EFFECTS OF PRO-INFLAMMATORY CYTOKINES ON IN VITRO MEASURES OF GENE EXPRESSION, PROTEIN EXPRESSION, AND PROCESS EXTENSION IN PRIMARY HIPPOCAMPAL NEURONS

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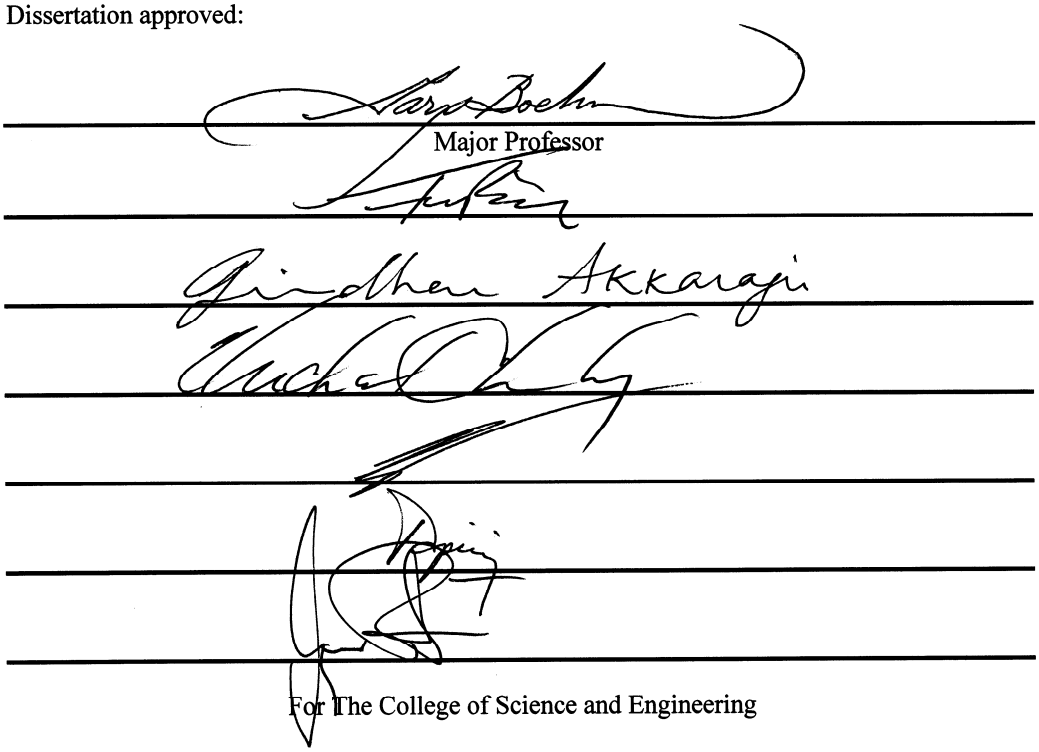

Major Professor

For The College of Science and Engineering

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1. INTRODUCTION

1.1 Neural-Immune Interactions

The immune and central nervous systems were thought to operate independently of one another until Ader and Cohen (1975) demonstrated that aspects of the immune response could be behaviorally conditioned. Specifically, using the conditioned taste aversion procedure developed by Garcia and colleagues (1955), Ader and Cohen conditioned an association between saccharin and cyclophosphamide, an immunosuppressive agent. Three days later, animals were injected with sheep erythrocytes to stimulate an immune response, and exposed thirty minutes later to either saccharin or water. Animals that were given saccharin, compared to those given water, showed conditioned immunosuppression, through decreased antibody titers in response to the immune stimulus. These data provided some of the seminal evidence for a direct association between the brain and the immune system.

Since Ader and Cohen discovered that the brain can modulate certain aspects of the immune system, there has been further inquiry into the opposing effect, whether the immune system can influence behavior, as well as cognition. A number of studies utilizing laboratory animals have reported that administration of lipopolysaccharide (LPS) or cytokines leads to deficits in learning/memory in a variety of paradigms (Aubert et al., 1995; Barrientos et al., 2002; Gibertini et al., 1995; Kent et al., 1996; Oitzl et al., 1993; Pugh et al., 1998, 1999; Shaw et al., 2005; Sparkman et al., 2005a, b). Despite a large number of studies characterizing the behavioral effects of immune stimulation, the cellular mechanisms that underpin this global cognitive impairment remain unclear.

1.2 Cytokines

The CNS and immune system interact with one another through endocrine function and through the release of cytokines, respectively. Cytokines are chemical messengers by which immune cells, and other cells, communicate with one another. Peripheral cytokines enter the central nervous system (CNS), typically an immune-privileged site, via three major pathways (Dantzer, 2004). First, cytokines can enter the brain through sites lacking a blood-brain barrier (BBB), such as the circumventricular organs (Konsman et al., 1999). Second, cytokine production can be induced centrally by peripheral cytokines stimulating the vagus nerve (Watkins et al., 1995). Finally, cytokines can enter through active transport into the brain across the BBB (Banks & Kastin, 1991).

Cytokines are typically divided into two categories: pro-inflammatory cytokines, which include interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), and anti-inflammatory cytokines, such as interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-10 (IL-10; Nomura et al., 2000). In times of infection, pro-inflammatory cytokines activate immune cells, increase tissue permeability, and induce fever (Harlan, 1985). In terms of behavior, the release of these pro-inflammatory cytokines leads to "sickness behavior", which includes anhedonia, decreased social exploration, sexual behavior, feeding behavior, and locomotor activity (Borowski et al., 1998; Yirmiya, 1996). These changes in an organism's behavior are not inflexible; rather, they represent a change in motivational state that enables an organism to better adapt to the needs of the current situation (Dantzer, 2004). For example, although locomotion is generally diminished, if an animal, or its offspring, are placed in a life-threatening situation they will still escape to safety, temporarily showing little or no locomotor impairment.

Pro-inflammatory cytokines have many local and systemic effects on the body including induction of fever and inflammation including increased vascular permeability, induction of acute-phase protein production, and recruiting neutrophils, basophils, and T-cells to the site infection. In addition to the natural benefits of cytokines, artificial administration of cytokines can be used to treat hepatitis C virus, various cancers, and human immunodeficiency virus (HIV) (Anisman & Merali, 2003). Despite these positive effects, cytokines are most notable in the neuroscience literature for their negative effects, such as exacerbating neurodegenerative disease, causing detrimental effects on memory, and their possible role in depression. For example, cytokines are implicated in aggravating neurodegenerative diseases such as Alzheimer's, HIV-related dementia, and prion diseases (Ader, 2007; Perry, 2005). Additionally, the induction of pro-inflammatory cytokines play a role in learning and memory deficits through their deleterious effects on acquisition (Oitzl et al., 1993; Aubert et al., 1995; Sparkman et al., 2005a,b) and memory consolidation (Pugh et al., 1997; Barrientos et al., 2002).

As described above, there are several different pro-inflammatory cytokines that can lead to a wide range of physiological and psychological effects. Arguably three of the best-studied pro-inflammatory cytokines are IL-1β, IL-6, and TNF-α. As these two cytokines are directly applicable to the proposed experiments, their properties are individually described in more detail below.

1.2.1 Interleukin-1β

IL-1β is a small molecular weight polypeptide (17.5 kDa) that is produced by macrophages, monocytes, and dendritic cells (Dinarello, 1984). Upon damage to the tissue or the onset of infection, IL-1β activates lymphocytes and increases effector cell access to the

damaged or infected tissue (Liao et al., 1984). Systemically, IL-1β leads to the induction of fever and also continues the cytokine cascade by initiating the production of IL-6, which in turn, induces antibody production.

IL-1β is the only known cytokine that has an endogenous receptor antagonist (IL-1ra; Liao et al., 1984). The expression of this antagonist is modulated by the inflammatory environment. During periods when inflammation is absent, the expression of IL-1ra is increased and is estimated to be bound to 90–95% of total IL-1 receptors (Opric et al., 2007). Under inflammatory conditions, transcription of IL-1ra is initially diminished and, concomitantly, IL-1 transcription and receptor binding is increased. The delicate balance between IL-1 and IL-1ra is important for normal function as well as pathological conditions (for review, see Spulber et al., 2009). IL-1β stimulation via LPS administration or through intraventricular injection induces sickness behavior and leads to a variety of deficits in learning and memory, as described below.

Despite the widely documented negative effects of IL-1β, Yirmiya has postulated a beneficial role for IL-1β in learning and memory. Evidence indicates that IL-1β follows an inverted "U"-shaped relationship, with under-expression and over-expression both causing negative effects on learning and memory. Three experimental models are often used to examine the effects of diminished IL-1β signaling. The first method is the administration of IL-1ra to block IL-1 receptors, preventing IL-1β from binding and stimulating the receptor. Secondly, many investigators utilize transgenic knockout mice with the gene coding for the IL-1 receptor deleted (IL-1rKO), and lastly, some experiments employ transgenic mice with that over-express IL-1ra. Recently, it has been shown that learning impairments observed in IL-1KO mice can be restored via exposure to an enriched environment (Goshen et al., 2009).

This effect is thought to be mediated through an increase in dendritic spine size that leads to a reinstatement of LTP, rather than through an increase in neurogenesis, trophic factors, or dendritic spine density.

1.2.2 Tumor Necrosis Factor-α

TNF-α increases tissue permeability and leakiness of the vasculature. This allows for influx of complement, IgG, and increases drainage of fluid to the lymph nodes. In addition, TNF-α has anti-tumoral effects, for which the cytokine is named, that have been known for almost 100 years (Starnes, 1992). Low levels of TNF-α are expressed in the CNS by microglia and astrocytes under 'normal' conditions (Hopkins & Rothwell, 1995). However, in circumstances of traumatic brain injury, such as an ischemic event, neuronal cell types can secrete elevated levels of TNF-α (Liu, Clark, McDonnell et al., 1994), as well as microglia (Gregersen et al., 2000) and macroglia (Yu & Lau, 2000). Once secreted, TNF-α signaling is transduced through two distinct cell surface receptors: TNF receptor type 1 (TNFR1) and TNF receptor type 2 (TNFR2). TNFR1 activates cell-death pathways that lead to apoptosis. TNFR2 receptor activation, however, has neuroprotective effects and uses NFκB cell signaling pathways (MacEwan, 2002).

1.2.3 Interleukin-6

IL-6 is a small molecular weight protein (26 kDa), originally known under the name, IFN-β_2. IL-6 is produced by lymphoid and non-lymphoid cells, including T-cells, B-cells, monocytes, fibroblasts, and endothelial cells (Kishimoto, 1989). Production of IL-6 can be stimulated or enhanced in response to T-cell mitogens, LPS, IL-1, TNF-α, and interferon-β, among others (Hirano et al., 1988). IL-6 itself is known to play a crucial role in the initiation of the acute phase response (Heinrich et al., 1990). Excessive levels of IL-6, either through

exogenous administration (Samuelsson et al., 2006), or transgenic over-expression can lead to learning and memory impairments (Heyser et al., 1997).

In addition to pathological conditions, IL-6 expression is also present under normal physiological conditions. Indeed, as with IL-1β, basal levels of expression are necessary to maintain normal learning and memory function. For example, Baier and colleagues (2009) demonstrated that IL-6 knock-out mice show impairments in both hippocampus-dependent and independent learning. These data indicate that subtle changes in IL-6 expression can lead to important physiological and cognitive effects.

1.3 Immune Influences on Learning and Memory

Learning is typically divided into two broad categories: associative and non-associative. Non-associative learning is a simple form of single-trial learning. Examples of this type of learning include sensitization and habituation. Associative learning (e.g., classical conditioning) involves the pairing of two stimuli that have temporal contiguity. The immune system has been shown to affect associative learning in a variety of paradigms. The relationship between these two phenomena is bidirectional; increased or decreased immune system activity can alter learning and, similarly, aspects of the immune response can be conditioned.

