

Do opioid-activated endothelial cells communicate with astrocytes and microglia in the central nervous system?

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ABSTRACT

Experiments in this thesis were undertaken to support the novel observation that endothelial cells of the brain and spinal cord are involved in the modulation and enhancement of opioid-induced central nervous system inflammation. This thesis will provide evidence that endothelial cells isolated from the central nervous system were activated by morphine's primary metabolite, morphine-3-glucuronide. Additionally, activated endothelial cells, injected into rats were shown to induce a pain response in the rats. Following these experiments involving just endothelial cells, primary astrocytes and microglia were added to *in vitro* studies. Astrocytes were shown to behave differently when cultured alone, compared to when they were cultured with CNS endothelial cells or with CNS endothelial cells and microglia. The difference in behavior and activation seen in these experiments suggests that there is important communication between these cell types, and further exploration of this topic is warranted

ABBREVIATIONS

<i>LPS</i>	Lipopolysaccharide, TLR4 agonist
<i>M3G</i>	Morphine-3-glucoronide, metabolite of morphine, TLR4 agonist
<i>TLR4</i>	Toll-Like Receptor 4
<i>ECs</i>	Endothelial cells
<i>LPS-RS</i>	Lipopolysaccharide from <i>Rhodobacter sphaeroides</i> , TLR4 antagonist
<i>aCSF</i>	Artificial Cerebral Spinal Fluid
<i>CNS</i>	Central Nervous System
<i>BBB</i>	Blood Brain Barrier
<i>GFAP</i>	Glial fibrillary astrocyte protein, Astrocyte activation marker
<i>TNFα</i>	Tumor Necrosis Factor-Alpha
<i>HMGB1</i>	High-mobility group protein-B1
<i>PGE₂</i>	Prostaglandin E ₂
<i>CD11b</i>	Cluster of differentiation molecule 11B, microglial activation marker

INTRODUCTION

Chronic pain affects millions of Americans, yet remains poorly controlled by currently available therapies. Until recently, neurons have been assumed to be the sole cell type responsible for the creation and maintenance of chronic pain. Yet, if neurons hold the key to chronic pain, why do all neuronally-targeted therapeutics fail to control chronic pain in the great majority of pain patients?

Within the past decade there has been growing recognition that non-neuronal cells modulate neurotransmission in the central nervous system (CNS) and importantly contribute to pathological pain states. The activation of non-neuronal cells in the brain and spinal cord called glia, including both microglia and astrocytes, has been directly linked to chronic pain. This project will expand beyond astrocytes and microglia to examine the potential role of CNS endothelial cells. Together, these various non-neuronal cell types can actively respond to and modulate signals within their local environment. When activated as a consequence of neural damage or other stimuli, glia and endothelial cells release a variety of substances that can increase the excitability of nearby neurons. These include pro-inflammatory cytokines, chemokines, ATP, excitatory amino acids, and nitric oxide (Watkins *et al.*, 2007; Nagyoszi *et al.*, 2010; Singh *et al.*, 2007). Glia are often exposed to excitatory neurotransmitters, but it is the excessive release or combination of signaling molecules that raises the alarm, thus signaling the release of glia-derived signaling molecules, such as pro-inflammatory cytokines including tumor necrosis factor α (TNF α), which is also released by activated peripheral immune cells (Owens *et al.*, 2005). When released outside the CNS, these substances serve as a signal to the immune system, so the immune system knows where to respond to the injury (Fields, 2009). These substances can also cause physical changes to the injured tissue and blood vessels as well as the

area surrounding the injury. When released in the CNS, this is known as neuroinflammation. The release of such substances can cause uninjured tissue to become hypersensitive to pain stimuli through dynamic modulation of pain transmission at spinal levels. The various substances secreted by these activated non-neuronal cells can be helpful, unless they are present for prolonged periods of time.

Microglia and Astrocytes

Microglia make up 5-12% of all the cells in the CNS and 5-10% of all glia, while astrocytes are 40-50% of all glia and actually outnumber neurons (Aldskogius *et al.*, 1998; Lee *et al.*, 2000). Activation of microglia results in changes in morphology and function (e.g., retraction of their processes, migration to area of damage, and release of pro-inflammatory mediators; Watkins *et al.*, 2007). Microglial activation can be measured by the upregulation of cluster of differentiation molecule 11B (CD11b). After microglia have been activated, they will either return to their basal state or enter a “primed” state (Perry *et al.*, 1985). These “primed” microglia over-respond to future stimulation by increasing the speed and magnitude in which they release pro-inflammatory signals (Perry *et al.*, 1985). Astrocytes under basal conditions provide neurons with energy sources and neurotransmitter precursors (Tsacopoulos, 2002). Astrocytes can be activated by trauma such as inflammation or infection. This activated state is indirectly reflected by the upregulation of glial fibrillary acidic protein (GFAP) expression (Aldskogius *et al.*, 1998).

Astocytes and microglia exist in close proximity to each other and therefore communicate. When stimulated together, microglia release substances that induce astrocyte activation, and then astrocytes release substances that stimulate microglial activation and proliferation (Watkins *et al.*, 2006). Research by Ledebuer *et al.* suggests that *in vivo*, microglia

are activated first. This activation causes the recruitment of astrocytes, with both cell types ultimately implicated in downstream effects (Ledeboer *et al.*, 2006).

Toll-Like Receptor 4

One class of receptors that plays a key role in neuropathic pain and trauma is the Toll like receptors (TLRs). TLRs are a highly conserved class of receptors found in the innate immune system, but are also expressed on glia and endothelial cells (Watkins *et al.*, 2007; Nagyoszi *et al.*, 2010). When TLRs are activated, they induce the release of neuroexcitatory products such as pro-inflammatory cytokines (Watkins *et al.*, 2009). The specific toll-like receptor, TLR4, is of particular interest in regards to opioid-induced CNS inflammation. TLR4 signaling is activated by signals of cellular damage, distress, and death (Watkins *et al.*, 2009). Until recently, microglia were thought to be the predominant CNS cell type expressing TLR4 (Hutchinson *et al.*, 2008), but current literature has begun to support TLR4 expression on other non-neuronal cells such as astrocytes (Gorina *et al.*, 2011). Experiments completed for this thesis will also provide evidence for expression of TLR4 on CNS endothelial cells, indicating that endothelial cells may play a larger role in pain amplification than is currently understood. The prime position of these cell types within the CNS, allow astrocytes and microglia to enhance pain resulting from damage or inflammation from the peripheral nerves (Watkins *et al.*, 2009). The experiments presented in this thesis will support and provide evidence for both the expression of TLR4 on astrocytes and CNS endothelial cells.

TLR4 activation causes signaling through at least three parallel pathways: the PI3K/Akt pathway, which affects cell motility and cell survival/apoptosis; and the NF κ B and MAPK pathways, which lead to release of pro-inflammatory products (Watkins *et al.*, 2009). Peripheral nerve injury leads to TLR4 activation on microglia and astrocytes in the spinal cord, which

causes the release of pro-inflammatory products leading to pain amplification. Recently it has been discovered that TLR4 is activated not only by endogenous danger signals, but also by opioids such as morphine. TLR4 activation, whether by endogenous danger signals or opioids, leads to the same downstream effects of glial activation and the release of pain enhancing substances (Hutchinson *et al.*, 2008).

Opioid-induced pro-inflammatory signaling

Several studies completed in the last decade have shown that glia cells can become activated in response to opioid treatment, leading to secretion of pro-inflammatory substances including cytokines (Watkins *et al.*, 2005; Hutchinson *et al.*, 2008). Researchers discovered an important link between TLR4 and opioid-induced glial activation when it was found that TLR4 knock-out mice treated with morphine had enhanced analgesia (pain relief; Hutchinson *et al.*, 2009). In *in vivo* studies, it was shown that treatment with “glial inhibitors” such as ibudilast (AV411) and minocycline prior to opioid treatment, potentiated morphine analgesia (pain relief; Hutchinson *et al.*, 2008; Hutchinson *et al.*, 2009). It was also discovered *in vivo* that the same enhanced morphine analgesia could be produced by blocking pro-inflammatory cytokine actions (Hutchinson *et al.*, 2008). This indicates that blocking the activation of glial cells can improve the efficacy of opioid treatment for pain. Morphine can bind to the classical opioid receptors (mu, kappa, and delta) but it also activates TLR4 (Hutchinson *et al.*, 2009). Interestingly, it has also been shown that morphine-3-glucuronide (M3G), morphine’s primary metabolite *in vivo*, activates TLR4 but not the classic opioid receptors (Labella *et al.*, 1979). For these reasons, experiments done for this thesis utilized *in vitro* stimulation with morphine and M3G, along with antagonists of TLR4 or the classical opioid receptors, in order to understand the receptor types implicated in the observed phenomena.

Blood Brain Barrier

The blood brain barrier (BBB) is comprised of endothelial cells within the capillaries (Figure 1). The endothelial cells form tight junctions that act as a selective barrier into the CNS. Astrocyte end feet surround the endothelial cells in the BBB. The function of the blood brain barrier is to

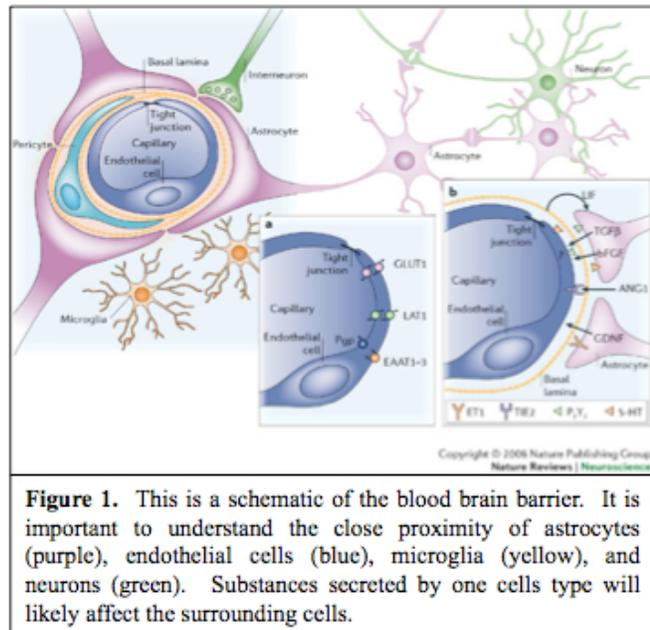


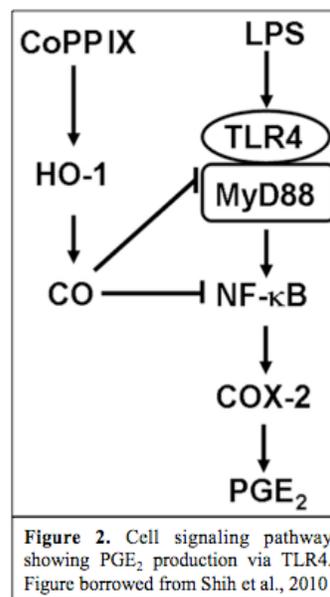
Figure 1. This is a schematic of the blood brain barrier. It is important to understand the close proximity of astrocytes (purple), endothelial cells (blue), microglia (yellow), and neurons (green). Substances secreted by one cells type will likely affect the surrounding cells.

supply the brain with nutrients while also mediating the efflux of waste and keeping unwanted molecules out of the CNS (Abbot, 2006). Because of their incredibly close proximity to each other (Figure 1), endothelial cells, astrocytes, and microglia likely communicate. *In vivo*, microglia and astrocytes interact and their secretions synergize (Watkins *et al.*, 2007). Astrocytic secretions play a role in regulating the integrity of the BBB by controlling the permeability of the tight junctions in the endothelium (Abbot, 2006).

Endothelial Cells

The majority of opioid-induced glial activation research focuses primarily on microglia and astrocytes, but there are several other cell types in the central nervous system that are likely involved in modulating neurotransmission. Specifically, this project will explore the role of the relationship between endothelial cells, astrocytes, and microglia in opioid-induced pro-inflammatory signaling in the brain and spinal cord. As noted above, endothelial cells (ECs) line

the capillaries within the CNS to form the blood-brain barrier. This position allows ECs to act as first-line responders to systemic opioids. As a result they secrete prostaglandin E₂, which is known to facilitate the production of inflammatory mediators (Ma *et al.*, 2008). Prostaglandin E₂ (PGE₂) is an arachidonic acid metabolite formed by PGE synthase from a precursor PGH₂ that is synthesized by both isoforms of cyclooxygenase (COX-1 & COX-2). COX-2 is downstream of TLR4 via the NFκB pathway (see figure 2; Shih *et al.*, 2010).



