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INVESTIGATING THE NEUROPROTECTIVE EFFECTS OF CANNABINOIDS AND  
INSULIN-LIKE GROWTH FACTORS ON GLIA WITH INDUCED INFLAMMATION.

By

Caleb Samuel Bloodworth

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

Oxford, MS

April 2022

Approved By

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## **ABSTRACT**

### **CALEB SAMUEL BLOODWORTH: INVESTIGATING THE NEUROPROTECTIVE EFFECTS OF CANNABINOIDS AND INSULIN-LIKE GROWTH FACTORS ON GLIA WITH INDUCED INFLAMMATION (Under the direction of Dr. Nicole Ashpole)**

Chronic inflammation is a driver of numerous neurodegenerative diseases that reduce quality of life for affected individuals. Non-psychoactive cannabinoids have begun to gain more interest in the world of anti-inflammatory medicine for chronically ill patients. Along with these cannabinoids, insulin-like growth factor-1 has been examined for its association with downregulation of inflammation. Our research aimed to investigate how neuroglia are affected by treatment with cannabinoids or IGF-1 in the face of inflammation from HIV-1 protein, Tat, or lipopolysaccharide (LPS). Preliminary studies in our laboratory showed that neither cannabinoids or IGF-1 treatment altered astrocyte morphology or overall astrocyte viability under baseline conditions, indicating no overt consequences of IGF-1 or cannabinoid treatment. Therefore, we hypothesized that cannabinoids and IGF-1 treatment would counteract astrogliosis induced by the key inflammatory mediators- tat and LPS. Numerous cannabinoids were assessed, with cannabidiol (CBD) showing significant effects across numerous measures of cell morphology and viability in the presence of Tat. No differences were observed when IGF-1 was administered. We also investigated whether astrocyte-derived IGF-1 would alter the number and size of microglia (Iba1+ cells) present in a sample of hippocampus tissue following systemic inflammation with LPS. We predicted that mice with astrocytic-IGF-1 knockout would suffer

more heavily from the neuroinflammatory effects of LPS. Surprisingly, the effects were minimal and indicated sex- and region-dependence, despite systemic inflammation in both males and females. Additional work is on-going to assess astrocyte response, as well as to assess the effects of cannabinoids on microglial and astroglial activation in mice exposed to chronic inflammation. Together, we hoped to clarify the protective capabilities of IGF-1 and cannabinoids on astrocytic and microglial cells, both of which hold an important role in neurological conditions.

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## **LIST OF ABBREVIATIONS**

AD	Alzheimer's Disease
CA1	Cornu Ammonis Zone 1
CA3	Cornu Ammonis Zone 3
CBC	Cannabichromene
CBD	Cannabidiol
CBN	Cannabinol
CCL2	C-C Motif Chemokine Ligand 2 Protein Coding Gene
CNS	Central Nervous System
DAPI	4',6-diamidino-2-phenylindole
DG	Dentate Gyrus
FBS	Fetal Bovine Serum
GFAP	Glial Fibrillary Acidic Protein
HIV	Human Immunodeficiency Virus
IFN	Interferon
IGF-1	Insulin- Like Growth Factor Type 1
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase

KO	Knock Out
LPS	Lipopolysaccharide
NF- $\kappa$ B	Nuclear Factor Kappa B
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PPAR	Peroxisome Proliferator-Activated Receptors
ROI	Region of Interest
S100B	S100 Calcium-Binding Protein B
TAT	Trans-Activator of Transcription
THC	$\Delta$ 9-Tetrahydrocannabinol
TNF	Tumor Necrosis Factor
WT	Wild Type

## **Background**

The brain is arguably the body's most important organ for survival, as it regulates vital processes such as respiration, cardiovascular output, and homeostasis. The nervous system integrates incoming information and processes it to allow for memory and learning, behavioral responses, pain perception, and overall affect. As an individual ages, systemic changes in inflammation and oxidative stress lead to alterations in functional processes in the central nervous system. Some processes, like the ability to learn new things and remember, decline. Other processes, like pain signaling, increase also as a result of neuropathic decline. The alterations in neurophysiology have become the heart of much pharmacological research, seeking a preventative measure to debilitating diseases and disorders. Underlying cellular changes have been studied at length and support the idea that reducing inflammation within the nervous system is an important neuroprotective measure. Therefore, understanding and identifying anti-inflammatory signals is critical.

### *Inflammatory Astrocytic Activation*

Proper functioning of the central and peripheral nervous systems relies on the presence and activity of glial cells such as astrocytes and microglia. It is estimated that glial cells exist in a 1:1 ratio with neurons in the human brain, while astrocytes in particular are the most numerous cell population of the central nervous system (Allen et al. 2009; Khan et al. 2020).

Astrocytes regulate growth of neurons, calcium and potassium ion uptake, glutamate, and are key cells in forming the blood brain barrier. Astrocytes work with other glial cells to respond to different signals within the CNS and often react to those signals in a manner termed ‘reactive gliosis’ (Kozela et al. 2017). Reactive gliosis leads to the release of pro-inflammatory factors that will be discussed further below. Unfortunately, this is a feed-forward interaction as increased inflammation leads to further reactive gliosis. Glial fibrillary acidic protein, GFAP, serves as an intermediate filament in astrocytic cells. GFAP holds a significant role in the structure of astrocytes, therefore it can be used as an excellent marker for reactive gliosis. High concentrations of the protein can indicate neurodegenerative diseases, signifying increased gliosis. Indeed, increases in the expression of GFAP and the overall number of GFAP+ cells are observed in numerous neurodegenerative diseases, including Alzheimer’s, Parkinson’s, HIV-associated neurocognitive disorder, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) (Palmer & Ousman, 2018). Increased GFAP expression is associated with alterations in overall astrocyte function within these disease states. For example, in Parkinson’s disease astrogliosis leads to a decline in extracellular buffering of toxins known to damage the dopaminergic neurons that cause symptom onset (Palmer & Ousman, 2018). This is similar to what has been observed in AD, where astrocytes are known to have weakened antioxidant defenses that lead to beta-amyloid induced cell death. In addition to a loss of key functions, reactive astrocytes increase inflammatory signaling within these disease states. For example, ALS models show that inflammatory NF- $\kappa$ B signaling within and from astrocytes ultimately causes neuronal death (Palmer & Ousman, 2018). Thus, the loss of key astrocyte functions and the gain of pro-inflammatory signaling establishes an environment of toxicity that is difficult to overcome.