One of the best known examples of the interplay between the brain and the immune system is the conditioning of certain aspects of the immune response to LPS through the conditioned taste aversion (CTA) paradigm (Oberbeck et al., 2003). Typically, CTA involves exposure to a novel flavored solution, followed by gastrointestinal discomfort caused by exposure to a toxin, high-speed rotation, or x-rays. On subsequent exposures to the flavored solution, the animal avoids the solution. CTA can occur in one trial and produce stable and

prolonged conditioning. Several symptoms or features of the acute phase response to LPS can be conditioned by CTA, such as fever and sleep alterations (Bull et al., 1994), plasma iron concentrations (Exton et al., 1995b), and anorexia (Exton et al., 1995a).

The conditioning of immune responses extends beyond the rodent literature to work done with humans. The conditioning of the human immune response was first described by Turnbull almost fifty years ago. Turnbull (1962) demonstrated that individuals with asthma could develop symptoms in the absence of allergens through exposure to a set of environmental cues. Furthermore, evidence of associative learning of drug side-effects has been shown in patients undergoing chemotherapy. Through the use of environmental cues, patients can show signs of conditioned nausea (Andrykowski, 1988), anxiety (Jacobsen et al., 1993), and fatigue (Bovbjerg et al., 2005). At the cellular level, some reports show that the number of natural killer cells, cells partially responsible for the innate immune response to viral infection, can be increased using the CTA paradigm (Buske-Kirschbaum, 1992), although at other times the same researchers could not replicate this effect (Kirschbaum et al., 1992).

The preceding experiments demonstrated the ability of the brain to influence immune system function. As stated previously, the relationship between these two systems is bidirectional. Activation of the immune system has been shown to impair cognition in a variety of paradigms (Pugh et al., 1998, 1999; Barrientos et al., 2002; Sparkman et al., 2005a,b; Kohman et al., 2007a,b). In these experiments, the immune system is stimulated either directly, through the administration of pro-inflammatory cytokines such as IL-1β, or indirectly through the administration of LPS. LPS is a glycolipid portion of the cell wall of gram-negative bacteria, and is an endotoxin that elicits a strong immune response

characterized by the release of pro-inflammatory cytokines (Borowski et al., 1998). LPS is not itself a replicating pathogen and, therefore, provides a controlled, safer, and potentially less variable model of immune activation. The effects of LPS on the CNS typically develop two to four hours after exposure and typically last as long as 24 hours (Dantzer, 2004). During this time, the release of cytokines can produce noticeable deficits in learning and memory. Administration of LPS (or cytokines) negatively affects memory acquisition and consolidation in a variety of behavioral paradigms, such as two-way active avoidance, Morris water maze, and autoshaping (e.g., Aubert et al., 1998; Pugh et al., 2001; Sparkman et al., 2005a). Furthermore, observed cognitive deficits are distinguishable from and not due to sickness. Such cognitive effects are not surprising, because of the high density of IL-1 receptors in the hippocampus, which is a brain structure that is widely known for its role in some tasks involving specific types of learning and memory (Schneider et al., 1998).

Pugh et al. (1998) demonstrated that LPS administration impairs memory consolidation in contextual (hippocampus-dependent), but not auditory fear conditioning (hippocampus-independent). The same deficits were also seen with intracerebroventricular (i.c.v.) administration of IL-1β, and the effects appear to be specific to tasks that depend on the hippocampus, such as contextual fear conditioning (Rachal-Pugh et al., 2001). Barrientos et al. (2002) further demonstrated that IL-1β specifically blocks learning of the context, and that exposure to the context prior to IL-1β administration can ameliorate these effects. Furthermore, recent studies with the two-way active avoidance paradigm indicated that the administration of LPS immediately following the first training session led to impairments in memory consolidation for the task in aged animals compared to young animals (Tarr et al.,

2011). These deficits corresponded with increased mRNA expression of IL-1β, both centrally and peripherally.

In addition to memory consolidation deficits, exposure to cytokines can impair memory acquisition as well. In an autoshaping task, animals injected with LPS during acquisition of a lever-pressing task performed fewer lever presses than control animals that were administered saline, indicating that these animals failed to associate the lever with the administration of a food pellet (Aubert et al., 1995). Sparkman et al. (2005) administered LPS 4 h prior to two-way active avoidance conditioning. The two-way active avoidance conditioning box consists of two compartments. At the start of the trial, the compartment housing the mouse is illuminated. Once this light turns on, the mouse must learn to cross to the other compartment to avoid the onset of a mild shock. The animals that received LPS performed fewer avoidance responses to the shock, compared to control animals. As these animals did not have any meaningful decrements in latency to cross, and an increased number of inter-trial interval crossings, these results do not appear to be due to fatigue, motor deficits, sensory deficits, or reduced motivation. Collectively, these data indicate that the fewer avoidance responses were not due to non-learning performance factors, but rather to a diminished ability to acquire the association between the light and ensuing shock.

One potential explanation for cognitive disruptions in the wake of an immune challenge is through diminished long-term potentiation (LTP) or synaptic plasticity. LTP is a prominent neurobiological model of learning. Hebb (1949) postulated the concept of neuronal plasticity which was developed further experimentally by Lømo and colleagues (Bliss & Lømo, 1973). The principle behind LTP is that associations between two stimuli are strengthened through the frequent concurrent firing of appropriate neural connections.

Likewise, Sastry and Goh (1984) found that the weakening of neural connections occurs through the infrequent concurrent firing of appropriate connections, an effect referred to as long-term depression (LTD). Interleukin-1β decreases LTP in the hippocampal slice preparation. Evidence indicates this effect may be due to decreased phosphorylation of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Bellinger et al., 1993; Lai et al., 2006). The effect of LPS-induced inflammation on LTP has been demonstrated in both NMDA-dependent and NMDA-independent forms of LTP (Min et al., 2009). One of the primary methods by which cytokines may decrease hippocampal LTP is through decreased expression of brain-derived neurotrophic factor (BDNF) in the CNS. Barrientos and colleagues (2004) were the first to demonstrate that central administration of IL-1β led to diminished BDNF mRNA in many regions of the hippocampus, including CA1, ventral CA2, and the dentate gyrus, in a time-dependent manner. The onset of these deficits corresponded with diminished memory for the context in the contextual fear conditioning paradigm.

Despite the overwhelming evidence that over-expression of cytokines leads to cognitive impairments, it is also known that basal levels of pro-inflammatory cytokines such as IL-1 are necessary for memories to be formed. Spulber and colleagues (2009) demonstrated that LTP for long-term rather than short-term memories was impaired in transgenic mice that over-express IL-1 receptor antagonist (IL-1ra). Transgenic mice that over-express IL-1ra show impairments in long-term but not short-term memory. These effects are associated with diminished expression of activity-related cytoskeleton-associated protein (Arc) at basal levels and during novelty-induced learning in the dentate gyrus (Spulber et al., 2009), and an impaired BDNF-signaling pathway. The deficits seen in mice

with the IL-1 receptor knocked out and with a CNS-specific over-expression of the IL-1 receptor can be abrogated by exposure to an enriched environment (Goshen et al., 2008).

Clearly, immune activation can influence the CNS and lead to cognitive impairments in various hippocampus-mediated testing paradigms, including the Morris water maze (Oitzl et al. 1993, Sparkman et al., 2005b,c), autoshaping (Aubert et al., 1995), contextual fear conditioning (Pugh et al., 1998), and two-way active avoidance conditioning (partially hippocampus-dependent) (Sparkman et al., 2005a). However, the relationship is a complex one, as basal levels of IL-1 are necessary for normal cognitive function.

Despite much inquiry into this line of research, the cellular mechanisms that govern the normal and deleterious neural effects of cytokines remain largely unknown. Because many individuals suffer from the cognitive effects of long-term inflammatory conditions or treatment with cytokine-based therapies, further research examining the potential mechanisms or means of attenuating these effects is warranted.

1.4 Molecular Mechanisms–in vivo

Relatively few articles have explored the molecular mechanisms underlying behavior *in vivo*. Among them, Palin et al. (2008) found that a c-jun N-terminal kinase (JNK) pathway inhibitor, D-JNKI-1, can abrogate the effect of a central injection of TNF-α on the induction of sickness behaviors and memory impairments in an operant learning task. These data implicate the JNK pathway and subsequent transcription factor activation in the formation of sickness behavior. The JNK pathway is responsible for apoptosis, cell proliferation and cytokine production through the transcription factor activation protein-1 (AP-1). These data help to elucidate a potential signaling pathway responsible for TNF-α-mediated effects on cognition and behavior.

TNF-α is known to bind to two endogenous receptors, TNF-R1 and TNF-R2, yet which receptor-type is responsible for inducing sickness behavior was not known until recently. Utilizing mice that had deletions of either TNF-R1 or TNF-R2 and i.c.v. injections of TNF-α, Palin and colleagues (2009) were able to determine that TNF-R1 is the receptor responsible for the induction of sickness behavior. Furthermore, TNF-R1 signal induction is dependent upon the activation of FAN (factor associated with neutral sphingomyelinase; Palin et al., 2009).

It should be noted that much of the work examining potential molecular mechanisms has been done *in vitro* using cell culture techniques, because it is easier to manipulate and control cell-signaling pathways in tissue cultures.

1.5 Neuroinflammation in vitro

IL-1β has an inverted-U shaped relationship with learning and memory. IL-1β in either excessive or below basal levels can lead to diminished learning and memory in a variety of paradigms. One way excessive levels of IL-1β may cause cognitive disruptions is through inhibiting the production of brain-derived neurotrophic factor (BDNF). BDNF is a trophic factor found in the brain that is important for neuronal growth, survival, and differentiation (Acheson et al., 1995). LPS or IL-1β has been shown to increase the expression of BDNF in the hippocampus. Specifically, in the dentate gyrus (Lapchak et al., 1993), though conflicting results have been observed *in vivo* (Barrientos et al., 2004). These studies indicate a complex relationship between IL-1β and BDNF.

Despite the negative inflammatory effects of IL-1, a variety of positive effects have been reported using cell culture techniques. For example, IL-1β has been reported to increase survival of primary dopaminergic neurons isolated from the substantia nigra (Akaneya et al.,

1995). In peripheral nerve cultures, it has been shown that IL-1 increases nerve growth factor (NGF) production in primary co-cultures of fibroblast and rat sciatic nerve tissue. IL-1 augments NGF production by increasing both the rate of gene transcription and the stability of resultant NGF mRNA (Lindholm et al., 1988). As further evidence of the seemingly unlikely benefit of IL-1β, results have shown that IL-1β increases neurite outgrowth in dorsal root ganglionic (DRG) and cerebellar granule neurons (CGN) through inhibition of RhoA, a negative regulator of neurite growth, as well as p38 MAPK and not NF-κB-mediated pathways (Temporin et al., 2008). IL-1 has demonstrated a therapeutic function in decreasing rejection of sympathetic ganglionic tissue grafts. Pretreatment with IL-1 resulted in an NGF-mediated neurotrophic effect on sympathetic superior cervical ganglionic (SCG) neurons, and resulted in enhanced tissue graft survival (Nakao et al., 1994).