Shih *et al.* documented the release of PGE₂ from brain endothelial cells when treated with lipopolysaccharide, a known TLR4 agonist (LPS; see methods below).

There are conflicting reports as to whether CNS endothelial cells express TLR4. Nagyoszi *et al.* (2010) reported that TLR4 mRNA was not detectable in primary brain endothelial cells; however, the cells were isolated from the brains of two-week old rats, and receptor expression in adults may not be predicted by expression in pups. Singh and Jiang (2004) reported that TLR4 mRNA was not detected in primary endothelial cells isolated from adult rat brains, but their protocol utilized a 24 hour isolation procedure and the cells were analyzed 24 hour after plating. This time point might not be sufficient time for the cells to recover normal protein expression following the isolation procedure. In contrast, Shih and Yang (2010) and Verma *et al.* (2006) provided evidence for TLR4 expression in mouse brain endothelial cells by measuring TLR4 mRNA expression and cytokine secretion in response to LPS, respectively.

The overall aim of the present experiments is to gain a better understanding of the interaction between non-neuronal cell types across the blood brain barrier. This project will investigate how activated endothelial cells affect astrocytes and microglia. Once these molecular pathways are better understood, this could reveal insights into how to improve opioid efficacy while limiting opioid-induced CNS inflammation and the resulting side effects associated with chronic opioid use.

METHODS

SUBJECTS

The subjects of the experiments were pathogen-free adult male Sprague–Dawley rats (325–350 g; Harlan Laboratories) for the isolation of endothelial cells. Pathogen-free female Sprague-Dawley rats were mated with pathogen free male Sprague-Dawley rats. The pups were born in the Watkins’s lab colony room. The male pups (1-2 days old) were used for the isolation of astrocytes and microglia. The endothelial cells isolated for intrathecal injection and the rats used for the intrathecal EC injection were adult male Lewis rats (325-250 g; Harlan Laboratories) The adult male rats were housed two per cage, while the females were housed one per cage (other than during mating, at which time a single male was housed with each individual female) in a temperature-controlled environment (23+/-2 °C) with a 12 h light/dark cycle (lights on at 7:00 AM), and with standard rat chow and water available *ad libitum*. All procedures occurred in the light phase. All adult rats were allowed 1 week of acclimation to the colony room before being mated. The Institutional Animal Care and Use Committee of the University of Colorado at Boulder approved all procedures.

DRUGS

Morphine-3-glucuronide (M3G), a major naturally occurring, long-lived non-opioid (fails to bind classical opioid receptors) metabolite of morphine that the Watkins laboratory has shown to be a TLR4 agonist (Labella *et al.*, 1979), was purchased from SigmaAldrich (St. Louis, MO, USA). Endotoxin-free morphine sulfate was a gift from Mallinckrodt, Inc. (St. Louis, MO, USA). Endotoxin-free status was confirmed with a limulus amoebocyte lysate (LAL) assay. M3G and morphine (which activates both classical mu-opioid receptors and TLR4) were reconstituted in 0.9% endotoxin-free saline (Abbott Laboratories, North Chicago, IL), diluted to stock concentrations of 10 mM and stored at 4°C, prior to dilution for use as described below. Lyophilized lipopolysaccharide (LPS; a classical TLR4 agonist; 25 mg, Escherichia Coli, serotype 0111: B4, Sigma Lot No. 072k4096) was reconstituted with 0.9% endotoxin-free saline (25 mL) to make a 1 mg/mL stock solution that was then aliquoted and stored at -20°C. Frozen aliquots were thawed at the time of experimentation and diluted with 0.9% endotoxin-free saline (Abbott Laboratories, North Chicago, IL) yielding various final concentrations as described below. Purified lipopolysaccharide from *Rhodobacter sphaeroides*, LPS-RS a classic TLR4 antagonist, was purchased from InvivoGen (San Diego, CA). The lyophilized LPS-RS was reconstituted in 1 mL of endotoxin-free water to make a 5-mg/mL stock solution. The stock solution was aliquoted and stored at -20°C. Stock aliquots were thawed the day of the experiment and diluted to various concentrations.

CELL CULTURES

Mixed Glial Isolation

Mixed glia and neuron primary cell cultures were isolated from the cerebral cortices of newborn male Sprague-Dawley rat pups. Between 1 and 2 days old, the pups were rapidly decapitated and the brains were dissected and placed in cold HBSS w/ Ca²⁺ and Mg²⁺. The

dissected brains were then brought into the sterile cell culture hood. In the hood there were four 60 mm petri dishes on ice, each with 2 mLs HBSS. The brains were placed in one of these dishes. First, the cerebellum and brainstem were gently removed and discarded. Next the cortices were easily separated by gently slicing them using curved tip forceps. One at a time, the hippocampal hemispheres were removed, leaving just the cortex. The meninges were gently pulled off the cortices and moved into the fourth and final petri dish with 2 mLs HBSS. Once all the cerebral cortices were in the final petri dish, the cortices were minced, resuspended in HBSS, and centrifuged. Next the tissue underwent enzymatic digestion in DNase I and Liberase Blendzyme III. Prior to putting the tissue into the flasks, the tissue was centrifuged and put into fresh medium and passaged through a 70-micron filter and then through a 40-micron filter to eliminate any remaining tissue chunks. (Viviani, 2006)

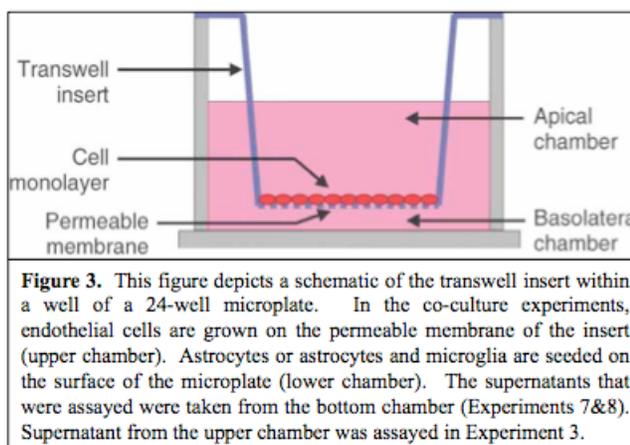
Endothelial Cell Isolation

Primary cultures of endothelial cells were isolated from adult pathogen-free male Sprague-Dawley and Lewis rats using a previously reported protocol. (Perriere *et al.*, 2005). This method yields >98% pure endothelial cell cultures. Tissue and cells isolated from the two rat breeds were treated and seeded separately. The protocol for isolation was the same. Rats were anesthetized using isoflurane and decapitated. The brain was dissected out of the skull and the spinal cord removed from the vertebral column by hydraulic extrusion using ice-cold saline 0.9% endotoxin-free saline (Abbott Laboratories, North Chicago, IL). The tissue underwent two enzymatic digestions and is then centrifuged through a density gradient to isolate microvessels. The isolated microvessel fragments were cultured for 48 hours in medium containing 4 µg/mL puromycin. Puromycin is a translation inhibitor that selectively kills non-endothelial cell types present in the microvessel culture so to achieve a pure endothelial cell culture. Four adult rats were used for each isolation. The brains and spinal cords from the 4 rats were combined prior to

enzymatic digestion. The collected microvessels were plated onto 10 cm culture dishes treated with a collagen I/collagen IV/fibronectin coating matrix. Once the endothelial cells reached 100% confluence (~1 week), the cells were passaged using .25% Trypsin in EDTA (Invitrogen, CA, USA) and seeded onto 24 (4 x 6) well microplates treated with the same collagen I/collagen IV/fibronectin matrix. Cells were grown to confluence to insure the most accurate replication of their *in vivo* presence. For each treatment condition run on these plates the n=6.

SEEDING CELLS ONTO TRANSWELL INSERTS

Polyester Transwell Inserts, purchased from Corning, Corning, NY, were used in the model of the blood brain barrier. The inserts fit inside a typical 24-well plate. The membrane at the bottom of the insert (Figure 3) is permeable. The pores are 0.4 μm in diameter. This is wide enough to let molecules through, but small enough to prevent the cells from migrating through. The total surface area available for cell growth is 0.33 μm . In the co-culture experiments, endothelial cells are grown on these inserts. It is important to note that the two chambers' media do not mix. The endothelial cells are grown to 100% confluence, so nothing can pass through the membrane to get to the lower chamber without being transferred by the endothelial cells themselves.



The following steps were taken to prepare the cells and the inserts for seeding. The inserts were coated in a collagen IV and fibronectin mixture. The coating matrices were removed and the inserts were rinsed with PBS. Endothelial cells were passaged from 10 cm

dishes using 0.25% Trypsin in EDTA to release the adherent cells while minimizing damage to them, in an incubator for 5 minutes. The trypsin was inactivated by the cell culture medium containing FBS and the cells were spun down at 200 x g for 5 minutes. FBS contains inhibitors of trypsin, and therefore halts the enzymatic action. The cells were then resuspended in culture medium containing 500 nM hydrocortisone, which is the physiological level at which it is present *in vivo* (Weidenfeller *et al.*, 2005). Hydrocortisone helps maintain the tight junctions made by the endothelial cells once seeded on the transwell inserts (Hoheisel *et al.*, 1998). The well below the transwell insert contained 1 mL of medium containing hydrocortisone, so that the cells had medium on both sides. In this set up, the upper chamber within the insert serves as the luminal side, or blood side, and the lower chamber (the 24 well plate) serves as the abluminal, or CNS side. Once the ECs were seeded onto the inserts, they were left to grow to 100% confluence (2 days).

ELISA

Prostaglandin-E₂

Prostaglandin-E₂ (PGE₂) concentrations were measured using an enzyme-linked immunosorbent assay (ELISA). The assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA) and used to measure the amount of PGE₂ secreted into the culture media. Samples of the medium were collected and frozen at -80°C prior to use. This kit is based on the competition between PGE₂ (from the samples) and PGE₂-acetylcholinesterase (PGE₂ tracer added by the kit) for antibody binding. The concentration of tracer is held constant, while the PGE₂ levels are variable within each sample. Thus there is an inverse relationship between the tracer and PGE₂; the more PGE₂ there is in a sample, the more displacement of PGE₂ tracer will occur. After the 18-hour incubation, the plate is washed and developed using Ellman's reagent

(included in kit). The Ellman's reagent undergoes an enzymatic reaction and develops a distinct yellow color that absorbs strongly at 412 nm. The more intense yellow color is inversely proportional to the amount of PGE₂ present during incubation. Concentrations are measured at 412 nm using a spectrophotometer.

TNF α

TNF α secretions were measured using the R&D immunoassay kit (R&D, MN, USA). This assay uses a monoclonal antibody specific for rat TNF α that has been pre-coated onto the bottom of a microplate. Samples from cell supernatants were frozen at -80°C prior to use. Cell supernatants were pipetted into the pre-coated wells, any TNF α present in the sample binds the immobile antibody already present in the well. After washing off any unbound substances, an enzyme-linked polyclonal antibody specific to rat TNF α was added. Following a wash to remove any unbound enzyme-antibody, a substrate solution was added to the wells. The ensuing enzymatic reaction creates a blue product that turns yellow upon addition of the Stop Solution . The intensity of the color is proportional to the amount of TNF α present in the sample.