There are numerous endogenous mediators known to regulate or associate with the expression of GFAP and other markers of astrogliosis. GFAP levels are inversely related to the circulating levels of IGF-1, a key growth factor in the body (Khan et al., 2020). While the exact relationship between IGF-1 and GFAP is not fully understood, it is clear that reductions in IGF-1 lead to increased inflammatory signaling in the brain, increased GFAP expression, and reduced neuronal function (Khan et al, 2020; Park et al., 2011). Other hormones, such as estrogen, also regulate GFAP expression under certain pathological conditions, as do endocannabinoids (Avraham et al., 2014; Mendez et al., 2006). Therefore, in this study, we will investigate whether various cannabinoids and IGF-1 affect the reactivity of astrocytes in the presence of the inflammatory inducers lipopolysaccharides (LPS) and HIV-1 Tat. **ADD DESCRIPTION OF HIV AND TAT**

### *Inflammatory Microglial Activation*

Microglia are another form of glial cells known for their overall role in regulating the inflammatory response within the CNS. They are the resident macrophages and are critical for cytokine production and overall homeostatic regulation of the brain. Microglia are also known for their response to oxidative stress and reactive oxygen species production. Microglia become ‘activated’ in response to damage and distress. Microglial activation is robust following acute neurological insults such as ischemic stroke, as well as chronic neurological issues such as HIV-associated neuropathic pain (Lu et al., 2021). Studies have shown association between microglia and risk genes for neurodegenerative disorders such as AD (Jansen et al., 2019; Li & Barres, 2017). Upon activation, microglia typically undergo hypertrophy and thickening of their soma and redistribution of their cellular processes/filopodia. While many studies discuss the changes in microglial activation as a sign of exacerbation of damage, microglia can also contribute to

natural physiological functioning along with its exacerbatory effects (Ma et al., 2017, Hayes et al., 2021). This duality in function has led to further research on the many roles of these glial cells. Microglia and IGF-1 have been associated with an indirect relationship; as IGF-1 circulation levels decrease, there is an increase in activation of glial cells, specifically microglia. The influence of cannabinoids on microglial activation has also been studied more extensively than cannabinoid regulation of astrocytes. While both cell types are glial cells similar in many functions, their differences hold the possibility of unexpected differential responses.

### *Neuroinflammation*

General inflammation is due to small proteins that are secreted to affect intracellular communication (Zhang et al. 2007). Neuroinflammation describes inflammation within the central nervous system and is not restricted to activation of inflammatory cascades specifically in neurons. Most inflammatory mediators within the central nervous system are derived from reactive glia. As described earlier, activated microglia can release pro- and anti-inflammatory cytokines and chemokines, with many of these having pro-inflammatory effects during periods of cellular distress. In addition to microglia, astrocytes can act as part of the complement system in any of three pathways, releasing these pro-inflammatory cytokines (Allen et al. 2009; Stevens et al., 2007; Boisvert et al., 2018). Some of the most commonly studied pro-inflammatory cytokines include tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)- $1\beta$ , and IL-6. Resulting inflammation from these molecules has been found to be associated with disorders involving neurodegeneration, such as Alzheimer's, multiple sclerosis, and dementia (Zhang et al. 2007).

TNF- $\alpha$  has been shown to trigger processes such as necroptosis, as well as play a role in ischemia, pain, and depression (Chitnis, 2017; Burstein, 2015). TNF- $\alpha$  has also been recognized

as a factor of activity in synaptic and axonal degeneration within the nervous system, which commonly occurs in HIV-associated neurocognitive disorder (HAND) and CNS injuries. The temporal upregulation of TNF- $\alpha$  can occur prior to increases in other pro-inflammatory signals, such as interleukins. Therefore, TNF- $\alpha$  has been suggested as a key initiation point in the inflammatory cascade that leads to axonal and synaptic manifestations of neurodegeneration (Myers & Shubayev, 2011). Interleukins were first discovered within leukocytes but have been shown to be involved with many immune and inflammation processes. IL-6 levels have been found to be significantly higher in individuals with neurodegenerative disease versus those without any Alzheimer's or dementia (Helmy et al. 2011). In HIV infections, microglia release TNF- $\alpha$ , IL-6, and numerous other cytokines and chemokines that cause neuroinflammation. HIV is not known to directly infect neurons, but instead can infect and control glial cells. The HIV-1 Tat protein (transactivator of transcription) directly interacts with glia to induce inflammation and secondarily precipitate hyperexcitability states, such as that seen with hyperalgesia. This interaction manifests one of the key clinical phenotypes of HIV+ individuals- increased pain response. Mechanistically, HIV-1 proteins are able to dysregulate neuronal calcium homeostasis and activate chemokine receptors (CXCR4) to activate the NF- $\kappa$ B pathway and associated downstream signaling cascades (Lu et al., 2021). A 2017 study found that HIV-1 Tat protein activated the NF- $\kappa$ B pathway and induced an increase in IL-1 $\beta$  levels and secretion. This same study established that Tat is able to prime the NLRP3 inflammasome, which is directly related to the expression of microglia *in vitro* (Chivero et al., 2017). When this inflammasome was blocked, IL-1 $\beta$  decreased secretion, implicating Tat's large role in induced neuroinflammation within HIV-1 neurotoxicity. While numerous studies have focused on understanding how HIV-1

dysregulates neuronal function and increases neuroinflammation, additional studies exploring pharmacotherapies capable of reducing inflammation long-term are still needed.

Neuroinflammation is often accompanied by increased oxidative stress within the surrounding tissue. In activated glial cells, an accumulation of ROS has been seen, and is known to induce an overload in calcium ions associated with cell death signaling (Palmer & Ousman, 2018; Ishii et al., 2017). Common neurotoxic insults, such as beta-amyloid and Tat have also been linked to oxidative stress in glial cells (Abramove et al., 2004; Palmer & Ousman, 2018). This is a bi-directional relationship between ROS and neuroinflammation, as oxidative species have also been known to mediate the effects associated with pro-inflammatory cytokines. Together, oxidative stress increases the likelihood of inflammation leading to neurodegeneration (Chitnis et al., 2017).

In this study, HIV-1 Tat protein and lipopolysaccharide (LPS) are used to model inflammation within astrocyte cultures. Tat has been shown to cause an influx of neutrophils, macrophages, and lymphocytes, ultimately leading to inflammation, microglia and astrocytic cell activation, and neurotoxicity (Jones et al. 1998). Lipopolysaccharide is a common model of innate immune activation, as these bacterial antigens induce secretion of proinflammatory cytokines in cells throughout the body. LPS stimulation leads to robust glial activation in vivo where it changes gene expression through activation of the toll-like receptor 4 (Kozela et al., 2010). LPS-mediated receptor activation and transcriptional regulation eventually activates the nuclear factor kappa B pathway (NF- $\kappa$ B), a regulator for immunity, and the toll-interleukin-1-receptor domain, which goes on to increase interferon- $\beta$  production. IFN- $\beta$  uses autocrine signaling to initiate the expression of multiple chemokines, like CCL2 (Kozela et al., 2010). LPS has been observed to successfully mimic neuroinflammation found in Alzheimer's disease



brought on by glial activation (Eddleston & Mucke, 1993; Hauss-Wegrzyniak et al., 1998; Reisenauer et al., 2011). Due to these pathways that induce cytokine secretion, LPS and Tat serve as effective models for neuroinflammation, and their toxicity will be investigated on viability and size of treated astrocytic cells.

### *Cannabinoids as Anti-Inflammatory Agents*

Exogenous cannabinoids, such as  $\Delta$ -9-tetrahydrocannabinol (THC) and cannabidiol (CBD) found in the plant *Cannabis sativa* have long been investigated for their medicinal use. In recent years, non-psychoactive cannabinoids have been more frequently researched to discover their effectiveness for decreasing inflammation and pain in chronically ill patients. CBD has been shown to have anti-inflammatory and sedative properties, with neuroprotective effects on Ab-induced neurotoxicity both *in vitro* and *in vivo*. (Esposito et al., 2006; Esposito et al., 2007). CBD has been shown to have effects on inflammation in many different pathways. These include decreasing monocyte attraction via CCL2, reducing IL-1B and IL-6 release induced by LPS, decreasing GFAP release, attenuating reactive species production from iNOS, and more (Kozela et al., 2010; Marin-Moren et al., 2011; Mecha et al., 2013; Stella et al., 2010). In other glial cells, such as microglia, CBD has been proven to reduce the expression of IL-1b, iNOS, and other cytokines induced by LPS (Burstein et al. 2015). CBD produced a reduction of gliosis triggered from the NF- $\kappa$ B pathway through a Peroxisome proliferator-activated receptor (PPAR)- $\gamma$ ; this reduction was demonstrated by a decrease of GFAP and S100B expression (Esposito et al., 2011). While CBD is the most well-known, non-psychoactive chemical constituent of *Cannabis*, CBD is not the only phytocannabinoid with anti-inflammatory potential being investigated.

