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The Effects of Insulin-like Growth Factor-1 (IGF-1) and Insulin-like Growth Factor Receptor (IGFR) Regulation on Cognition and Structure of Astrocytes

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By

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A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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I would like to thank Dr. Ashpole for her continuous guidance and support as my advisor and mentor for so many years. I would also like to thank my readers for taking the time to help me with this project and the Sally McDonnell Barksdale Honors College for the opportunities and experiences it has provided me during my time at The University of Mississippi. Finally, I extend many thanks to everyone in the lab for their constant encouragement and to my family and friends who supported me through the entirety of this process.

Abstract

Insulin-like growth factor-1 (IGF-1) is a neuroendocrine signaling hormone that plays an integral role in bone and tissue growth and development. Inhibition of this hormone is known to disrupt the chemistry of the brain, resulting in cognitive impairments such as those seen in many common neurodegenerative diseases. While much research has been conducted on neurons and their relation with IGF-1, the role of astrocytes still needs to be explored. Our research investigates how astrocytes are affected as a result of IGF-1 regulation. Preliminary studies in our laboratory established a connection between IGF-1 and glial fibrillary acidic protein (GFAP), and in this study we focused on understanding these changes in GFAP expression and astrocyte structure. We hypothesized that the mice lacking IGF-1 or its receptor, IGFR, would have increased number and size of GFAP positive (GFAP+) cells in the hippocampus, which is associated with cognitive dysfunction. The value of this research can be noted in its efforts to increase the understanding of astrocytes, a group of cells that contribute significantly to the maintenance of the brain's environment and cognitive function. By investigating how astrocytes respond to particular changes, we will be clarifying aspects of an under-researched group of cells that have an undeniably important role in cognitive dysfunction.

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Background

The brain is one of the key organs of the body needed for basic survival. Without it, respiration and cardiovascular function would not be regulated, and overall homeostasis would be difficult to maintain. As a major component of the central nervous system, it also integrates many other aspects of life such as learning, memory, and depression. The brain, therefore, is critical not only for life but for quality of life as well, and there is much left to uncover about it. The brain undergoes changes as time progresses, and the aging brain has been a particular area of interest for researchers. While not all individuals develop dementia, most individuals show signs of age-related cognitive decline. Studies on aging have yielded valuable insight on the major components of neuronal function and cognitive decline, but more research needs to be done to target and prevent this age-related dysfunction.

Astrocytes

While the brain holds great importance with its role in controlling the nervous system, it is important to acknowledge that this major organ would not be able to work as efficiently without proper maintenance of neurons by glial cells. A testament to this is the fact that approximately 90% of the human brain consists of glial cells (Allen et al. 2009). Astrocytes are a specific type of glial cell that populate the central nervous system in a complex, yet organized, manner. They are capable of signaling to blood vessels, affecting the rate of blood flow to certain regions of the brain, and they also communicate with neurons and

respond to synaptic activity (Allen et al. 2009). Astrocytes express Excitatory Amino Acid Transporter 1 and 2 (EAAT1 and EAAT2) which are glutamate transporters responsible for bringing glutamate into astrocytes from the extracellular space. Glutamate is an excitatory amino acid, and the disruption of glutamate levels (either too high or too low) in synapses results in impairment and dysfunction. Astrocytes also help in regulating levels of glutamate by taking the amino acid into the cell and further converting it to glutamine which can be used in various ways by astrocytes or other cells. If glutamate is not regulated, neurodegenerative diseases such as Alzheimer's, Huntington's, stroke, and Traumatic Brain Injury (TBI) could occur.

Glial fibrillary acidic protein (GFAP)

GFAP functions as the main intermediate filament in mature astrocytes. Due to its prominent role in stabilizing astrocyte structure, this protein also serves as a marker for astrocyte activity (Eng et al. 2000). High expression of GFAP indicates astrogliosis - an increase in the number or size of astrocytes as a response to damage that has occurred to the central nervous system (Sofroniew 2015). Additionally, high concentrations of GFAP have been detected in aging brains and connected with cases of degenerative dementia such as Alzheimer's disease (Hol et al. 2003; Mecocci et al. 1995). Expression of GFAP is also connected to insulin-like growth factor 1 receptor (IGFR-1) expression. Preliminary studies in our laboratory indicate that reduced levels of circulating IGF-1 lead to increased GFAP expression. Additionally, previous studies have shown that levels of GFAP increase when astrocytic IGFR is reduced (Logan et

al. 2018). This inverse relationship has been identified as a way for the brain to promote delivery of sufficient IGF-1 to areas of brain damage or aging, as the growth factor has healing effects (Madathil et al. 2013). In this study, we will investigate the particular ways that an increase in GFAP levels is expressed in cells. An increase in the number of GFAP+ cells, larger GFAP+ cells, and/or higher GFAP content within cells are all possible manifestations of a rise in GFAP levels.

Insulin-like growth factor 1 (IGF-1) and its receptor IGFR

IGF-1 and IGFR are, respectively, a hormone and its receptor found throughout the body. IGF-1 is predominantly produced in the liver and is capable of crossing the blood-brain barrier. We recently showed that IGF-1/IGFR signaling influence expression of EAAT 2 and, consequently, glutamate uptake (Prabhu et al. 2019). IGF-1 regulates cell development and function not just in the brain but all through the body. However, significant changes in brain structure and function particularly can be seen as a result of the decrease of IGF-1 that occurs with aging. Animal studies have indicated that consequences of low levels of circulating IGF-1 include reduced brain size, loss of myelination, and impaired brain and central nervous system development overall (Ashpole et al. 2015). Additionally, increasing levels of IGF-1 in older animals results in an increase in neurogenesis, vascular density, and glucose utilization. This leads to improvements in learning and memory (Sonntag et al. 2005).

Based on the research that has been conducted on astrocytes, we hypothesize that IGF-1 and IGFR are critical regulators of GFAP. We set out to

investigate the regulation of GFAP by reducing IGFR in astrocytes and liver-derived IGF-1 in mice and analyzing the subsequent effects. While changes in behavior and cognition of the animals were monitored, particular attention was given to examining the way that an increase in GFAP levels was expressed. In this way, the size of astrocytes and the number of astrocytes were both measured to determine if GFAP levels were affected by abundance of cells or by the area of cells in the brain.

Methods

Animals

The procedures utilized in this study were approved by and conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Mississippi. One group of animals were derived from $igfr^{f/f}$ male and female mice, homozygous for LoxP sites around exon 3 of the igfr gene, that were purchased from Jax laboratories and bred in house. This research used three litters of male and female pups that had an average of 8-10 pups per litter, and their genotypes were confirmed using qPCR. On the first post-natal day, male and female pups were euthanized via swift decapitation and cortical tissue was extracted to make *in vitro* astrocyte culture for genetic experimentation.

A separate group of animals used was mice with inducible astrocyte-specific IGFR knock-out raised from the breeding of female $igfr^{f/f}$ and male

Cre/ERT mice that were purchased from Jax laboratories. Because the GFAP-Cre/ERT transgene does not follow mendelian genetics, the number of pups with the preferred genotype varied among each litter. After three months, the mice were genotyped and the selected ones were injected intraperitoneally with either corn oil or tamoxifen. Tamoxifen was diluted in corn oil, heated, and upon cooling, 75mg/kg dose injections were administered to induce Cre recombinase expression to target the removal of exon 3. These animals were used for behavior analysis and research on the effects of astrocytic IGFR reduction on cognition. Two months after injections, the group of animals was euthanized via swift decapitation and brains were isolated for analysis.