In addition to the positive effects of IL-1β on neurite growth and survival, a positive role of another pro-inflammatory cytokine, IL-6, has been demonstrated both centrally and peripherally. Satoh (1988) showed that administration of IL-6 led to a level of neuronal differentiation in PC12 cells similar to those that received NGF. In addition to increased neurite growth and branching, IL-6 was able to induce "NGF-like" functional changes such as the formation of voltage dependent Na^+ channels. Despite the similarities, different neuronal properties were observed in IL-6-differentiated cells. Differences included a longer onset of clear morphological change (2–3 days), a shorter time-period of maintainable differentiation (10 days), and decreased production of acetylcholinesterase activity compared to NGF-treated cell populations. Importantly, these data are the first to demonstrate the role of IL-6 as a trophic factor for PC12 cells. Later, IL-6 was found to work synergistically with NGF to induce neurite outgrowth in the original line of PC12 cells developed by Greene, and

in a variant line (E2; Wu & Bradshaw, 1996a). Given the evidence that cytokines can increase nerve regeneration following trauma, Edoff and Jerregård (2002) examined the effects of IL-1β, IL-6, and LIF on neuritogenesis and cell survival using primary sensory dorsal root ganglion (DRG) cells co-cultured with fibroblast-like cells. Specific concentrations of IL-1β and IL-6 (5 ng/mL) supported DRG cell survival and increased neuritogenesis through NGF-dependent mechanisms.

Most surprising of all, IL-6 was found to be neuroprotective for dopaminergic neurons after treatment with 1-methyl-4-phenylpyridintium (MPP^+), the toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Administration of MPP^+, an agent commonly used to induce a pharmacological model of Parkinson's disease, typically results in the selective death of dopaminergic neurons in the substantia nigra of the mesencephalon. Administration of IL-6, but not IL-1β or IL-3, attenuated MPP^+-induced neurotoxicity, though this effect was not through increased glial proliferation (Akaneya et al., 1995). Other findings, however, demonstrated that administration of LPS in conjunction with MPTP doses normally too low to produce effects showed dopamine depletion and evidence of parkinsonian symptoms, such as gait instability (Byler et al., 2009).

The effects of TNF-α on a cell are seemingly contradictory to one another. TNF-α pushes the cell toward apoptotic pathways while simultaneously acting to negatively regulate apoptosis. Depending upon the pathways activated, TNF-α can act to kill or save the cell. Although TNF-α is known to activate programmed cell-death pathways (Laster et al., 1988), we will first focus on the positive effects of TNF-α, when TNF-α acts upon the NF-κB pathway, it induces NF-κB to transcribe a set of genes that prevent the cell from undergoing apoptosis (van Antwerp et al., 1998). The activation of NF-κB pathways may be responsible

for the neuroprotective effect of TNF-α observed in response to excitotoxic insults, which is relevant to ischemic events and seizure recovery (Cheng et al., 1994). The addition of neurotrophic factors NGF and BDNF increased TNF-α-induced activation of NF-κB pathways in PC12 cells (Furuno & Nakanishi, 2006). The co-operative action of cytokines and neurotrophic factors are important for neuronal survival, differentiation, and death. Indeed, NGF and TNF-α share a complex relationship with one another. TNF-α, through activation of TNFR2, and NGF have been shown to promote cell survival but suppress differentiation of neuroblastoma cells through convergence on Akt signaling pathways (Takei & Laskey, 2008).

However, not all of the data support a beneficial role of TNF-α. TNF-α administration was shown to decrease neurite outgrowth and branching in primary hippocampal neurons co-cultured with glial cells. In order to test whether glia could produce similar concentrations of TNF-α, glial cells were induced, through the administration of IL-1β and IFN-γ, to produce endogenous TNF-α. Upon stimulation, glia produced high levels of TNF-α and, once again, decreased neurite extension and branching were observed. These effects were hypothesized to be due to a Rho-dependent mechanism. Indeed, trituration of cells in the presence of C3 transferase successfully adenosine diphosphate (ADP)-ribosylated and inactivated Rho, which led to the abolition of the deleterious effects of TNF-α on neurite outgrowth and branching (Neumann et al., 2002).

One possible explanation for the seemingly contradictory findings regarding the effect of pro-inflammatory cytokines on neurite outgrowth, is the importance of cellular context. Rage and colleagues (2006) demonstrated completely opposite effects of IL-1β on BDNF expression, depending upon the cellular milieu. Administration of IL-1β to primary

hypothalamic neurons increased BDNF mRNA. However if astrocytes were co-cultured with the neurons, IL-1β decreased BDNF mRNA. Astrocytes are known to release prostaglandins in response to IL-1β. The presence of glia caused neurons to increase expression of an inhibitory (EP3) versus stimulatory (EP1, EP2, or EP4) prostaglandin receptor.

Given the mostly unexplained mechanisms of pro-inflammatory cytokine-mediated cognitive deficits, this line of research clearly warrants further inquiry. In an effort to better understand the possible mechanisms at work, we searched the literature for research that might provide a suitable method for modeling these effects. Das and colleagues (2004) developed PC12 cells as a neuronal model for detecting potential neurotoxicants. They measured the expression of growth associated protein-43 (GAP-43) and synapsin-I, proteins associated with differentiation and neurite extension. In addition to these neurochemical measures, morphological measures of neurite length and differentiation were obtained using computer-based quantitative image analysis. These data are not only potentially useful in determining the possible deleterious effects of the contents of the neuronal milieu in the developing brain, but also are relevant to adulthood neurogenesis.

1.6 Role of Synapsin-I and GAP-43 in Learning and Memory

Synapsin is a phosphoprotein that is associated with the synaptic vesicle fusion and involved in synaptic maturation, axonal elongation, and neurotransmitter release (Greengard et al., 1993). Induction of LTP through perforant path stimulation was associated with increased hippocampal expression of synapsin-I mRNA (Morimoto et al., 1998). Rapanelli and colleagues (2009) utilized an operant task to assess gene transcription of synapsin-I during different phases of learning. Elevated synapsin-I mRNA expression was associated with memory acquisition for the task (when animals performed at 50–65% of expected

responding), and returned to basal levels after complete training (performance at 100% of expected rate) or in the absence of any training. Additionally, spatial learning is associated with increased expression of synapsin-I mRNA and protein compared with yoked controls (Gómez-Pinilla et al., 2001).

GAP-43 is a protein that is important to the development of growth cones (Neve et al., 1987). Further, GAP-43 expression is important in the formation of hippocampus-dependent memories. Indeed, GAP-43 protein expression and phosphorylation levels were elevated following exposure to contextual fear conditioning compared with untrained controls, and remained elevated for 72 hours (Young et al., 2002). Additionally, diminished expression of hippocampal GAP-43 using heterozygote knockout mice ($GAP43^{+/-}$) was associated with learning deficits in the contextual, but not cued, fear conditioning paradigm, indicating a crucial role for GAP-43 in hippocampus-dependent memory (Rekart et al., 2005). Furthermore, these GAP43-deficient mice show "autistic-like features" including a lack of novelty preference and increased anxiety (Zaccaria et al., 2010).

The relevance of synapsin-I and GAP-43 in hippocampal learning and memory, and related structural plasticity, make them a relevant target for the possible deleterious effects of inflammation on learning and memory that have been observed *in vivo*.

1.8 Hypotheses

The present study utilized primary hippocampal cell culture to examine the effects of IL-1β, IL-6, and TNF-α on process extension and expression of relevant proteins. Specifically, we hypothesized that administration of IL-1β, IL-6, or TNF-α would lead to morphological alterations (as evidenced by decreased process length, process number, and density of processes), as well as decreased protein and mRNA levels of GAP-43 and

synapsin-I. Furthermore, in addition to replicating the prior findings of Neumann et al (2002), our goal was to extend this line of investigation to potential effects of IL-1β and IL-6.

2. METHOD

2.1 Animal Subjects

Subjects were male and female C57BL/6J mice bred at the TCU vivarium from breeding stock obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were used as breeders, and their offspring will be used as subjects in Experiment 1. Breeding pairs were housed, with free access to food and water under a 12:12 light/dark cycle, in standard polycarbonate mouse cages. All animals were housed and treated in accordance with the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996), and in accordance with a general protocol approved by the TCU Institutional Animal Care and Use Committee.

2.2 Primary Hippocampal Cell Culture

Primary hippocampal culture of neurons from newborn mice was performed in a modified version of the protocol designed by Nuñez (2008). Hippocampi were dissected from 6 mouse pups on Postnatal Day 1. In an effort to minimize culture contamination, each mouse pup was briefly submerged in 70% ethanol followed by HBSS+ (10mM HEPES pH 7.3, 100 units of penicillin per mL and 100 ng of streptomycin per mL [Sigma-Aldrich, Saint Louis MO, Cat No. P4333] in Hank's balanced salt solution calcium and magnesium free [Invitrogen, Carlsbad CA, Cat No. 14185052]). As each hippocampus was harvested, it was placed into 5 mL of HBSS+. Once all of the hippocampi had been harvested, 55 µL of 2.5% (10x) trypsin (Invitrogen, Cat No. 15090046), was added and then incubated for 15 minutes at 37°C. HBSS+/trypsin solution DNAse I (Sigma-Aldrich, Cat No. DN25) in 0.15M NaCl was added to the remaining 4.5 mL of HBSS, in order to denature any genomic DNA that

may have spilled from damaged cells. The solution was then triturated using a serological pipette tip several times to achieve a homogenous single-cell suspension. Next, the cells were distributed evenly among four 35 mm tissue culture dishes (VWR International, Radnor PA, Cat No. 25382), each containing a 22x22 mm square coverglass (VWR International, Cat No. 48366-067) that had previously undergone acid washing with hydrochloric acid and treatment with poly-L lysine (Sigma-Alrich, Cat No. P6282), in order to provide an improved substrate for cell adherence (for procedure, see Section 2.3). In addition to the HBSS+ cell suspension, 2 mL of plating medium (10% horse serum [Sigma-Aldrich, Cat No. H1138], 3 mL of 20% glucose (sterile filtered), 100 mM pyruvic acid [Sigma-Aldrich, Cat No. P2256] in Minimum Essential Medium [Invitrogen, Cat No. 51200038]), was added to each tissue culture dish and gently swirled to mix. Dishes containing the newly plated hippocampal neurons were incubated for 4 hours at 37°C with 5% CO_2 in order to allow time for the cells to attach. After the four hour incubation period, the HBSS+/MEM plating medium was aspirated and 2 mL of Neurobasal+, (200 µL of B-27® serum-free supplement [Invitrogen, Cat No. 17504044], 2,000 units of penicillin/2 mg streptomycin [Sigma-Aldrich], 0.5mM L-glutamine [Sigma-Aldrich, Cat No. G7513], in Neurobasal® medium [Invitrogen, Cat No. 12348017]) was added to each culture dish. The cells were then allowed to co-culture for 7 days. On the 7^{th} day, cells were treated with either 100 ng/mL of IL-1β (Sigma-Aldrich) 100 ng/mL of IL-6 (Sigma-Aldrich), or 10 ng/mL of TNF-α (Sigma-Aldrich) of mouse recombinant protein, or remained untreated.

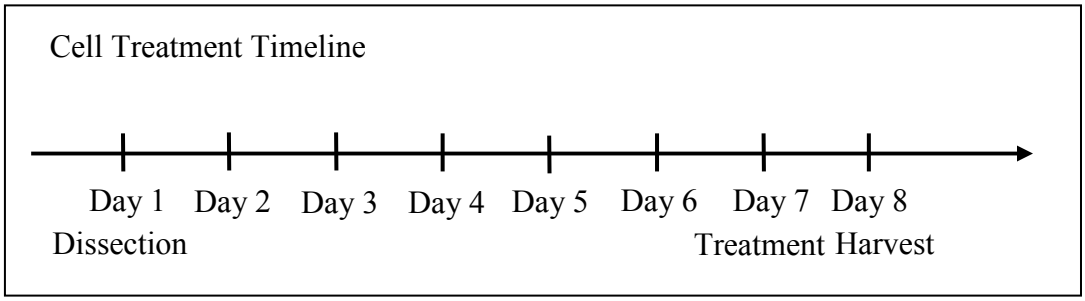

Figure 1. Primary Hippocampal Cell Culture Timeline – Hippocampal tissue was dissected and plated on Day 1 and co-cultured to Day 7, when cells are treated with cytokines, incubated for 24 hours, and harvested on Day 8.