Cannabichromene (CBC) is another terpenophenolic compound derived from *Cannabis*, more well known for its use in epilepsy treatments than other uses in medicine (Anderson et al., 2021). Reports have long suggested that recreationally consumed *Cannabis* actually contains a higher concentration of CBC than its more famous relative, CBD (DeLong et al., 2010). At high doses, CBC produced significant effects in the tetrad assay of locomotor suppression, catalepsy, antinociception, and hypothermia (DeLong et al., 2010). CBC also showed significant benefits in a study on LPS-induced inflammatory swelling of mouse paws, where its beneficial effects were suggested to be mediated by non-CB<sub>1</sub>/CB<sub>2</sub> pathways (DeLong et al., 2010).

Another phytocannabinoid, Cannabinol (CBN), has been investigated for its potential medicinal uses. In a model mimicking ALS, CBN was found to delay symptom onset, however it did not affect survival rate (Weydt & Witting, 2005). While investigating neuroinflammation pathways, it was discovered that CBN possesses the ability to prevent accumulation of intracellular amyloid beta, demonstrating its role in attenuating inflammatory pathways (Schubert et al., 2019)

In addition to cannabinoids, *Cannabis* produces numerous terpenes and other natural products that individuals consume for potential health benefits. Terpenes represent a class of naturally occurring compounds in various plants that are often associated with odors and flavors of the plant. They have been used as analgesics and anti-inflammatory products for a long time but are becoming increasingly common within essential oils and products containing CBD and CBC. Beta-myrcene has been shown to reduce oxidation effects with anti-inflammatory pathways. In ischemia models, myrcene showed repair in damage caused to mice brain tissue, leading to more questions about its possible benefits and uses (Kumar et al., 2021); in behavior and biochemical analyses, myrcene significantly impacted results; notably, TNF- $\alpha$ , and IL-6

were protected against. In treated tissues with both high and low dosages, myrcene demonstrated its ability to maintain synaptic plasticity and cause the modeled AD symptoms to not harm treated tissue. In memory testing, patients treated with myrcene demonstrated a slower decline in cognitive function than untreated AD or PD individuals (Kumar et al., 2021).

$\beta$ -Pinene is one of the most prominent terpenes in *Cannabis*, with concentrations in hemp found to be up to 21% (Weston-Green et al., 2021).  $\alpha$ -Pinene and  $\beta$ -pinene do not have a significant binding affinity to CB1 or CB2 receptors but have been shown to target GABA and serotonin receptors, suggesting antidepressant properties. While  $\alpha$ -pinene is more well-studied than its counterpart,  $\beta$ -pinene has also been linked to some neuroprotective properties. Pre-treatment of rodent subjects with the terpenoid offered some ability to protect against ischemia, stroke, and seizures (Weston-Green et al., 2021). Pinene is commonly found in essential oils, of which some have shown protective effects:  $\beta$ -pinene-rich essential oil from *Salvia lavandulaefolia* showed strong protective effects against bovine liposomes, as well as demonstrating anti-inflammatory activities in rat models (Perry et al., 2001). Thus, we are interested in whether cannabinoids and terpenes found within *Cannabis* are able to reduce neuroinflammation and associated neurodegenerative disease processes.

#### *Insulin-like Growth Factor as a Modulator of Neuroinflammation*

As stated earlier, hormones are also associated with glial activation and neuroinflammatory responses. We are particularly interested in insulin-like growth factor (IGF-1). Its production is stimulated by growth hormone release into circulation. IGF-1 is mainly synthesized in the liver but is also synthesized in muscles and the brain, while being capable of crossing the blood-brain barrier (Armstrong et al., 2000; Ashpole et al., 2015; Hayes et al.,

2021). IGF-1 is associated with synapses, neurotransmission, neurogenesis, and development (Hayes et al., 2021) With aging, IGF-1 levels diminish; these can be the result of reduced brain size and loss of myelination (Ashpole et al. 2015). Higher levels of IGF-1 have been markers of learning and memory, the result of increasing vascularity and efficiency of glucose utilization. In a study examining IGF-1 role in hypoxic-ischemia, animals showed reduced somatosensory deficits for 3 weeks when contrasted with animals that went untreated. Neuronal loss followed the same pattern and was markedly decreased for 20 days after injury (Guan et al., 2001; Sonntag et al., 2005). IGF-1 has implications of GFAP regulation, which will be examined in this study. In microglia, IGF-1 circulation has been found to be inversely related to microglial activation, decreasing the production and release of proinflammatory cytokines (Hayes et al., 2021).

### *Overall Goals*

This study was designed to investigate the protective roles of cannabinoids and IGF-1 in glial cells when faced with induced inflammation. Together, our study had four aims:

1. Investigate the effect of cannabinoids on astrogliosis induced from the HIV-1 protein Tat
2. Investigate the effect of cannabinoids on astrogliosis induced from LPS
3. Investigate the effect of IGF-1 on LPS induced astrogliosis
4. Determine whether a reduction in astrocyte-derived IGF-1 leads to increased microglial activation.

We expected Tat and LPS to cause inflammatory responses in astrocytes and microglia, and that cannabinoids and IGF-1 would ablate those responses. Our laboratory hypothesized that cannabinoids and IGF-1 application would attenuate LPS- and Tat-mediated astrocyte

hypertrophy (as measured by changes in overall area, perimeter, and diameter) to levels equivalent to or below the control groups. In another set of experiments using cell-specific IGF-1 KO mice, we hoped to explore the effects of local IGF-1 production on glial activation *in vivo* in wildtype and KO mice on inflammation response. By reducing IGF-1 levels locally, through targeting IGF-1 production, we expected to see increased glial activation and an exacerbation of LPS-mediated neuroinflammation.

## Methods

### *Animals*

The following procedures were approved by the Institutional Animal Use and Care Committees of the University of Mississippi and performed in accordance with their approved guidelines. For in vitro astrocyte cultures, timed-pregnant Sprague Dawley rats (16-18 days postictal plug) were purchased (Envigo) and were housed in 19 x 11.5 x 11-inch polycarbonate cages until euthanasia. Sprague Dawley rats were provided standard rat chow (Teklad 7001) and water *ad libitum*. Pregnant rats were anesthetized with isoflurane and cervical dislocation and rapid decapitation was performed. Embryonic rat pups (E17-19) were excised and rapidly decapitated for astrocyte cultures (see below). Male and female pups were not separated and all cerebral tissues from isolated embryonic rat pups were homogenized. Hence, these studies were indicative of astrocytic response from males and females.

**Genotyping:** All mice were genotyped for the presence of the *igfrf/f* exon 4 loxp sites and the iGFAP-Cre transgene. Two-three millimeters of tails were clipped immediately after weaning and RNA was extracted using Sigma REDExtract-N-AMP Tissue PCR Kit (XNAT) per manufacturer's recommendations. The resulting PCR product was run on a 2% agarose gel with Greenglo (Denville CA3600) for 1 h at 100V. All agarose gels were run with positive and negative controls and a 100 base pair DNA ladder.

