Stroke and associated cardiovascular diseases are a leading cause of mortality and disabilities worldwide, with few effective treatments for minimizing the long-term deficits precipitated by ischemic stroke. Currently, the most effective post-ischemic stroke treatment is the administration of tissue plasminogen activator (tPa) to restore blood flow to the ischemic tissue; however, the three-hour therapeutic window post-stroke severely limits its feasibility for many patients. While the ischemic core is considered irreversibly damaged, the surrounding penumbral region is thought to be salvageable if effective pharmacological intervention/novel therapeutics were to be identified. There is a wealth of supporting clinical and preclinical data suggest that insulin-like growth factor-1 (IGF-1) may be a potential target for treating stroke in the days following insult. In humans, low levels of IGF-1 increase the risk of stroke and severity of post-stroke deficits. Preclinical studies show that administration of exogenous IGF-1 reduces neurological deficits and tissue damage caused by ischemic stroke. However, the cellular and molecular mechanisms underlying this protection are poorly understood. Thus, it is critical to delineate the effectors of IGF-1 can be targeted for effective therapeutic treatment of ischemic stroke. Without these advances, stroke will remain one of the largest causes of death and disabilities worldwide.

Our **long-term goal** is to determine how neuroendocrine modulators influence the cerebrovasculature and overall brain health. Our laboratory has an extensive history of studying IGF-1 in various pathologies associated with advanced age. IGF-1 regulates brain homeostasis, promotes neuronal development and function, and reduces glutamate-associated excitotoxic damage- which is arguably the most detrimental contributor in exacerbating stroke damage in the penumbra region. There remains a need to ascertain *how* IGF-1 protects the brain and aides in stroke outcome. As a growth factor, IGF-1 itself is not an ideal therapeutic due to increased cancer risk; therefore, there is an essential need to understand the cellular mechanisms by which IGF-1 exerts in benefits. Preliminary evidence from our laboratory indicates that reducing IGF-1R specifically in astrocytes impairs their ability to buffer glutamate-induced excitotoxic damage. Considering this, we presume that the loss of IGF-1 regulation of astrocytes contributes to tissue damage following ischemic stroke. On the other hand, IGF-1 may be directly acting on neurons to induce neuroprotection, as studies have shown significant protection in pure neuronal cultures subjected to stroke-like insults. *The objective of this application is to understand if astrocytic or neuronal IGF-1R signaling is responsible for the beneficial outcomes associated with exogenous IGF-1 treatment following ischemic stroke.* We have developed transgenic models that allow for cell-specific and timed regulation of IGF-1 signaling in astrocytes and neurons. **We hypothesize that the regulation of both neurons and astrocytes by IGF-1 modulates the extent of damage following ischemic stroke.** We further hypothesize that because astrocytic IGF-1R deficiency impairs glutamate handling, inflammation and stroke damage will be worse in mice deficient in astrocytic IGF-1R than neuronal IGF-1R. We will rigorously evaluate these hypotheses through these aims:

- **Aim 1 will examine the role of IGF-1R in astrocytes following ischemic stroke**. We will induce middle cerebral artery occlusion (MCAO) (or sham) in our inducible transgenic mouse model of astrocytic IGF-1R knockout *(igfap-cre/igfr$^{fl/fl}$)* and subsequently treat with exogenous IGF-1 (or vehicle). Acute and long-term changes in neurological deficit score (NDS) and infarct size will be measured. Motor coordination, balance, cortical-driven movement regulation, and locomotor asymmetry will be examined using pole test, cylinder test, balance beam, and rotarod tests. Histological changes in astrocyte size/number, glial activation, and neuro-glio-vascular structure will be assessed. Expression of astrocyte-derived growth factors, cytokines, and the machinery critical for glutamate uptake will be quantified. **This aim determines if astrocytic IGF-1R knockout exacerbates damage caused by ischemic stroke/reperfusion** (Yr 1-2)**.**
- **Aim 2 will determine the function of neuronal IGF-1R post-stroke.** Neuronal IGF-1R deficiency will be induced in adult transgenic mice (*icamk2a/igf1$^{fl/fl}$),* which will subsequently be subject to MCAO (or sham) and supplemented with IGF-1 (or vehicle). Similar to Aim 1, NDS, infarct size, behavioral deficits expected to be caused by stroke will be assessed, in addition to inflammatory signaling and neurogliovascular changes described. **This aim is independent of Aim 1 as it delineates if neuronal IGF-1R is a necessary and sufficient for maintaining brain health following ischemic stroke/reperfusion** (Yr 2-3)**.**

Scientific Premise: The results of this study will elucidate important major glial and excitatory cellular targets for future stroke therapies modulating IGF-1. We have successfully bred colonies of the inducible neuronal and astrocytic IGF-1R knockout mice needed for this study, and have an extensive history of conducting behavioral tests and various cellular and molecular techniques. The proposed project will allow the trainee to gain experience in maintaining transgenic models, microsurgical techniques, behavior analyses, and *ex vivo* analyses. *We have established a strong mentoring team to help achieve the goals of this project and foster the growth of the applicant to transition to a post-doctoral position, and ultimately to an independent researcher*.

A. Doctoral Dissertation and Research Experience

1. Pre-Graduate

University of Mississippi Undergraduate Research Assistant August 2016 - May 2019
Mentored by Dr. Nicole Ashpole Dr. Jordan Zjawiony, and Dr. Jason Hoeksema

As an undergraduate, I conducted research in several disciplines and took part in an international summer research program in Poland. As an initial member of the Ashpole laboratory, I assisted in organizing laboratory equipment, maintaining the animal colony, and conducted several behavioral studies. In addition, I obtained skills in stereotaxic surgery in addition to several techniques in microscopy, protein quantification, and cell culture. I successfully completed one project focused on characterizing the role of neuronal IGF-1R in the hippocampus and its impact on learning and memory. I am the first-author for a publication currently under review at *Hormones and Behavior.* A second critical research experience was conducted **Under Dr. Zjawiony**. I was selected as one of four students to participate in a six-week International Research Experience Hajnowka and Bialystok, Poland. During this research tour, I conducted field research collecting fungi for natural product isolation, collected and analyzed ectomycorrhizal (symbiotic) fungi, organized an international conference between 7 countries, and learned a variety of extraction techniques such as thin-layer chromatography, column chromatography, and liquid chromatography. This program enabled me to conduct field research and gain experience in various pharmacognosy techniques used to extract natural products that could function as potential cancer therapeutics. A last important research experience occurred during my time as an undergraduate, where I was mentored in the **Biology Department under Dr. Jason Hoeksema.** Under his guidance, I compiled data on mycorrhizal fungi for a large meta-analysis study. The data I collected is being used to develop a mycorrhizal open-source database. Compiling data for a meta-analysis before the start of graduate school strengthened my ability to read and understand scientific literature, especially in a field outside of my main research area. Under Dr. Hoeksema's guidance, I gained foundational training on how to organize data, perform meta-analysis, and applying biostatistics to a large data set.

2. Graduate Projects Serving as PI/1st Author

University of Mississippi Graduate Research Assistant August 2019 – Present
Mentored by Dr. Nicole Ashpole

The role of astrocytic IGF-1R in learning and memory: *This project is currently under review at Brain and Behaviour, and I am the first-author.* My role in this project was to stain and image astrocytes within our novel astro-IGFR-KO transgenic mice, compile and rigorously analyze behavioral data collected by a former graduate student, interpret the results and draft the manuscript. In this project, we highlighted a continued role for astrocytic IGF-1 signaling on maintaining cognition after development. Astrocytic IGFR was reduced in cohorts of male and female mice that subsequently underwent behavioral evaluation for learning and memory, affective changes in depression/anxiety, and motor coordination. We found that astrocytic IGFR impacts cognition and anxiety in a sex-specific manner, and that the glutamate signaling machinery was reduced with astrocytic IGFR deficiency.