In a subsequent set of studies, pups from male and female homozygous IGF-1^{flox} mice were raised in house. The first-generation parents were purchased from Jax laboratories. After three months, the mice were injected retro-orbitally with either AAV8-TBG-GFP or AAV8-TBG-Cre, adeno-associated viral vectors. Approximately four months after the injection, the mice were euthanized via rapid decapitation and brain tissue was harvested. This tissue was then sliced, stained, and analyzed.

Pregnant wild-type Sprague-Dawley female rats were purchased from Envigo for *in vitro* studies. The study included six litters of rats, averaging at 10-15 pups per litter. Soon after the birth of the pups, P1-P3, they were euthanized by rapid decapitation and cortical tissue was extracted. The tissue was used for making *in vitro* primary neuron and astrocyte cell culture for pharmaceutical experimentation.

All animals in this study had housing with enriched bedding, and they received standard rodent chow (5053 Pico Lab, Purina Mills, Richmond, IN) and had access to as much water as desired. The mice lived in SuperMouse 750 (12x5.5 inch) ventilated cages with three to five per cage. The rats resided in (Ancare R20 10.5x19x8 inch) static cages with a maximum of two per cage.

Astrocyte culture

Two astrocyte cultures were done, with the intentions that one would be used for genetic reduction of IGFR and the second for pharmacological manipulation. Cortical brain tissue was harvested from the $igfr^{f/f}$ mice for the genetic experiment, and for the pharmacological experiment, tissue was isolated from the cortex of wild-type Sprague Dawley Rats. Astrocytes were derived from the cortex and hippocampus using an established protocol (McCarthy and De Vellis 1980). The tissues from both areas were mixed, and papain was used to enzymatically digest the tissue. Trituration was used to mechanically break down the tissue. Then, the cells were resuspended in growth media which was composed of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, penicillin/streptomycin (10 units/mL), L-glutamine (29.2 μg/mL) and N15 supplement (Schildge et al. 2013). The astrocytes were grown in 10cm dishes coated with poly-L-lysine, and the media was changed every three to five days. When confluency was 80-90%, microglia were separated by removing the media and adding trypsin to the plate. The separated cells were then centrifuged into a pellet and resuspended in new media. Changing the media helped kill neurons because 10% FBS is toxic to neurons but not astrocytes. These actions were

repeated until the number of neurons was <5% in the dishes (Prabhu et al. 2019).

Genetic and Pharmacological Reduction of IGFR

In order to genetically inhibit IGFR in the astrocytes, the igfr$^{f/f}$ astrocyte cultures were transduced with AAV5-CMV-Cre or control AAV5-CMV-GFP. These adeno-associated viruses transduce cells, and the cytomegalovirus (CMV) promoter causes the overexpression of Cre recombinase in those cells. In the igfr$^{f/f}$ cells, Cre recombinase causes the removal of exon three which is the exon responsible for binding IGF-1. The viral vectors, which were purchased from Penn Vector Core, were diluted 1:10,000 in cell growth media. Seventy-two to eighty-six hours before the cells were tested, they were treated with the viruses. IGFR in wild-type rat astrocyte cultures was then inhibited pharmaceutically by treating cells with picropodophillyotoxin (PPP), an antagonist of IGFR.

Viability Staining and Counting

The viability of cells after treatment and experimentation was analyzed with the use of ethidium homodimer, calcein-AM, and DAPI cell counting. The ethidium homodimer worked to label cells with affected membranes, and the DAPI stain allowed for quantification of the total number of nuclei. The coverslips with attached cells that were treated with PPP were washed in phosphate-buffered saline (PBS). They were then stained with a Live/Dead Cytotoxicity/Viability Kit (Molecular Probes) in accordance to the manufacturer's protocol. To analyze the effects of treatments, the coverslips were imaged in

three areas using 200X magnification on a Nikon Ti-2 inverted microscope. A TRITC filter identified ethidium homodimer+ cytotoxic cells, and a FITC filter noted the calcein-AM cleaved FITC+ cells.

Retro-orbital Injections

Research was also done with three-month-old IGF-1^{flox} mice. A 24-gauge needle and syringe were used with 100μL of injectate. The injectate was phosphate-buffer saline (PBS) mixed with the adeno-associated viral vector AAV8-TBG-GFP or AAV8-TBG-Cre. The thyroxine-binding globulin (TBG) ensures that the viral vector will target the liver, specifically. Once the AAV8-TBG-Cre virus transduces the liver, the hepatocytes begin making Cre recombinase which excises exon four, the flox exon. Prior to injection, the mice were anesthetized one at a time with inhalant anesthetic by placing them in a plexiglass chamber with isoflurane. The mouse was laid on its side, and gentle pressure was applied to partly protrude the right eyeball from the eye socket. A needle was then inserted, and once it reached the base of the eye, the liquid was slowly injected into the venous sinus of the animal. After the needle was gently retracted, a drop of ophthalmic solution was placed on the eye to prevent dryness and irritation, and the mouse was returned to its cage to recover.

Tissue Isolation and Sample Preparation

Brains that were removed from seven-month old liver IGF-1 deficient (LID) mice and cut longitudinally down the middle in order to separate the hemispheres. The left hemisphere tissue was fixed in 4% paraformaldehyde

(PFA) overnight at 4°C. The tissue was then embedded with 30% sucrose in phosphate buffer for three days at 4°C. The brains were then washed and rinsed with PBS. A thin layer of optimal cutting temperature compound (OCT) was used to coat the bottom of a cube-shaped mold, and the fixed left-brain hemisphere was placed with the inner surface down in the mold. More OCT was added to completely cover the top and sides of the tissue. The molds were placed in a dry ice bath before being stored at -80°C. The brain molds were then transferred onto a cryosectioning sample holder using OCT to attach, and a Leica cryostat at –18 to –21°C was used to obtain 25 micron thick slices of the tissue. The slices were placed in cryopreservative in 24 well plates.

Immunohistochemistry of Brain Tissue

In order to stain the slices from LID animals, they were transferred from the cryopreservative, using a small paintbrush, into a 24 well plate that contained 500μLs of PBS in each well. The slices were carefully rinsed three times with PBS before being permeabilized in PBS tween 20 (0.10%) for 20 minutes at room temperature. Antigen retrieval was required for these samples, so citrate buffer was boiled and put into each well. The plate was then incubated at 95°C for 30 minutes. After a cooling period of 20 minutes, the slices were rinsed with PBST (0.10%) twice for 2 minutes. The sections were blocked with PBS 0.5% bovine serum albumin (BSA) for 30 minutes at room temperature. Mouse primary antibody to GFAP (goat anti-mouse, abcam) was diluted in PBS 0.1% BSA before it was used to incubate the tissue overnight at 4°C. The sections were rinsed with PBST (0.10%) twice for 2 minutes. They were then incubated with a

secondary antibody (donkey anti-goat 546, abcam) for 2 hours at room temperature. After rinsing the slices with PBST (0.10%) twice for 2 minutes, the tissue was mounted onto glass slides and covered with Prolong Gold Antifade (Molecular Probes) with DAPI. Cover slips were placed on each slice, and the slides were placed in darkness to dry overnight. A Nikon Ti-2 inverted microscope was used to analyze the astrocytes. The secondary antibody that was utilized highlighted GFAP red. Magnification of 100X was used to obtain the number of hippocampal astrocytes present, and a magnification of 600X was used to determine the area of three to four astrocytes in the CA1 region of the hippocampus.