**Tamoxifen Injections:** At three months of age, mice enrolled in this study received Tamoxifen to induce Cre recombinase expression. All mice received Tamoxifen to confound for Tamoxifen neurogenesis effects (Smith et al., 2022). Tamoxifen was diluted in corn oil, heated to 50°C to

solubilize, cooled, and administered via intraperitoneal injection in a 75 mg/kg dose or 20 mg/mL concentration as recommended by Jax laboratories. Specifically, corn oil increases the solubility of tamoxifen and decreases inflammation at the intraperitoneal injection. Mice received 100  $\mu$ L of tamoxifen for five days consecutively.

Astrocytes were subcultured every 3-4 days (90-100% confluent) to passage cells and select for astrocytes. To subculture, all growth media was removed, cells were washed with 1X PBS, and 0.05-0.25% of Trypsin-EDTA 1X (Gibco 25200-072) was added to dislodge cells from PDL coated 10cm plates. Following astrocyte dislodgement, the cells were centrifuged (1500 xg; 5 min) and suspended in growth medium for further plating. All astrocyte cultures were passaged multiple times prior to experimentation, and the cell morphology was monitored at each passage to determine if cells were exhibiting replicative senescence. Cells were not passaged more than 6 times to avoid confounds of senescent phenotypes.

### *Astrocyte Culture*

As stated briefly before, cortical tissue from male and female rat pups were isolated, digested, and pelleted. To enhance astrocytic growth, the digested tissue was centrifuged, cell pellets were suspended in astrocyte growth media containing Neurobasal medium (Gibco 21103-049) with 10% fetal bovine serum (Corning 35-010-CV), penicillin/streptomycin (10 units/mL; Life Technologies, 15140122), and 1x L-glutamine (250303-081). The isolated cell pellet was plated on PDL-coated 10cm dishes and media was completely exchanged the day following plating.

## *Treatments*

Neuroinflammation was induced using purified HIV-1 Tat protein acquired from the NIH HIV Reagent Program. Astrocytes were treated with 0.6  $\mu\text{L}$  of HIV-1 Tat per 100  $\mu\text{L}$  of astrocyte media. *Cannabis*-derivatives (Cannabichromene (CBC), cannabidiol (CBD), beta-pinene, and myrcene) were investigated for neuroprotection against Tat induced neuroinflammation. All in vitro treatments were performed in 96-well plates. Specifically, all treatments were provided to astrocytes in triplicates. All treatments were administered at a 2x concentration to avoid stressing astrocytes by removing 100% of the cellular media. After the compounds had been applied, 0.6  $\mu\text{L}$  of Tat was added to every well. The concentrations of cannabinoids were halved starting at 0.5  $\mu\text{M}$  down to 0.01625 $\mu\text{M}$ .

In a separate study, exogenous lipopolysaccharide (LPS) at 100 ng/mL was also administered to induce inflammation, and subsequently, the anti-inflammatory potential of cannabinoids against LPS was assessed. LPS (100 ng/mL) has been previously shown to induce glial cell activation (Kozela et al., 2010); hence, the justification of this concentration used in the studies.

Moreover, we investigated if a neuroendocrine hormone, insulin-like growth factor-1 (IGF-1), could exert neuroprotective properties against LPS-induced neuroinflammation. The concentration of IGF-1 used in this study was 100nM.

All treatments were performed in a similar timeline. At 60% confluency, astrocytes were treated with the designed compound and their cell count and size was subsequently assessed at



24 hours. Similar to the other treatments described above, these cells were incubated for 24 hours prior to assessment of glial activation.

### *Staining*

After the 24-hour treatment, cell viability was assessed using the live dead assay (Thermo Fisher Scientific L3224). Calcein-AM and ethidium homodimer were diluted (1:1000-2000; 1:500-1000) in PBS. After washing, the live dead solution was added to each well and incubated for 20 minutes. Cells were imaged on the Nikon Ti2-E HCA inverted fluorescent microscope using the JOBS automated image acquisition (Nikon). Magnification was set to 200x (20x, extra-long working distance objective) and samples were excited with the LED Triggered acquisition exposures using excitation/emission filters for 470-FITC and 540-TRITC. The JOBS program selected fields at random per well for imaging, and the total number of live and dead cells were quantified in 2-3 images using the Nikon Elements Cell Analysis plug-in features. The total number of cells and viability were calculated by observers blinded to treatment groups by dividing the number of live stained cells by that of the total cell number.

Additionally, viability was normalized to the number of live cells in each field and standardized our data based on our structural quantifications. For example, in our study with hippocampal slices with different genotypes and treatments, we quantified the number of cells present within an ROI, as well as the area statistics, and found the relative number of cells per unit area to assess overall viability.

Along with adding our secondary antibody to astrocyte tissues, phalloidin was added using a 1:400 dilution. Phalloidin binds to f-actin, prevents its degradation, and acts as a fluorescent marker.

## *Immunohistochemistry*

For our astrocyte immunohistochemistry studies tissues were fixed using 4% PFA and permeabilized using 0.1% PBS-Triton and 0.1% PBS-Tween. Cells were then washed using 1X PBS and blocked using 10% BSA for 90 minutes. The primary antibody, anti-GFAP ASTRO6 (#MA5-12023), was applied using a 1:200 dilution. The next day, the plates were rinsed with 0.1% PBS-Tween and incubated with a secondary antibody, rabbit anti-mouse 488 (#A27023) using a 1:1000 dilution. After washing the plates three times with 1X PBS, the tissues were incubated with 1:1000 DAPI to stain cell nuclei. Imaging was then carried out for these plates as described below.

For our microglia immunohistochemistry study, the slices were washed 2 times with PBS, and antigen retrieval was performed in Citrate buffer at 95 °C for 30 minutes, and the slices were then cooled down for 20 minutes, washed with 1X PBS, and blocked using 1% BSA for 2 hours. The primary antibody, rabbit anti-Iba1 FUJIFILM Wako (019-19741), was applied using a 1:500 dilution. The following day, the tissues were rinsed with 0.1% PBS-Tween twice for two minutes and incubated with a secondary antibody for two hours. The secondary antibody, 647 gt anti-rabbit (#A27040), used a 1:1000 dilution and was applied for 1-2 hours in complete darkness. After the secondary antibody was incubated, the tissues were again rinsed with 0.1% PBS-Tween twice and incubated for 30 minutes with 1:1000 DAPI in 0.1% PBS-BSA. Slices were then mounted using Prolong anti-fade DAPI mounting medium (#66248) and sealed with a coverslip. Imaging was then carried out using an inverted Nikon T2 microscope.

## *LPS + IBA1 Microglia slices*

Brains were removed from our mice and their tissues were fixed with 4% PFA overnight in a 4 °C environment and then embedded with 30% sucrose until the brains had sunk. Following tissue fixation, sagittal sections of brains were washed with 1X PBS and placed in plastic molds and frozen using O.C.T. (#4585). Brain molds were maintained at -80 °C until ready to slice using a cryostat. All brains were sliced using a cryostat at 45 microns and placed in a 24 well-plate containing 500 µL of a cryopreservative in each well.

### *Data Analysis and Graphs*

The figures associated with this research were constructed using Sigma Plot software. Data was imputed from Microsoft Excel worksheets into Sigma Plot to graph data in simple scatter plots with error bars on a logarithmic scale with individual compounds labeled. Unpaired t-tests, as well as one-way and two-way ANOVA tests were used to determine statistical significance as defined by values of  $p > .05$ .