The modulation of IGF-1 regulates protection in the neurogliovascular unit: *This project is currently under review at Frontiers in Aging Neuroscience and I am the first-author.* In the A0 submission of this application, we proposed a variety of *in vitro* studies looking at the response of neurons and astrocytes to overstimulation with glutamate when IGFR was reduced, as excitotoxicity is a major contributor to expansion of damage following ischemic stroke. We hypothesized that IGFR deficiency in the neurogliovascular unit increases susceptibility to insults such as glutamate-induced toxicity. To test this hypothesis, I used pharmacologic interventions to reduce IGF-1R in individual cell cultures and assessed glutamate-induced cellular toxicity, reactive oxygen species (ROS) production, and mitochondrial dysfunction. Our results confirmed that neurons are highly susceptible to excitotoxicity compared to astrocytes or endothelial cells, and a reduction in IGF-1 does not lead to overt toxicity. While astrocytes and endothelial cells are highly resistant to glutamate-induced toxicity, reduced IGF-1R signaling in astrocytes causes increased ROS, as measured by DCFDA, in the hours following insult. Mitochondrial dysfunction was also seen when IGFR was inhibited in astrocytes, and the loss of astrocyte IGFR signaling impaired their ability to protect neurons from excitotoxic glutamate in co-cultures. IGFR inhibition reduced endothelial cell division, but did not alter glutamate sensitivity or ROS. Thus, we conclude that IGF-1 differentially modulates the neuro-glio-vascular unit and cells within this entire system may be targets for therapeutic development. These findings provide further support for our central hypothesis. Overall, I learned to conduct multiple *in vitro* assays using primary cell cultures, such as viability staining, and DCFDA for ROS quantification, and microscopy techniques to image and quantity these types of data. Furthermore, this project enhanced my manuscript preparation skills as far as figure preparation, data organization, and writing/editing.

AGE/RAGE Project: Our laboratory recently received funding to examine the effects of advanced glycation end products (AGEs) on change in cognitive function, inflammation, and oxidative stress in the aged brain. I gathered instrumental preliminary data for this project and am assisting in overseeing an undergraduate student carrying out a major portion of the study. One of the well-characterized aging changes is an accumulation of AGEs which alters protein and lipid structure/function and ultimately increase inflammation and oxidative stress. We hypothesize that systemic knockout of the receptor for AGE (RAGE) will delay the onset of cognitive and physical impairments, and accompanying molecular changes, in advanced age. To test the hypothesis, transgenic mice are undergoing tests of learning/memory, depression/anxiety, and muscle function/coordination throughout their lifespan. AGE accumulation, levels of inflammatory and senescence signals, and neuroglial structure will be assessed. For this project, I established the breeding colony and learned about various genotypinc techniques. I also trained on the confocal microscope and developed cellular analysis skills following immunohistochemical staining and imaging, which will enhance my training in the ex vivo component of the proposed project. In the future, I will assist our undergraduate researcher and lab technician in the behavioral and ex vivo assessments.

The previous three projects enhance the feasibility of the proposed aims while positioning me as a unique candidate for a post-doc with backgrounds in both *in vitro and in vivo* studies with backgrounds in neuroendocrine modulation, learning/memory, aging, glycobiology, and mechanistic stroke research.

University of Mississippi Medical Center Graduate Technical Education May 2020 – Present
-Mentored by Dr. Roland Thorpe, Dr. Jennifer Reneker, and Dr. Mandip Dhamoon

Inflammation and stroke in the Jackson Heart Study: I am currently a Smith Scholar at the University of Mississippi Graduate Training Educational Center. This is a supplemental honors program for graduate students interested in cardiovascular diseases. As a Smith Scholar, we analyze previously collected data from the NIH-funded Jackson Heart Study, the largest single program for monitoring cardiovascular disease of African Americans. A manuscript proposal I developed, aimed at understanding if there is an association between inflammatory levels (high sensitivity-C Reactive Protein) and stroke incidence in African Americans, has been approved by the JHS committee. The manuscript and data analysis are currently underway, and I expect to submit a first author publication within the next few months. Upon completing this two-year program, I will have training in biostatistics and study design using observational data.

Graduate Dissertation/Proposed Aims and Projects
-Mentored by Dr. Nicole Ashpole

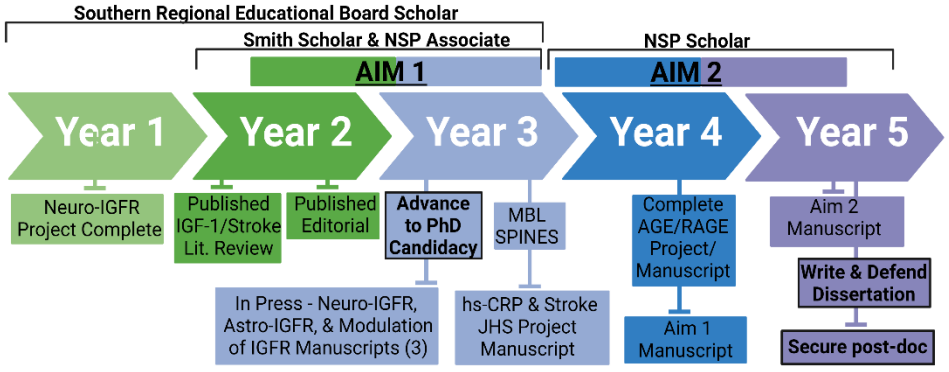

Figure 1: Visual representation of graduate trainee background, milestones, and future timeline under award year including expected manuscripts and aims completion. Course work was omitted considering trainee has completed all major coursework.

The role of astrocytic and neuronal IGF-1R on stroke outcome: For my graduate studies, I want to learn new skills and pursue a project that closely aligns with my passions to understand the interplay between cardiovascular and neuronal health. As I was reading the literature, I found that many studies corroborate that IGF-1 is beneficial in reducing tissue damage and improving neurological outcomes following ischemic stroke, but there was an apparent lack of mechanistic studies aimed at understanding the cellular and molecular mechanisms that provide this protection. Our lab has the tools to elucidate this mechanism.

In the proposed project, we hypothesize that the regulation of both neurons and astrocytes by IGF-1 modulates the extent of damage following ischemic stroke. The overall goal of the proposed aims is to characterize which cell type is responsible for the beneficial effects of IGF-1 following ischemic stroke. Methods needed to complete this project are the maintenance of transgenic mouse models; middle cerebral artery occlusion surgery; sensorimotor behavior analysis; tissue analysis with staining and immunohistochemistry; cellular/molecular analysis using qPCR, cytokine arrays, and ELISAs. IGF-1 is an essential component of embryonic and postnatal development, thus developing an inducible model to reduce IGF-1R in adulthood is key. Our lab had previously generated the astrocyte-specific inducible IGF-1R knockout mice; however, I had to develop a new model for

the neuronal component of this project. In the past year, I crossbred IGF-1R flox mice with inducible neuron-specific Cre recombinase mice (both validated and purchased from Jackson laboratories) to reduce IGF-1R in neurons in adulthood. The second obstacle for me in this project was learning how to induce ischemic stroke via middle cerebral artery occlusion. I met with multiple experts that routinely use mouse models of stroke and worked with our in-house veterinarian and Thaddeus Nowak, a member of our mentoring team, to induce ischemic stroke and reperfusion via temporary middle cerebral artery occlusion. Upon completion of this project, I will have gained skills in using and developing transgenic rodent models, inducing stroke via MCAO, and assessing sensorimotor function. In addition, I will have an extensive background in cellular/molecular analysis, *in vitro* experimental design, immunohistochemistry, microscopy, and statistical analysis using various programs such as R, Matlab, and Sigmaplot. We expect to see that reductions in astrocytic IGF-1R will worsen stroke damage, sensorimotor function, and inflammation in the weeks following ischemic stroke, even when exogenous IGF-1 is administered. In addition, we hypothesize that reductions in neuronal IGF-1R before inducing ischemic stroke will also increase neuronal death and sensorimotor impairment; although, we expect to see fewer changes in our neuronal KO study as astrocytic glutamate handling will continue to be intact in this model.

B. Training Goals and Objectives

After completing my Ph.D., my goal is to secure a post-doctoral position that will provide an interdisciplinary skillset different from my doctoral training specifically cellular mechanisms of cardiovascular disorders or behavioral neuroscience/endocannabinoid biology in which I can build on the skills in my graduate training highlight in section B. Long term, I hope to become an independent research scientist at an R1 level institution using both *in vitro* and *in vivo* studies to answer scientific questions. My goal is to develop novel therapeutics to decrease the health disparities gap that African Americans experience with these issues. Additionally, I am determined to not only understand and become an expert in STEM, but also develop an extensive background in researching observational and longitudinal studies using previously collected data in studies such as the Reasons for Geographic and Racial Differences in Stroke (REGARDS) project and the Jackson Heart Study (JHS). My plan includes receiving advanced training in experimental design and neuroscience techniques with an interdisciplinary background of science communication skills, public engagement, and effective teaching/mentoring skills**.**

At the University of Mississippi, I have a stalwart support system ranging from my research mentor, Dr. Nicole Ashpole, to my dissertation committee members, and my undergraduate professors in Biology, Sociology, and the Classics departments who still mentor me and provide insight to topics outside of my graduate studies. I also have an exceptional external support system of tenured academic scientists outside of the University of Mississippi -- Harvard (Jacob Hooker, a chemical neuroscientist), Vanderbilt (Steven Townsend, a chemist and member of executive board for UofM Glycore), John Hopkins (Roland Thorpe, an epidemiologist and public health disparity researcher), Boston University (Alberto Cruz-Martin, a neuroscientist), and University of Tennessee Health Sciences Center (Thaddeus Nowak, ischemic stroke specialist) who will actively have roles in my graduate training. I meet regularly with them to ensure that I am on the right path to achieve my short-term and long-term goals. Receiving the Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship will broaden the available resources for my graduate education training while creating the foundation for my career as an independent scientist.