2.3 Acid Washing and Poly-L Lysine Treatment of Coverglass

Coverglass were placed into a loosely covered glass beaker containing 1M HCl and incubated for 16 hours at 50–60°C, cooled, and then washed with distilled H_2O followed by Millipore H_2O, rinsed in ethanol, and dried between sheets of Whatman paper. Next, the acid washed coverglass was submerged in 1 mg/mL poly-L lysine (Sigma-Aldrich, Cat No. P8920) and rotated for at least 30 minutes in a 10cm tissue culture dish. The coverglass were washed in at least 5 changes each of distilled H_2O and Millipore H_2O to wash away any free poly-aminoacids, which can be toxic to the cells, and then rinsed in ethanol.

2.4 Western Blot: GAP-43 and Synapsin-I

2.4.1 Cell Harvesting and Processing–Western Blot

Cells were scraped off of the coverglass and pipeted into a microcentrifuge tube and centrifuged for five minutes at 1,500 RPM. The supernatant was aspirated and the pellet washed with 500μL of 1x phosphate-buffered saline (PBS; 2.7 mM KCl, 1.4 mM KH_2PO_4, 137 mM NaCl, 4.3 mM Na_2HPO_4) cooled to 4°C, and centrifuged at 8,000 RPM for five minutes. The supernatant was aspirated and either stored at -20°C until it was ready to be used or immediately processed. Next, the cell pellet was resuspended in 70 μL of cold lysis buffer (20 mM Tris HCl pH 7.5, 50 mM NaCl, 25 mM β-glycerophosphate, 1 mM sodium

orthovanadate, 10% glycerol, 1% triton-X-100, 1mM dithiothreitol (DTT), 10 µg/mL leupeptin, 1mM PMSF, and 1 tablet Roche Complete Mini EDTA-free protease inhibitor cocktail [Roche Applied Science, Mannheim, Germany, Cat No. 1873580001]), and incubated for 15 minutes at 4°C. After incubation, the supernatant was centrifuged at 8,000 RPM for 10 minutes at 4°C. For each sample, the supernatant was transferred to fresh tubes, 12 µL of 6x SDS loading buffer (30% glycerol, 70% 4x Tris HCl pH 6.8, 100 mg/mL sodium dodecylsulfate (SDS), 93 mg/mL DTT, 12 ng/mL Bromophenol Blue) was added, and boiled for five minutes at 95°C in the DriBath (Thermo Scientific, Waltham, MA), then immediately frozen. The remaining pellet was kept for an estimation of overall protein concentration using a Bradford protein assay (Biorad, Hercules, CA). Samples were run in triplicate and the protein concentration was used to determine the amount of sample to be loaded.

2.5.2 Preparation of SDS-PAGE Gel

A 9% SDS Page gel was prepared (4X Tris buffer pH 8.8, 30% acrylamide/0.8% bisacrylamide, 10% (w/v) ammonium persulfate, 0.01% TEMED). Gels were run in SDS running buffer (0.125M Tris base, 0.96M glycine, 0.5 w/v SDS) at 100v for 1 hour or until the bromophenol blue band reached the bottom of the gel. Proteins were then transferred to polyvinylidene difluoride (PVDF, Millipore) membranes in transfer buffer (0.04M Tris base, 0.29M glycine, 20% methanol) at 100v for 1 hour.

2.5.3 Immunoblotting

Equal amounts of sample (normalized for protein concentration, based on Bradford results) and pre-stained SDS broad range ladder (New England BioLabs Inc., Ipswich MA,

Cat No. P7709V) were loaded, and the gel was run. Membranes were washed in PBST (2.7mM KCl, 1.4mM KH_2PO_4, 137mM NaCl, 4.3mM Na_2HPO_4, 0.1% Tween 20).

Once the transfer was completed, the membranes were incubated for 1 hour in 2% BLOTTO solution (10g Non-fat dry milk, 500mL PBST) and primary antibody specific to GAP-43 (1:1,000 in 2% BLOTTO; Sigma-Aldrich; Cat No. G9264) and synapsin-I (1:1,000; Sigma-Aldrich; Cat No. S193). After one hour, the membrane was placed in a sealed tray with the primary antibody solution for 30 minutes and then incubated at 4ºC overnight. The membrane was subjected to three 10-minute washes in 2% BLOTTO.

Secondary antibody (Promega, Madison WI, anti-rabbit: Cat No. S373B, anti-mouse: Cat No. S373B) was prepared (1:10,000 in BLOTTO) and added to the membrane for one hour. The membrane was again subjected to three, 10 minute washes in 2% BLOTTO, and given a final wash in 1X PBS. Alkaline phosphatase staining reagents, NBT and BCIP (Promega, Cat No. S3771) were added, as per manufacturer's instructions, to alkaline phosphate buffer (100mM Tris pH 9.5, 5mM $MgCl_2$, 100mM NaCl) for membrane staining.

2.5.4 Densitometry

Each western blot underwent analysis of band optical density using a Kodak Gel Logic 100 Imaging System (Kodak, Rochester, NY). Band densities were obtained for the proteins of interest (GAP-43 or Synapsin-I) and normalized against a control blot (α-tubulin or β-actin).

2.6 Immunofluorescence

Cells were grown on acid washed poly-L lysine coated coverglass using the timeline described in Figure 2. On Day 8, the culture medium was aspirated and washed with 1mL of 1x PBS. Next, cells were fixed with 1 mL of methanol chilled to -20°C, and incubated at

room temperature for two minutes, followed by five washes with 1 mL of 1x PBS. Primary antibodies specific to GAP-43 and synapsin-I were diluted in 1x PBS with 3% goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove PA, Cat No. 005-000-121) to a 1:250 concentration. 0.5mL of the appropriate primary antibody was added to the wells and incubated for two hours at room temperature. After incubation, the wells were washed four times with 0.7 mL of 1x PBS in 3% goat serum. As part of a series of steps that were conducted in the dark, 0.5 mL of the appropriate secondary antibody coupled with a fluorochrome (Invitrogen, anti-rabbit Cat No. A1108, anti-mouse Cat No. A1103) and was diluted in 1x PBS with 3% goat serum to a 1:250 concentration, and added to the cells. The plate was protected from light and incubated for 30 minutes at room temperature. Afterwards, the wells were washed three times with 1 mL of 1x PBS, and the coverglass was removed from the tissue culture dish using forceps, mounted on a microscope slide using Fluoromount-G (Southern Biotechnology, Birmingham AL, Cat No. 0100-01), and viewed under a fluorescent microscope (Carl Zeiss Microimaging, Thornwood, NY, Zeiss ApoTome Fluorescence Microscope).

2.7 RNA Isolation and Real-Time Polymerase Chain Reacton

Cells were dissociated from the poly-L lysine-coated coverglass by aspirating the cell culture medium, washing the cells once with sterile 1x PBS, and adding 0.5 mL of 2.5% trypsin. The cells were then monitored under the microscope until most cells were dissociated from the coverglass, and then 1.5 mL of Neurobasal medium with B-27 serum was added to stop the enzymatic reaction. The sample was transfered into microcentrifuge tubes and centrifuged at 8,000 RPM for 5 minutes, and the supernatant was aspirated. Next, the pelleted cells were resuspended in 350 μL of kit-provided lysis buffer and homogenized

using a TissueTearor™ (BioSpec Products Inc., Bartlesville, OK). The RNA was then isolated from the cells using an RNeasy Micro spin column kit (Qiagen, Valencia CA, Cat No. 74004), in accordance with manufacturer instructions, then quantified and assessed for purity using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The amount of GAP-43 and synapsin-I mRNA present was determined utilizing TaqMan™ probe and primer chemistry (Applied Biosystems, Foster City, CA) specifically designed to bind to reverse transcribed GAP-43 and synapsin-I cDNA. The relative amount present was then compared across the treatment conditions, using the $-2^{\Delta\Delta ct}$ method of analysis (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008).

2.8 Morphological Measures

Cells were photographed under a fluorescent microscope (Zeiss ApoTome Fluorescence Microscope) at 63x magnification. Seven separate visual fields were chosen in a pseudorandom fashion from predefined regions of the coverglass from each treatment group per experiment. Utilizing ImageJ software (NIH), the length and number of processes were taken by tracing individual processes using the segmented line tool and obtaining the length of each process in the visual field, as well as the number of processes (see Figure 10). Furthermore, a measure of the total area of processes was derived by removing all sections of the image that were larger in area than the process (e.g., cell bodies), and all portions that were smaller (e.g., debris or noise). From these analyses, the total area of the processes, as well as the area fraction of the image total were derived (see Figure 13).

2.9 Statistics

Western blot densometric data, relative gene expression using PCR, and morphometric data were analyzed using standard analysis of variance (ANOVA) procedures

(Statview 5.0, SAS, Cary, NC). The alpha level used for all statistical analyses was 0.05. Significant omnibus effects were followed by Fisher's PLSD post hoc tests.

3. RESULTS

3.1 Developing Primary Hippocampal Culture Technique

The first step was to develop the primary hippocampal cell culture technique and characterize the cell culture. The resultant cultures were mixed cultures containing hippocampal neurons and glial cells. Bright field images of a representative culture were taken on Day 7 at 20x and 40x magnification (see Figure 2).

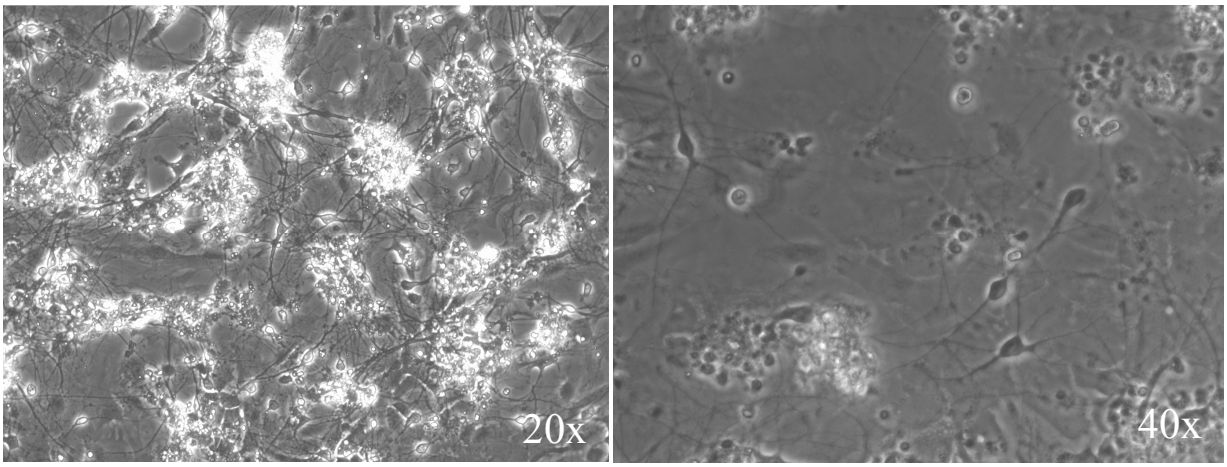

Figure 2. Untreated Primary Hippocampal Neurons. Cells were photographed with a bright field microscope at 20x and 40x magnification.