Behavioral Analysis of IGFR knock-out mice

Blindness Tests

Multiple tests were used to assess astrocytic IGFR knock-out (aIGFR-KO) mice for their level of blindness. First, mice were held by their tails at approximately two feet above a platform and were rapidly lowered. If the animal stretched out its paws in anticipation of the platform as it got closer to it, then it was not considered to be blind as it seemed to be able to see the approaching object. A second test conducted involved an open field and a hollow red tube like the ones already in the mice cages. The animals were placed one at a time in an open field with the red tube located near the center of the exposed surface. If the animal was able to see the tube and go towards it in a short amount of time, then it was considered to not be showing signs of blindness. A final test was completed by suspending mice from their tails and holding them near a vertical

rod. If the mouse reached for the bar in an attempt to latch onto it, then it was noted as having normal vision. Levels of blindness were measured on a scale of 0 to 1 by a blinded observer. Positive behavioral results for blindness resulted in a 0, 0.5 was assigned to animals that showed weak signs of vision, and 1 was reported for animals who did not exhibit blindness in a particular trial.

Tail Suspension

In order to test possible depression-like phenotypes of reducing astrocytic IGFR, mice were tested using a tail suspension method. The tails of the mice were taped to the center of the top panel of a box structure, so that the mouse was suspended upside down during the duration of the trial. A camera was situated to record the behavior of the mice as they attempted to become upright. The amount of time each animal struggled continuously without stopping once was recorded, and the amount of time each mouse took to reach full immobility was documented, as well. Each trial lasted a maximum of six minutes.

Barnes Maze

The Barnes maze was utilized as a way to measure spatial learning and memory. This maze is constructed as a platform with several circles marked around the perimeter. Open spaces and lights are aversive to rodents, motivating them to find an escape. One of the circles marks a hole that is the entrance to an escape box for the mouse to leave the open platform. The maze was situated in a well-lit room and surrounded by dark curtains. A camera was placed above the maze, facing down, in order to record the trials. The animals were exposed to the

escape box for 30 seconds on the day of the trials. Each mouse experienced 4 trials of acquisition for 4 days. In this phase of the experiment, the mice used visual cues on the curtains to find the escape box. A probe trial was conducted on the fifth day. During the probe trial, there was no escape box for the animals. Another probe trial was held on the tenth day and 4 trials of reversal on the eleventh day. In the reversal phase, the escape box was moved to a different position. An animal was directed to the escape box if it had not reached the correct hole by the end of the trial. Typically, rodents use a particular strategy such as random, serial, or spatial in order to learn the escape location. Each trial had a duration of 90 seconds. The Noldus Ethovision Software was utilized to measure path length, latency, velocity, number of errors, and strategy for each animal. While all of these parameters were assessed, my focus in this project was to determine strategy.

Data Analysis

The graphs made with this research were constructed using Sigma Plot graphing software. In order to determine statistical significance, unpaired t-tests were run on the collected data. Noldus Ethovision Software was used to analyze behavioral tests, and Microsoft Excel was utilized to calculate percentages. The animals were randomly assigned to treatment groups. Block randomization was utilized with random cages receiving a specific treatment.

Results

The four animal cohorts utilized in this study are described in Figure 1. We genetically inhibited IGFR in mice astrocyte cultures by transducing them with AAV5-CMV-Cre or control AAV5-CMV-GFP. The reduction of the receptor in the igfr$^{f/f}$ mice was confirmed through qPCR analysis, and the expression of exon 3 of IGFR was reduced by 82% (Prabhu et al. 2019). This particular exon is connected with ligand-binding and activation of IGFR, making it a desirable target for inhibition. After the AAV5-CMV-Cre treatment, no significant change was observed in cell number and viability of knock-out or control cells (Figure 2).

We then reduced IGFR through pharmacological means with PPP, a small molecule inhibitor of IGFR. The viability of astrocytes was recorded after this treatment. Similar to the results of the genetic regulation of IGFR, the PPP treatments did not cause a significant difference in the number of living cells. Interestingly, though, a change in distribution was observed in these cultures. The cells were visibly more aggregated (Figure 3).

To determine whether changes in astrocyte number or aggregation was visible *in vivo*, we then examined astrocyte number and size in mice lacking IGFR specifically on their astrocytes. The description of these animal cohorts can be found in our recent publication (Prabhu et al. 2019). Immunohistochemistry was performed and astrocytes were visualized in the hippocampal area. The astrocytes of control and aIGFR-KO animals did not exhibit notable differences in size and count (Figures 4 and 5). These results were similar between both male

and female cohorts which suggests that astrocytic IGFR-KO did not significantly change astrocytic size or shape.

While astrocytic IGFR did not significantly affect astrocyte structure, our preliminary findings that reduced IGF-1 leads to increased GFAP encouraged us to explore astrocytes in the LID mice next. Mice were retro-orbitally injected with AAV8-TBG-Cre to knockout IGF-1 in the liver, and then the brain tissue was harvested. We next conducted immunohistochemistry of the brain tissues in order to obtain a clearer view of astrocytes in both groups of animals. We were able to quantify and examine the distribution and size of astrocytes using this method. The LID males and females both exhibited a trending increase in number of astrocytes, but the difference was not statistically significant (Figure 6). There were significant sex-specific differences in regard to the area of astrocytes, though. The LID males showed a significant increase in GFAP+ cell area compared to their respective controls, while the LID females showed a significant decrease in astrocyte area when compared to the areas of astrocytes of control mice (Figure 7).

In collaboration with other members of our lab, behavioral and cognitive aspects of the aIGFR-KO animals were considered as well. Considering that GFAP was used as a promoter to drive knock out and retinal ganglion cells express robust levels of GFAP, we conducted tests to assess the blindness of the mice as a control for other behavioral experiments. Overall, more females showed visual deficits than males. When comparing the control to knock out animals, there was no significant difference between them. Approximately 65.6%

of the male KO mice expressed blindness, and 71.4% of male control mice were blind. For the female animals, 65.6% of female KO were blind, and 66.7% of the female control mice showed blindness (Figure 8).

A tail suspension test was used to assess the amount of depressive-like behavior among the animals. If an animal initially stops trying to challenge its upside-down position in a shorter amount of time, then that can be interpreted as depressive-like behavior. If a mouse struggles for a longer period of time, then the animal is considered to have lower depressive tendencies. The data collected from this test show that there was no significant difference in how long the aIGFR-KO and control mice took to become immobile. This was consistent for both males and females. However, it was interesting to see that the aIGFR-KO mice took significantly longer than control animals to reach their initial moment of immobility (Figure 9).

The Barnes maze tested the spatial learning and memory capabilities of the animals. The type of strategy each animal used throughout the trials to learn and relearn the location of the escape hole gave insight on the state of each animal's cognitive abilities. Male and female animals from each type of treatment used the serial path strategy the most, and the random path strategy was used the least (Figure 10). Looking at this data, no significant changes were seen in the learning and memory processes after astrocytic IGFR reduction.