Experimental Design and Neuroscience Techniques Training:

- **Microsurgery:** My research proposal requires extensive technical experience. Considering that the project focuses on inducing ischemic stroke through middle cerebral artery occlusion, it is essential that I successfully add this technique to my surgical repertoire. Within the last few months, I have been working closely with our veterinarian staff and Thaddeus Nowak to successfully and consistently perform middle cerebral artery occlusion on mice. Becoming a microsurgeon has been one of the most challenging tasks of my graduate training; however, I can say that I have spent an abundant time refining the technique, which significantly increases the likelihood of success in the proposed project.
- **Statistical analysis:** Neuroscience is becoming more computational, so I have committed to learning statistical language and coding using Matlab. I have previously completed our required course, Biometry, which provided me with a solid foundation for understanding statistics and how to use "R." I will continue to hone these skills as I believe this will be valuable in my future positions and strengthen my candidacy as a well-rounded scientist. I will also apply to programming courses offered by Cold Springs Harbor Laboratory (courses change each year, although computational courses are almost always offered). In my final year of

PhD training, I intend on taking the self-training course for programming and coding in Python offered by Python For Biologists.

- **Cellular/Molecular Techniques:** Lastly, I intend on reinforcing my understanding of biochemical and molecular assays. Recently, I was enrolled in a course, Techniques in Biomolecular Sciences, which provided me with the opportunity to strengthen my understanding of techniques used throughout our department. Dr. Ashpole has guided me in planning and practicing early, understanding my goals, and ensuring my knowledge of a technique spans both theory and practice. In the proposed project, I intend on learning how to conduct a multiplex array, perform ELISAs, use immunohistochemistry to characterize the neurogliovascular unit and its changes post-stroke, and learning confocal microscopy through our Imaging Core. Additional opportunities for training in analytical methods of protein analysis using mass spectrometry are available should issues arise with our proposed ELISAs or multiplex.

Professional Goals and Training: The professional goals outlined below are critical for the development of the graduate trainee. The path for achieving these goals during the award period is discussed in section C. Science communication, public engagement, teaching, mentoring, diversity/equity advocacy, and writing are all enhanced by and dependent upon my success in the laboratory. Hence, I highlight that these professional goals are secondary to the research-focused training that I will gather from the proposed project.

- **Science in Layman Terms:** During the COVID-19 pandemic, it has becoming obvious to see how scientists struggle to effectively communicate science to the community. I believe it is critical to be able to communicate all parts of research to not only the scientific community but the general population as well. I never knew the importance of science communication until I was approached by an African American man at a poster session. He first asked me to explain my project and then explain my educational background along with my family's. Then he said, "Can you explain your research to me as if I have no scientific background and am one of your friends or family members who did not go to college?" At the end of our conversation, he gave his card and asked me to reach out to him for a post-doctoral position at Yale. This is how scientists should be questioned. Within in past six months, I have had several interviews with science communicators to communicate what I do to the public. In addition, I published an editorial highlighting the "black scientists experience" in *ACS Chemical Neuroscience*. In my graduate training, I hope to continue giving scientific presentations and posters at conferences, in lab meetings, and during department seminars; however, I believe the harder aspect is communicating my science to the outside world. I will also continue honing my sci-communication skills through video interviews and podcasts just as I have already been doing with Adam MA Simpson and Dr. Sherry Nouraini. *I will continue giving presentations to high schools all across the state of Mississippi on the importance of biomedical science research. To improve my science communication, I have also applied to become a pen pal for letters to a pre-scientist through the Black in Neuro community. Upon completion of my qualifying exams, I intend on writing commentaries for the public bridging the gap between the science and public community.*
- **Public Engagement Bridging the Gap:** A large component of my identity is an African American male from a rural town in Mississippi with a population of 250 people who decided to change the narrative of my own story. I never envisioned myself going to college, let alone enrolling in a Ph.D. program, but now that I have, I am determined to bring awareness that people like me who are considered "disadvantaged" deserve the same opportunities as others. I make it my mission to reach out to the high school that I attended to try and give presentations each year on "How To Get To College" and mentor other students across the state who have personally reached out to me. I will continue giving speeches to high schools across Mississippi just as I have done in the past (Byhalia, Murrah, and Morton High School). *Speaking publicly to the future generation will benefit my internal goal of wanting to help "my" people. Poster sessions, speed networking, oral presentations are all components of science that are translatable to other pathways that I firmly believe be enhanced by this fellowship.* Please refer to the “Science in laymen terms” section above.
- **Teaching and Mentoring, Next-Gen Changes:** Although we are not required to teach and pursue any type of mentor activities in our graduate curriculum, I have made it a personal goal to do both as a Ph.D. student. I currently serve as a mentor for several students across the state of Mississippi. I also serve as a graduate school ambassador, which allows me to meet hundreds of students interested in graduate education. I currently serve as a graduate research mentor to two undergraduate research students in our laboratory, which will continue throughout the training period. *I will be taking the Teaching in Pharmacology course with Dr. Noa Valcarcel, an award-winning educator and dissertation committee member, in Spring 2022 to develop and deliver lectures to undergraduate and professional PharmD students.* This will provide an opportunity for development of an academic teaching portfolio.

- **Diversity and Inclusion:** The University of Mississippi lacks diversity both in the student and faculty population. Thus, the School of Pharmacy recently created a Diversity and Inclusion committee composed of Deans, Chairs, and Directors within the School of Pharmacy. Until recently, this committee lacked graduate students to serve as a point of reference and contract to target this issue. *Being one of the only African Americans in the graduate program, I now serve as the graduate representative on the Diversity and Inclusion Committee. I have also authored an editorial titled "Black Scientists Are Not the Door To Diversity" in ACS Chemical Neuroscience with an almetric score within the top 5% of articles viewed and shared.*
- **Writing:** Communication is the most essential skills a scientist can have, especially when seeking a career in academia. My first research paper was written when I was 16, and as a biology major in college, I did not gain any experience in scientific writing. At the start of graduate school, writing was my greatest obstacle, and it was evident that it would be a barrier to an academic career. Throughout my first two years of graduate school, I have made tremendous strides to perfecting my writing pertaining to manuscripts and grants. For example, in a year, I was able to write a literature review on IGF-1 and stroke. Two major strides that I voluntarily made to better my scientific writing skills was having weekly meetings with Dr. Noa Valcarcel while I was writing the literature review, and I attended a one-week writing course during spring break offered by the SOP and Department of Writing and Rhetoric. Currently, my primary mentor, Dr. Nicole Ashpole, and other mentors (Drs Alberto Cruz-Martin, Jacob Hooker, and Steven Townsend) assist in me in my writing skills at different draft stages. To date, I have crafted five manuscripts, and five grant applications, and 10+ external awards and program applications, many of which have been successful. My commitment to writing is evident by my voluntary attendance to a manuscript writing course hosted by the School of Pharmacy and the Rhetoric Department. In addition, I also attended a grant writing workshop offered by the School of Pharmacy and I will be attending a grant writing workshop sponsored by the Neuroscience Scholars Program. I will also gain valuable grant writing experience in the MBL SPINES program when I participate in the summer of 2022. *Outside of programs and workshops, I have an extensive list of well-known scientists at the University of Mississippi (Nicole Ashpole, Jason Paris, Soumyajit Majumdar), Harvard (Jacob Hooker), Vanderbilt (Steven Townsend), John Hopkins (Roland Thorpe), and Boston University (Alberto Cruz-Martin) who will be actively helping me perfect my writing skills for both manuscripts and grants.*

C. Activities Planned Under This Award (Table 1)

My graduate training plan extends beyond the realm of the scientific community but into broader communities that are often forgotten. I am adamant about pursuing opportunities that will not only make me a better scientist but a better mentor, professor but most of all, a person who believes each student deserves equal and equitable opportunities to pursue higher education. Each plan I listed is not contingent upon receiving this fellowship but on my personal goals, which will only be enhanced by receiving this fellowship.