In addition to characterizing the basic morphology of the culture, it was important to image the overall cell population using immunofluorescent antibodies specific for α-tubulin, a protein present in neurons and glia (see Figure 3), as well as neurofilament a protein present in mature neurons (see Figure 4). Finally, we were interested in visualizing the cellular localization of the two proteins of interest, GAP-43 and synapsin-I, in the absence of any experimental manipulation (see Figure 5).

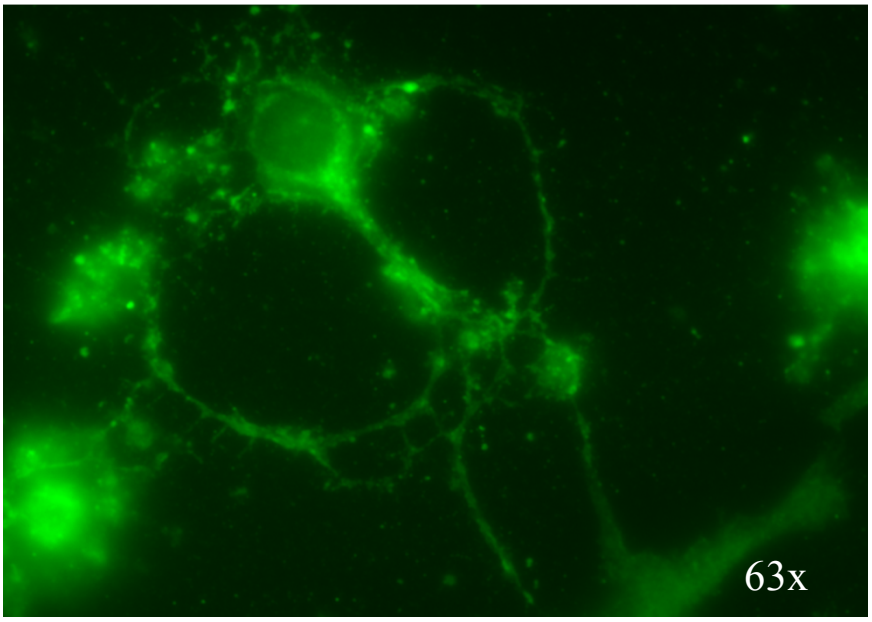

Figure 3. Untreated Cells immunolabeled for α-tubulin. Cells were fluorescently labeled with an antibody specific to α-tubulin and photographed at 63x magnification using a fluorescence-detecting microscope.

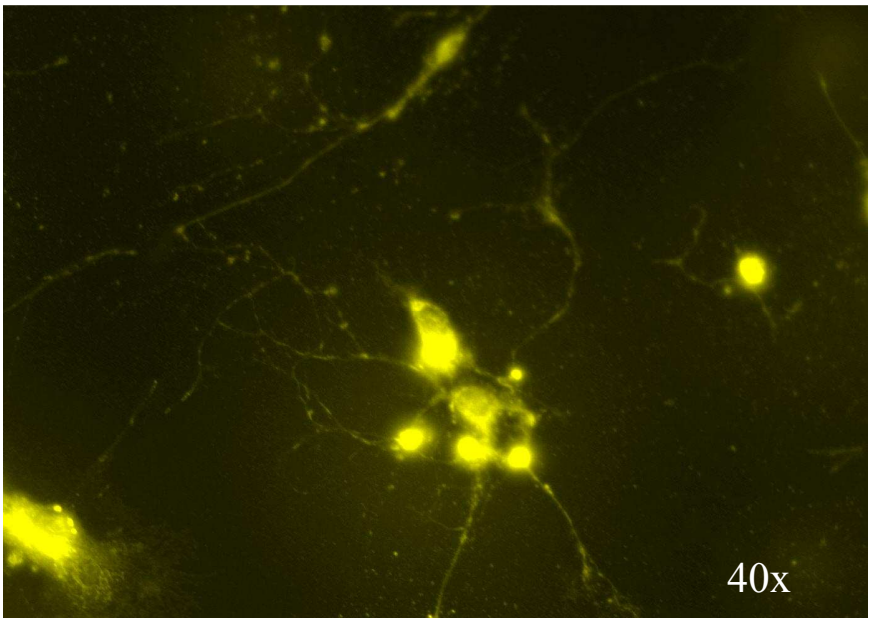

Figure 4. Untreated cells immunolabeled for neurofilament. Cells were fluorescently labeled with an antibody specific to neurofilament and photographed at 40x magnification using a fluorescence-detecting microscope.

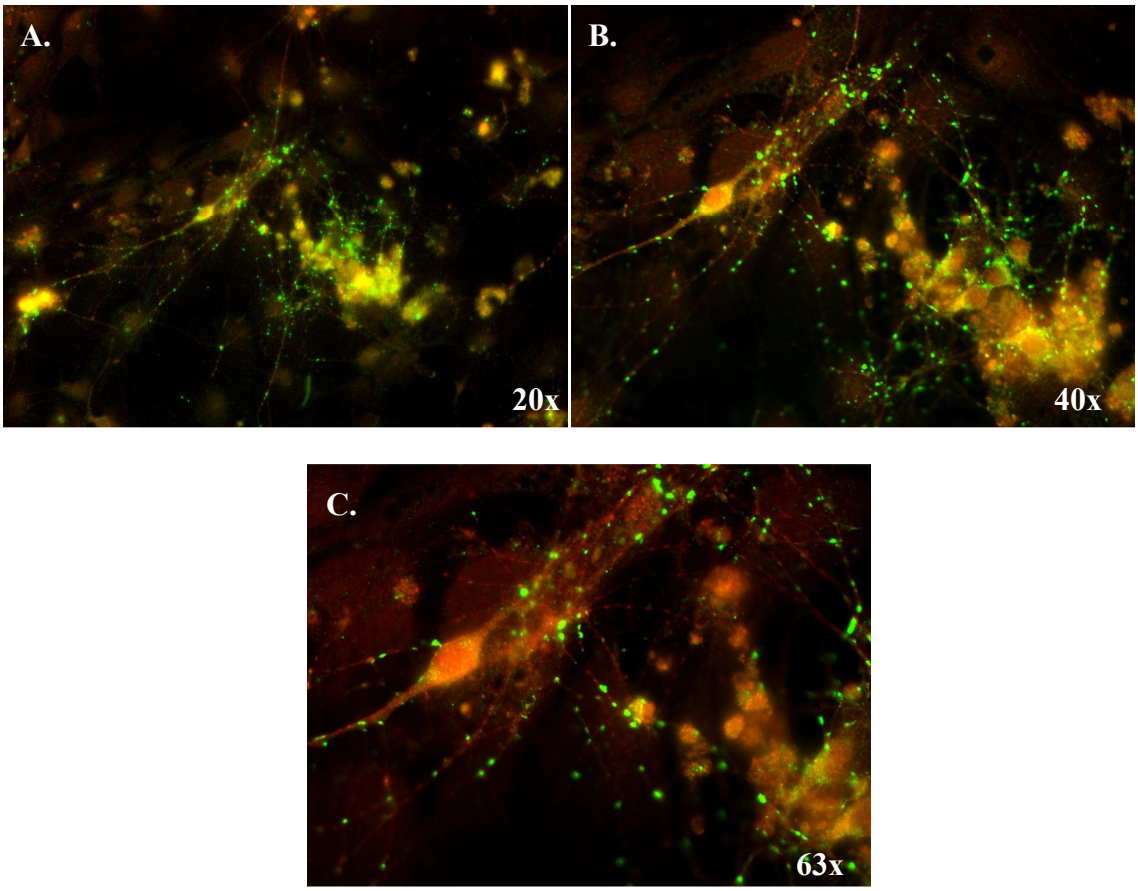

Figure 5. Untreated cells immunolabeled for GAP-43 and Synapsin-I. Cells were fluorescently labeled with an antibody specific to GAP-43 (red) and Synapsin-I (green) and photographed at 20, 40, and 63x magnification using a fluorescence-detecting microscope.

3.2 Effects of Pro-inflammatory Cytokine Exposure on mRNA Expression

After characterizing the hippocampal culture, we could now assess potential effects of pro-inflammatory cytokines beginning at the level of gene expression. We utilized RT-PCR to examine the relative presence of mRNA expression in response to administration of pro-inflammatory cytokines. Cells were treated in accordance with the timeline shown in Figure 1. After 24 hours incubation with pro-inflammatory cytokines, the cells were harvested and the total RNA was isolated and measured. Next, RT-PCR was performed to measure changes in mRNA expression of synapsin-I and GAP-43 compared with β-actin, an endogenous control protein. Graphs represent data pooled across four independent experiments.

In regard to alterations in synapsin-I expression, a main effect of Treatment was observed ($F(3,8)=6.014$, $p<.05$), indicating that cells administered pro-inflammatory cytokines showed increased expression of synapsin-I mRNA (see Figure 6). The effect of Treatment on synapsin-I expression was largely driven by increased expression among cells that were administered TNF-α. Indeed, post-hoc analyses revealed that cells administered TNF-α, had significantly increased expression of synapsin-I compared with all other groups, including those that were administered IL-1β or IL-6.

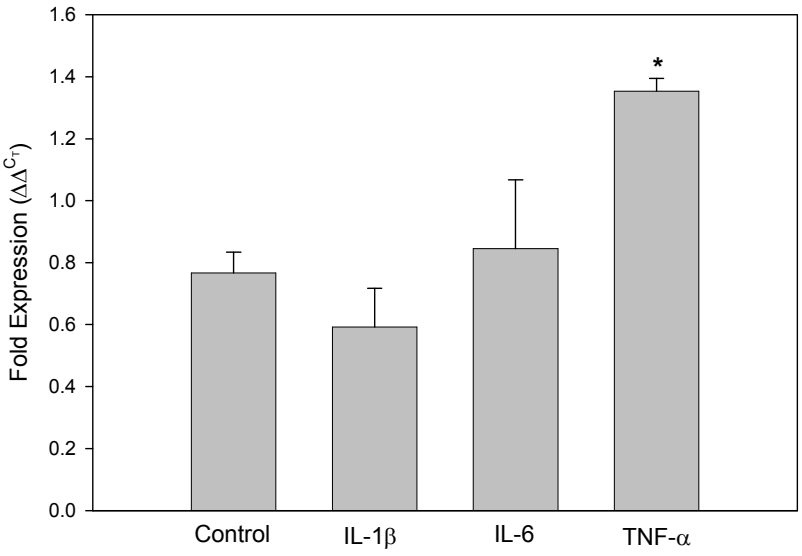

Figure 6. Effect of Pro-inflammatory Cytokine Treatment on Synapsin-I mRNA Expression. A significant main effect of cytokine treatment was observed, with the cells that were administered TNF-α expressing significantly increased levels of synapsin-I mRNA compared to all other groups. * $p<.05$. Error bars represent ± standard error of the mean.

However, in terms of GAP-43 mRNA expression, no significant main effect of cytokine treatment was observed ($F(3,8)=0.12$, *ns*; see Figure 7).