Figure 1

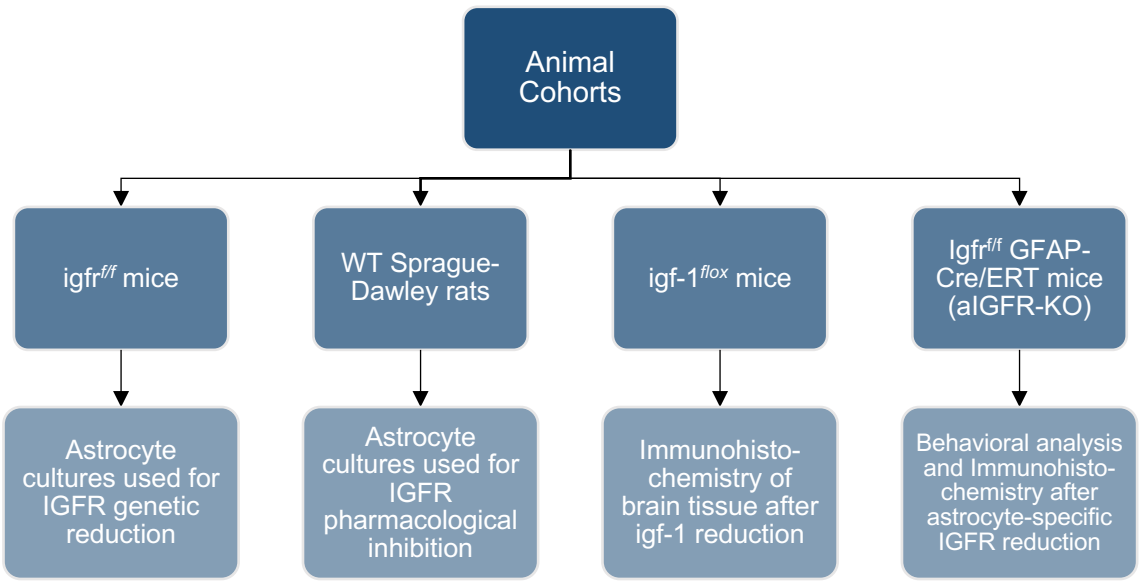

Figure 1: Animals Cohorts Used

Four different animal cohorts were used in this study. Astrocytes were derived from igfr$^{f/f}$ mice and used in genetic manipulation of IGFR. Astrocytes from wild-type Sprague-Dawley rats were treated pharmacologically to reduce IGFR in the cells. Brain tissue from igf-1^{flox} mice was stained in order to analyze astrocyte size and count after igf-1 was reduced in these animals. The aIGFR-KO mice were tested behaviorally to assess the effects of IGFR reduction on cognition, and the size and count of hippocampal astrocytes were measured through immunohistochemistry of their brain tissue.

Figure 2

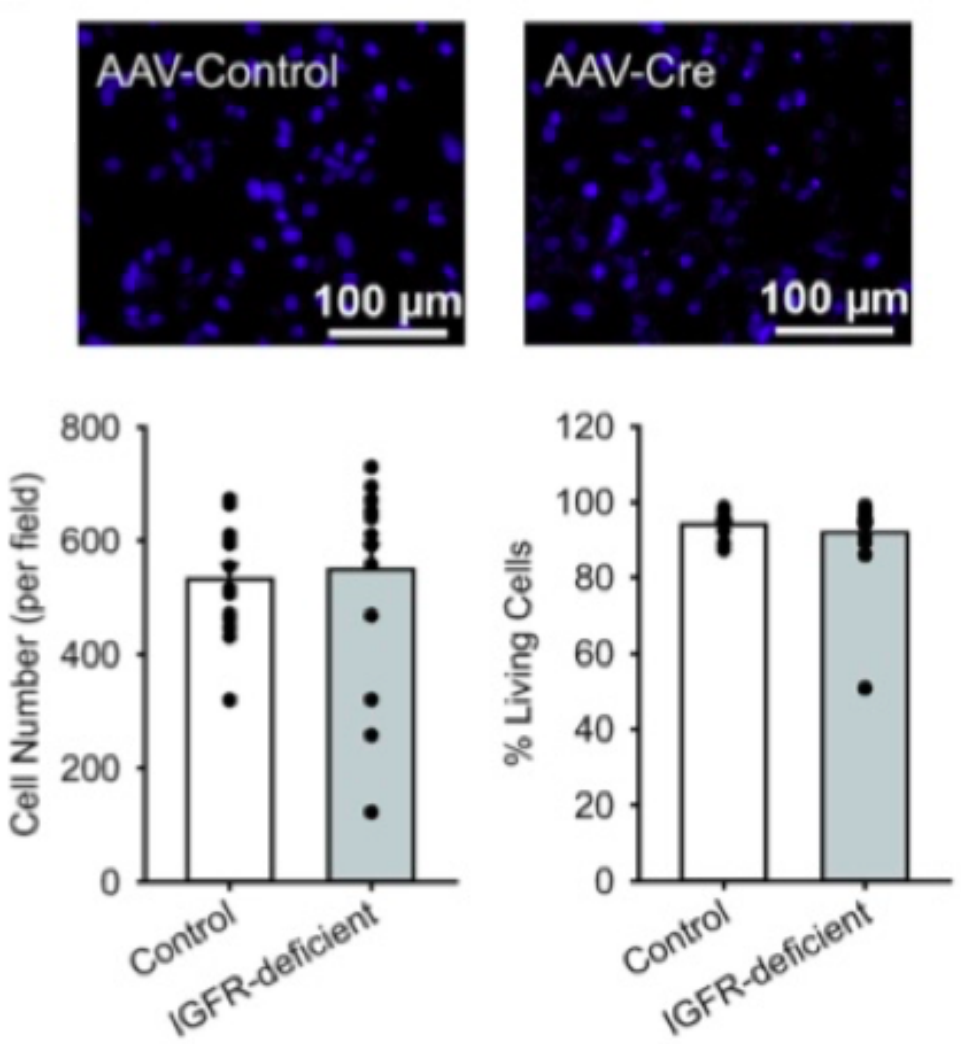

Figure 2: Cell Count after Genetic Reduction

Images of control and IGFR-deficient astrocytes that were labeled with ethidium homodimer and DAPI were captured with an inverted microscope. The total number of cells and % living cells were quantified. No significant differences were noted.