The overall project capitalizes on my previously learned techniques to take them to the next level of difficulty with more advanced techniques, which are used in both aims. Aim 1 will be completed in year one and the beginning of year 2. Aim 2 will be completed throughout year two and until graduation in year three. These experiments are set up rigorously to help me master the technical skills outlined in the training proposal. Although some of these techniques are novel to the Ashpole laboratory, I have shown in the preliminary data that these experiments are feasible and can be completed successfully with my hands. To increase feasibility, I have been in contact with several stroke researchers at the University of Mississippi Medical Center, the University of Tennessee, and Louisiana State University. Other aspects of the training that Dr. Ashpole will directly train me on are confocal microscopy, immunohistochemistry, and cytokine array. Dr. Ashpole will also help me develop my manuscript and grant writing techniques with our individual weekly meetings to discuss research and career development. Some aspects of my training (epidemiology, biostatistics, and population health disparity study design) are not outlined in the proposal since they are components of other external fellowships, the Southern Regional Education Board and UMMC-GTEC and MBL SPINES. From these programs, I will gain a significant amount of experience that other graduate students do not have access to, like collaborating with outside researchers in different fields and attending regular training and conferences on pursuing a career in academia and teaching. Overall, with my previous research experience and combined with the proposed project and as a recipient of other fellowships, I will graduate with extensive skills that will give me a strong foundation to pursue a career an independent researcher.

As an undergraduate researcher, I attended numerous conferences presenting both poster and oral presentations, which have garnered me innumerable accolades. At the Mississippi Academy of Sciences, I received awards for the best poster and oral presentation. I was selected to give an oral presentation and a travel

award recipient at the largest minority conference in the US, The Annual Biomedical Research Conference for Minority Students (ABRCMS). I also received the Trainee Professional Development Award from the Society of Neuroscience in 2019. Outlined above are other conferences (SfN, BlackinX, BlackinNeuro, Glial Biology in Medicine Conference 2021, ABRCMS, Gordon Conference, ITM, and IAPHS) that I plan to attend to strengthen my scientific networking skills and broaden my knowledge in neuroscience, aging, and teaching. My participation in such a wide array of conferences provides me with a unique opportunity to make connections across many disciplines. I will continue applying for awards and giving poster and oral presentations at conferences outlined in the table.

Activity		Years
Research (80%)	**Aim 1 & 2** -Establishing cohorts of male and female *iGFAP-cre/igf$^{f/f}$* and *iCamk2a-cre/igf$^{f/f}$* mice -IGF-1R reduction via tamoxifen administration -Middle cerebral artery occlusion -Infarct analysis -Sensorimotor/behavioral deficit testing -IHC to assess reactive astrogliosis and microglial activation - Qpcr, and cytokine array to quantify inflammatory markers: high sensitivity reactivity protein (hs-CRP), ROS, TNF-α, caspase 3, and 9, and pro-inflammatory cytokines -Glutamate transporter assessment	1-3
	Data Analysis: -Infarct size and sensorimotor deficits between groups (latency, quantity of failed attempts, forepaw place preference) -Neuron/astrocyte/microglial analysis via confocal microscopy -Protein and inflammatory marker quantification -Sigma Plot v14 software; R Version 64 3.6 + to RStudio; Matlab v2020a; Image J. -Python For Biologist Course and CHL courses	1-3
	Writing -Manuscript and grant writing workshops **(completed)** -Currently under review 1st author manuscripts **(3)** -Manuscript and dissertation preparation **(2 manuscripts)** -Other expected 1st author manuscripts **(2)** and co-author manuscripts **(1)** -NSP,SOP, MBL Grant & Manuscript Writing Workshops	1-3
Meetings (8%)	Weekly lab meetings, weekly one-on-ones with Dr. Ashpole, and Dissertation Committee Meetings (1 per semester) -Neuroscience Journal Club and Graduate Journal Club (each once per month) -Department Seminars (once per week, with me presenting once a year) -Diversity and Inclusion Committee (1 per month) -UMMC-GTEC Training and Research (weekly)	1-3
Courses (2%)	Teaching in Pharmacology I and II; ITPD3 603 (Responsible Conduct of Research). (all other coursework is completed)	1-2
Conferences (5%)	Society for Neuroscience, Black in Neuro, Black in X, Institute on Teaching and Mentoring, Gordon Conference Biology of Aging, Glial Biology in Medicine Conference 2021,The Annual Biomedical Research Conference for Minority Students	1-3
Mentoring (5%)	Undergraduate Research Assistants (2) Undergraduate Luckyday Scholar Minority advancement in STEM (The Louis Stokes Mississippi Alliance for Minority Participation)	1-3

Table 1: Activities planned under this award period.

Other components of my education consist of applying for external funding, as this is an integral skill set needed for independent researchers. I received a University of Mississippi graduate school pilot grant to generate preliminary data used in this application. In addition, I have received two honorable mentions for applications submitted to the National Science Foundation GRFP and Ford Foundation Predoctoral Fellowship. I am also a receipt of several nationally known program such as the MBL SPINES program, Neuroscience Scholars program sponsored by SfN, Southern Regional Education Board Scholars program, and UMMC-GTEC Smith Scholars program. Thus, I have a significant amount of experience pursuing external opportunities using my writing skills to secure these opportunities. *In the future, I will reapply to the neuroscience scholars program to become a scholar, travel awards sponsored by conferences, and international brain research fellowships to attend conferences and courses offered by MBL and/or Cold Spring Harbor Laboratories.*

As described above, I will be attending many seminars and journal clubs in neuroscience and biomedical sciences. I will also be attending our weekly lab meetings. My dissertation committee will meet once per semester to ensure that I am progressing in my project and receive guidance on pursuing the remaining components of my research. We utilize the individual development plan (IDP) to ensure we are on track with our goals. I also intend to spend a small amount of time teaching through our courses "Teaching in Pharmacology," which are similar to preparing future faculty programs. I will continue to mentor undergraduate students. I currently mentor two honor students, one of which is an undergraduate researcher in the Ashpole laboratory. Lastly, I will continue developing my overall skill set to prepare me better to pursue post-doctoral positions and become an independent investigator.

As a trainee, I have already developed an extensive network within the scientific community across the nation. As a 3rd year Ph.D. student, I have already received multiple offers for post-doctoral positions at Harvard utilizing human neuroimaging or Vanderbilt studying oligosaccharides and novel glycans role in premature health with the possibility of incorporating several aging aspects. Both of these offers deviate from my training and are largely chemistry based with high-level neuroscience. These opportunities are a direct result of my networking and connection skills which I plan to continue to develop. Receiving the NIH F31 Ruth Kirschstein will exponentially increase my feasibility of my success by connecting me with a larger success system, NIH, inherently providing the opportunity and doors that would otherwise be closed.

SIGNIFICANCE:

Stroke and IGF-1: Stroke and other cardiovascular disorders are the fifth leading cause of mortality and disabilities in the United States[1,2]. Current ischemic stroke treatments, such as exogenous tissue plasminogen activator (tPA), rely heavily on the latency to intervention and focus on restoring blood flow. Unfortunately, tPA fails to stop the cascade of damage in the penumbra region caused by the initial ischemia, which ultimately leads to progressive impairments in the days following the insult. Thus, there is a critical need for more effective post-stroke treatments that reduce secondary damage after blood flow has been restored.

IGF-1, previously known as Somatomedin-C, is a 70 amino acid polypeptide with endocrine, autocrine, and paracrine functions. In the brain, all major cell types respond to IGF-1 via the IGF-1-receptor (IGF-1R)[3-5]. The levels of circulating IGF-1 decline in advanced age, when the risk of stroke is highest. This temporal correlation, and the known pro-survival actions of IGF-1 during development, led many to postulated that IGF-1 may serve as a possible biomarker for predicting ischemic stroke outcomes in patients, as we recently reviewed[6].