IN-CELL WESTERN BLOTS

Cells (ECs, astrocytes and microglia) were grown in 24 well microplates and stimulated as explained in each experimental design, below. After stimulation, In-cell Western blots were performed. Culture media was removed and 1 mL fresh 4% paraformaldehyde was added to each well and incubated at room temperature for 5 minutes. The paraformaldehyde was removed and the cells underwent five washes in PBS + 0.1% Triton X-100 at room temperature to permeabilize the cells. The cells then were incubated in Odyssey blocking buffer (LI-COR, Lincoln, NE) for 1 hour on a shaker at room temperature. The blocking buffer was removed and a solution containing primary antibody diluted in fresh Odyssey blocking buffer was put on to

the cells and incubated overnight on a shaker at 4°C. Each plate had 1 well that did not have primary antibody, to serve as a negative control for the plate. Primary antibodies used were raised against glial fibrillary acidic protein (GFAP; astrocyte activation marker; Mouse IgG (Cell Signaling) and CD11b (detected with monoclonal OX42 antibodies; a complement receptor subunit that is a microglial activation marker; Mouse IgG). After the overnight incubation, the microplates were washed 4 times with PBS + 0.1% Tween-20 at room temperature on a shaker. The plates were then incubated with an Odyssey blocking buffer solution containing secondary antibody, 0.2% Tween-20, and the cell stains DRAQ5 (Biostatus Limited, Leicestershire, United Kingdom) and Sapphire700 (LI-COR). DRAQ5 is a cell permeable DNA-interactive agent that can be used for stoichiometric staining of DNA in live or fixed cells. Sapphire700 is a non-specific cell stain that accumulates in both the nucleus and cytoplasm of fixed or dead cells, but not live cells. Staining cells with Sapphire700 and DRAQ5 expands the linear range, which allows more accurate normalization of cell number across various cell densities. The control well solution did not contain the cell stains. Secondary antibodies used were IRDye 800CW conjugates of goat-anti-mouse-IgG (LI-COR). The plate incubated in the secondary antibody solutions for 1 hour on a shaker at room temperature protected from light. Then the plates were washed 4 times with PBS + 0.1% Tween-20 in the dark, on a shaker at room temperature. After the last wash the wash buffer was removed and the excess was tapped against paper towels to remove excess moisture. The plates were scanned and analyzed with an Odyssey IR scanner with Odyssey imaging software (LI-COR). The antibody signals were analyzed as the 800-channel integrated intensities normalized to the 700-channel signal integrated intensity to correct for well-to-well variations in cell number. Results are expressed as percent relative responses compared to vehicle-treated control wells.

VON FREY TEST FOR TACTILE ALLODYNIA

Rats were habituated to the wire grill testing apparatus for 1 h per day on 4 consecutive days. The von Frey test (Chaplan *et al.*, 1994) was performed within the sciatic innervation area of the plantar hindpaw as described previously (Milligan *et al.*, 2000), using calibrated Semmes-Weinstein monofilaments (Stoelting, Wood Dale, IL). The von Frey hairs used range from 0.407 – 15.136 g, with $\log \text{ stiffness} = \log_{10} (\text{milligrams} \times 10)$. This range of stimuli produces a logarithmically graded slope (Chaplan *et al.*, 1994). The von Frey hairs were applied randomly to the left and right hindpaws to determine the stimulus intensity required to elicit a paw withdrawal response. For all experiments, von Frey testing was performed blind with respect to treatment group. The raw behavior data were used to calculate the absolute thresholds by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method, which allows for parametric statistical analyses (Harvey, 1986; Treutwein and Strasburger, 1999; Milligan *et al.*, 2000). Data are presented as the left and right paw responses averaged together for each rat.

mRNA EXTRACTIONS and cDNA SYNTHESIS

Total RNA was extracted from the isolated and stimulated cells using the standard phenol/chloroform extraction with TRIzol Reagent (Invitrogen, CA, USA) according to the guidelines set out by the manufacturer. Total RNA was reverse transcribed into cDNA using Superscript II First-Strand Synthesis System (Invitrogen). cDNA was synthesized using random primers (5ng/ μ l) and 1mM dNTP mix (Invitrogen) and incubated at 65°C for 5 min. The samples were placed on ice for 2 minutes. Next, cDNA synthesis buffer (Invitrogen,) and dithiothreitol (DTT- 10 mM) was added to the total RNA mixture and incubated at 25°C for 2 min. Finally reverse transcriptase (Superscript II; 200 U; Invitrogen) was added and incubated

for 10 min at 25°C, 50 min at 42°C, and deactivating the enzyme at 70°C for 15 min. cDNA was diluted twofold in nuclease-free water and stored at -80°C.

RT-PCR

Primers were designed to span an intron to eliminate genomic interference because the samples did not undergo DNase treatment given the small cell numbers analyzed. Amplification of the cDNA was performed using QuantiTect SYBR Green PCR kit (QIAGEN) in an iCycler iQ 96-well PCR plate (Bio-Rad) on a MyiQ single Color Real-Time PCR Detection System (Bio-rad). The reaction mixture was composed of QuantiTect SYBR Green, 10 nM fluorescein, 500 nM of each forward and reverse primer (Invitrogen, CA, USA), nuclease-free water, and 1 µl of cDNA from each sample. Each sample was measured in triplicate. The incubation times were as follows: hot start at 95°C for 25 min, followed by 40 cycles of 15 seconds at 94°C (denaturation), 30 sec at 55–60°C (annealing), and 30 sec at 72°C (extension). The PCR products were measured using SYBR Green I fluorescence in MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad).

STATISTICS

Data were analyzed using One Way ANOVA tests, with Dunnett's Post-hoc test. A p-value of .05 was used as criteria for significance. The data for Experiments 1 and 2 were analyzed using a Two Way ANOVA.

EXPERIMENTAL DESIGN and RESULTS:

EXPERIMENT 1:

The effects of endothelial cells stimulated with M3G and TLR4 antagonist, LPS-RS, on gene expression.

The aim of this experiment was to provide support for the hypothesis that endothelial cells in the CNS express TLR4. Endothelial cells were isolated (as seen above), trypsinized and passaged onto 24 well microplates and allowed to grow to confluence (~2 days). Then cells were stimulated with either the control (saline) or LPS-RS (10 ng/mL) for 1 hour and then treated with M3G (0.001 μ M, 0.01 μ M, 0.1 μ M, 1 μ M) for 4 hours. After 4 hours incubation, plates were prepared for mRNA extractions and ultimately underwent PCR to examine TNF α gene expression. LPS-RS is a classic TLR4 antagonist and M3G selective binds TLR4. If ECs express TLR4, it would be reasonable to expect a downregulation of TNF α mRNA in cells treated with LPS-RS prior to M3G treatment. Additionally, the cells treated with M3G would upregulate TNF α mRNA (pro-inflammatory cytokine).

EXPERIMENT 1 RESULTS:

The effects of endothelial cells stimulated with M3G and TLR4 antagonist, LPS-RS, on gene expression. (Figure 4)

The data from this experiment support the hypothesis that endothelial cells do indeed express TLR4. The cells treated with the control and then M3G (0.01 μ M, 0.1 μ M, 1 μ M) significantly ($P < 0.0001$; $F_{(1,49)} = 21.41$) upregulated TNF α mRNA expression (Figure 4). Endothelial cells that were treated with

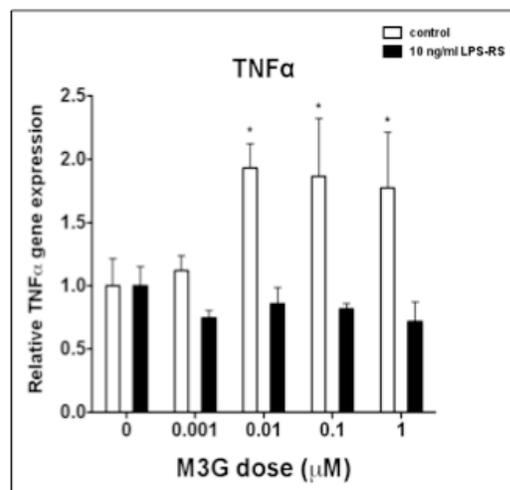


Figure 4. Endothelial cells were pretreated with LPS-RS or control followed by M3G. TNF α gene expression was measured. $n=6$ for each condition * $p < 0.0001$. This figure the cells treated with the control and then M3G (0.01 μ M, 0.1 μ M, 1 μ M) significantly (* $P < 0.0001$; $F_{(1,49)} = 21.41$) upregulated TNF α mRNA expression. Endothelial cells that were treated with LPS-RS (10 ng/mL) and M3G (0.001 μ M, 0.01 μ M, 0.1 μ M, 1 μ M) did not differ from the control/control stimulated cells in their TNF α mRNA expression.

LPS-RS (10 ng/mL) and M3G (0.001 μ M, 0.01 μ M, 0.1 μ M, 1 μ M) did not differ from the control/control stimulated cells in their TNF α mRNA expression. These data are evidence that endothelial cells can mediate TLR4 specific substances and express TLR4. This data also conferred that endothelial cells are activated by M3G.

EXPERIMENT 2:

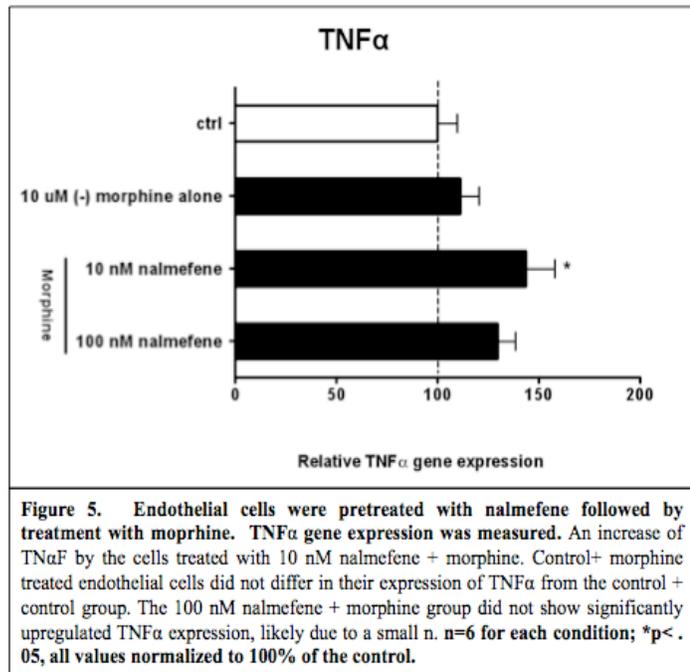
The effects of endothelial cells stimulated with morphine and pan opioid receptor antagonist, nalmefene, on TNF α gene expression in endothelial cells.

This experiment investigates TLR4 expression on endothelial cells. Nalmefene is a pan opioid receptor antagonist that blocks all types of classic opioid receptors (μ , Δ , κ) but does not modulate TLR4. Endothelial cells express μ_3 opioid receptors, which function in an anti-inflammatory role (Stefano *et al.*, 1996). Morphine is capable of binding both TLR4 and classical opioid receptors, but these pathways have confounding effects when stimulated together. By stimulating endothelial cells with nalmefene, to block the μ_3 opioid receptor, prior to treating the cells with morphine, the pro-inflammatory effects of the TLR4 cascades should be uncovered. Endothelial cells were isolated and passaged onto 24 well microplates. Cells were treated with control (saline)+ control, control + morphine alone (10 μ M), 10 μ M nalmefene + morphine (10 μ M), or 100 nM nalmefene + morphine (10 nM) (n=6 in each condition). Cells were stimulated for 1 hour with either the control or nalmefene (10 nM, 100 nM) and then with either control or morphine (10 μ M) for 4 hours. After 4 hours, the cells were prepared for mRNA extractions and were ultimately measured for changes in TNF α mRNA expression.

EXPERIMENT 2 RESULTS:

The effects of endothelial cells stimulated with morphine and pan opioid receptor antagonist, nalmefene, on TNF α gene expression in endothelial cells. (Figure 5)

Endothelial cells were stimulated for 1 hour with either the control or nalmefene (10 μ M, 100 μ M) and then with either control or morphine (10 μ M) for 4 hours. Upon analysis of the mRNA expression for TNF α there was found to be a significant ($p < .05$; normalized to 100% control; $n=6$ in each treatment condition) increase by the cells treated with 10 nM nalmefene + morphine. This suggests



that morphine bound to TLR4 and initiated a pro-inflammatory response. Control+morphine treated endothelial cells did not differ in their expression of TNF α from the control + control group. This data also supports the confounding results of morphine binding both TLR4 and the classic μ_3 opioid receptor. The data from this experiment and Experiment 1 provide suggest the CNS endothelial cells express TLR4 and can be activated by treatment with morphine.

EXPERIMENT 3:

The effects of opioid stimulation of endothelial cells on PGE $_2$ secretion.

This experiment investigated the secretion of PGE $_2$ from polarized ECs. Endothelial cells were polarized by seeding the cells onto transwell inserts (see methods above; Figure 3 for transwell schematic). aCSF was put in the bottom chamber, while serum containing medium was in the upper chamber (blood side). This set up provides an environment that is similar to endothelial cells *in vivo*. The aim was to observe if there were differences in the secretions abluminally (into CNS) compared to luminally (into blood). Endothelial cells on inserts were treated with control

(saline) and M3G (0.001 μ M, 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M) for 24 hours. After 24 hours, samples were collected from both upper and lower chambers and were assayed using the PGE₂ ELISA kit (Cayman).