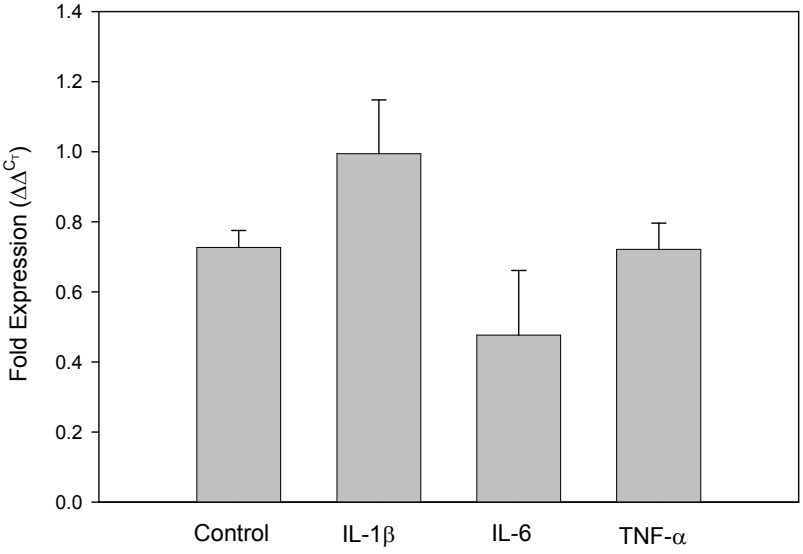

Figure 7. Effect of Pro-inflammatory Cytokine Treatment on GAP-43 mRNA Expression. No significant effect of pro-inflammatory cytokine treatment was observed on GAP-43 expression. Error bars represent ± standard error of the mean.

3.3 Effects of Pro-inflammatory Cytokine Exposure on Protein Expression

Cells were treated with either 100 ng/mL of IL-1β, 100 ng/mL of IL-6, 10 ng/mL of TNF-α, or remained untreated. The cells were incubated for 24 hours post-treatment and then harvested. The relative protein expression of synapsin-I or GAP-43 was normalized versus an endogenous control protein (α-tubulin) and analyzed by western blot. The protein bands underwent densometric analysis. The normalized amount of protein was transformed based as a function of the expression levels in untreated cells and expressed as "Normalized Fold Expression Over Untreated". Graphs depict data pooled across six independent experiments (n=6).

Treatment with IL-1β, IL-6, or TNF-α led to a 2-fold or greater increase in synapsin-I expression relative to untreated cells (see Figure 8).

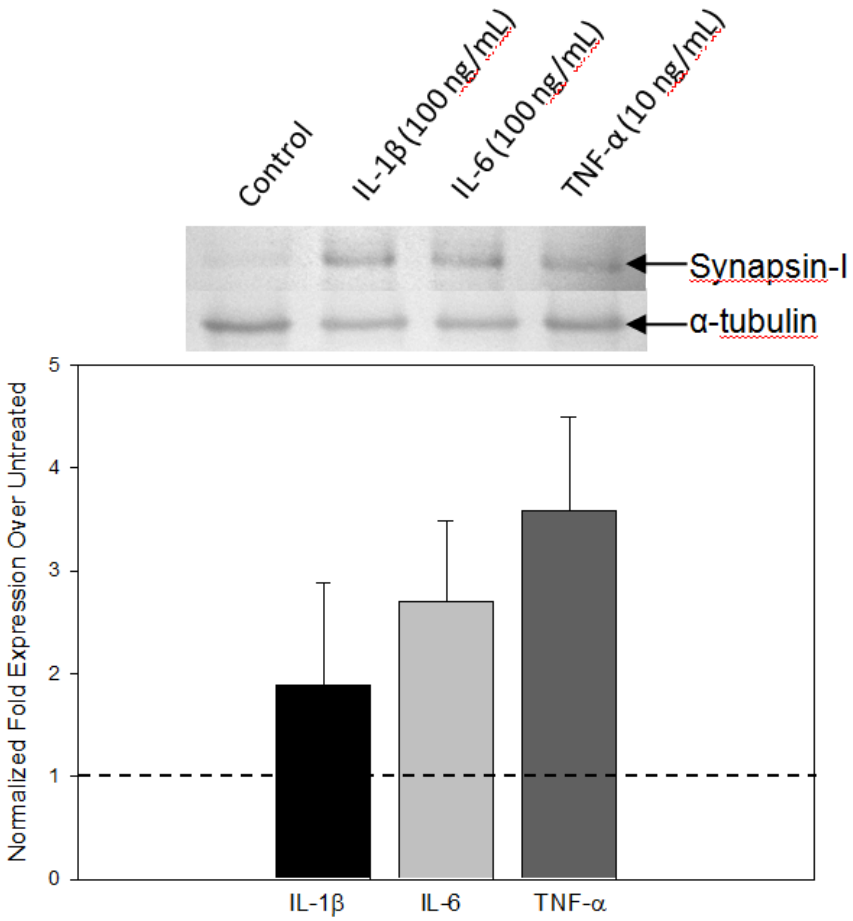

Figure 8. Effect of Pro-inflammatory Cytokine Treatment on Synapsin-I Expression. Western blot data indicate that cells administered pro-inflammatory cytokines were expressing a 2–3.75 fold increase in expression of synapsin-I. Western blot data were normalized by α-tubulin expression and transformed to represent fold increase relative to untreated cells (dotted line represents the expression level of untreated cells). Error bars represent ± standard error of the mean.

Treatment with IL-1β, TNF-α led to no significant change in GAP-43 expression compared with untreated cells. Treatment with IL-6 led to a modest, 1.5-fold increase, in GAP-43 expression relative to untreated cells (see Figure 9).

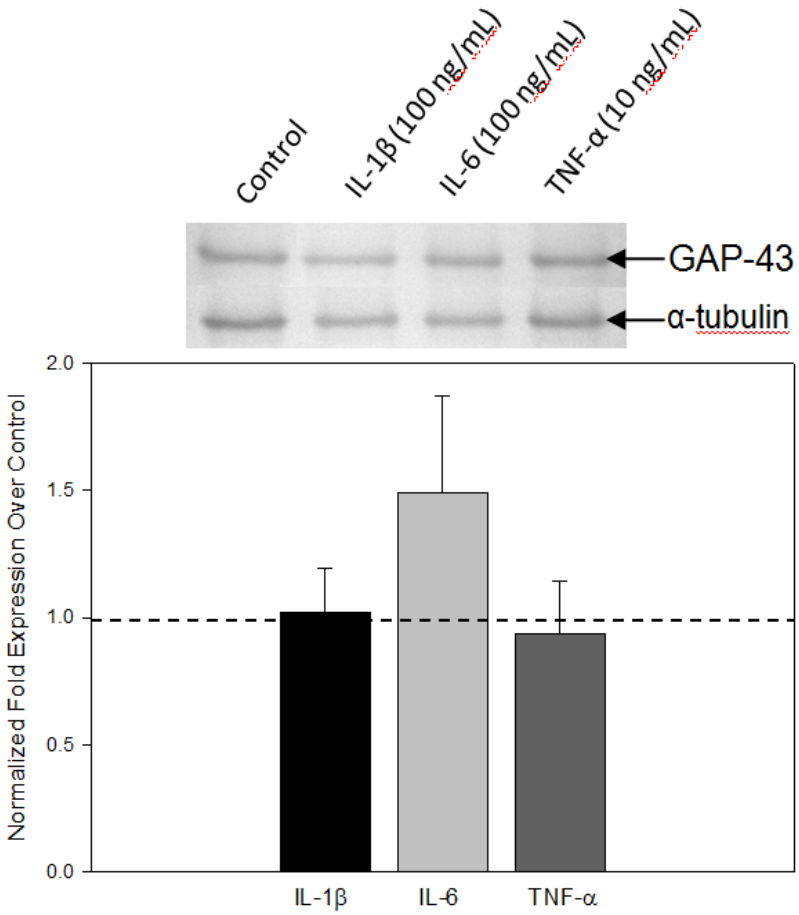

Figure 9. Effect of Pro-inflammatory Cytokine Treatment on GAP-43 Expression. Western blot data indicate that cells administered pro-inflammatory cytokines, were expressing a similar (IL-1β and TNF-α) or modestly increased expression (IL-6) of GAP-43. Western blot data were normalized by α-tubulin expression and transformed to represent fold increase relative to untreated cells (dotted line represents the expression level of untreated cells). No significant effects were observed. Error bars represent ± standard error of the mean.

3.4 Effect of Cytokine Administration on Morphology

Cells were treated with either 100 ng/mL of IL-1β, 100 ng/mL of IL-6, 10 ng/mL of TNF-α, or remained untreated. The cells were incubated for 24 hours post-treatment, harvested and then dual-labeled for GAP-43 and synapsin-I. Under the fluorescent microscope, 7 pictures were taken pseudorandomly from each condition. The images were then analyzed in ImageJ (NIH) for length and number of processes (see Figure 10) by an

experimenter blind to the treatment condition. Graphs represent data pooled across three independent experiments.

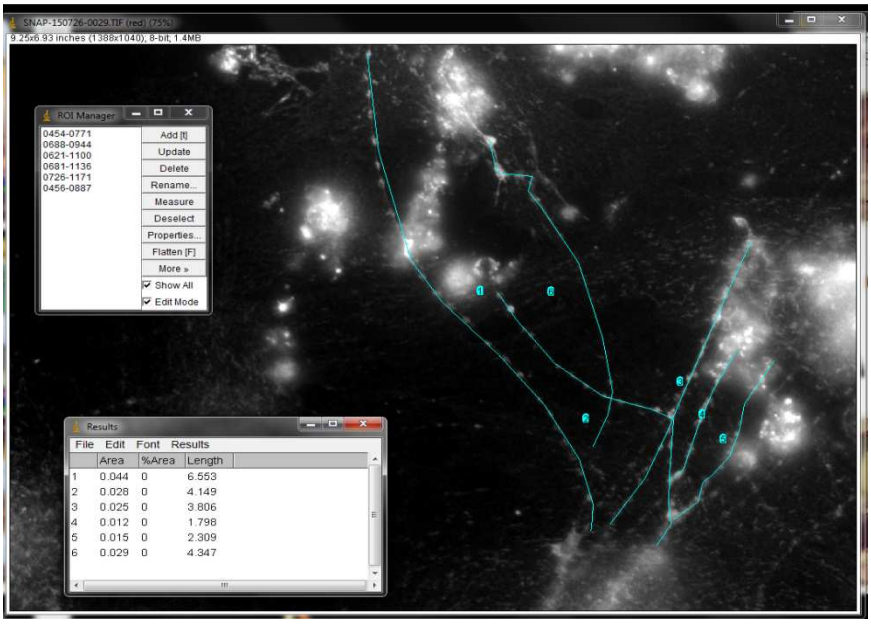

Figure 10. Example of Process Counting and Measurement. ImageJ was utilized to obtain measurements of process length and number by tracing the length of a process in microns.

No statistically significant effect of cytokine treatment was observed on the mean number of processes ($F(8,3)=0.980$, *ns*), though a trend was observed with control cells having, on average, more processes compared with those that were treated with IL-1β, IL-6, or TNF-α (see Figure 11).

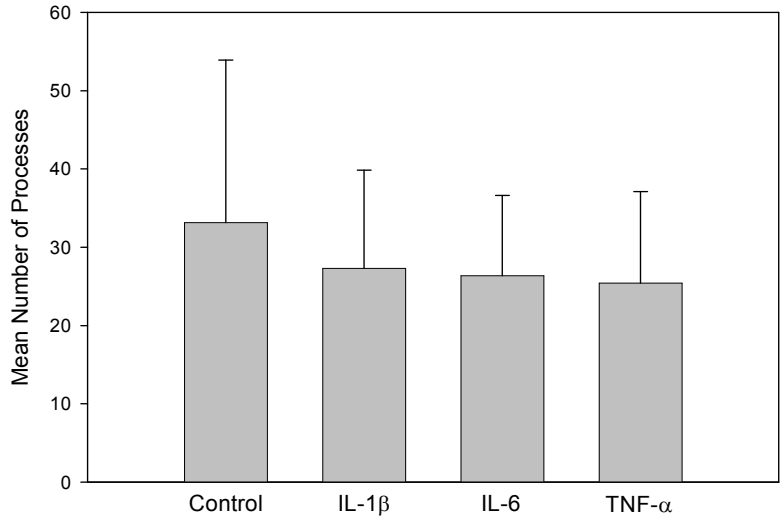

Figure 11. Effect of Pro-inflammatory Cytokine Treatment on Mean Number of Processes. No significant effect of pro-inflammatory cytokine treatment was observed. Error bars represent ± standard error of the mean.