Clinical and Preclinical Correlates Between IGF-1 and Stroke: A large observational study revealed an inverse correlation between baseline IGF-1 levels and the incidence of stroke in the following years (low IGF-1, higher stroke incidence)[7,8]. Furthermore, other studies have shown that lower levels of IGF-1 are associated with increased stroke mortality rate[9-11] and increased neurological deficits classified by the National Institutes of Health Stroke Score and the Modified Rankin Scale[10,12,13]. While there have been some conflicting findings where no associations were observed[14,15] many studies corroborate the theme that lower IGF-1 levels correlate with increased stroke deficits and poor neurological outcomes. Preclinical studies in rodent models of ischemic stroke support clinical evidence that IGF-1 attenuates infarct damage, neurological deficits, and behavioral defects. Administration of exogenous IGF-1 in the time surrounding the insult via various routes of administration (intravenous, intranasal, and subcutaneous) and various doses reduces infarct size, sensorimotor impairments, coordination, and working memory deficits caused by ischemia[16-26].

The mechanism by which exogenous IGF-1 exerts beneficial outcomes following stroke is poorly understood. The protective effects of IGF-1 could be mediated through any or all of the components of the neuro-glial-vascular unit as each cell type expresses IGF-1R. For example, IGF-1 protects cultured neurons from excitotoxic damage, inflammation, and hypoglycemia[3,27-29]. IGF-1 also reduces oxidative stress in endothelial cells and promotes glucose transport in astrocytes[30,31]. We recently showed that reductions in IGF-1R in astrocytes impaired their ability to buffer extracellular glutamate (Fig. 1), which would likely exacerbate a neurotoxic environment since impaired glutamate transport is one of the key contributors to the cascade of neuronal damage within the penumbra[32]. *Therefore, we postulate that exogenous IGF-1 may be acting on multiple cell types to provide neuroprotection, reduce infarct size, and improve behavioral outcomes animal models of ischemic stroke.*

Preliminary data: As mentioned, knockout of IGF-1R specifically in astrocytes (Fig. 1)[31] dysregulated their ability to buffer extracellular glutamate. Mechanistically, this was due to reductions in glutamate transporter expression and availability on the cell surface[31]. We have since observed a number of glutamate signaling related gene changes in the brains of astrocyte-specific IGFR KO mice (Fig. 2A). Moreover, glutamic acid levels were 1.73 fold higher in astro-IGFR-KO brains, suggesting large-scale changes in glutamate handling with astrocytic IGFR deficiency. In the previous submission of this F31, we proposed a series of preliminary studies using primary cell cultures of neurons and astrocytes to examine whether IGFR deficiency alters cellular responses to glutamate-induced toxicity. We completed that proposed aim and verified that neuron cultures are much more sensitive to glutamate excitotoxicity, than astrocytes or endothelial cells (Fig. 2B). Interestingly, pharmacological inhibition of IGFR in astrocytes increased their susceptibility to glutamate-induced death, increased reactive oxygen species production, impaired mitochondrial respiration, and ultimately reduced their ability to protect neurons from excitotoxicity (Fig. 2C-F). IGFR inhibition in neurons did not exacerbate glutamate excitotoxicity. Thus, the loss of IGF-1 signaling on astrocytes resulted in significant impairments in cellular function, which could wreak havoc in ischemic tissue and surrounding penumbra.

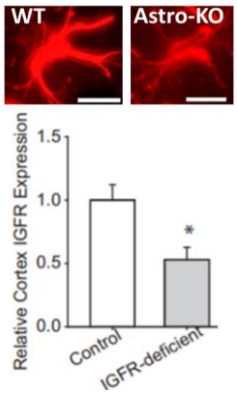

Figure 1: Representative GFAP+ astrocytes of astro-IGFR-WT and KO mice. IGFR expression in the cortex of astro-IGFR mice compared to control.

Central Hypothesis: Our long-term goal is to determine how neuroendocrine modulators like IGF-1 influence the cerebrovasculature and overall health in the aged brain so novel, selective therapeutics can be developed. There remains a lack of knowledge regarding which cell type(s) in the brain are directly modified by the exogenous IGF-1 to ultimately provide the observed neuroprotection. The central objective of this proposal is to assess whether IGF-1 benefits stroke outcomes by acting directly on neurons and/or indirectly by modulating astroglial cells. Considering

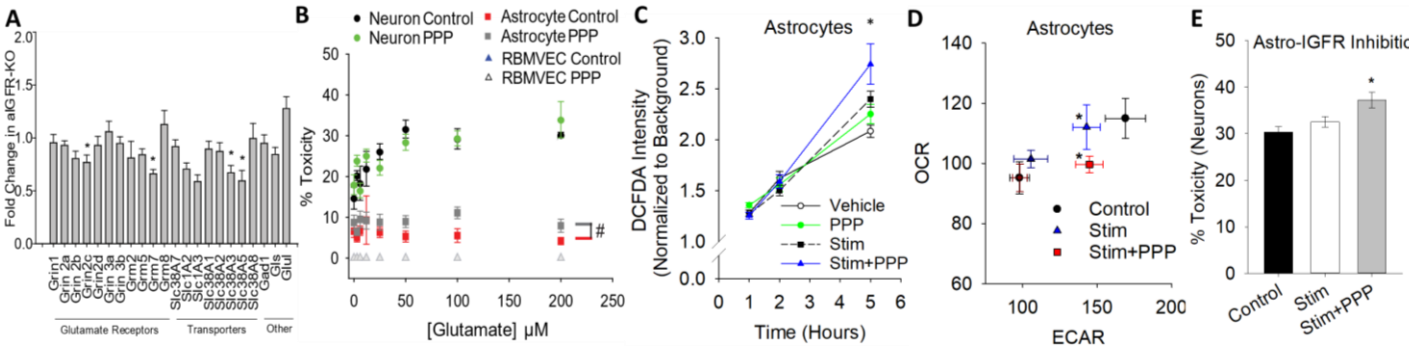

Figure 2: Changes in glutamate machinery mRNA expression in male astro-IGFR-KO mice (t-test *p<0.05)(A). IGFR inhibition with picropodophyllin (PPP) increases astrocyte and not neuron or rat brain microvascular endothelial cell susceptibility to toxicity from glutamate stimulation in culture (Two-way ANOVA, # main effect PPP treatment) (B). IGFR inhibition + glutamate stimulation increases ROS levels (RM-ANOVA *p<0.05)(C) and decreases maximal mitochondrial oxygen consumption rate (One-way ANOVA, *p<0.05)(D) in astrocytes. IGFR inhibition specifically in astrocytes reduces neuroprotection from glutamate stimulation in triple cultures of neurons, endothelial cells, and astrocytes (One-way ANOVA, *p<0.05)(E).

glutamate excitotoxicity is a major contributor to stroke damage, we presume that the loss of IGF-1 signaling in astrocytes will contribute to stroke damage due to failures in glutamate buffering and accompanying metabolic stress and inflammation. It is also possible that IGF-1 is directly regulating neurons by blocking oxidative stress and glutamate excitotoxicity. **Hence, we hypothesize that IGF-1 reduces damage from ischemic stroke by modulating both neurons and astrocytes.**

Innovation: The status quo in the field has been to administer exogenous IGF-1 (pre- or post-insult) to various models of ischemic stroke and subsequently assess global outcomes in sensorimotor behavior and tissue death. One barrier to assessing the underlying mechanisms of IGF-1-induced protection, was the inability to circumvent compensatory changes in development precipitated by genetic manipulations of IGF-1 early in life. However, genetic advances have made inducible, cell-specific knockout models easily attainable. Thus, our approach, in our opinion, provides an innovative departure from the status quo by altering the endogenous IGF-1 signaling cascades in a cell-specific, inducible manner. We have established two transgenic mouse lines to reduce IGF-1R in neurons or astrocytes after the developmental surge of circulating IGF-1. Many studies have relied on one or two sensorimotor tests in a single-sex experimental design, but we propose to use a comprehensive battery of sensorimotor tests (rotarod, pole test, cylinder, and balance beam) to rigorously assess acute (3 days) and long-term (30 days) effects on tissue damage and behavioral outcomes in male and female cohorts. Moreover, our proposed aims deviate from past literature by assessing specific cellular changes within the ischemic tissue and penumbra following the initial insult. Together, our study will discern whether IGF-1R signaling in astrocytes and neurons is *necessary* and/or *sufficient* for IGF-1 to protect against ischemic stroke.