Figure 3

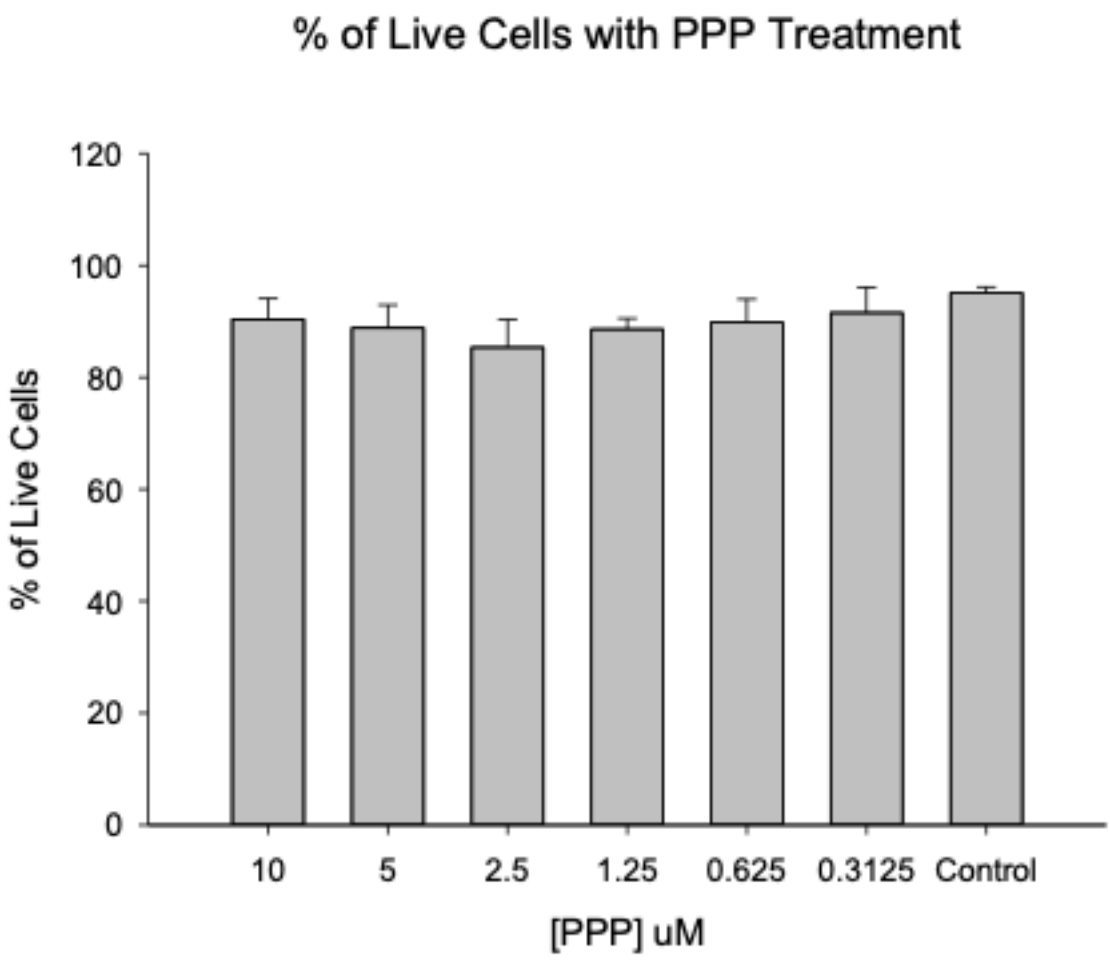

Figure 3: Cells after Pharmacological Inhibition

The percent of live astrocyte cells after PPP treatment was quantified. No significant effect on the number of living cells was observed after increasing concentrations of PPP were administered to the cells in order to decrease IGFR.

Figure 4

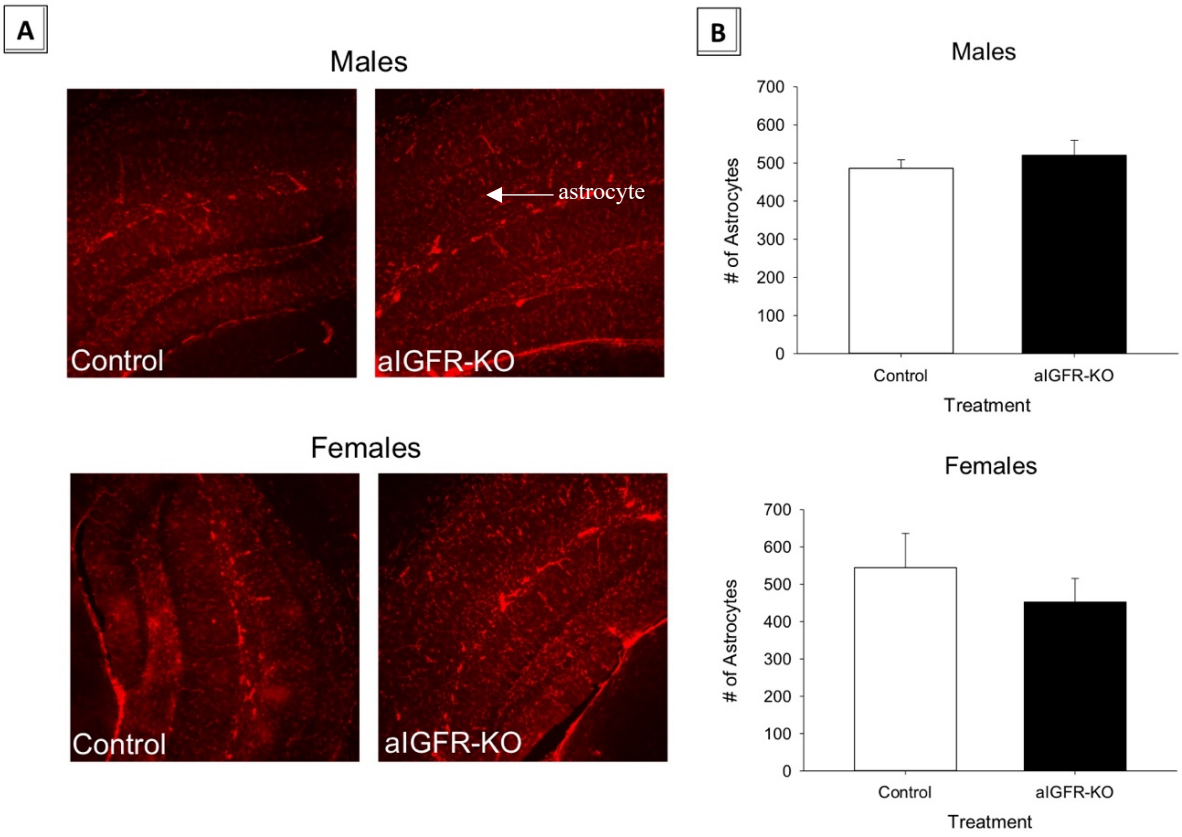

Figure 4: Astrocyte Count in aIGFR-KO Animals

A) Slices of brain tissue from IGFR-KO and Control animals were stained and imaged at 10X. The astrocytes (seen as bright red dots) were counted for both control and knock-out animals. **B)** The number of astrocytes was recorded for treated and untreated animals. No significant difference was found between the male and female cohorts.

Figure 5

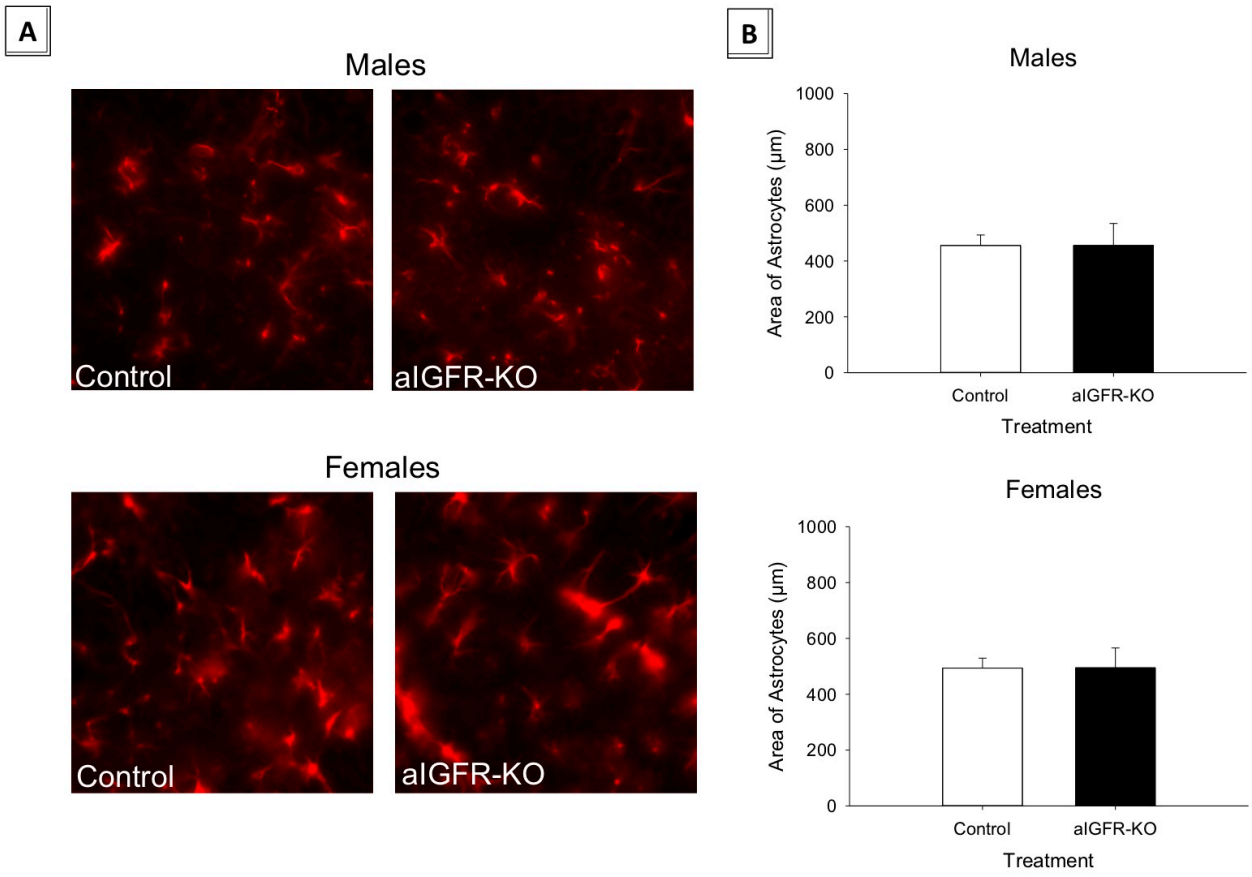

Figure 5: Area of Astrocytes in aIGFR-KO Animals

A) Stained tissue slices of the hippocampi of Control and IGFR-KO animals were analyzed at 600X magnification. The areas of the bright red astrocytes were measured to see if any difference existed between control and IGFR-KO animals.