EXPERIMENT 3 RESULTS:

The effects of opioid stimulation of endothelial cells on polarized PGE₂ secretion. (Figure 6)
Using a culture design in order for

endothelial cells to secrete substances both abuminally (into CNS) and luminally (into blood), ECs were stimulated on transwell inserts and measured for PGE₂ protein secretion after 24 hours of incubation with control (saline) and M3G (0.001 μ M, 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M). Overall, there seemed to be a trend in the data that more

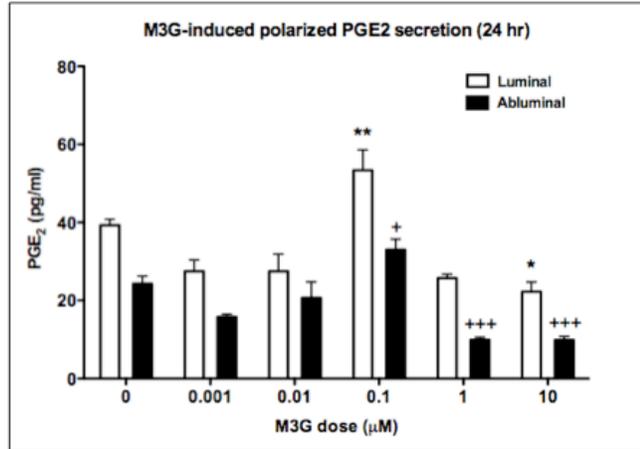


Figure 6. Endothelial Cells were stimulated on transwell inserts and measured for PGE₂ protein secretion after 24 hours of incubation with control (saline) or M3G (0.001 μ M, 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M). Endothelial cells treated with 0.001 and 0.01 μ M M3G did not differ from the control in their secretion of PGE₂ luminally or abuminally. The 0.1 μ M dose of M3G significantly enhanced the secretion of PGE₂ luminally and abuminally. The 1 μ M and 10 μ M doses of M3G significantly reduced abuminial secretion of PGE₂, possibly indicating negative feedback onto the ECs. n=6 for each treatment condition. *P=.05, **P=.01 compared to luminal control; +P=.05, +++P=.001 compared to abuminial control

PGE₂ was secreted luminally compared to abuminally. Endothelial cells treated with 0.001 and 0.01 μ M M3G did not differ from the control in their secretion of PGE₂ luminally or abuminally. The 0.1- μ M dose of M3G significantly enhanced the secretion of PGE₂ luminally (p=. 01) and abuminally (p=. 05). The 1 μ M and 10 μ M doses of M3G significantly (p=. 001) reduced abuminial secretion of PGE₂, possibly indicating negative feedback onto the ECs. Additionally, through other experiments, minocycline (thought to be a glia specific inhibitor) was found to cause a decrease in PGE₂ secretion by endothelial cells (Ramos *et al.*, manuscript in preparation).

EXPERIMENT 4:

The effects of intrathecal injection of stimulated endothelial cells on tactile allodynia.

Endothelial cells were treated with M3G (0.1 μ M), morphine (10 μ M), and a control (saline) for 18 hours. The cells were prepared for intrathecal injection into male Lewis rats. The rats were then behaviorally tested for allodynia (pain response to a non-painful stimuli) using von Frey testing (see above) at 1,2,3, and 24 hours post intrathecal injection of stimulated cells. To prepare endothelial cells for injection, the medium was removed following drug stimulation (0.1 μ M M3G, 10 μ M morphine, and control (saline)) and the cells were rinsed twice with PBS. The cells were then incubated with 0.05% trypsin containing EDTA (Invitrogen) for 2 min at 37°C. DMEM/F-12 containing 10% FBS was added to stop the enzyme, and the cells were centrifuged for 5 min, 180 \times g. The supernatant was removed and cells were resuspended in PBS at a density of 1,000 cells per 10 μ l.

For injection of the stimulated cells, rats were lightly anesthetized with isoflurane. The lumbar region of the back was shaved and cleaned with 70% ethanol. An 18-gauge guide needle, with the hub removed, was inserted into the L5/6 intervertebral space. A PE-10 catheter was threaded through the guide needle, pre-marked such that the distal end of the PE-10 tubing rested over the L4–L6 lumbar spinal cord. Cells were injected over 15 s (10 μ l of PBS containing 1,000 cells followed by 6 μ l of sterile PBS flush) with a 30 s delay before removing the catheter and guide needle. Each animal was anesthetized for a maximum of 5 min, and none exhibited neurological damage from the procedure.

EXPERIMENT 4 RESULTS:

The effects of intrathecal injections of stimulated endothelial cells on tactile allodynia in Lewis rats. (Figure 7)

After months of modifying this experiment to reduce confounding data, allodynia was finally

produced by intrathecal injection of endothelial cells treated with 0.1 μM M3G.

This group was significantly different than the other groups (vehicle (PBS), saline-stimulated cells, and 10 μM morphine-stimulated cells) at 1,2,3 hours post-injection ($p < 0.0001$; interaction of treatment and time, $F_{(12, 160)} = 5.121$). This

indicates that M3G activated endothelial cells are sufficient to cause pain *in vivo*.

Additionally, these data and the polarized endothelial cell secretion of PGE₂ data saw

the most significant effects occur with treatment of 0.1 μM M3G. Because of the significance seen using 0.1 μM M3G, this concentration was used repeatedly in the following experiments.

Following the promising data collection from endothelial cells, both *in vitro* and *in vivo*, the role endothelial cells play in relation to astrocytes and microglia was examined (Experiments 5-10).

EXPERIMENT 5:

The effects of four-hour opioid stimulation of astrocytes, cultured alone, on gene expression.

In Experiment 1, astrocytes were seeded alone in a microplate and stimulated with saline (control), 100 ng/mL LPS, 0.1 μM M3G, or 10 μM morphine. Astrocytes were isolated from

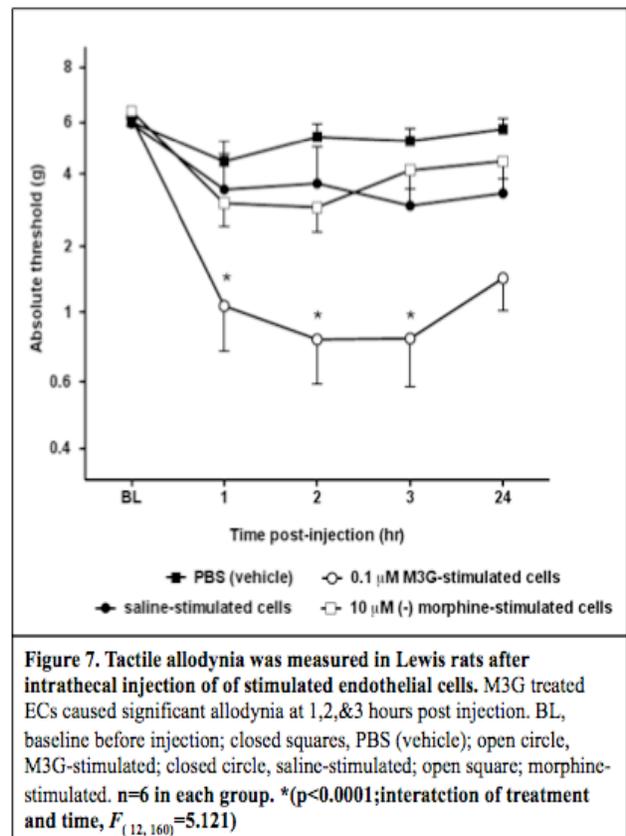


Figure 7. Tactile allodynia was measured in Lewis rats after intrathecal injection of of stimulated endothelial cells. M3G treated ECs caused significant allodynia at 1,2,&3 hours post injection. BL, baseline before injection; closed squares, PBS (vehicle); open circle, M3G-stimulated; closed circle, saline-stimulated; open square; morphine-stimulated. n=6 in each group. *($p < 0.0001$; interaction of treatment and time, $F_{(12, 160)} = 5.121$)

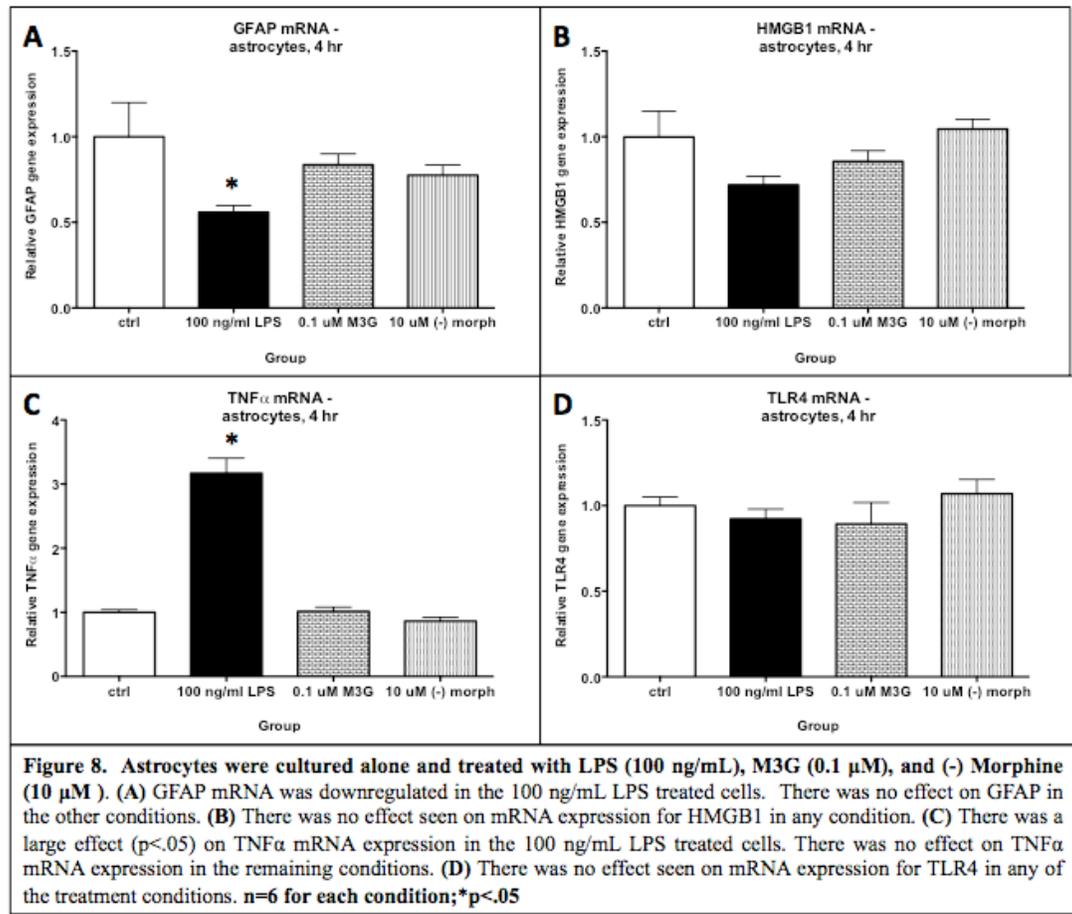
neonatal Sprague Dawley rats as described above. After the flask reached confluence (approx. 8 days), microglia were shaken off and an L-leucine methyl ester solution was applied and incubated for 2 hours to kill the remaining microglia, to achieve a pure sample of astrocytes. The astrocytes were resuspended, plated at ~200,000 cells/well and incubated in a CO₂ incubator at 37°C for 2 days prior to stimulation with drug. Drugs were administered to each well using a repeat pipettor, and the plate was placed back into the CO₂ incubator for the duration of the stimulation. After 4 hours of incubation, the cell media containing the respective drugs were removed. 800 µL of Trizol (Invivogen) was added to each well of cells and the plate was frozen at -80°C until mRNA extractions, cDNA synthesis, and RT-PCR was performed. The cells were tested for changes in expression of mRNA for HMGB1, TNFα, TLR-4, and GFAP. HMGB1 and TNFα are pro-inflammatory cytokines that were measured to understand the pro-inflammatory secretions that are implications of TLR4 signaling.