RESEARCH DESIGN:

Aim 1: Examine the role of IGF-1R on astrocytes in regulating stroke damage

Aim 1 Rationale: Upon completion of Aim 1, we will understand whether the beneficial effects of IGF-1 on stroke outcomes seen in previous *in vivo* studies are mediated directly through astrocytes. This study provides significant insight into the response of astrocytes to ischemic stroke and the impact of astrocyte regulation on the exacerbation of damage within the ischemic core and penumbra. Astrocytes are the most abundant glial cell in the nervous system, and are critical for maintaining homeostasis in the brain microenvironment. Ischemic stroke leads to increased astrocyte proliferation, alterations astrocyte size/structure, increased pro-inflammatory cytokine production, and changes in key gliotransmitters/growth factors produced in the astrocytes [33,34]. This study will examine these hallmark changes in transgenic animals where astrocyte-specific IGF-1R is reduced, which will address the gap in our understanding of the cellular mechanisms underlying the beneficial effects of IGF-1, and will better delineate the roles of both astrocytes and IGF-1 as regulators of stroke damage.

Aim 1 Objectives: Objective 1: Determine whether selectively reducing IGF-1R on astrocytes prior to ischemic stroke exacerbates neurological and behavioral deficits. Objective 2: Determine if the beneficial effects of exogenous IGF-1 are mediated through astrocytes. Objective 3: Explore whether the number, structure, and localization of activated astrocytes following ischemic stroke are influenced by IGF-1. Objective 4: Examine whether characteristic changes in astrocyte function post-stroke are modulated by IGF-1.

Aim 1 Hypothesis: Reductions in IGF-1R in astrocytes attenuate neuroprotective functions of IGF-1 in stroke.

Aim 1 Research Design: Animals: Transgenic mice expressing iGFAP-igfr$^{fl/fl}$ will be divided into 5 groups (Fig. 3): (1) SHAM-wildtype (WT), (2) MCAO-WT + saline, (3) MCAO-astro-IGFR-KO + saline, (4) MCAO-WT + IGF-

1, and (5) MCAO-astro-IGFR-KO + IGF-1. To induce KO in adult mice, igfap-Cre-igfr$^{fl/fl}$ mice will receive five intraperitoneal (i.p.) injections of tamoxifen, as we recently described[31]. See **Extended Methods** for experimental details for all underlined techniques. Control mice will receive i.p. injections of tamoxifen vehicle (corn oil). One month following KO, mice will be subjected to middle cerebral artery occlusion (MCAO) to induce stroke or SHAM surgery (control) and given an intranasal dose of exogenous IGF-1 or saline control. The dose (150µg) and route is consistent with reports of reduced infarct size when administered immediately upon completion of MCAO[35]. Endpoint comparisons described below will be made using two factors: 1- genotype (astro-IGF-KO or WT controls) and 2- treatment (intranasal IGF-1 or saline). Separate cohorts of male and female mice will be used to evaluate sex as an independent biological variable. Sequential vaginal cytology will be performed to ensure stroke is induced when females are in diestrus- the phase with low estrogen (more clinical relevance to post-menopause where stroke incidence is higher, and more comparable to males).

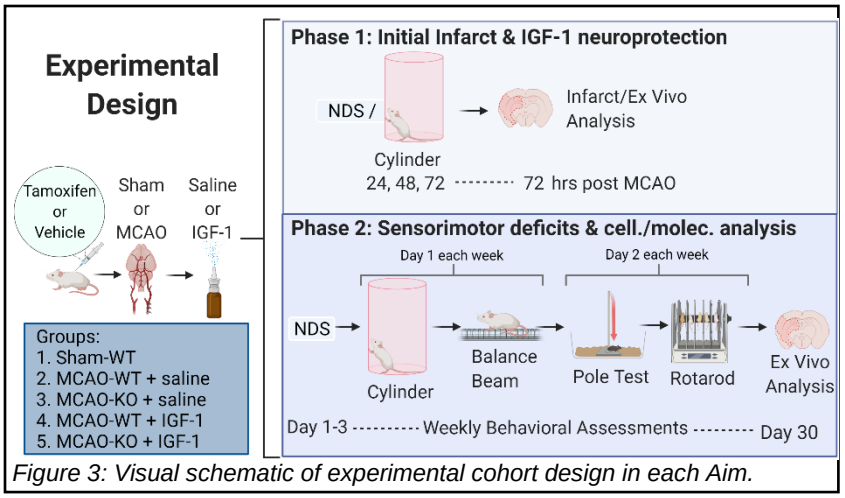

Figure 3: Visual schematic of experimental cohort design in each Aim.

In phase 1 of outcome assessment, we will examine acute changes in neurological deficit score (NDS), locomotor asymmetry, and infarct size 72 hours following insult (Obj. 1 & 2) (n=8 mice/group), as this time point if often used for pathology endpoints post stroke[36,37]. Infarct size will be measured in TTC-stained tissue (Fig. 4). Long-term damage will be assessed in Phase 2, wherein sensorimotor changes will be evaluated in the weeks following stroke in a separate cohort of mice (Fig. 3). Phase 2 mice (n=16 mice/group) will perform a battery of behavior tests each week (over 2 days) post-stroke to assess motor coordination (rotarod), balance (balance beam), locomotor asymmetry (cylinder test), and movement disorders caused by cortical damage (pole test) (Fig. 4). These assays have been used to highlight deficits in function in the weeks following ischemic stroke.

One month following MCAO, brain tissue will be isolated to assess if astro-IGF-1R-KO increased stroke damage (Obj. 1 & 2), astrocyte activation (Obj. 3), and astrocyte function (Obj. 4). Stroke damage and astrocyte activation will be assessed with immunohistochemistry and confocal microscopy (Fig. 1). Consistent with recent reports[38], colocalization of the loss of neurons (MAP2), astrocyte scar boundary formation (GFAP), microglial activation (Iba1), and endothelial cell loss (CD31), will be used for infarct size assessment. These same samples will be used to quantify the number of astrocytes, the volume of individual astrocytes, the structural complexity of astrocytes within the scar and surrounding penumbra, and the extent of juxtavascular localization of the astrocytes (GFAP/CD31 colocalization), as each of these endpoints are altered in rodent models of MCAO and/or brain trauma[38]. Further ex vivo analyses of isolated tissue will examine pro-inflammatory cytokine and growth factor production via commercially available multiplex bead arrays or enzyme-linked immunoabsorbance assays (ELISAs). Signals of interest include, but are not limited to, TGF-β, IL-1β, IL-15, GDNF, and BDNF, since they are known to be differentially produced by astrocytes following ischemic stroke[39]. Changes in the expression of glutamate transporters/receptors (Fig. 2A) and key astrocyte structure and communication machinery (including GFAP, vimentin, Aqp4, and Cx43) will be quantified in brain tissue using quantitative polymerase chain reaction (qPCR). Because the selected molecular endpoints are markers of critical astrocyte function and astrocytes form a communicative syncytium across the brain, we propose to quantify these signals in both the infarcted and non-infarcted sides of the brain, independently, to determine if astrocytic IGF-1R reductions and stroke alter the "healthy" contralateral side, which remains vastly understudied.

Expected Results/Outcomes: As the key functions of astrocytes include uptake of extracellular glutamate, secretion of growth factors, maintenance of ion homeostasis, and responding to inflammation, we anticipate maintenance of IGF-1 regulation is critical for astrocytic protection against ischemia. Thus, we do not expect exogenous IGF-1 supplementation will provide protection to the astro-IGFR-KO mice. Further, we expect that the loss of IGF-1R in astrocytes will significantly exacerbate the tissue damage and behavioral deficits caused by MCAO, particularly in the long-term cohort, since penumbral damage expansion is primarily driven by glutamate toxicity[40,41]. It is unclear whether many of the characteristic stroke-induced changes in astrocyte structure and function directly contribute to the damage or are an endogenous attempt at compensating for the damage. Since this remains unclear, we anticipate astro-IGFR-KO may amplify some mechanistic changes (glutamate handling expression) and dampen others (astrocyte proliferation).

Potential Problems & Alternative Strategies: We have established, verified, and maintain a large breeding colony of our astro-IGFR-KO line. While MCAO is a technically challenging procedure, we have successfully induced MCAO in preliminary studies to increase our probability of success (Fig 4). Dr. Thaddeus Nowak, an expert in mouse models of brain ischemia, provided feedback on our standard operating procedures and will continue to provide guidance through the training period. In case inducing MCAO becomes unreliable, other methods to induce ischemic stroke are available and are protected by with IGF-1, including photothrombotic injury or endothelin-1-induced occlusion[42-45]. The full-time rodent behavioral experts within our COBRE-funded Neuropharmacology Core will assist any issues encountered with intranasal drug administration and/or behavioral assessments. If intranasal admin of IGF-1 fails to rescue in wild-type animals, we will instead switch to i.p. administration as both routes have been shown to be efficacious[6] . Recent shifts towards less invasive approaches like intranasal administration of small polypeptides like this are appealing for drug delivery to the brain, which is why we selected this avenue. Our Imaging Core facility will assist with IHC and cell analyses as needed (see Letter from Dr. Majumdar), and the biochemical techniques are ones we routinely perform and do not expect issues.