B) Graphing software SigmaPlot was utilized to compare astrocyte area values. The areas of control and knock-out animals were very similar in both males and females.

Figure 6

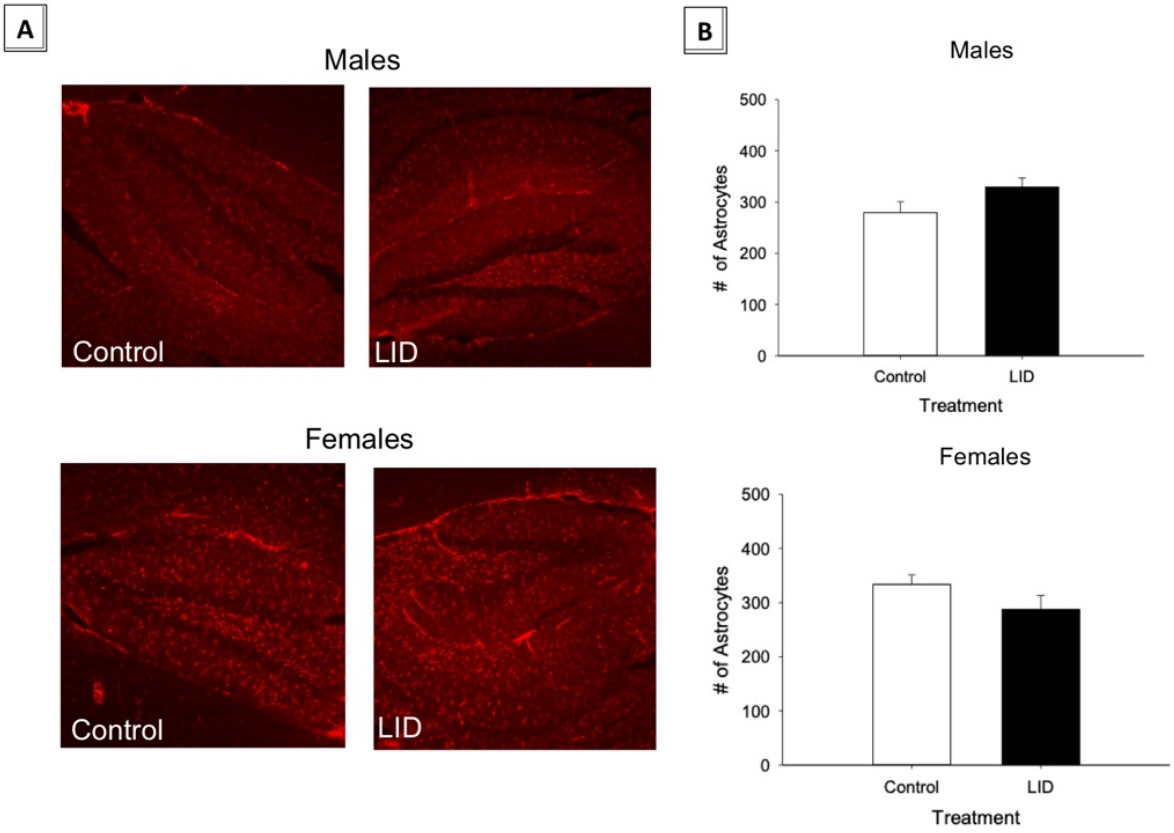

Figure 6: Astrocyte Count in Liver IGF-1 Deficient Animals

A) Images of Control and IGF-1 deficient mice brain tissues were analyzed with a TRITC filter on an inverted microscope after staining via immunohistochemistry. Astrocytes were highlighted bright red. **B)** The number of astrocytes in the hippocampus was quantified. No significant changes were observed between control and LID animals in male or female mice.

Figure 7

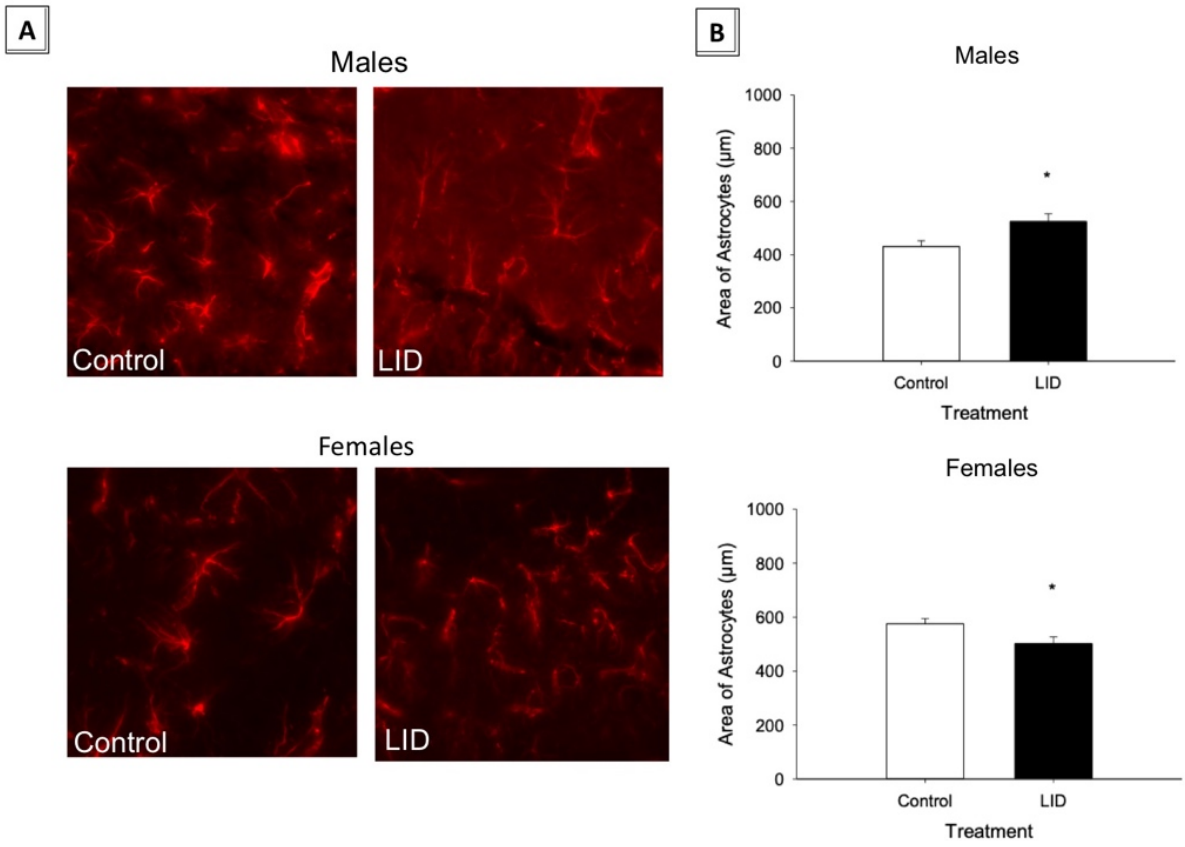

Figure 7: Area of Astrocytes in Liver IGF-1 Deficient Animals

A) Images of mice brain tissue treated with AAV8-TBG-Cre (LID) and AAV8-TBG-GFP (Control) were taken at 600X magnification for male and female animals. **B)** The areas of three to four astrocytes were recorded for each animal, and the values from the male and female control and knockout mice were compared. A sex-specific difference was observed.