No significant effect of cytokine treatment was observed on the mean length of processes ($F(8,3)=0.442$, *ns*), though a trend was observed with cytokine-treated cells having, on average, longer processes compared with untreated cells (see Figure 12).

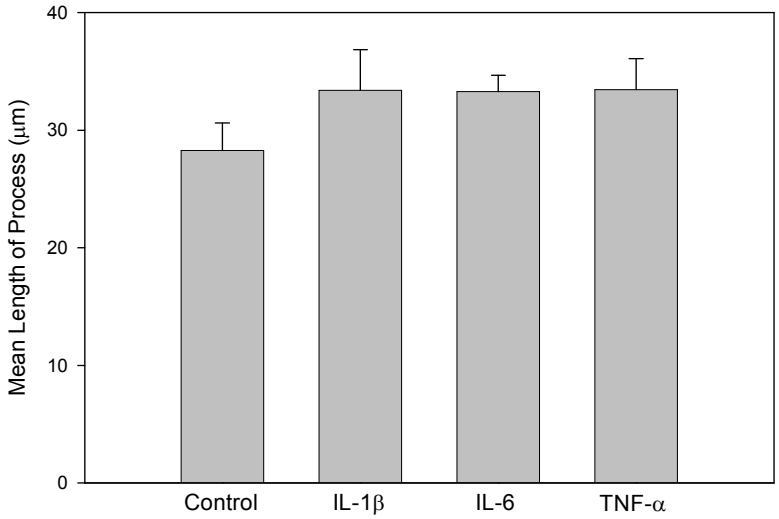

Figure 12. Effect of Pro-inflammatory Cytokine Treatment on Process Length. No significant effect of pro-inflammatory cytokine treatment was observed. Error bars represent ± standard error of the mean.

In addition to measuring the length of processes, ImageJ was utilized to obtain measurements of process density using the same images utilized above (see Figure 13).

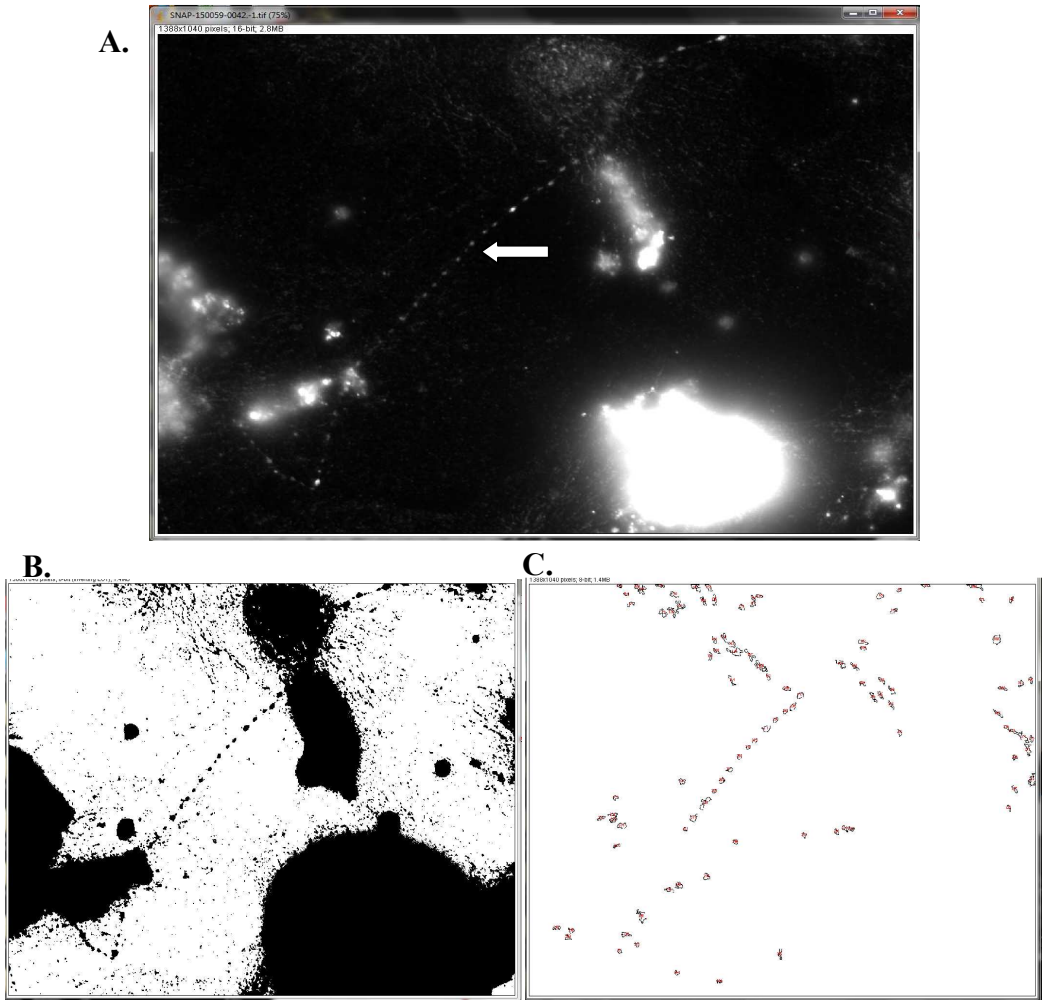

Figure 13. Example of Process Density Measurement. ImageJ was utilized to obtain measurements of process density by using the threshold tool to convert the original image (A) to a black and white contrast image show the relevant process (arrow) (B) excluding particles either larger or smaller than the processes and excluding these from the image. Finally, the bounded image (C) is used to obtain measurements of particle number and total area of the image.

No significant effect of cytokine treatment was observed on the mean area of processes density ($F(8,3)=0.913$, *ns*), though a trend was observed, with cytokine-treated

cells having, on average, an increased portion of each image taken up by processes (see Figure 14).

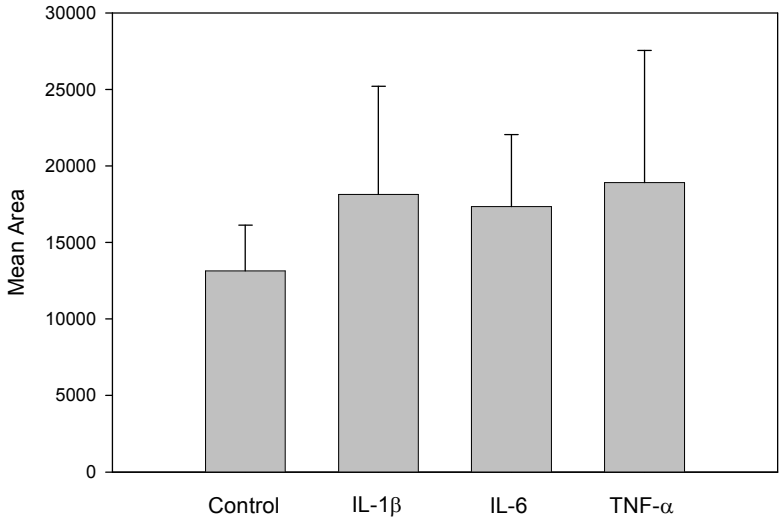

Figure 14. Effect of Pro-inflammatory Cytokine Treatment on Process Density. No significant effect of pro-inflammatory cytokine treatment on the mean area of the image that was comprised of processes (process density) was observed. Error bars represent ± standard error of the mean.

No significant effect of cytokine treatment was observed on the average number of processes obtained in each image ($F(8,3)=3.646$, *ns*), though a trend was observed with cytokine-treated cells having, on average, an increased number of processes compared with untreated cells (see Figure 15).

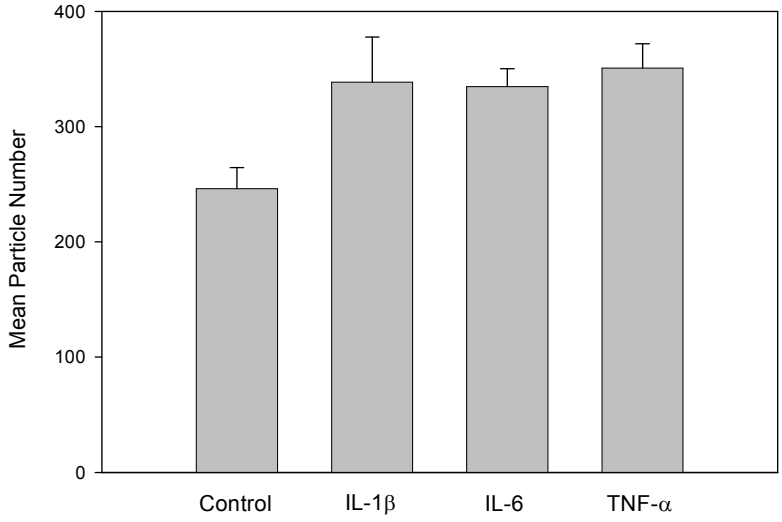

Figure 15. Effect of Pro-inflammatory Cytokine Treatment on Process Number. No significant effect of pro-inflammatory cytokine treatment was observed on the number of process particles present in each image (process number). Error bars represent ± standard error of the mean.

4. Discussion

Our experimental goal was to characterize the effects of pro-inflammatory cytokines at the level of gene expression, protein expression, and morphological cellular changes in process extension. The present set of studies is the first to characterize the effects of IL-1β and IL-6 in primary hippocampal cell culture. Based on results observed *in vivo* regarding the effects of central cytokine production on learning and memory, we hypothesized that exposure to IL-1β, IL-6, and TNF-α would lead to impaired outgrowth and lead to diminished neuronal plasticity. Specifically, we hypothesized that administration of these cytokines would lead to decreased mRNA and protein expression of synapsin-I and GAP-43, and that these alterations would coincide with decreased process extension, number, and density, compared with untreated cells.

Before assessing the effects of inflammatory cytokines on outgrowth, it was necessary to develop the methodology of primary hippocampal cell culture and characterize the cells. Our first goal was to use immunofluorescence to assess the phenotype of the culture population. We initially wanted to visual the entire cell population so we immuno-stained for α-tubulin (see Figure 3), a protein that is ubiquitously expressed among neurons and glial cells. Our next goal was to image mature neurons in isolation, using antibodies specific for neurofilament protein (see Figure 4). Finally, we imaged untreated cultures stained for our proteins interest, GAP-43 and synapsin-I, in an effort to observe the cellular localization of these proteins prior to any manipulation (see Figure 5).

We first examined the effect of cytokine exposure on gene expression using RT-PCR with probes and primers with sequences complimentary to synapsin-I and GAP-43 mRNA transcripts. After seven days of co-culturing, cells were treated with either, IL-1β, IL-6, TNF-α, or remained untreated and incubated for 24 hours. After 24 hours, the cells were harvested and the total RNA was isolated and measured (see Figure 1). RT-PCR was performed to measure the fold change in GAP-43 and synapsin-I mRNA expression compared to control gene expression.

In terms of synapsin-I expression, a significant main effect of cytokine treatment was observed. However, this effect was largely driven by TNF-α-treated cells, which showed significantly increased expression of synapsin-I compared with all other groups, including those that were treated with IL-6 and IL-1β (see Figure 6). However, no significant effects of cytokine treatment were observed on GAP-43 expression. These findings were not in line with our original hypothesis that cytokine administration would decrease gene transcription

of GAP-43 and synapsin-I. Rather, we found no changes on GAP-43 expression and increased expression of synapsin-I in response to TNF-α administration (see Figure 7).