EXPERIMENT 5 RESULTS:

The effects of four-hour opioid stimulation of astrocytes, cultured alone, on gene expression. (Figure 8)

The aim of this experiment was to determine the effect on astrocytes when these primary cells were stimulated directly with opioids (morphine, which binds both TLR4 and mu opioid receptors; M3G which binds only TLR4) for 4 hours. No prior study has explored whether astrocytes will respond to M3G, and no prior study has explored morphine-induced effects on these endpoints in astrocytes. Until recently, the literature generally assumes that astrocytes do not express TLR4 either at an mRNA or protein level. Gorina *et al.*, in early 2011 published a paper suggesting the primary spinal astrocytes *do* express TLR4. Quantitative RT-PCR was completed to test for changes in mRNA for HMGB1, TNFα, GFAP, and TLR4 (Figure 8). These genes were chosen for analysis because TNFα and HMGB1 are both pro-inflammatory cytokines

that are important in the downstream effects of TLR4 activation; GFAP is an activation marker for astrocytes; and TLR4 is the receptor of interest that is suggested to be the culprit in these situations of pro-inflammatory signaling. To avoid contamination with fibroblasts (which can also express GFAP), the meninges were removed from the cerebral cortices prior to enzymatic digestion. This method provides >90% pure astrocyte culture (Viviani, 2006). There was no difference between the treatment groups and the control for the expression of HMGB1 or TLR4. This easily detected TLR4 mRNA expression is especially interesting as there is so little literature on astrocyte expression of TLR4. There was a large effect on TNF α mRNA expression in the LPS-stimulated cells (P<. 05). This result is consistent with the fact that LPS is a TLR4 agonist and astrocytes are recently found to express TLR4 receptors. The astrocytes in culture are rapidly dividing. The apparent downregulation in GFAP expression was not a proliferation effect; rather there was true downregulation of GFAP. This down regulation of GFAP could be due to negative feedback. There is precedence of LPS stimulation downregulating astrocyte proliferation (Letournel-Boulland *et al.*, 1994).



EXPERIMENT 6:

The effects of twenty-four-hour opioid stimulation of astrocytes, cultured alone, on gene expression.

In Experiment 2, the astrocytes were prepared as in Experiment 1. In this experiment, the cells were incubated for 24 hours. One plate was stimulated with morphine (1 μ M, 10 μ M, and 100 μ M) and M3G (0.01 μ M, 0.1 μ M, and 1 μ M). Each plate was prepared for mRNA extractions as in Experiment 1. mRNA changes were quantified for HMGB1, TNF- α , TLR-4, and GFAP. On a third plate, cells were incubated in 100 ng/mL LPS, 0.1 μ M M3G, and 10 μ M morphine. After 24 hours, samples were taken from each well for a cytokine ELISA measuring secreted levels of TNF- α . The remaining media was removed and the cells were used for an In-

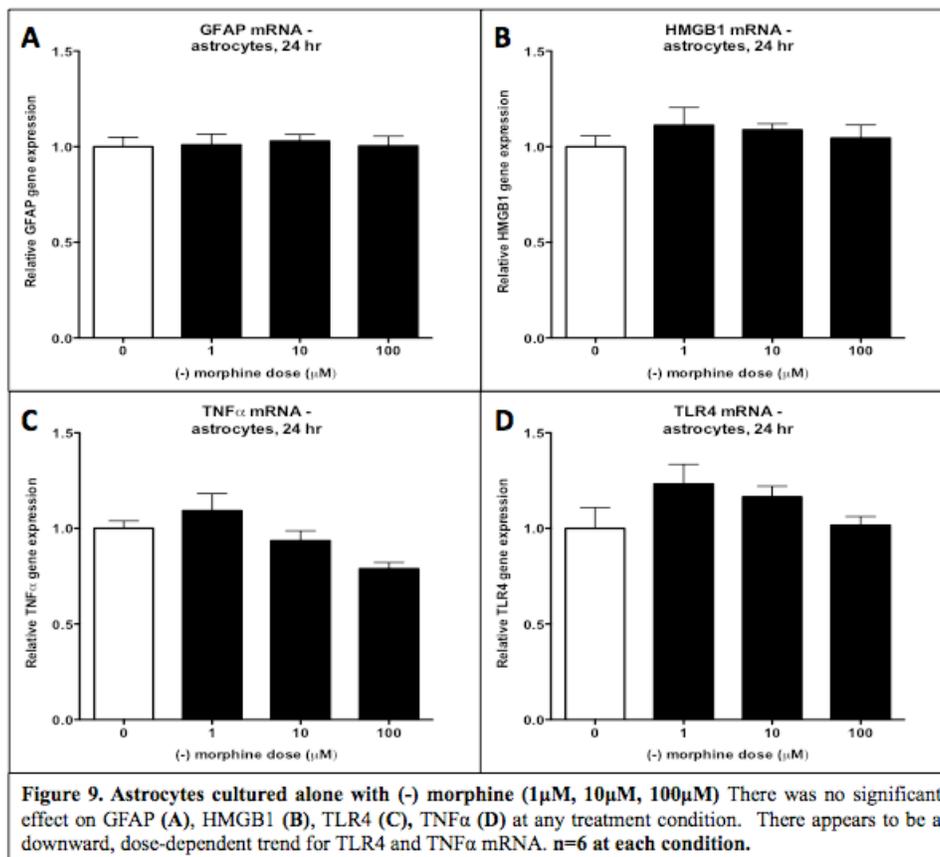
cell Western blot using an antibody against GFAP. The in-cell western protocol was completed as described above.

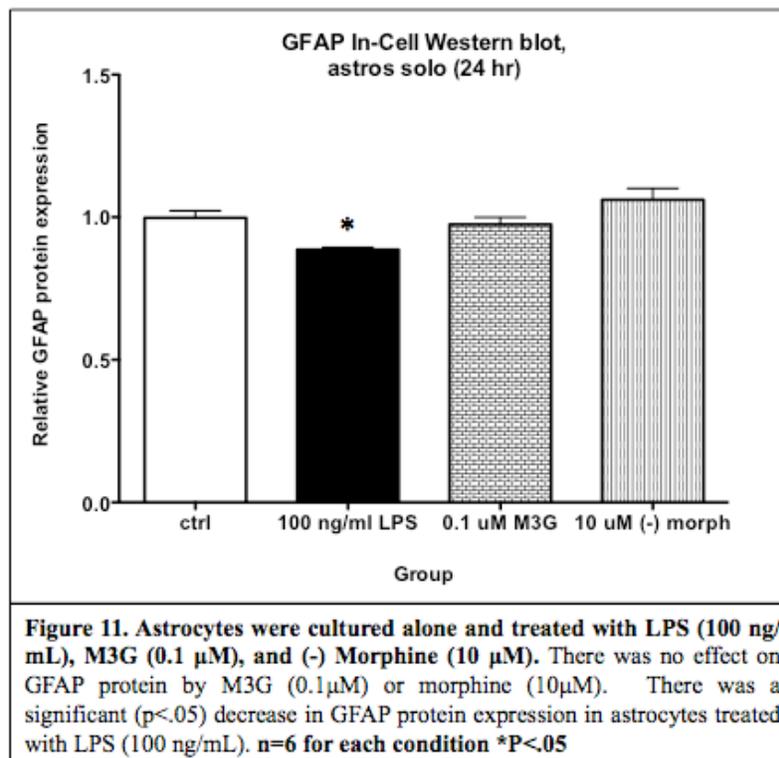
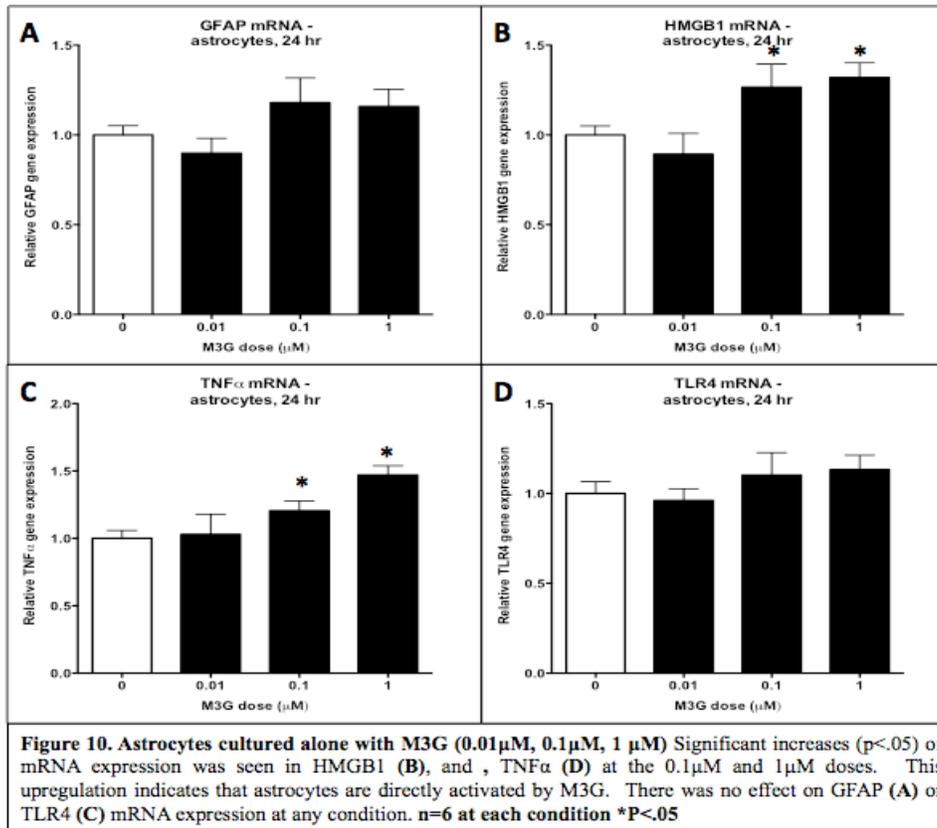
EXPERIMENT 6 RESULTS:

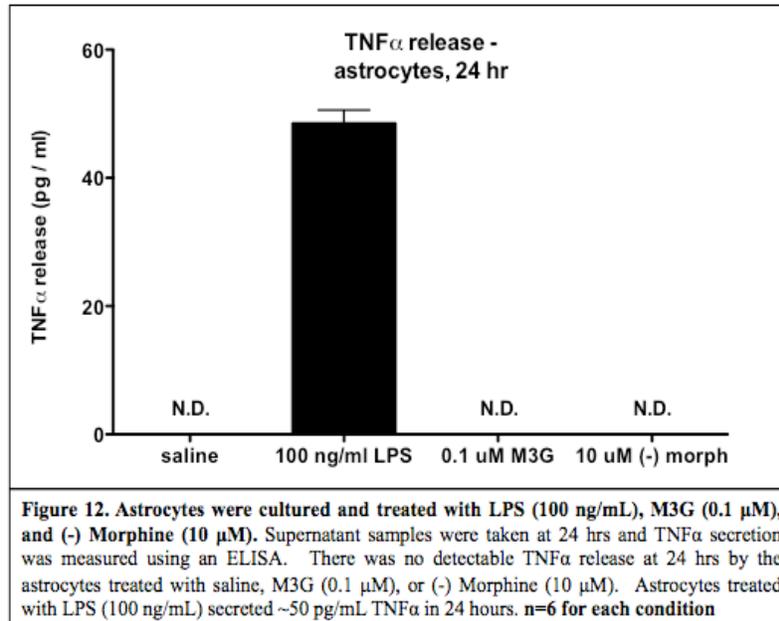
The effects of twenty-four-hour opioid stimulation of astrocytes, cultured alone, on gene expression. (Figures 9-12)

Similar to Experiment 1, this experiment examined the direct affect of opioids on astrocytes, but for a 24-hour stimulation. Stimulations were extended from the 4 hours seen in Experiment 1 to 24 hours to see if the effects of direct opioid stimulation of astrocytes were different than data collected at 4 hours. Morphine and M3G were administered in a dose response as to allow a better understanding of the interaction of opioids with astrocytes. Again, contrary to much of the available literature, evidence was accrued for basal expression of TLR4 mRNA in primary astrocytes isolated from neonatal cerebral cortices. After 24 hours of stimulation with morphine (1 μ M, 10 μ M, 100 μ M; agonist at both mu opioid receptors and TLR4) there was no significant change in mRNA for HMGB1, GFAP, and TLR4 while there appeared to be a downward trend for the expression of TNF α mRNA (Figure 9). A different pattern of results was apparent upon stimulation with M3G (agonist at TLR4, but not mu opioid receptors). The astrocytes stimulated with M3G (0.01 μ M, 0.1 μ M, 1 μ M) for 24 hours upregulated mRNA expression for HMGB1 (Significant in One-Way ANOVA, but not significantly different from control in post-hoc test $p > .05$) and TNF α ($p < .05$) No effect was seen on GFAP mRNA expression (Figure 10). There was no effect on TLR4 mRNA. This indicates that M3G can activate astrocytes directly, at least at the level of gene expression. The GFAP In-cell Western blot showed no change in GFAP protein by M3G or morphine. The LPS-stimulated cells showed a significant decrease in GFAP protein expression ($p < .05$; Figure 11), but no effect by either opioid at these doses. The TNF α ELISA detected 50 pg/mL TNF α

release by the LPS-stimulated cells (Figure 12), a significantly elevated difference from the other groups ($p < .05$). There was no detected $\text{TNF}\alpha$ release by the astrocytes receiving the other treatment conditions.