Figure 8

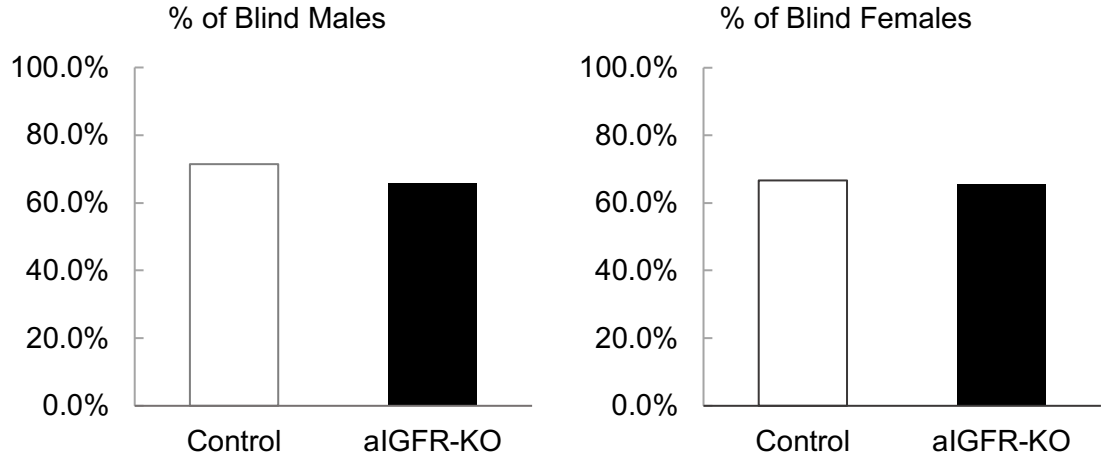

Figure 8: Blindness of Animals

Blindness levels of animals were tested with ratings of 0,1, or 2. Animals that showed strong signs of vision received a 2 rating, and animals that exhibited clear blindness received a rating of 0. The mice were rapidly lowered to see if paws would stretch outward, were placed in an open field to see if they would move toward a familiar object, and were suspended near a cylinder to see if they would attempt to grab the nearby object. No significant difference was seen between control and aIGFR-KO mice.

Figure 9

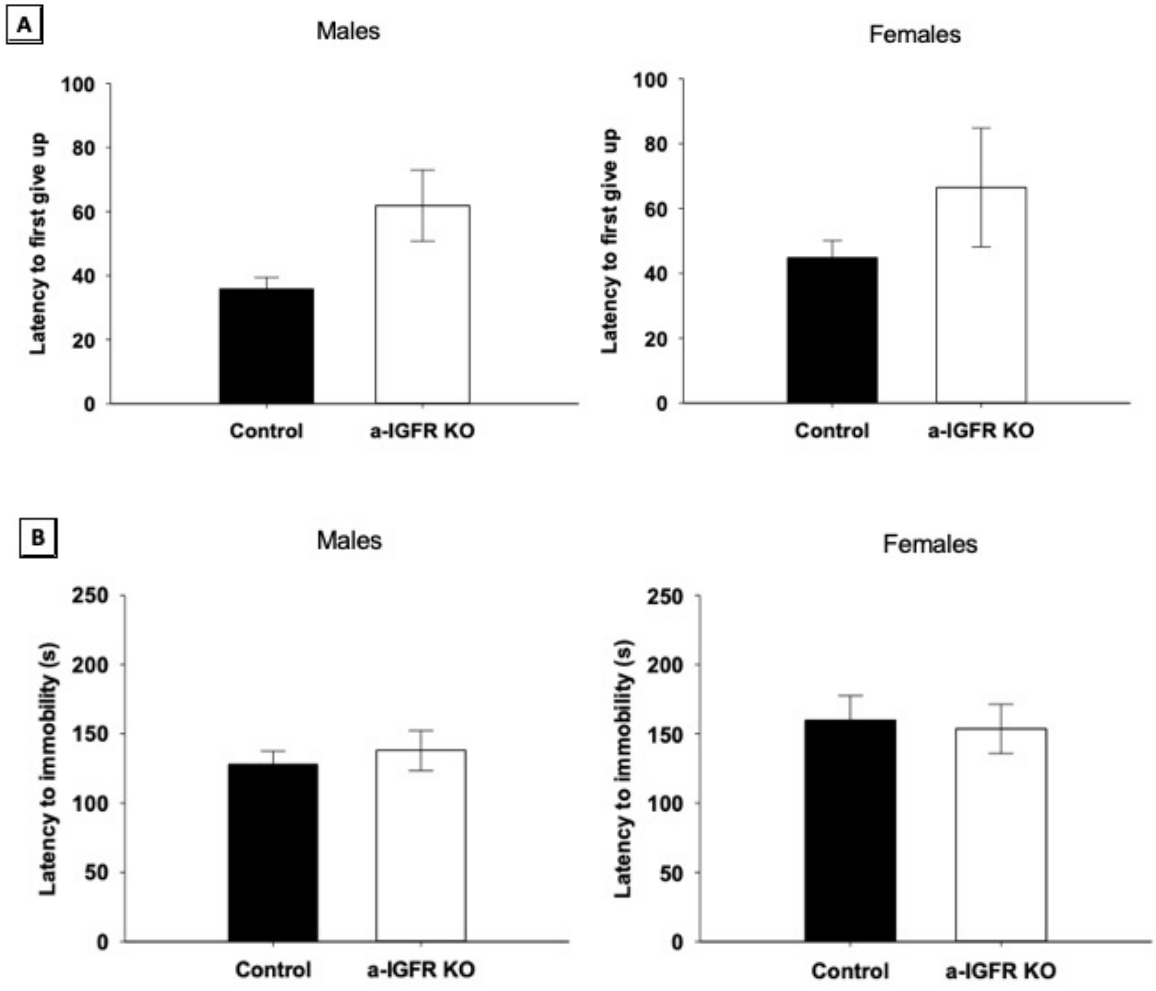

Figure 9: Tail Suspension Behavior

Tail suspension tests were conducted with astrocytic IGFR deficient animals. **A)** The knockout animals were noticeably resistant in regard to the first instance of immobility, or the first moment the animal stopped struggling to become upright, but overall there was no change between the control and treated groups. **B)** The latency to immobility graphs show how many seconds the animals in each cohort took to stop fighting their suspension. Larger intervals between latency and instances of immobility indicate fewer depressive tendencies in the animals.