## **Results**

The Tat treatment group when compared to the control group showed a significant decrease in area of astrocytes (Figure 1). CBC and Myrcene showed a significant decrease in astrocyte area when co-administered with Tat (Figure 1). No significant difference was observed between Tat and CBC or Tat and Myrcene (Figure 1). However, CBD restored astrocyte area to control levels in the presence of Tat (Figure 1). Astrocyte diameter was then assessed with a 0.5 µM screen of cannabinoids (Figure 2). Astrocyte diameter of the Tat treatment group was decreased when compared to the control group (Figure 2). CBD treatment group showed a significant increase in astrocyte diameter when compared to both control and Tat treatment groups (Figure 2). No significant difference was observed between CBC and Myrcene when

compared to control (Figure 2). A concentration curve for cell count of astrocytes was conducted with Tat as a vehicle and no significant difference was observed between groups (Figure 3).

When observing the diameter of astrocytes of control treated and LPS treated groups, there is a significant decrease in the diameter of astrocytes for the LPS treated group ( $p=.03$ ) (Figure 4A). There is no significant difference between control treated and LPS treated groups for the number of astrocytes (Figure 4B). However, there is a significant difference in the area of astrocytes with the LPS treated group showing a decrease in astrocyte area ( $p=.04$ ) (Figure 4C). No significant difference was observed in the perimeter of the astrocytes between the two groups (4D).

Examination of diameter, perimeter, cell count, and area of astrocytes with the addition of a cannabinoid treatment co-administered with LPS (Figure 5). No significant differences were observed in any of the assessments with the exception of CBC showing a significant decrease in cell count when compared to the control treatment group (Figure 5).

When LPS is co-administered with IGF-1, there is a significant decrease in cell count when compared to the control group (Figure 6). A significant decrease in perimeter was also found in the LPS treated group when compared to the co-administered LPS + IGF-1 group (Figure 6).

Cell number in hippocampal slices of males and females was then examined (Figure 7). A significant decrease in the number of Iba+ cells was observed in KO+LPS female mice when compared to WT ( $p=.010$ ) (Figure 7).

Figure 1: 1 $\mu$ M Screen of Cannabinoid Area in Astrocytes

Area of Astrocyte Cells with No Treatment, Treatment with Tat for 24hrs, and Treatment with Tat + 1 $\mu$ M Cannabinoids for 24hrs

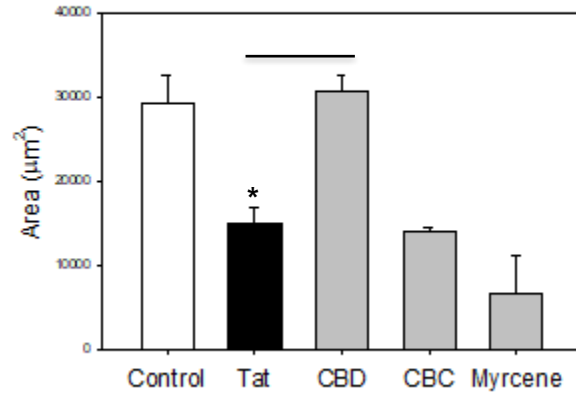


Figure 1: A 1 $\mu$ M screen of cannabinoid protective effects against HIV-1 tat protein in astrocytes. Data was compared using a One-Way ANOVA and Tukey's post hoc. All data are expressed as mean + SEM. \* indicates  $p < 0.05$

Figure 2: 0.5  $\mu\text{M}$  Screen of Cannabinoid EqDiameter in Astrocytes

EqDiameter of Astrocyte Cells with No Treatment, Treatment with Tat for 24hrs, and Treatment with Tat +  $1\mu\text{M}$  Cannabinoids for 24hrs

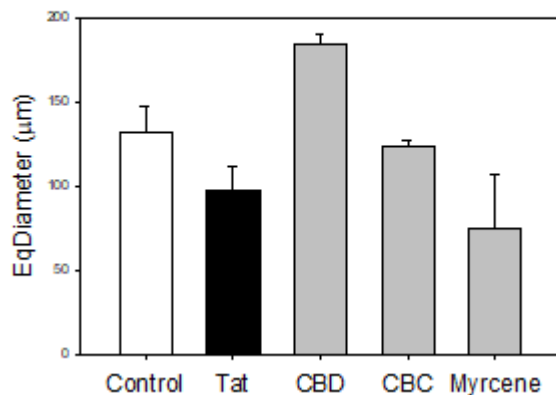
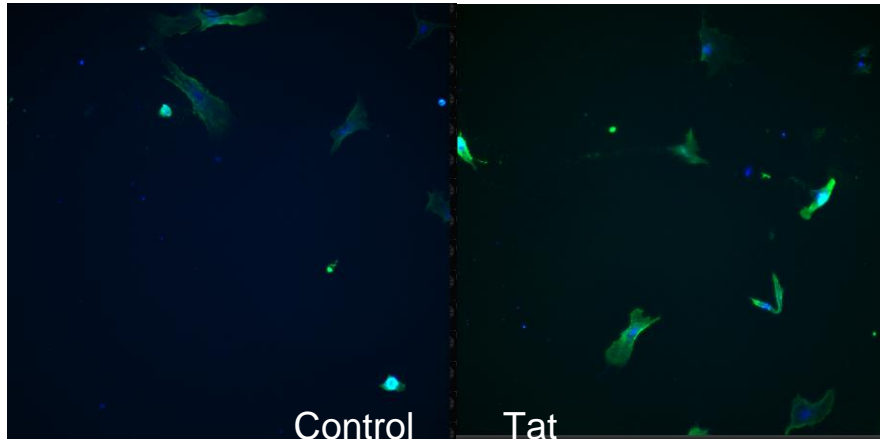


Figure 2: Equivalent spherical diameter measurements in astrocytes for control, vehicle, and treatment (0.5  $\mu\text{M}$ ) cannabinoid groups. Data was compared using a One-Way ANOVA and Tukey's post hoc. All data are expressed as mean + SEM Abbreviations: Cannabidiol (CBD), Cannabichromene (CBC)

Figure 3: Full Concentration Curve for Cell Count of Astrocytes with Treatments of Cannabinoids + Tat

A



B

Cell Count of Astrocyte Cells in Wells with No Treatment, 24hrs Treatment Tat, and 24hrs Treatment Tat + Cannabinoids

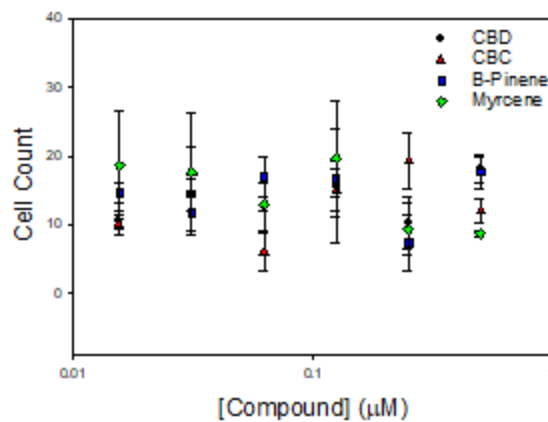


Figure 3: **A**) Representative images of control treatment (Left) and vehicle treatment of Tat (Right). **B**) Full concentration curve of cells treated with various cannabinoids and tat, assessed for cell count. No significant differences were found between groups. Data was compared using a One-Way ANOVA and Tukey's post hoc. All data are expressed as mean +/- SEM. \* indicates  $p < 0.05$  Abbreviations: Cannabidiol (CBD), Cannabichromene (CBC)

