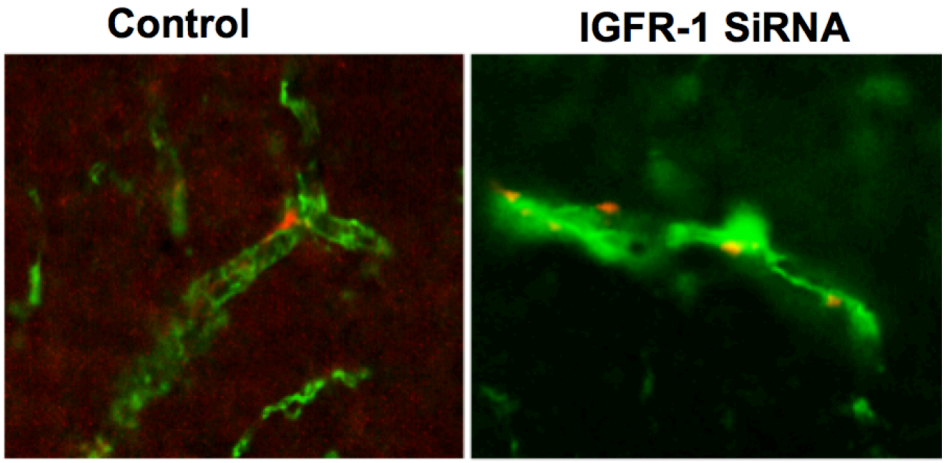

Figure 2: Confirmation of the Viral Integration in BMECs. Brain sections stained with fluorescein conjugated lectin (green) showing endothelial cells on cross sections of blood vessels. The mCherry reporter gene (red) indicates viral integration within endothelial cells of brain vessels. Images were taken at 40X magnification.

Microvessels

In order to assess the impact of rAAV9 on IGFR expression, brain microvessels were extracted and probed for the IGF-1 receptor using an immunostaining staining protocol. As shown in Figure 3, colocalization of mCherry (rAAV; red fluorescence) and IGFR immunoreactivity (green) resulted in the presence of yellow-orange cells (indicated by arrows) in control microvessels. In contrast, there was little overlap in rAAV positive cells and IGFR label in microvessels from animals that received the siRNA construct, consistent with the possibility that IGFR expression was inhibited in these cells.

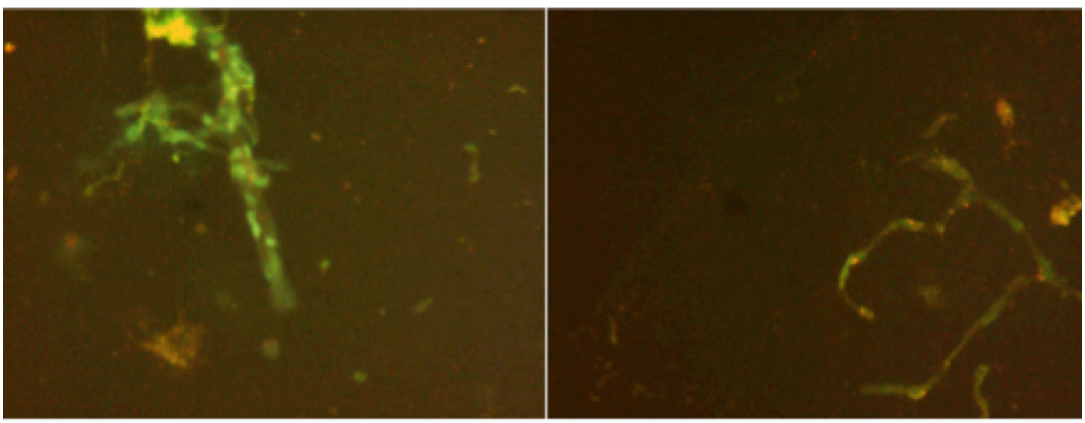

Figure 3: Immunohistological Comparison of IGF-1 Receptor Expression in Brain Microvessels. BMECs were extracted and immunostained with an IGFR antibody labeled with green fluorescence. The red color indicates mCherry reporter gene expression in these microvessels. The images were taken at 20X magnification. Note the colocation of IGFR in mCherry positive cells in the control microvessels, and the reduced IGFR (green) expression in mCherry positive cells in the vessels from the shRNA-IGF1R construct injected animals.

qPCR Analysis

IGFR gene expression was further assessed by qPCR analysis (Fig. 4).