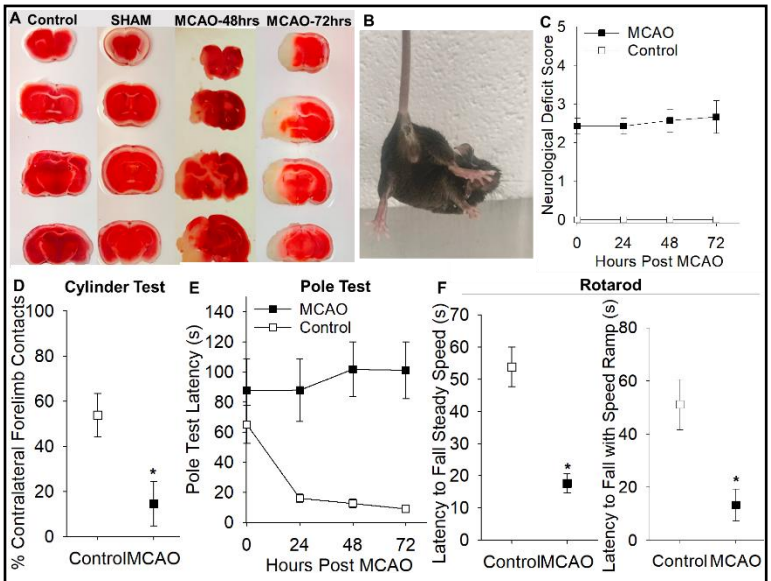

Figure 4: Tissue damage and behavioral impairment with MCAO. TTC-stained brains (A), image of post-stroke contralateral twist (24hrs) (B), neurological deficit scores (various times)(C), forelimb asymmetry (72hrs) (D), latency to reorient and transverse a pole following MCAO (E), and latency to fall on a rotarod at 3rpm (left) or during a ramp of 3-10rpm (right) (72hrs)(F). n=4-8/group, *p<0.05.

AIM 2: To determine the function of neuronal IGF-1R on stroke outcome.

Aim 2 Rationale: Upon completion of Aim 2, we will understand if the beneficial effects of IGF-1 on stroke outcomes are mediated by *direct modulation of neurons*. As mentioned, this will address the current knowledge gap regarding the cellular and molecular underpinnings of protective capabilities of exogenous IGF-1 following ischemic stroke. Moreover, this study will provide significant insight regarding whether endogenous IGF-1 signaling in neurons substantially impacts the ischemic core and surrounding penumbra. In a similar experimental design to Aim 1, we propose to evaluate the behavioral, cellular, and molecular changes in ischemic stroke response within our novel transgenic mouse line, this time focusing on our neuro-IGFR-KO mice.

Aim 2 Objectives: Objective 1: Determine whether selectively reducing neuronal IGF-1R prior to ischemic stroke exacerbates neurological and behavioral deficits. Objective 2: Determine if the beneficial effects of exogenous IGF-1 on infarct size and behavioral deficits are mediated through neurons. Objective 3: Explore whether neuron loss and glial activation following stroke are influenced by neuronal IGF-1R. Objective 4: Examine whether characteristic increases in neuroinflammation post-stroke are modulated by neuronal IGF-1R.

Aim 2 Hypothesis: Loss of neuronal IGF-1R will worsen stroke damage, but exogenous IGF-1 will still provide neuroprotection against ischemia, as IGF-1 can still support the structure/function of surrounding glia.

Aim 2 Research Design: We will utilize our inducible transgenic mice where IGF-1R can be selectively removed from neurons in adulthood (icamk2-Cre-igfr$^{fl/fl}$), and subsequently induce ischemic stroke in those mice via MCAO. As in Aim 1, we will assess infarct size and neurological deficits in the days immediately following MCAO with and without intranasal administration of exogenous IGF-1 (Obj. 1 & 2). A second phase (Fig. 3) of analyses will examine behavioral deficits, histological changes in neurons and glia, and neuroinflammation markers in the month following a stroke (Obj. 1-4). Methodology for transgene induction, MCAO surgery, intranasal IGF-1 administration, neurological deficit scores, sensorimotor assays, and infarct size staining (IHC and TTC) are consistent with Aim 1 (Extended Methods). To further explore glial activation (Obj. 3), the number and volume of astrocytes (GFAP+) and microglia (Iba+) within and surrounding the infarct will be quantified using confocal microscopy (Fig. 1). Changes in the expression of glutamate transporters (Fig. 2A), astrocyte functional proteins and growth factors (Aqp4, CX43, BDNF, GDNF, VEGF), and markers of astrocyte and microglial activation that are known to increase with stroke (GFAP, vimentin, Iba1, CX3CR1, CD40, etc), will be compared in both the infarcted and non-infarcted hemispheres of the brains using qPCR and/or ELISAs. Levels of canonical neuroinflammatory mediators (hs-CRP, TNF-α, IL-1B, IL-6, CCL2/3/5, CXCL1/2/8) known to be altered by stroke[46-48] will also be compared using qPCR and ELISAs/multiplex cytokine/chemokine arrays (Obj. 4).

Aim 2 Expected Outcomes: The astrocytic IGF-1 signaling cascade will remain intact in our neuro-IGFR-KO mice, which should allow for maintenance of astrocytic glutamate buffering during and following ischemia. Neuro-IGFR-KOs will have an intact glial system; thus, we believe the supplementation of exogenous IGF-1 will continue to reduce stroke deficits in the neuro-IGFR-KO mice, similar to neuro-IGFR-WT controls. We anticipate that neuronal IGFR deficiency will result in the canonical increase in neuroinflammation and glial activation post-stroke, and may even exacerbate this inflammatory state given the inverse relationship between IGF-1 levels and ROS[49]. It is possible that neurons, rather than astrocytes are the key effector cells or equally contribute to the protection observed with IGF-1. Regardless of the outcome, data gathered through these studies are of interest for future stroke treatments manipulating IGF-1 signaling.

Aim 2 Potential Pitfalls and Alternative Approaches: We have successfully bred the transgenic line needed for this aim; thus, the potential problems likely to be encountered are consistent with those in Aim 1. Since tissue levels of glutamate are 1.73 fold higher in astro-IGFR-KO mice, we may also include assessments of glutamate concentration within the tissue in neuro-IGFR-KO mice as well to better-understand the involvement of glutamate signaling in any changes observed. For this, tissues will be sent to the Vanderbilt Analytical Services Core.

JUSTIFICATION AND FEASIBILITY: The transgenic lines of inducible astro-IGFR-KO and neuro-IGFR-KO were developed in house by the mentor and applicant, and the astro-IGFR-KO line has been utilized in our published study and one currently under review. The difference between the genetic models lies in the Cre recombinase promoter (*camk2a*-neuron; *gfap*- astrocyte), which are both driven by tamoxifen-Ert2 activation. Our approach to reducing IGF-1R in adulthood allows IGF-1 to properly function throughout development, and then mimic the age-related loss of IGF-1 signaling in a cell-specific manner after recombination. Independent cohorts of males and females are included, and the estrous cycle will be tracked in females to administer stroke during diestrous phase. Thaddeus Nowak, an expert in rodent stoke models, guides our experimental procedures to ensure reproducibility. In-house core facilities may assist with the behavioral and cellular analyses, increasing the feasibility of the proposed studies and providing training and networking opportunities for the applicant.