Figure 4: Comparison of Cell Count and Morphology in Astrocytes with

No Treatment and 24 hours Treatment with LPS

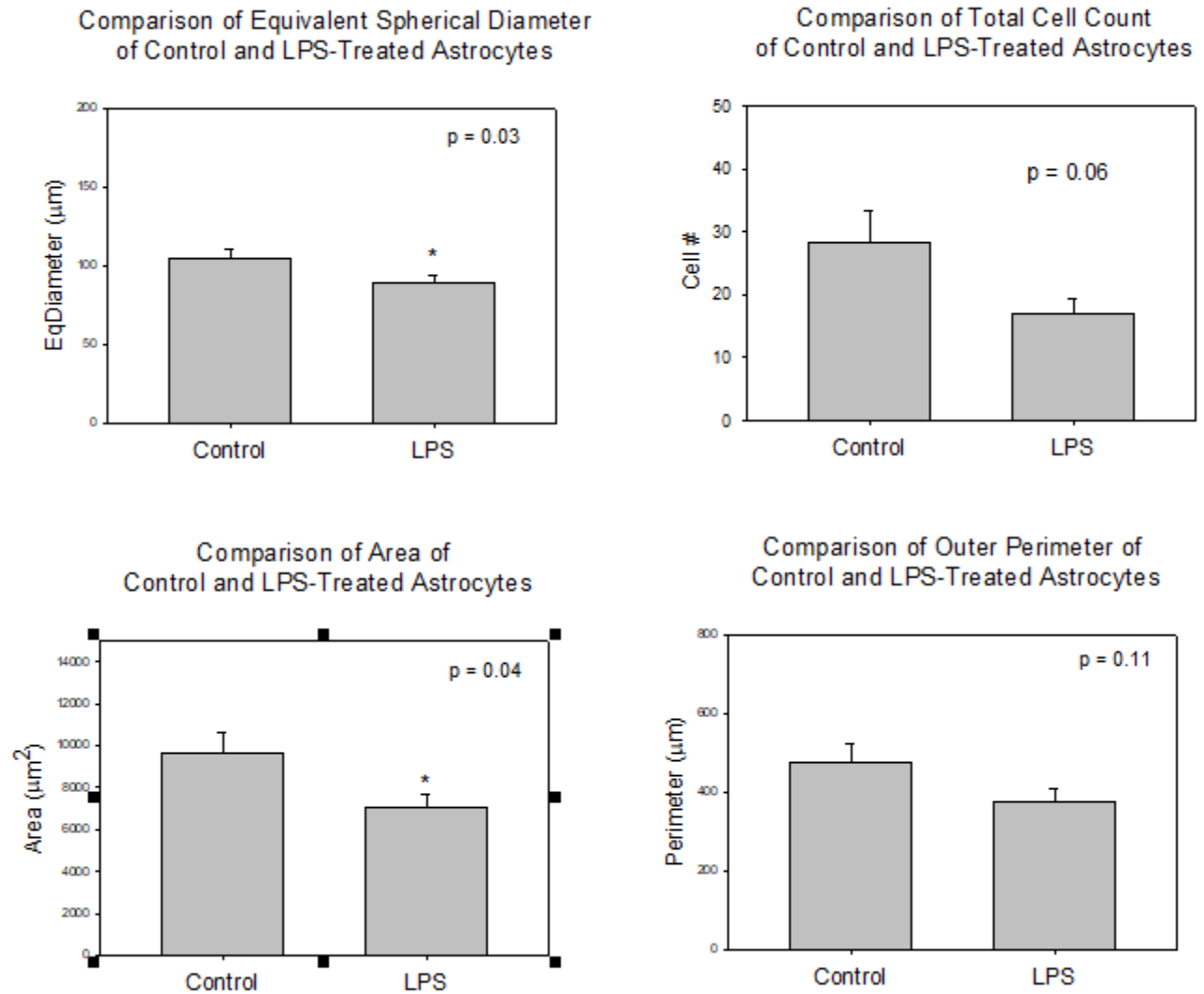


Figure 4: Quantification of equivalent spherical diameter (A), cell number (B), area (C), outer perimeter (D) in astrocyte cultures comparing a 24-hour LPS treatment to control. Data was compared using an unpaired t-test. All data are expressed as mean + SEM. \* indicates  $p < 0.05$

Abbreviations: Lipopolysaccharide (LPS)



Figure 5: Number and Size of Astrocyte Cells with No Treatment, LPS Treatment, and LPS + Cannabinoid Treatment

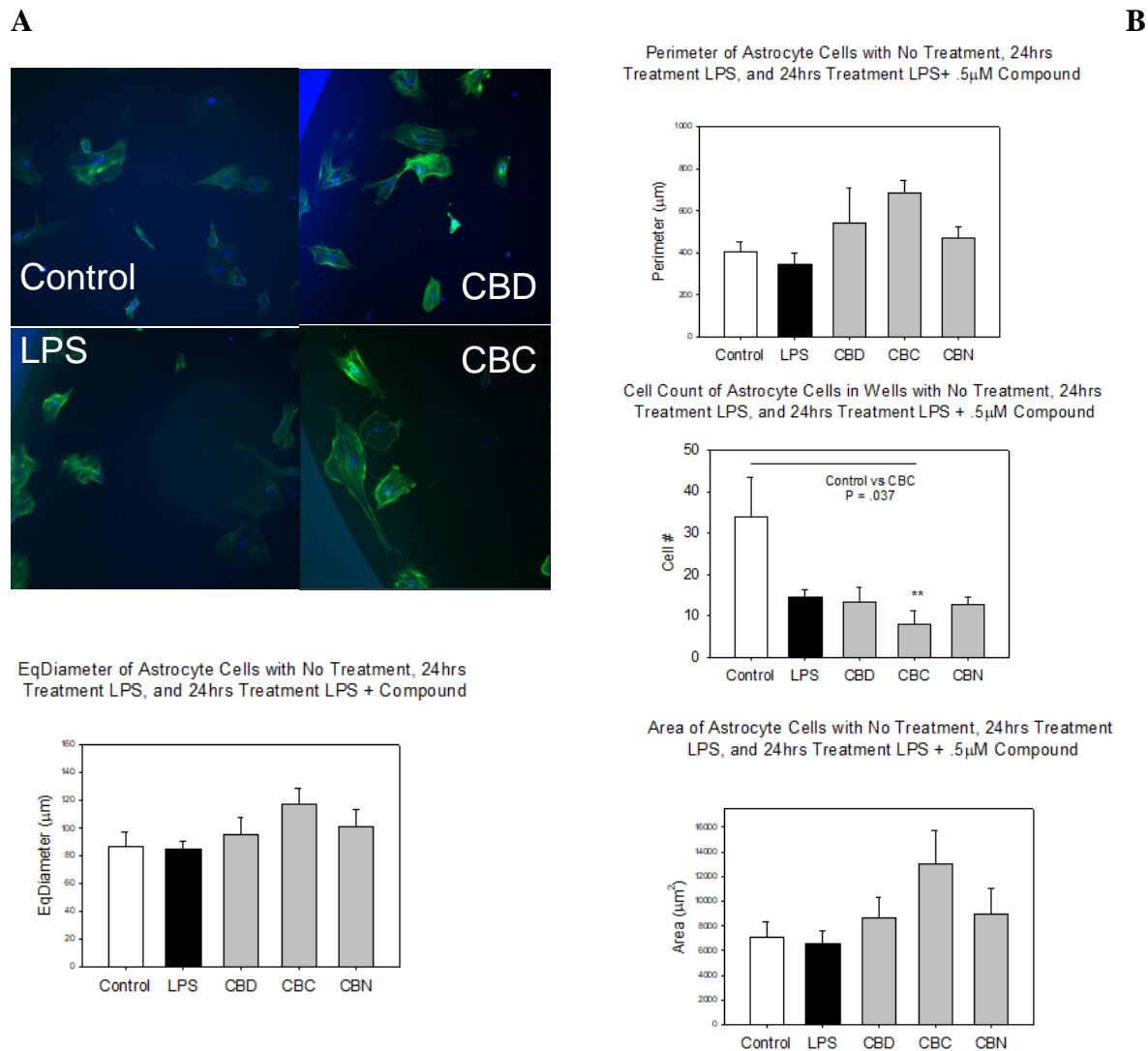


Figure 5: A) Representative images of astrocyte cultures treated with LPS and cannabinoids of interest. The CBD and CBC treatments are at concentrations of 0.125  $\mu$ M and 0.0625  $\mu$ M respectively. B) A screen of perimeter, cell number, equivalent spherical, and area; treatment groups shown are at 0.5  $\mu$ M concentrations. Data was compared using a One-Way ANOVA and