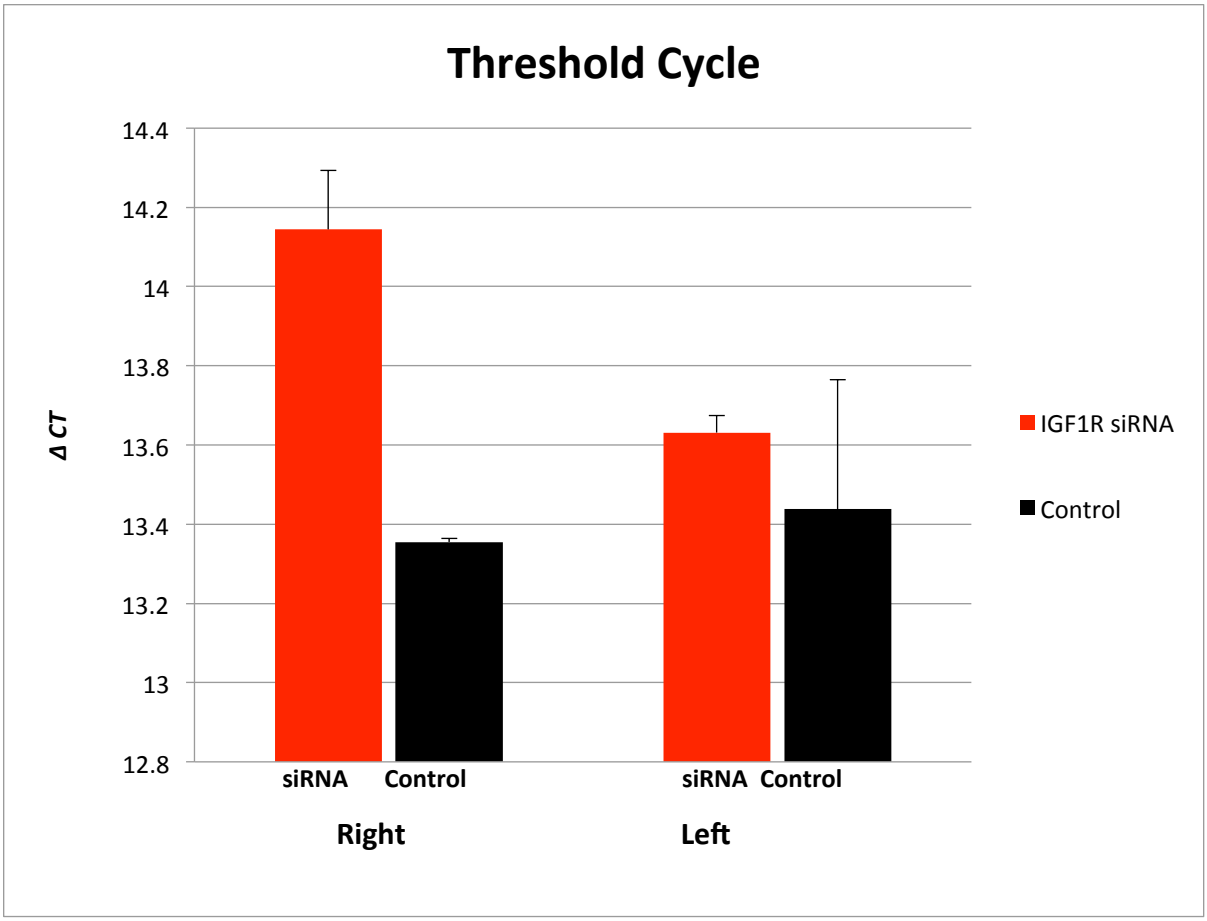

Fig. 4. Comparison of IGF-1 Receptor Gene Expression. qPCR analysis was perfomed and the threshold cycles (C_T) were quantified for samples receiving the IGF1R Rat Primer and 18S Housekeeping Gene Primer (standard). The threshold cycle was normalized by calculating $\Delta C_T = C_{T(IGF1R)} - C_{T\,(18S)}$ shown in the y-axis. There were two groups compared: 1) AAV9-IGF1R –shRNA and 2) AAV-9-control vector . The left and right hemispheres were also compared, left being the non-injected site (control) and right being the injected site. Our sample size was 2 and had standard error values = 0.148 (Right-siRNA), 0.043 (Right-control), 0.088 (Left-siRNA), 0.327 (Left-control). Note that the threshold cycle has an inverse relationship; therefore the higher the ΔC_T the less IGF1R gene expressed.

Threshold cycles (C_T) were quantified for IGFR amplicons and normalized to 18S for the injected and non-injected hemisphere of control and AAV9-IGF1R-shRNA injected animals. The AAV9-IGF1R-shRNA injected group had a higher ΔC_T value, which indicates a lower IGFR gene expression, in comparison to the control group, and statisitical analyses indicated a trend ($p< 0.075$) towards a significant difference. Interestingly, no such trend was detected on the non-injected side, where the control and AAV9-IGF1R-shRNA animals displayed similar levels of IGFR gene expression ($p<0.825$). One possibility for the statistical 'trend' may be due to the small sample size for these experiments (n=2 per group).

CHAPTER IV

CONCLUSION

The main objective of this research project was to develop a successful experimental strategy to suppress IGF-1 receptors in brain endothelial cells. Images taken from the immunohistochemistry analysis on the brain sections show that rAAV9 is localized to cells in lectin stained vessels, which confirms the integration of the viral contents within brain microvessel endothelial cells. In order to test suppression of the IGF-1 receptor, microvessels were extracted and stained for IGFR. Images taken from the microvessels showed inhibition of the IGF-1 receptor for animals injected with the shRNA-IGF-1R construct in comparison to the control vector group. A qPCR analysis on microvessel RNA showed a trend toward down regulation of the IGFR gene in animals injected with the AAV9-IGF1R-shRNA construct. These pilot findings will be studied in additional animals to confirm the utility of viral mediated suppression of IGFR in BMECs. If this approach is successful, future studies will determine how endothelial IGFR impacts stroke outcome in adult female rats.

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