EXPERIMENT 7:

The effects of opioid stimulation of astrocytes co-cultured with endothelial cells on the secretion of TNF α and gene expression.

In this experiment, endothelial cells and astrocytes were co-cultured using transwell inserts (Corning, Corning, NY). Endothelial cells were seeded onto transwell inserts (see methods above). To co-culture the ECs on the inserts with astrocytes, a separate 24 well plate contained astrocytes seeded as in Experiments 1 and 2. The day of the experiment, the medium was removed from the astrocytes and replaced with aCSF and the transwell inserts with the ECs were transferred to the astrocyte plate. The combined cells were left to rest for 2 hours and then stimulated. Drugs were added to the luminal (blood) side and left for 24 hr. RT-PCR and TNF α ELISAs were completed on the astrocytes. This approach allowed investigation of the effect activated ECs had on the astrocytes plated below them. One plate of endothelial cells was stimulated with M3G (0.01 μ M, 0.1 μ M, and 1 μ M) and another with (-) morphine (1 μ M, 10 μ M, and 100 μ M.)

EXPERIMENT 7 RESULTS:

The effects of opioid stimulation of astrocytes co-cultured with endothelial cells on the secretion of TNF α and gene expression. (Figures 13&14)

In this co-culture experiment, the endothelial cells were stimulated with the same doses of M3G as seen in Experiment 6, and the astrocytes (seeded in the separate chamber from the stimulated endothelial cells) were measured for effects on mRNA expression. In the M3G-stimulated co-cultured endothelial cells (drug administered to “blood side”) there was no effect on astrocyte mRNA expression for HMGB1, TLR4, or TNF α (Figure 13). GFAP mRNA trended to be decreased (0.01 μ M, 0.1 μ M, 1 μ M M3G doses, $p=0.06$). In morphine treated cells, there was an increase in TLR4 (100 μ M; Figure 14).

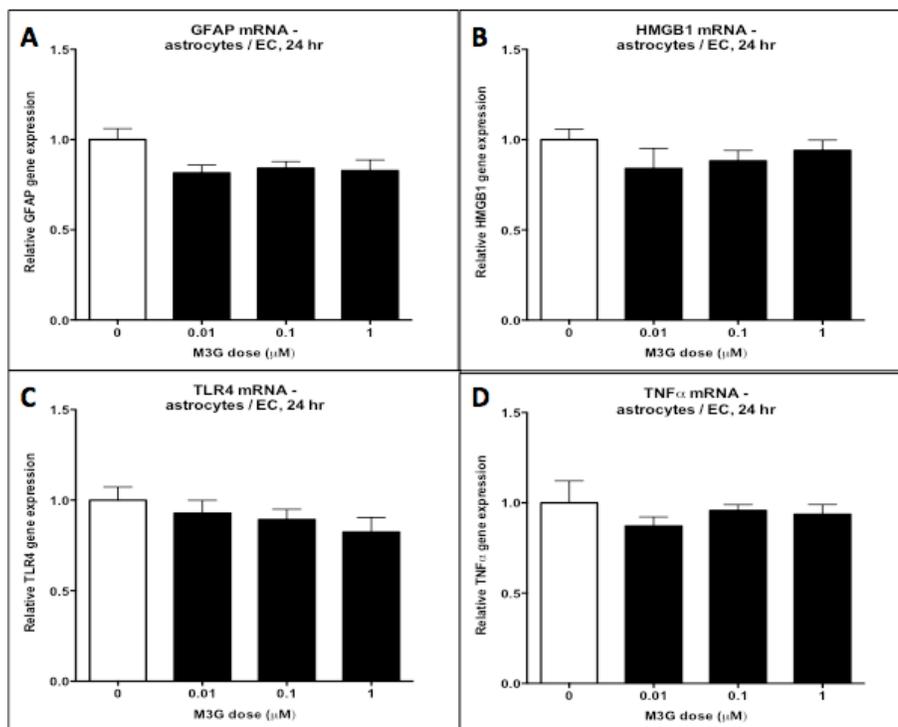
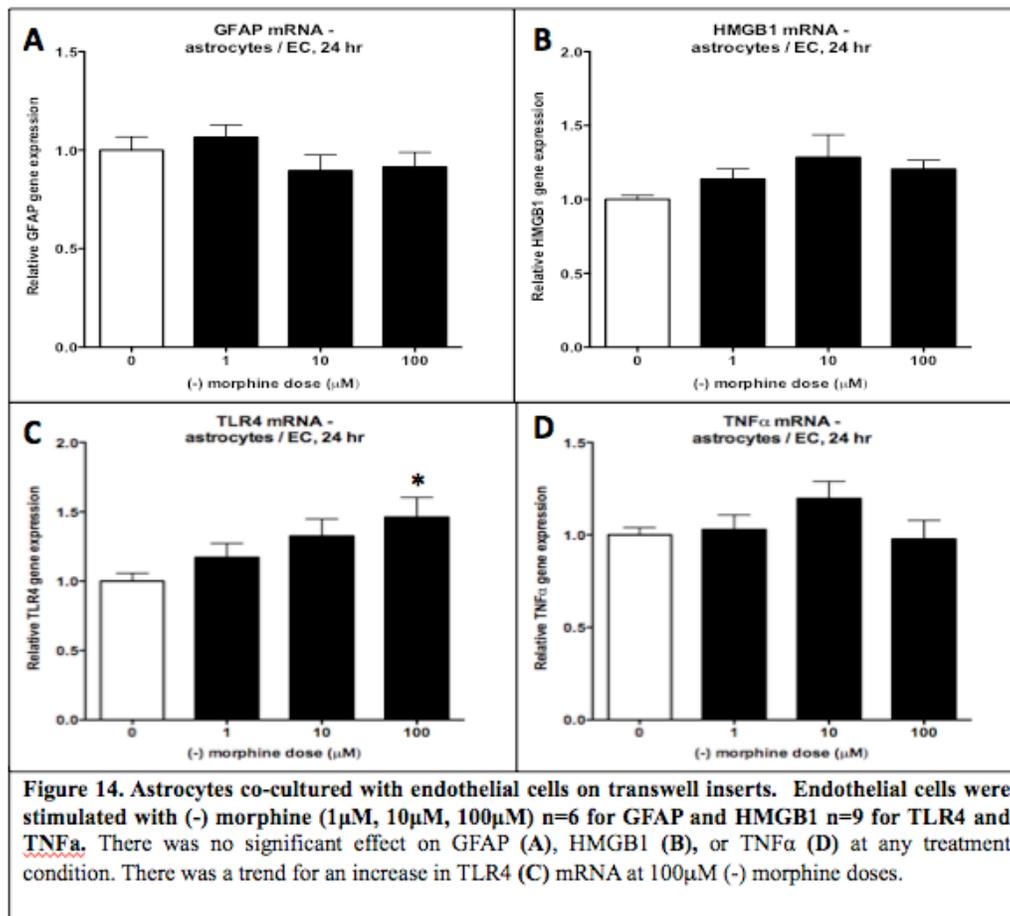


Figure 13. Astrocytes co-cultured with endothelial cells on transwell inserts. Endothelial cells were treated with M3G(0.01 μ M, 0.1 μ M, 1 μ M).There was no effect of mRNA expression in HMBG1 (B), TLR4 (C), or TNF α (D) at any of the treatment conditions. The decrease seen GFAP mRNA expression at all treatment conditions was close to being significant, as none of the M3G doses differed, they were pooled and a significant increase was found. (* $p=.05$, upon pooling) $n=6$ at each condition.



EXPERIMENT 8:

The effects on astrocytes co-cultured with stimulated endothelial cells followed by direct stimulation with LPS on gene expression and TNFα secretion.

In this experiment, endothelial cells and astrocytes were combined as in Experiment 3A. The upper chamber with ECs was stimulated with 100 ng/mL LPS, 0.1 μM M3G, and 10 μM (-) morphine and incubated for 24 hours. At the 24-hour time point, the inserts were removed and discarded and a sample of the aCSF was taken for an ELISA. Then the astrocytes were directly stimulated with 100 ng/mL LPS and incubated for another 4 hours. After 4 hours, another aCSF sample was taken for ELISA analysis and the cells were prepared for mRNA extractions for RT-PCR gene analysis. The purpose of this experiment was to look at the idea of glia priming. The

signals released by the activated endothelial cells may not be enough to activate the astrocytes, but perhaps the signals they receive act to prime the astrocytes so that they become more reactive to direct TLR4 binding.

EXPERIMENT 8 RESULTS:

The effects on astrocytes co-cultured with stimulated endothelial cells followed by direct stimulation with LPS on gene expression and TNF α secretion. (Figures 15&16)

After 24 hours of incubation with stimulated endothelial cells, the astrocytes were directly stimulated with LPS (100 ng/mL) for 4 more hours. This experiment aimed to further investigate the role of glial priming. Specifically, is it possible that the endothelial cells that were stimulated secreted substances onto the astrocytes that would cause them to become more sensitive to direct LPS (TLR4 agonist)? Samples for a TNF α ELISA were taken at 24 hours from the astrocyte supernatant and there was no detectable release from cells in any condition. Then the inserts with the endothelial cells were thrown out and all astrocytes were stimulated with LPS (100 ng/mL) for 4 hours. After the direct LPS stimulation another sample was taken from the astrocyte supernatant for a TNF α ELISA. There was no difference in TNF α secretions from the astrocytes incubated with endothelial cells stimulated with vehicle, M3G (0.1 μ M) or morphine (10 μ M; Figure 15). In the astrocytes that had been co-cultured with LPS-stimulated ECs, there was a significant difference from the control cells in TNF α secretion after the 4-hour stimulation with LPS (100 ng/ml). It is important to note that the astrocytes co-cultured with the vehicle-stimulated ECs secreted 300 pg/mL TNF α after the subsequent 4 hour stimulation with LPS (100 ng/ml), while astrocytes cultured alone and stimulated with LPS (100 ng/ml) only secreted 50 pg/mL in 24 hours (Experiment 2). The ECs in the co-culture are communicating with the astrocytes and must be sending signals that cause the sensitization of TLR4 on the astrocytes. When these astrocytes are treated with LPS, the sensitized TLR4 causes less

secretion of TNF α . The astrocytes stimulated with the LPS (100 ng/mL) treated ECs secreted significantly less TNF α . It is not likely that this is the result of endotoxin tolerance. Banks *et al.*, (Banks *et al.*, 2010) report that peripherally administered LPS is not taken up into the BBB at a level that would stimulate the CNS. Rather, they explain that the brain endothelial cells sequester LPS, and then mediate the secretions to the brain side. This supports the hypothesis that the endothelial cells in the in vitro BBB model are communicating to the astrocytes.

The astrocytes were also examined for change of GFAP gene expression (Figure 16). The astrocytes that were co-cultured with morphine-stimulated endothelial cells have significantly ($p < .05$) more activation in response to LPS than astrocytes co-cultured with endothelial cells receiving any other treatment. This gene expression fits with the earlier recorded observation (Experiment 7; Figure 14) that morphine treated endothelial cells upregulated TLR4 mRNA when co-cultured with astrocytes. Additionally, M3G treated endothelial cells (Experiment 7; Figure 13) had no significant effect on TLR4 mRNA in co-cultured astrocytes, which also is supported because astrocytes co-cultured with M3G-treated ECs showed no significant change in LPS-induced activation.