RIGOR AND REPRODUCIBILITY: Experiments are designed to be double-blinded, and *a priori* tests were conducted to estimate sample size based on the statistical endpoint of interest. Sample size estimates were performed in SigmaPlot v14 using power of 0.8, a mean difference of 15%, and a type-1 error of 0.05 for ANOVA comparisons. While 6-8 mice/group are needed for infarct analysis, 12-15 mice/group would be necessary for behavioral assays. Treatment groups are assigned with block randomization for equal n. Fewer samples (n=4-6 mice/group) are needed to detect a difference in ex vivo analyses due to increased within-subject sampling. Wild-type controls, sham surgery controls, and intranasal saline controls are included in each cohort. In many, a two-way ANOVA (or one-way if appropriate) will be utilized with appropriate post hoc (Tukey's HSD or Fisher's LSD). Normality (Shapiro-Wilk) and equal variance (Brown-Forsythe) are tested and reported. A p-value ≤ 0.05 will be considered statistically significant. As noted, sex will be considered independently. To increase the rigor of our IHC and microscopy studies, lesioned and peri-lesioned areas in the striatum and cortex will be used as a reference, and images will be thresholded and subsequently binarized. Image of the boundary between the penumbra and core will be defined by the absence/presence of MAP-2 and delimited using the astrocyte barrier. Ex vivo analysis will be analyzed by blinded undergraduate research assistants, and data will be decoded upon completion of experiments at the time of statistical comparison and graphing. Data will be analyzed using SigmaPlot v14, Matlab v2020a, Image J, and R Version 64 3.6.1 with guidance from Dr. Paris or the Behavioral Core Biostatistician. Behavioral studies are video recorded and tracked with Noldus EthoVision software when relevant. All data will be published following the ARRIVE guidelines.

EXTENDED METHODS: **Animals**: All mice are provided standard rodent chow and water ad libitum while housed on a 12:12 h reverse light-dark cycle to ensure behavioral analysis is conducted during the active phase. **Transgenic Models**: B6.Cg-Tg(GFAP-cre/ERT2)505Fmv/J mice are bred with B6;129-Igf1rtm2Arge/J mice to generate tamoxifen-inducible astrocytic IGF-1R knockouts (astro-IGFR-KO; iGFAP-igfr$^{fl/fl}$). Similarly, male (B6; 129S6-Tg(Camk2a-cre/ERT2) Aibs/J) are bred with female (B6;129-Igf1rtm2Arge/J) mice to generate an inducible neuronal knockout of IGF-1R (neuro-IGFR-KO; iCAMK2A-igfr$^{fl/f}$). Both transgenic lines are backcrossed against B6;129-Igf1rtm2Arge/J to maintain homozygosity of igfr$^{fl/fl}$. Following weaning, male and female mice are genotyped using PCR protocols by JAX laboratories to ensure transgene insertion and flox homozygosity[31]. **Vaginal Cytology[50]:** Female mice estrous cycling is tracked based on previously published methods by committee member, Dr. Jason Paris. Briefly, epithelial tissue is collected and assessed using light microscopy (50X and 200X). Mice are cycled at the start of the light phase for 7 consecutive days and those that do not demonstrate at 4-5 day cycle are excluded. Females will undergo surgeries during the diestrus phase. **IGF-1R Reduction[32]**: Tamoxifen induces Cre recombinase expression in the transgenic mice, which removes the floxed sequences of the target gene. Tamoxifen is diluted in corn oil, solubilized at overnight at 50°C, cooled, and

administered via i.p. injection for 5 consecutive days in a 75mg/kg dose per JAX protocol. Control mice receive injections of corn oil. **Middle Cerebral Artery Occlusion and Assessment[51]:** Ischemic stroke will be induced 1 month following knockout using the established Koizumi unilateral middle cerebral artery occlusion (MCAO) technique (Fig 4) (both Kozuimi and Longa methods resulted in similar survival and damage). Briefly, mice are anesthetized with isoflurane, and a sterile surgical field is prepped. The common, internal, and external carotid arteries are exposed, sutured, and a silicon-tipped 0.21mm monofilament, 9-10mm in length is guided up to the middle cerebral artery through the internal carotid (Doccol 6021910PK10Re). After 60 minutes of occlusion, the monofilament is removed to allow reperfusion. Recovery in a warmed chamber is observed and clear H2O diet gel is placed in their home cage to encourage hydration. The standard operating procedure was developed with the Attending Veterinarian and Dr. Thaddeus Nowak. **Exogenous IGF-1 Administration:** Recombinant IGF-1 (R&D Techne #291-G1) is reconstituted in sterile PBS and 5µls is administered during inspiration to the intranasal passages of mice immediately following the MCAO procedure. Assistance will be provided by the Neuropharmacology Core who routinely perform intranasal administration of test compounds (see letter from Dr. Majumdar). **Neurological Deficits Score [52]:** Neurological deficits will be scored by a blinded observer at 1, 24, 48, 72-hours post-reperfusion with a six-point scale (0=normal, 1= mild circling behavior with <50% contralateral rotation attempts, 2= mild circling with >50% rotating to the contralateral side, 3= consistent and immediate circling, 4= severe rotation and progression into barreling, or a 5= coma/moribund (Fig. 4). **Infarct Quantification[51,53]:** Brains are isolated following euthanasia, sectioned in 100µm slices in brain matrixes, and stained used the 2,3,5-triphenyl tetrazolium chloride (TTC, #T8877 Sigma) for 20-30 minutes (Fig. 4), and imaged on the Zeiss Discovery Steromicroscope (Imaging Core). Infarct size throughout the slices is quantified using ImageJ. Previous studies report significant differences using n≤8 for this endpoint.

Behavioral Analysis: A battery of behavioral studies will assess sensorimotor function once per week following transient MCAO; tests are split across two days each week to avoid fatigue. Sample size estimation indicates n=12-15 is needed. A baseline assessment of all mice will be performed prior to surgery. The **Pole Test** is used to test movement disorders caused by cortical damage. Mice are placed on a head-up on a vertical pole placed in their home cage. Latency to reorient downward and descend to their home cage is measured (Fig. 4), with a maximal latency of 120sec for non-performers. The **Rotarod** is used to assess motor coordination and balance by placing the mouse on an adjustable speed rotating rod. Mice are acclimated to the rotating rod with a 60sec 3rpm trial, and 30 mins later a trial of increasing speed is performed (3-10rpm over 180sec). Latency to fall and distance travelled are compared (Fig. 4). The **Cylinder Test** assesses locomotor asymmetry and evaluates limb usage by comparing the number of independent and simultaneous placements of each forelimb on the wall of a plexiglass cylinder in which the mouse is placed. Animals with unilateral brain damage will exhibit paw placement preference (Fig. 4). A **Balance Beam** is used to assess muscle function and coordination. The mouse is placed in the center of a small, secured rod and has 60sec to scale the beam to a nearby safety platform. The process is repeated three times with rods of decreasing width, and latency to reach the safety platform is recorded.

Ex vivo Inflammatory Analysis: Immunohistochemistry (IHC): After behavioral analysis, serum and brain tissue will be isolated for immunohistochemistry, qPCR, and protein quantification. The numbers, size, and complexity of astrocytes, neurons, and microglia within the core and peri-infarct zones will be assessed with IHC. In brief, brains will be fixed in 4% PFA, sucrose embedded (30%), and cryosectioned (45µm). Brain slices are washed with PBS, undergo antigen retrieval, blocked, incubated in primary (MAP2- Millipore MAB3418; GFAP- Abcam ab7260; Iba1- Wako 019-19741; CD31- BD Pharma #550274) and secondary antibodies (all Invitrogen), and mounted using Fluoromount-G (Thermo #4958-02). Imaging is performed on our inverted Nikon Ti2-triggered fluorescent microscope or Zeiss confocal microscope (see Letter from Dr. Majumdar). The lesioned and peri-lesioned areas in the striatum and cortex are used as a reference to set threshold and subsequently binarized the image. The boundary between the penumbra and core is defined by the absence/presence of MAP-2 and delimited using the astrocyte barrier. Cell count, volume, complexity, and co-localization are assessed using NikonElements plug-ins or ImageJ. **Quantitative PCR (qPCR):** Total RNA is isolated from tissue using the RNAeasy mini kit (Qiagen, 74004) and converted to cDNA using High-Capacity RNA-to-cDNA kit (Thermo, 4388950). Real time polymerase chain reaction (RT-PCR) is then be performed using TaqMan Universal PCR Master Mis (Thermo Life Tech) and TaqMan validated primers on the CFX Connect Real-time PCR detection system (Bio-Rad). All results are normalized to two housekeeping genes, HPRT and B2M. The experimenter is blinded when conducting the analysis, and data are decoded when calculating ΔΔ-Ct. **ELISAs and Multiplex Bead Array:** Perfused brain tissue is lysed in RIPA buffer. A custom-designed cytokine/chemokine multiplex bead array for analytes of interest (R&D Techne) is utilized to quantify inflammatory markers of interest, per manufacturers recommendations, on the Luminex-200. BDNF (R&D DY248), GDNF (R&D DGD00), VEGF (MMV00) and any cytokines not available in the multiplex are measured using commercially available ELISAs.