Figure 10

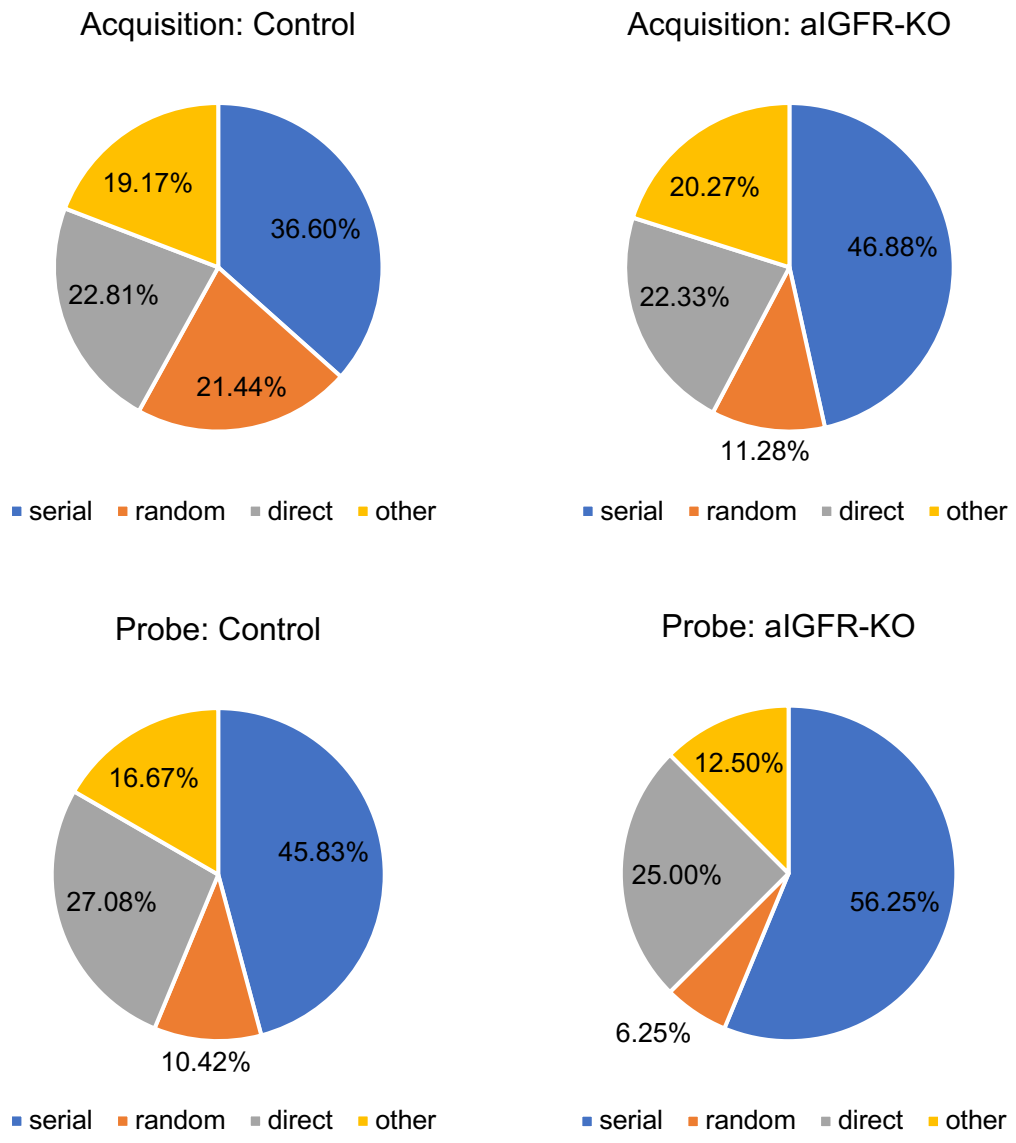

Figure 10: Barnes Maze Strategy Usage

The strategies used in Barnes Maze trials reflect how the animals attempted to find the escape hole. The serial strategy was used if the mouse resorted to searching the entire perimeter of the maze, passing each hole until the correct one was found. This was the strategy most used by both male and female mice in the probe and acquisition trials.

Discussion

When reducing astrocytic IGFR and liver derived IGF-1, we expected a rise in GFAP levels, and we wanted to determine if the rise occurred as an increase in astrocyte cell count or as an expansion of GFAP+ cell size. Our research yielded significant results in terms of cell size, but cell count was not greatly affected by the experiments. Cell size and cell count each serve as different indicators of brain health and function. A study by Oberheim et al. suggests that the size of astrocytes is positively correlated with complexity of brain functions. A possible reason is that an increase in area allows for a larger number of synapses to form, in turn allowing for more complex processing to occur per glio-neuronal unit (Oberheim et al. 2009). A decrease or increase in the number of astrocytes has been suggested to be linked with the plasticity of synapses and myelination of neurons (Williams et al. 2013).

Studies such as the one conducted by Tynan et al., which observed the effects of long-term stress have uncovered another interesting aspect of astrocyte size and number. In that study, a GFAP test indicated that astrocyte numbers had decreased, but by using a second test they learned that the number of astrocytes had not, in fact, decreased. The expression of GFAP had just declined in the cells. The results from our research coincide with the findings of the described studies in supporting the idea that GFAP levels are more closely regulated by and connected to the size of astrocytes rather than to the number of astrocytes.

We also studied the behavior of the animals to see if IGFR affected cognition. The difference in time in initial instance of immobility implied that knock-out animals experienced fewer depressive-like symptoms in the beginning of their tail suspension trials. From the results obtained after genetic and pharmacological experimentation on IGFR levels, a change in the number of astrocytes was not notable, but IGF-1 reduction did cause a sex-specific change in the size of GFAP+ cells. The male mice of this cohort showed an increase in GFAP levels which is congruent with the data presented in earlier studies that report GFAP increasing as IGF-1 levels decrease (Moloney et al. 2010). The data from the female mice does not share this trend, though, indicating that sex may influence the rise and fall of GFAP levels. A reason for this sex-specific difference in the GFAP and IGF-1 relationship could be the different levels of specific hormones in male and female animals. For example, estrogen, which has a higher expression in females than males, has been linked with the activation of the IGF-1 pathway (Kahlert et al. 2000). The same hormone does not seem to have a significant impact on GFAP (Trangue et al. 1987).

This difference in our male and female data reflects findings that support that sex may play a role in neurological differences. Aging studies that have observed sex-specific differences have noted hormonal changes and processes such as menopause as an important factor to consider for neurodegeneration (Miller et al. 2013). The physical structure of the brain is also known to be varied between male and female mice, with certain regions being larger or smaller than those of the opposite sex (Koshibu et al. 2004). Changes in brain structure could

cause physiological consequences such as particular areas of the organ receiving more or less microglial response or access to hormones. Additionally, sex differences can be seen in the prevalence of neurological conditions. For example, Parkinson's disease is more common in men, and Multiple sclerosis more often affects women. Women also experience poorer outcomes and a steeper decline in health in the situation of strokes (Hanamsagar et al. 2016). As microglia play a large role in response and environment of the brain in various conditions, sex-specific differences seen in neurological data should not be overlooked and could lead to more targeted and efficient therapy for age-related health issues that exhibit sex differences.

Limitations and Future Studies

There is still a large amount of information that we do not know about astrocytes and the effects of glial cells, in general. Another way that GFAP could increase or decrease its levels is by increasing concentration within a cell. Finding ways to measure GFAP concentration in a cell could provide more insight on not only the role of GFAP in astrocytes but also new ways that it could be regulated. Furthermore, the present study only focused on GFAP, but there are other markers of astrocytes that could be researched in order to uncover more about astrocytes and their regulation. In this study, the reduction of IGF-1 yielded a more significant response from the astrocytes than regulation of IGFR did, and the results also suggested that the size of astrocytes were more affected than the number of astrocytes. This encourages increased exploration of the relationship between IGF-1 and the area of astrocytes as we age. By expanding

our knowledge of these influential cells, we could potentially be able to unlock new ways to target and prevent the age-related progression of serious neurological degradation by effectively regulating GFAP, IGF-1, and its receptor.

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