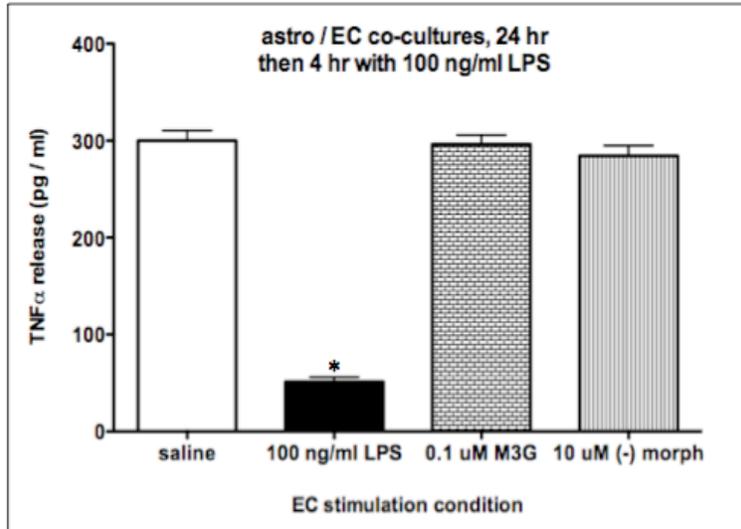


Figure 15. Astrocytes co-cultured with endothelial cells on transwell inserts were stimulated with LPS (100 ng/mL), M3G (0.1 μ M), and (-) Morphine (10 μ M). At 24 hours, transwell inserts were removed and the astrocytes were stimulated directly with LPS (100 ng/mL) for an addition 4 hours. At the 24 hour time point, there was no detectable TNF α secreted by any condition. This graph shows the results from 4 hours after the direct LPS stimulation. There was no difference in TNF α secretions from the astrocytes incubated with endothelial cells stimulated with vehicle, M3G (0.1 μ M) , or (-) morphine (10 μ M). There was a significant decrease in TNF α secretion by the astrocytes cultured with the endothelial cells treated with LPS (100 ng/mL). It is important to note that the astrocytes co-cultured with the vehicle-stimulated ECs secreted 300 pg/mL TNF α after the subsequent 4 hr stimulation with LPS (100 ng/ml), while the solo astrocytes stimulated with LPS (100 ng/ml) only secreted 50 pg/mL in 24 hours (Experiment 2). n=6 for each condition *P<.05

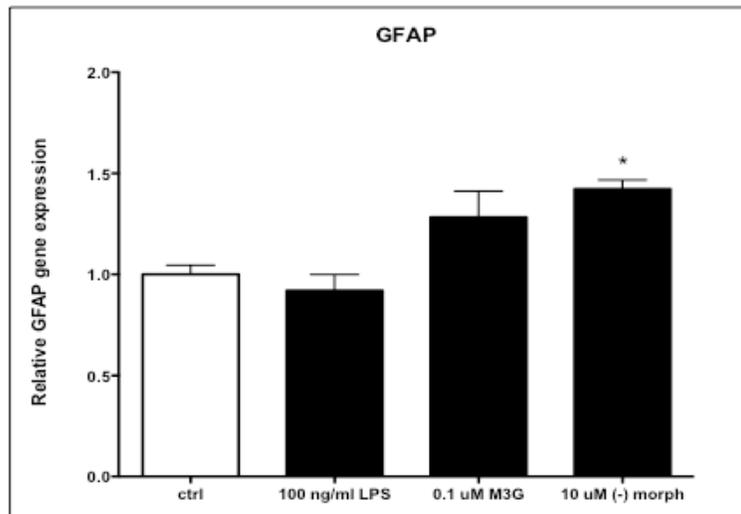


Figure 16. Astrocytes co-cultured with endothelial cells on transwell inserts were stimulated with LPS (100 ng/mL), M3G (0.1 μ M), and (-) Morphine (10 μ M). At 24 hours, transwell inserts were removed and the astrocytes were stimulated directly with LPS (100 ng/mL) for an addition 4 hours. GFAP gene expression was measured. Astrocytes that were co-cultured with morphine-stimulated ECs show significantly more activation in response to LPS (100 ng/mL) treatment. M3G-treated ECs showed no significant change in LPS-induced activation. n=6 for each condition *P<.05)

EXPERIMENT 9:

The effects on gene and protein expression by opioid stimulation of astrocytes co-cultured with microglia.

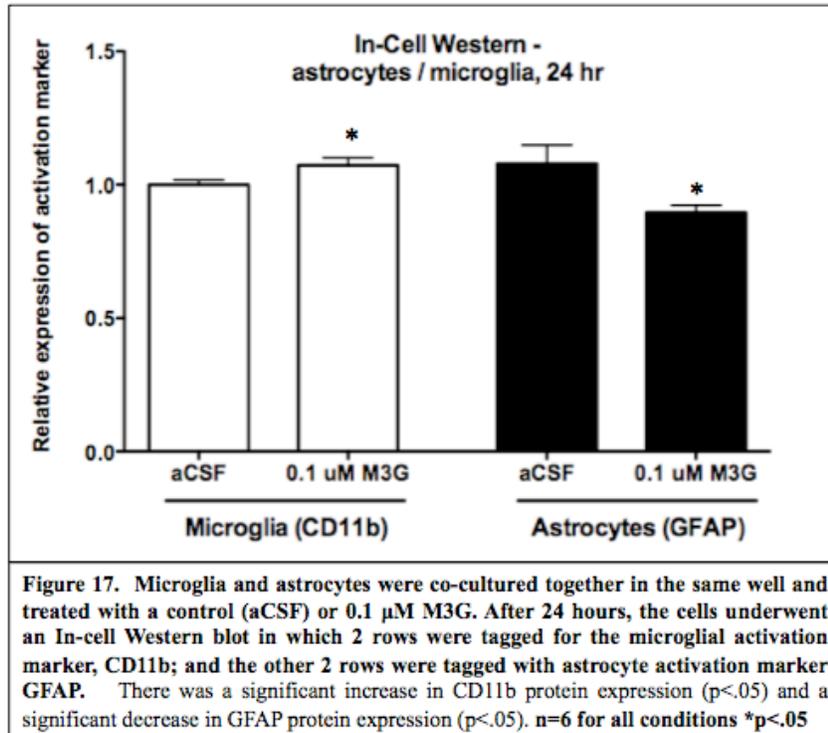
Astrocytes are in close proximity to microglia in the CNS, so it is logical to look at the role of microglia in this model of the blood brain barrier. In this experiment microglia and astrocytes were isolated from neonatal pups as outlined above, but after reaching confluence, the microglia were saved and counted. The astrocytes were passaged (as above) and seeded (~200,000 cells/well) into a 24 well plate. The microglia (~23,000/well) were seeded on top of the astrocytes. All isolated cells were used in the culture. These cells came directly from the animal; the ratio that they were seeded at is approximately what would be physiologically observed. The day of the stimulation the cell media was removed and replaced with aCSF and allowed to sit for 2 hours prior to stimulation. Cells were stimulated with aCSF as a control and 0.1 μ M M3G. An in-cell western was completed (as seen above). 2 rows were tagged with astrocyte activation marker; GFAP and 2 rows were tagged with microglia activation marker CD11b.

EXPERIMENT 9 RESULTS:

The effects on gene and protein expression by opioid stimulation of astrocytes co-cultured with microglia. (Figure 17)

Microglia and astrocytes were co-cultured together in the same well and were treated with aCSF as a control or 0.1 μ M M3G. In-cell Western blots (n=6) measured the changes in protein expression of CD11b and GFAP, which are activation markers specific to microglia and astrocytes respectively. CD11b protein expression was upregulated while GFAP protein expression was downregulated in M3G-stimulated co-cultures (Figure 17). It is important to note that when astrocytes alone were stimulated for the same time and with the same dose of

M3G, there was no effect on GFAP protein. This indicates that there must be communication between the microglia and the astrocytes.



EXPERIMENT 10:

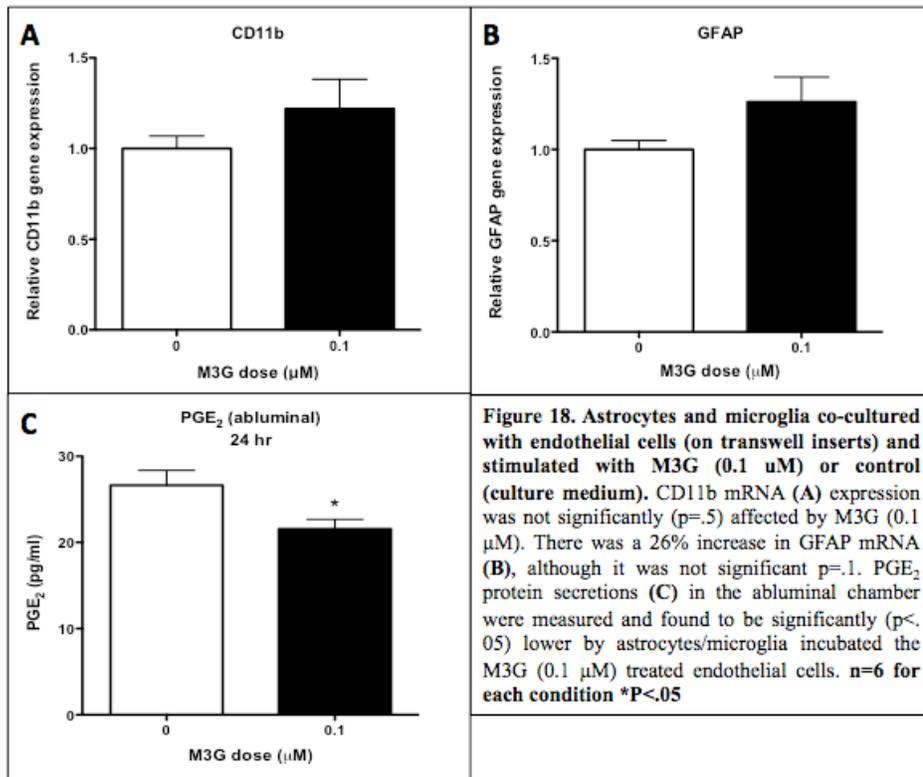
The effects of opioid stimulation on TNF α secretion and gene expression of astrocytes and microglia co-cultured with stimulated endothelial cells.

Microglia and astrocytes were treated as in Experiment 9. Additionally, ECs seeded on to inserts as seen in Experiment 7, were added to the microglia/astrocyte co-culture. The inserts and the mixed glial culture were combined in the same fashion previously outlined. In this experiment, the endothelial cells on inserts were stimulated with either M3G (0.1 μ M) or the control (aCSF). Changes in astrocyte and microglial activation were measured using RT-PCR on GFAP and CD11b (astrocytes and microglia; respectively). PGE₂ secretion was measured in the abluminal chamber.

EXPERIMENT 10 RESULTS:

The effects of opioid stimulation on TNF α secretion and gene expression of astrocytes and microglia co-cultured with stimulated endothelial cells. (Figure 18)

The progressive addition of cell types to each culture design allows for better control over the experiment conditions. This is the most complex of the *in vitro* experiments completed for this project. By co-culturing microglia and astrocytes with endothelial on inserts, it adds a new level of complexity that is found *in vivo*. Endothelial cells were stimulated with M3G (0.1 μ M) or the control (culture medium). mRNA expression by the microglia and astrocytes was measured for CD11b (microglial activation marker) and GFAP (astrocyte activation marker; Figure 18, boxes A&B). Additionally, aCSF was assayed for abluminal PGE₂ protein secretions (Figure 18, box C). n=6 for each of the treatment conditions. CD11b mRNA expression was not significantly (p=.24) affected by M3G (0.1 μ M). Although it appears that with more trials, this might become a significant increase in CD11b mRNA. GFAP mRNA expression was almost significantly increased (p=.1). There was a 26% increase in GFAP mRNA in astrocytes when co-cultured with both microglia and endothelial cells. This experiment provides the most evidence to support the hypothesis that activated endothelial cells can activate glia. PGE₂ protein secretions in the abluminal chamber were measured and found to be significantly (p<.05) lower by astrocytes/microglia incubated the M3G (0.1 μ M) treated endothelial cells. Since the abluminal chamber contains secretions from three cell types, it is unknown which cells were secreting PGE₂ and how much each cell type contributed to the overall PGE₂ measured. Experiment 3 (Figure 6) showed that there is more PGE₂ secreted lumenally, than there is abluminally. This may be because ECs secrete PGE₂ into the blood to recruit peripheral immune cells to the CNS.