Tukey's post hoc. All data are expressed as mean + SEM. \* indicates  $p < 0.05$  Abbreviations:

Cannabidiol (CBD), Cannabichromene (CBC), Cannabinol (CBN)

Figure 6: Analysis of Cell Count and Morphology in Astrocytes with Control, LPS, and LPS + IGF-1 Treatments

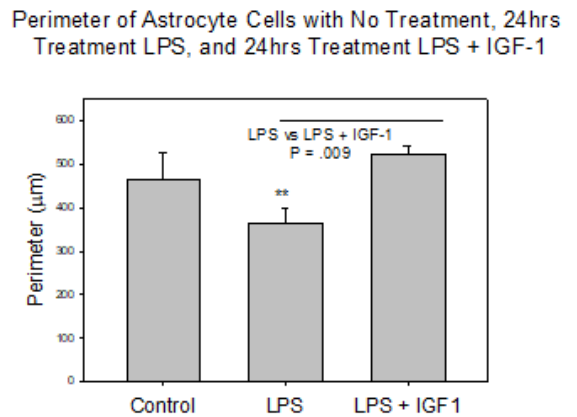
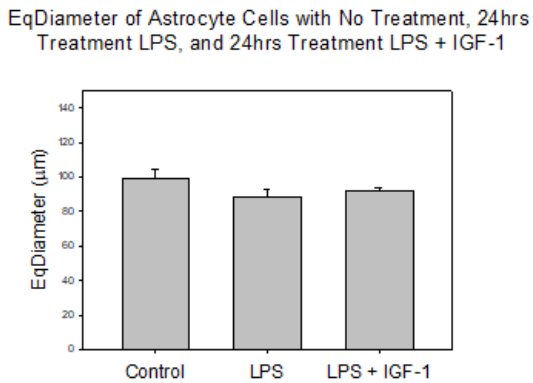
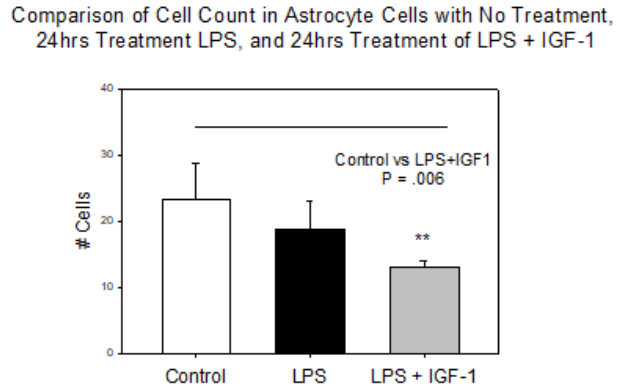
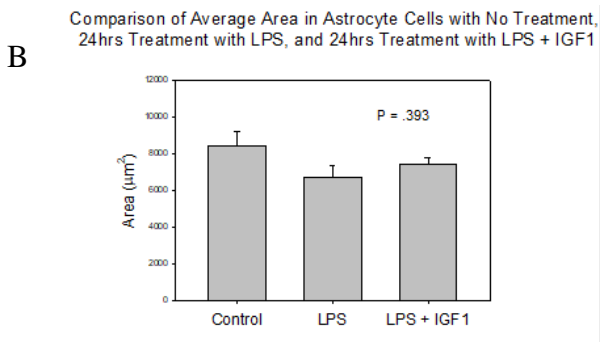


Figure 6: A) Representative Images of Control, LPS, and LPS + IGF-1 astrocyte cultures B) The area, total cell number, equivalent spherical diameter, and perimeter of coverages was quantified as an indicator of cell morphology changes. Data was compared using a One-Way ANOVA and Tukey's post hoc. All data are expressed as mean + SEM. \* indicates  $p < 0.05$  Abbreviations: Lipopolysaccharide (LPS), Insulin-like Growth Factor 1 (IGF-1)