Having characterized the effects of cytokine expression on gene transcription, the next step was to examine how these changes in mRNA expression impacted protein expression. We utilized western blots to examine relative expression of target proteins compared with a control protein that is expressed ubiquitously and expressed the data as fold change relative to control cells.

Contrary to our hypothesis that administration of pro-inflammatory cytokines would result in diminished expression of growth associated proteins, we observed increased or unaltered expression relative to control cells. Specifically, we observed that administration of IL-1β, IL-6, or TNF-α led to a two-fold, or greater, increase in levels of synapsin-I protein expression compared with untreated cells (see Figure 8). Furthermore, IL-1β and TNF-α treatment led to no change in GAP-43 expression relative to control, though treatment with IL-6 led to a 1.5-fold increase (see Figure 9).

Although these findings may not fit within our original hypotheses, they are not necessarily surprising. Pro-inflammatory cytokines, especially IL-1β have been shown to play a beneficial, though complex, role in learning and memory at the cellular and organismal level. Song and colleagues (2003) demonstrated that intracerebroventricular (i.c.v.) administration of IL-1β enhanced conditioned fear memory. The observed cognitive enhancement was associated with the induction of an anti-inflammatory response (peripheral production of IL-10) and increased corticosterone and prostaglandin E_2, possibly representing an adaptive response that may enhance survival during illness. Additionally, transplant of neural precursor cells to IL-1 receptor knockout neonates lead to an astrocyte-

mediated rescue of memory for the contextual fear and Morris water maze paradigms, as well as leading to a partial recovery of LTP (Ben Menachem-Zidon et al., 2011). Therefore, some experimental evidence argues in favor of the beneficial effects of pro-inflammatory cytokines on cognition and LTP.

Finally, it was important to determine whether these alterations in gene and protein expression, led to morphological changes within the cell. A clear way to image cell morphology is by utilizing immunofluorescence to stain for the presence of particular proteins within the cell. Fluorescence microscopy allows finer, more delicate, processes to be imaged than what can be observed under bright field microscopy. To measure changes in cell morphology, we utilized two separate modes of image analysis within the program NIH ImageJ. The first method measures the length and number of processes present in each image (see Figure 10). The second method measures the density of processes within each image, with cell bodies and other fluorescent debris excluded from the analysis (see Figure 13).

Despite observing alterations in protein expression, we failed to find significant morphological differences among cells administered pro-inflammatory cytokines, and control cells. Specifically, we observed a trend towards untreated cells showing increased number of processes, compared with cytokine-treated cells (see Figure 11). In contrast, we measured an opposite trend in the length of processes, with cells treated with IL-1β, IL-6, or TNF-α exhibiting longer processes than control cells (see Figure 12). These morphological data indicate that cytokine-treated cells trend toward leading to longer, but less profuse processes, though not significantly so. Similar effects were observed in measurements of process density. Cytokine administration did not significantly affect the number (see Figure 15) or

density of processes (see Figure 14), though there was a trend toward cytokine treatment leading to increased number and density of processes.

In general, our morphological data do not support the findings that Neumann and colleagues (2002) reported, indicating that incubation with TNF-α for 24 hours led to decreased process length. Rather, we found no significant change in process number or density, and observed a trend toward increased expression. There are many plausible explanations for these findings. For example, although alterations in synapsin-I expression at the levels of gene and protein expression were observed, perhaps 24 hours of incubation is not long enough for alterations within the cellular architecture to sufficiently manifest themselves. Another alternative is that important ceullular changes may be occurring at the synaptic level that may not necessarily impact the gross morphology of the cell. For example, IL-1β has been implicated in altered regulation of AMPA receptors (Lai et al., 2006). Moreover, IL-6 expression is associated with the formation of excitatory synapses (Wei et al., 2011). Additionally, IL-1β (Li et al., 1997), IL-6 (Jankowsky et al., 2000), and TNF-α (Butler et al., 2004) have been associated with impaired LTP, which generally affects the overall morphology of the cell, yet is manifested initially only at the synaptic level. These findings provide a small example of the myriad cellular changes that could be occurring in response to cytokine exposure, but would not necessarily impact process number, length, or density. Another possible explanation is that our experiments were designed to model the effects of acute inflammation in the context of pre-existing cellular architecture (i.e., not intended to development), similar to a single injection of LPS or some other immune stimulant. It is conceivable that although acute inflammation can lead to learning and memory impairments in several paradigms (e.g., Aubert et al., 1998; Pugh et al., 2001;

Sparkman et al., 2005), it does so without impacting cellular morphology in the same way that a chronic inflammatory environment, or administration during development, might. Perhaps changes that are induced by pro-inflammatory cytokines are limited to transient alterations in gene and protein expression that do not lead to structural changes within the cell. Finally, the observed alterations in protein expression may not have been sufficiently large enough in magnitude to impact the cytoskeletal architecture (i.e., we have a small effect size, and insufficient statistical power to uncover the effect).

How these overall results fit within the context of results from *in vitro* experiments performed in other labs is more complex. We have evidence that cytokine exposure may lead to increased expression of proteins related to process extension and outgrowth. Despite conflicting with reports of Neumann and colleagues (2002), our data are in agreement with work that has been performed using peripherally-derived nerve cells. As discussed earlier, IL-1β has been shown to increase neurite outgrowth in the dorsal root ganglion and cerebellar granule neurons (Temporin et al., 2008). Furthermore, in immortalized cell lines, as well as primary dorsal root ganglion cells, IL-6 exposure was associated with increased cell survival and process extension (Satoh et al., 1988; Edoff & Jerregard 2002). Exposure to TNF-α can promote cell survival (Takei & Laskey, 2008) and act in a neuroprotective fashion (Cheng et al., 1994). Much of the variability observed in these studies could be due to differences in dosage or the timing of cytokine administration. Additionally, the milieu of the culture could significantly impact the response to external signals, such as cytokines. For example, if a mixed culture is used, as it was in this study, the surrounding glia and support cells respond to an inflammatory environment differently, than a solely neuronal culture (Chang et al., 2001).

Within the context of *in vivo* work examining the effect of cytokine induction on cognition with rodents, the present results appear somewhat inconsistent with our hypotheses regarding putative mechanisms behind impaired learning in contextual fear conditioning (Pugh et al., 1998), two-way active avoidance (Sparkman et al., 2005a), Morris water maze (Sparkman et al., 2005b), autoshaping (Aubert et al., 1995), and Y-maze and T-maze (Sanderson et al., 2008), among others. However, a growing body of literature has begun to characterize instances when infection or immune stimulation does not lead to cognitive dysfunction. Swanepoel and colleagues (2011) tested the effects of a simulated *Mycoplasma pneumonia* infection using fibroblast-stimulating lipopeptide-1 (FSL-1) on sickness behaviors, spatial memory, and central cytokine production. Administration of FSL-1 was associated with dose-dependent increases in many common manifestations of "sickness behavior", including core body temperature, lethargy, and anorexia. Furthermore, increases in IL-1β mRNA expression were observed in the hippocampus and hypothalamus. However, despite these physiological changes, no significant impairments in spatial learning and memory were observed. Furthermore, among FSL-1-treated animals the swim speed was not altered. These data indicate that although FSL-1 induced production of hippocampal IL-1β that was sufficient to mediate sickness behavior, lethargy, and anorexia, it did not impair spatial learning in the Morris water maze. In addition to these findings, other researchers have observed similar effects with different immune stimulants, including following an intraperitoneal (i.p.) injections of LPS (Huang et al., 2010), Staphylococcal enterotoxin A (Woodruff et al., 2010), and IL-1β (Thomson & Sutherland, 2006). In humans, i.v. administration of LPS caused an increase in body temperature, though it led to no impairments in memory recall (Grigoleit et al., 2010). These data indicate that the effects of

immune activation on hippocampus-dependent learning and memory are complicated and depend upon a number of complex factors. Exactly why infection or immune activation can lead to memory impairments and some experimental situations and not others remains to be investigated (Swanepoel et al., 2011). It is possible that a similar effect may also be at work at the molecular and cellular level. For example, it may be possible that the concentrations of pro-inflammatory cytokines might be sufficient to induce "sickness behaviors" or other inflammation-related responses, but not impact certain cellular correlates of learning and memory (e.g., changes in morphology). Indeed, although exceedingly small doses of LPS (1 μg/kg; Teeling et al., 2007) can lead to profound sickness behavior, typically a much higher dose (125□250 μg/kg) is necessary to elicit learning and memory deficits (Pugh et al., 1998; Sparkman et al., 2005a,b).

Given the foregoing, we can conclude that the effects of inflammation *in vivo*, as well as *in vitro*, lead to complex changes that are not easily characterized and are, at times, seemingly contradictory. Future research should focus on characterizing the specific experimental parameters or cellular context in which inflammation-induced cytokine production promotes the seemingly beneficial antecedents (e.g., process outgrowth, neuroprotection, cognitive benefits) or the more harmful ones (e.g., cell death, exacerbated neurodegeneration, cognitive decline).

In conclusion, we have demonstrated that exposure to TNF-α led to significantly increased expression of synapsin-I mRNA, and that exposure to IL-1β, IL-6, and TNF-α led to alterations in expression of synapsin-I protein. However, pro-inflammatory cytokine treatment did not significantly impact gene or protein expression of GAP-43. Further, these changes in synapsin-I did not significantly impact cellular morphology, in terms of process

number, length, and density. The present study is the first to investigate the effects of IL-1β or IL-6 on primary hippocampal neurons, though considerably more work is needed to adequately characterize the cellular and molecular correlates of cytokine-induced cognitive deficits.

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Awards

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ABSTRACT

EFFECTS OF PRO-INFLAMMATORY CYTOKINES ON IN VITRO MEASURES OF GENE EXPRESSION, PROTEIN EXPRESSION, AND PROCESS EXTENSION IN PRIMARY HIPPOCAMPAL NEURONS

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A bi-directional relationship exists between the immune system and the central nervous system. A number of *in vivo* studies have reported that immune stimulation through administration of endotoxin or pro-inflammatory cytokines can lead to cognitive deficits in a variety of paradigms. However, despite a large body of work characterizing the behavioral effects of immune stimulation, the cellular mechanisms that underlie this global cognitive impairment remain unclear. The present study utilized primary hippocampal cell culture to examine the effect of pro-inflammatory cytokines on process extension and expression of relevant proteins. Specifically, we hypothesized that administration of interleukin-1β (IL-1β), interleukin-6 (IL-6), or tumor necrosis factor-α (TNF-α) would lead to morphological alterations (as evidenced by decreased process length, process number, and density of processes), and decreased protein and gene expression of GAP-43 and synapsin-I, two proteins important in synapse formation and process extension. Furthermore, in addition to replicating the prior findings of Neumann et al (2002) with regard to TNF-α, our goal was to extend this line of investigation to IL-1β and IL-6. The observed results were generally inconsistent to our original hypotheses. Rather than

decreasing expression, exposure to TNF-α led to significantly increased expression of synapsin-I mRNA, and exposure to IL-1β, IL-6, and TNF-α led to significant increases in expression of synapsin-I protein. However, pro-inflammatory cytokine treatment did not significantly impact gene or protein expression of GAP-43. Further, these changes in synapsin-I, though correlated, did not significantly impact cellular morphology, in terms of process number, length, and density. The present study is the first to investigate the effects of IL-1β or IL-6 on primary hippocampal neurons.