DISCUSSION:

Experiments in this thesis were undertaken to support the novel observation that endothelial cells of the brain and spinal cord are involved in the modulation and enhancement of opioid-induced CNS inflammation. The progression of experiments shows that CNS endothelial cells can be activated by M3G, which can induce the secretion of PGE₂. Treatment of CNS endothelial cells with TLR4 and opioid receptor antagonists (LPS-RS and nalmeferne, respectively) followed by treatment with M3G and morphine (respectively), show that this activation is consistent with TLR4-mediated pathways. M3G-activated CNS endothelial cells were also sufficient to induce pain *in vivo*. Following these experiments, primary astrocytes and microglia were added to *in vitro* studies. Astrocytes were shown to behave differently when cultured alone, compared to when they were cultured with CNS endothelial cells or with CNS

endothelial cells and microglia. The difference in behavior and activation seen in these experiments suggests that there is important communication between these cell types, and further exploration of this topic is warranted.

Through the data presented in Experiments 1-4, CNS endothelial cell activation by opioids was examined. There is no precedence of this in the literature. There was robust expression of TLR4 found in CNS endothelial cells (Ramos *et al.*, manuscript in preparation). It has previously been reported that CNS endothelial cells express the μ_3 opioid receptor (Stefano *et al.*, 1996). Experiments 1 and 2 explored the use of both opioid receptor (nalmefene) and TLR4 (LPS-RS) antagonists. The data collected in these experiments supported the original hypotheses. When stimulating the ECs with nalmefene (opioid receptor antagonist) followed by morphine (which can signal through both TLR4 and opioid receptors), it was expected that there would be an upregulation in pro-inflammatory gene expression. Indeed, this was the case (Experiment 2; Figure 5). The opposite was also true; when ECs were treated with LPS-RS (TLR4 antagonist) followed by treatment with M3G, it was expected that there would be suppression of pro-inflammatory gene expression. This was also supported (Experiment 1; Figure 4). Experiment 3 provides evidence that, upon stimulation of polarized ECs with M3G (0.001 μ M, 0.01 μ M, 0.1 μ M, 1 μ M, and 10 μ M), PGE₂ was secreted in different concentrations luminally compared to abluminally. PGE₂ secretion significantly increased by stimulation with 0.1 μ M M3G. This is the same dose of M3G that caused activation of ECs sufficient to cause allodynia when the cells were injected into the lumbar intrathecal space. PGE₂ is released by CNS ECs to induce fever in instances of bacterial or viral infection (Milton *et al.*, 1970; Elmquist, *et al.*, 1997). Interestingly, morphine can induce fever as well, but fever caused by this co-activator of mu-opioid receptors and TLR4 is not blocked by prostaglandin synthesis

inhibitors (Nikolov, 2010; Prakash *et al.*, 1981; Clark *et al.*, 1978). As there appear to be no studies of alterations in core body temperature by M3G, whether TLR4 activation by M3G is sufficient to induce fever via a PGE2-dependent mechanism is unknown. PGE2 also induces other pro-inflammatory responses in the body that cause pain. (Shih *et al.*, 2010).

Experiment 4 provided evidence that M3G-activated endothelial cells are sufficient to cause pain *in vivo* (Figure 7). In this experiment, endothelial cells were stimulated for 18 hours with M3G (0.1 μ M), morphine (10 μ M), or saline. After the stimulation, cells were prepared and injected intrathecally into male Lewis rats. The rats underwent von Frey behavioral testing at 1, 2, 3, and 24 hours post injection. The rats injected with 0.1 μ M M3G were more sensitive to stimulation at hours 1, 2, and 3. The rats that received morphine- or saline-stimulated cells did not differ in pain response from the control group that was injected with PBS. These studies provide key evidence that endothelial cells may play a larger role in opioid-induced CNS inflammation than has been previously appreciated. These data provided a platform to begin the next set of studies involving astrocytes and microglia.

Experiments 5-10 aim to gain a deeper understanding of whether there is communication between microglia, astrocytes, and endothelial cells derived from the spinal cord and brain. Data were collected using five different cell culture set-ups, that were designed to understand how treatment with LPS (100 ng/mL), M3G (0.01 μ M, 0.1 μ M, 1 μ M) and morphine (1 μ M, 10 μ M, 100 μ M) affected astrocytes alone for either 4 hours or 24 hours, astrocytes co-cultured with endothelial cells, astrocytes co-cultured with microglia, and astrocytes co-cultured with microglia and endothelial cells. Effects on the various cell types were measured using RT-PCR (to measure expression of mRNA for HMGB1, GFAP, TLR4, TNF α), In-cell Western blots (to measure GFAP and CD11b protein expression), TNF α ELISAs, and PGE₂ ELISAs. The data

presented provide evidence that there is indeed communication between CNS endothelial cells and the microglia and astrocytes present in the CNS.

The experimental design chosen for these next experiments was important to the relevancy of this *in vitro* work *in vivo*. Several experiments were undertaken to determine how opioid-activated endothelial cells would in turn affect co-cultured astrocytes and microglia. The experiment design utilized a cell culture model that approximates the blood brain barrier. Endothelial cells are polar cells, meaning that they have a luminal side that is exposed to the blood, and an abluminal side that faces the central nervous system. The endothelial cells used for all of the present experiments were primary cells isolated from the brain and spinal cord tissue of adult male Sprague Dawley rats. Endothelial cells were grown in confluent monolayers on the transwell inserts, such that their luminal sides faced an upper chamber containing cell culture medium while their abluminal sides faced a lower chamber containing artificial cerebrospinal fluid (aCSF; Figure 2). The primary astrocytes and microglia were isolated from the cortex of neonatal male Sprague Dawley rats. These cells were grown on the surface of the microplate itself in the aCSF. The permeable membrane at the bottom of the transwell insert allowed for signaling molecules to pass between the endothelial cells and the astrocytes and microglia. The endothelial cells were grown in culture medium containing 20% fetal bovine serum (FBS), which functioned as “blood” in this model of the blood brain barrier. The lower chamber of glia contained aCSF. The endothelial cells in the upper chamber (within the insert) were stimulated with various drugs including morphine and M3G.

It was observed in Experiment 6 (Figure 10), that stimulating astrocytes alone for 24 hours with M3G (0.01 μ M, 0.1 μ M, 1 μ M) resulted in increases of mRNA expression for HMBG1 and TNF α . This suggests that astrocytes can be activated by M3G directly.

Interestingly, there was no effect on GFAP protein expression in astrocytes alone treated with 0.1 μ M M3G for 24 hours, but when astrocytes were co-cultured with microglia with identical treatment (Experiment 9; Figure 17), there was a significant decrease in GFAP protein expression. This suggests that there must be communication between the microglia and astrocytes to cause this change. *In vivo*, Microglia are activated first and then recruit astrocytes (Ledeboer *et al.*, 2006). There were also differences seen between the astrocytes alone stimulated for 24 hours with M3G (0.01 μ M, 0.1 μ M, 1 μ M) and the astrocytes co-cultured with endothelial cells (on inserts). The astrocytes cultured alone (Experiment 6; Figure 10) had increased expression of mRNA for HMGB1 and TNF α , while the astrocytes co-cultured with ECs (Experiment 7; Figure 13) downregulated GFAP mRNA. Since only the endothelial cells were directly exposed to M3G, and the astrocytes were not, this suggests that the change in mRNA expression is due to signaling from the activated ECs to the astrocytes. Again, the decrease in GFAP is not due to a decrease in proliferation, rather it a decrease in the production of GFAP mRNA. In both of these examples, astrocytes behave differently alone than they do when co-cultured with CNS endothelial cells.

Another important observation of how endothelial cells alter astrocyte functioning relates to TNF α secreted by astrocytes in response to 100 ng/mL LPS. Astrocytes stimulated with 100 ng/mL LPS (Experiment 6; Figure 12) secreted 50 pg/mL of TNF α in 24 hours. Co-culturing the astrocytes with endothelial cells altered this responsiveness, as shown in Experiment 8: astrocytes co-cultured with saline-stimulated ECs for 24-hour subsequently secreted 300 pg/mL TNF α in response to a 4-hour stimulation with 100 ng/ml LPS. This is strong evidence that co-culturing astrocytes with endothelial cells changes the way the astrocytes respond compared to the response by astrocytes cultured alone and treated with the same treatment.

In Experiment 8, where TNF α secretions were measured from astrocytes that were co-cultured with endothelial cells for 24 hours and then stimulated with 100 ng/mL LPS, there was a significant decrease in TNF α secretion by the astrocytes co-cultured with LPS-stimulated endothelial cells (Figure 15). A question could be raised that this decrease is due to endotoxin tolerance. However, the literature supports that LPS does not cross the BBB in significant enough amounts to activate glia in the CNS (Banks *et al.*, 2010). It is hypothesized that peripheral LPS binds TLR4 on the brain endothelial cells, and the ECs then release secretions into the brain. This suggests that the effect seen is not due to endotoxin tolerance and supports the communication link between endothelial cells and astrocytes.

Further evidence of the hypothesized cellular cross talk is seen in Experiment 8. Astrocytes when co-cultured with M3G-stimulated ECs (Experiment 7; Figure 13) had no significant change in TLR4 mRNA expression and no significant change in subsequent responsiveness to LPS stimulation (as measured by GFAP mRNA; Figure 16). In contrast, when astrocytes were co-cultured with morphine-stimulated ECs, the astrocytes significantly increased expression of TLR4 mRNA and subsequently had a significantly great response to LPS stimulation (as measured by GFAP mRNA; Figure 16).

Since the involvement of endothelial cells and opioid-induced CNS inflammation via TLR4 is relatively new, the concept that ECs could contribute to the effects of other drugs of abuse (also linked to TLR4) has never been explored. While this series of studies has focused on M3G, as a well-characterized TLR4 agonist with no mu opioid agonist activities, there is growing recognition that TLR4 is activated by other drugs such as alcohol and cocaine (Wu *et al.*, 2011; Northcutt *et al.*, 2010). Endothelial cells are exposed to the highest levels of systemic drugs of any cell type in the CNS, and may have previously undiscovered roles in the effects of

many such drugs of abuse. These effects include neuroinflammation that has been linked to the suppression of morphine-induced analgesia enhancement of morphine reward, enhancement of morphine-induced drug dependence and withdrawal, morphine- and cocaine-induced reward, alcohol-induced sedation and ataxia, and morphine-induced respiratory depression (Watkins *et al.*, 2005; Johnston *et al.*, 2004; Raghavendra *et al.*, 2002; Raghavendra *et al.*, 2004). Until now, the possibility that these drug effects arise, at least in part, due to the activation of CNS endothelial cells has never been considered.

The contribution of CNS endothelial cells to opioid-induced CNS inflammation has been overlooked until now. Historically, the study of non-neuronal cell types an (Abbot, Ronnback, & Hansson, Astrocyte-endothelial interactions at the blood brain barrier., 2006) (Abbot, Ronnback, & Hansson, Astrocyte-endothelial interactions at the blood brain barrier., 2006) (Abbot, Astrocyte-endothelial interactions and blood-brain barrier permeability, 2002)d their influences on neuronal function have focused almost exclusively on microglia and astrocytes. Microglia and astrocytes readily express activation markers that can easily be quantified. The ease of measuring activation makes astrocytes and microglia easy to study. Schwann cells and oligodendrocytes have been studied for their interactions with the axons they enwrap. Their activation can be imaged in cell culture by treatment with calcium sensitive dyes that “light up” when the cells are activated. Endothelial cells lack classical activation markers, and therefore their involvement has often gone over-looked. The data presented in this thesis provide proof that endothelial cells become active when treated with M3G, and suggest that future studies of CNS inflammation should include study of endothelial cells.

While the studies included here have tried to accurately reflect the *in vivo* environments for the chosen cell types, there remains the question of whether cell culture adequately addresses

what happens *in vivo*. This difference between the *in vitro* and *in vivo* data suggests that *in vivo* signaling is more complicated than the experimental design allows. While the co-culture experiments aimed to more closely model *in vivo* signaling, the cultures only included, at most, three cell types. There is no question that *in vivo* signaling involves more cell types and molecules than the ones included in the cultures presented.

In future experiments, an even more relevant cell culture model could include neurons in the cultures, or at least physiologically relevant amounts of substances that are known neuron-to-glia signals. Fractalkine is an example of a neuron-to-glia signal. Fractalkine is expressed only by neurons and its receptor only by microglia (Verge *et al.*, 2004). Literature shows that fractalkine enhances responsivity by activating microglia via the release of pro-inflammatory cytokines (Milligan *et al.*, 2005; Johnston *et al.*, 2004). Further studies in support of the data presented in this thesis are exploring whether adding neurons to the co-cultures and mimicking the release of neuron-to-glia signals may amplify the effects observed in the present studies.

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