Figure 7: Cell Number per Millimeter in Hippocampus Slices

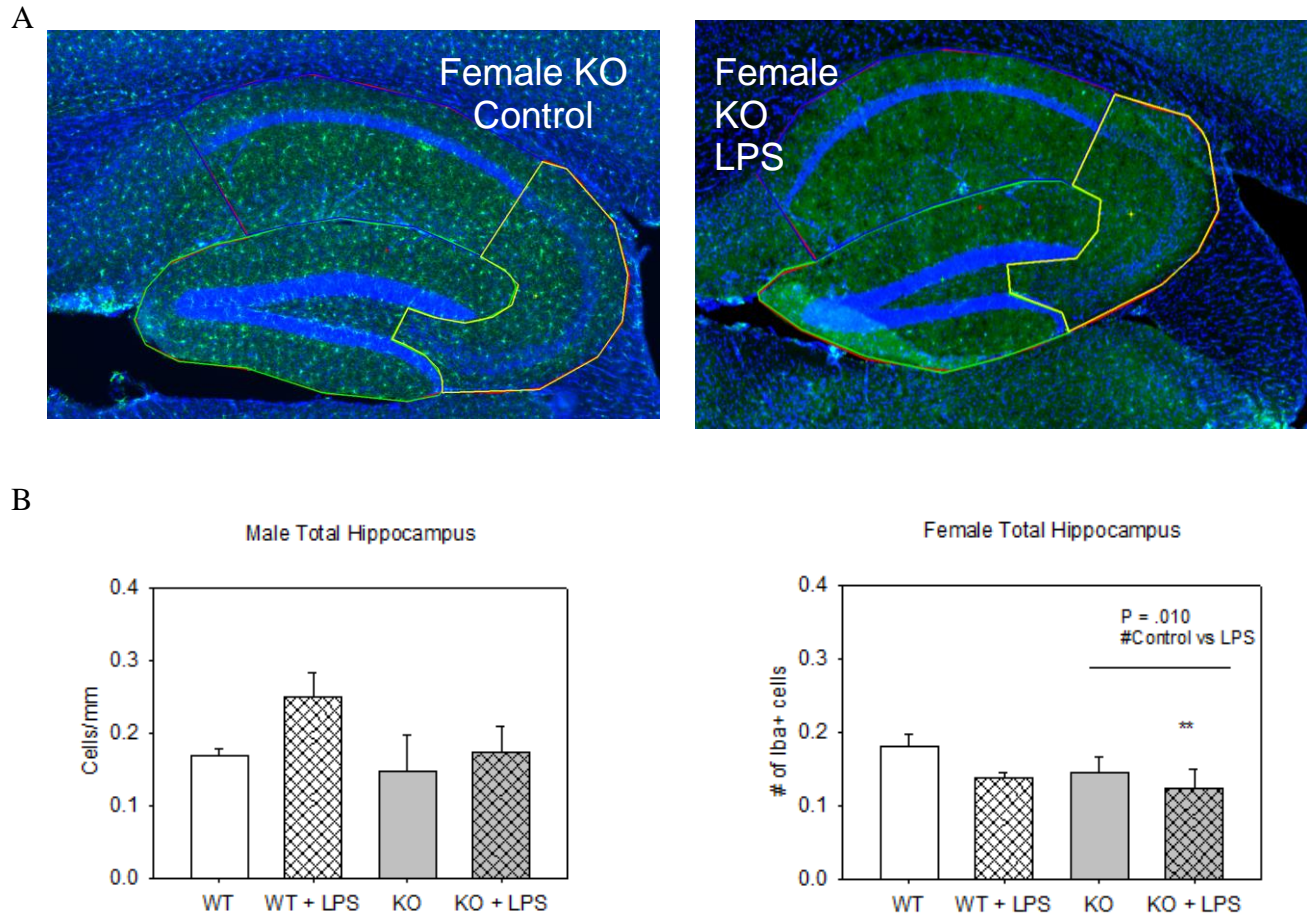


Figure 7: A) Representative images of hippocampal slices of female knockout mice with treatments of control and LPS. B) Quantification of the number of GFAP+ cells per millimeter in hippocampus slices of male and female IGF-1R knockouts and controls along with LPS treatments. Data was compared using a One-Way ANOVA and Tukey's post hoc. All data are expressed as mean + SEM. \* indicates  $p < 0.05$  Abbreviations: Knockout (KO), Lipopolysaccharide (LPS), Wildtype (WT)

## **Discussion**

In this study, we hypothesized that Tat would cause inflammatory effects within glial cells and that treatment with cannabinoids would decrease these effects. The results indicated that HIV-Tat attenuated astrocytic area and equivalent spherical diameter and that of all the cannabinoids of interest, CBD restored both variables to control levels. CBC and Myrcene did not alter astrocytic area and equivalent spherical diameter. Moreover, none of the cannabinoids of interest altered the total astrocyte number. On the other hand, when astrocytes were treated with LPS, we observed a reduction in equivalent spherical diameter and area but not the total cell number, nor perimeter. Furthermore, none of the cannabinoids could restore astrocyte perimeter, cell number, equivalent spherical diameter, or area to baseline control levels. In the context of growth factors, exogenous IGF-1 when given concurrently with LPS further reduced astrocyte number and increased astrocyte perimeter.

Briefly, exogenous HIV-Tat administration to astrocytes was able to alter multiple aspects of glial cell morphology. Researchers have shown that glial cells have varying morphology depending upon their activation state and response to their environment. Astrocytes are the most resistant neuroglia; thus, the observed changes caused by Tat and LPS are indicative of how potent neuroinflammatory stimuli can be. Of the cannabinoids of interest, CBD restored astrocyte area reductions caused by HIV-Tat which could suggest potential neuroglial protective properties of CBD.

Results from our study with LPS and cannabinoids yielded similar results to our investigations using HIV-Tat. Interestingly, LPS did not show a significant increase in any of our

measurements as we expected. These results may indicate that there was a change in overall morphology that did not lead to overt swelling that is sometimes associated with astrogliosis. A recent study observed a similar effect in which LPS caused morphological shifts in cultured astrocytes (Acáz-Fonseca et al., 2019). Specifically, LPS reduced the frequency of polygonal shaped astrocytes and increased the frequency of stellate and other elongated phenotypes (Acáz-Fonseca et al., 2019). Our observed decreases in equivalent spherical diameter and perimeter may support that astrocytes are undergoing a morphological change associated with neuroinflammation; however, we did not quantify the various inflammatory markers associated with reactive astrocytes. In addition, we did not categorize the cells by shape and rather used a mathematical readout indicative of cell shape; henceforth, other studies classifying cell shape/morphology are warranted which could be more intuitive of the type of inflammatory response. Along with potential changes in outer anatomical structure, CBC treatment resulted in a reduction in the number of astrocyte cells compared to the control group and LPS group, with a statistically significant difference from the control. These results can potentially support a Shinjyo et al. study from 2013 that reported a down-regulation of GFAP and nestin with application of CBC. Overall, further studies are essential to understanding how other cannabinoids and terpenes can alter neuroinflammation.

We also examined the effects of insulin-like growth factor-1 on LPS-induced inflammation. A 2011 Park et al. study provided support that exogenous IGF-1 downregulates cytokine production in microglia. Due to this evidence, we predicted that a similar effect would be seen in astrocytes, but there was no significant increase in cell count or area between the vehicle and control groups. Significant differences were between the total cell number in the

control and treatment groups, as well as in the perimeter of vehicle and treatment groups. Overall, these results do not emphasize the claim that cannabinoids or IGF-1 possess any neuroinflammatory protective abilities on reactive astrogliosis from a natural inflammatory promoter-LPS.

Due to the preliminary in vitro evidence from our study and previous findings from other laboratories, we hypothesized that mice with an IGF-1 knockout (KO) genotype would exhibit an increase in cell count and area in comparison to control mice. We observed no significant difference between genotypes that would result in an increase or decrease of cell count or size. Specifically, there was not a significant difference in a singular region nor the whole hippocampus suggesting that intraperitoneal administration of LPS does not induce neuroinflammation in the hippocampus by altering GFAP expression. Interestingly, the total cell number in female mice, when standardized for area, differed between control and LPS groups in KO mice. These results were also seen at an insignificant level in WT mice. Results in the studies of females suggest that IGFR KO is implicated in reactive astrocytes in response to circulating LPS which crosses the blood brain barrier. Interestingly, treatments among males showed a contrasting effect: LPS treatment upregulated the amount of cells/area in both WT and KO mice which was expected. Although these findings do not provide a plausible explanation, they do correlate with sex specific differences in the effects of IGF-1 especially genetic KOs. Conditions such as Multiple Sclerosis, Alzheimer's, and Parkinson's all appear more common in one sex compared to the other; as microglia contributed significantly to the onset of these diseases, different outcomes based on gender would support these claims (Hanamsagar et al., 2016).



### *Limitations and Future Studies*

There is much still to be learned about astrocytes, microglia, and how these neuroglia can modulate neurological inflammation and promote the onset or progression of diseases. One limitation of this study is the use of isolation astrocytes which could have heightened or lessened responses that that in vivo/in silico. We attempted to combat this limitation by quantifying GFAP+ cells in the hippocampus of genetic mutants. However, with this, the limitation is that LPS was administered in an extremely short time window which possibly could not have been enough time for LPS to reach the hippocampal area as it is deep in the limbic system. Future studies aimed at administering LPS directly into the hippocampus using the stereotaxic approach post-KO could provide insights on the true response that astrocytic derived IGF-1 plays on hippocampal reactive astrogliosis. Moreover, other areas and insults should be taken into consideration since hippocampi are primarily composed of neurons. Another limitation which should be investigated is if the knockout of astrocytic derived IGF-1 impacts neurogenesis from the dentate gyrus of the hippocampus and how neuroinflammation impacts that mechanistically. Lastly, future studies may utilize categorization of cells based on quantitative morphology, structural filamentary protein expression, and fluorescent intensity to investigate the differences of cells that have been treated with potentially protective compounds.

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