gamma-Aminobutyric acid inhibits synergistic interleukin-6 release but not transcriptional activation in astrocytoma cells

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γ-AMINOBUTYRIC ACID INHIBITS SYNERGISTIC INTERLEUKIN-6 RELEASE
BUT NOT TRANSCRIPTIONAL ACTIVATION IN ASTROCYTOMA CELLS

by

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ABSTRACT

γ-Aminobutyric Acid Inhibits Synergistic Interleukin-6 Release but not Transcriptional Activation in Astrocytoma

By

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A decline in the inhibitory neurotransmitter γ-aminobutyric acid (GABA) may enhance cytokine release in Alzheimer’s disease (AD) resulting in neuroinflammation. We investigated the GABA-mediated suppression of the synergistic release of interleukin-6 (IL-6) due to interleukin 1-β (IL-1β) and tumor necrosis factor α (TNF-α) synergy. Our results indicate that p38 and nuclear factor (NF)-κB are essential for the synergistic release of IL-6 by IL-1β and TNF-α. Both IL-1β and TNF-α stimulate p38 phosphorylation and each are able to enhance the degradation of the NF-κB inhibitor, IκB-α, with no change in IκB-β. Interestingly, co-treatments IL-1β and TNF-α induce a greater increase in IκB-α degradation compared to either cytokine alone. In addition, co-treatment increased IL-6 mRNA indicating synergy on a transcriptional level. In addition, Western blot analysis revealed that co-treatments of IL-1β and TNF-α resulted in an increase of IL-6 protein that can be reversed by GABA. Furthermore, co-
treatments of IL-1β and TNF-α resulted in activation of p38 and degradation of \( \text{IκB-α} \) at earlier time points compared to either cytokine alone. These data suggests that IL-1β and TNF-α synergizes by accelerating p38 and NF-κB activation, increasing IL-6 transcription. Although GABA suppressed the IL-1β and TNF-α synergistic release of IL-6, it did not affect \( \text{IκB-α} \) or \( \text{IκB-β} \) degradation, p38 phosphorylation or IL-6 transcription. Instead it appears that GABA may inhibit IL-6 translation. We have demonstrated that this synergistic release of IL-6 can be inhibited by GABA and its receptor agonists.
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ABBREVIATIONS


Aβ – β-Amyloid
AD – Alzheimer’s Disease
APP – Amyloid Precursor Protein
β-ME – 2-mercaptoethanol
BSA – Bovine Serum Albumin
CNS – Central Nervous System
DMSO – Dimethyl Sulfoxide
ECL – Enhanced Chemiluminescence
EDTA – Ethylenediaminetetraacetic Acid
ELISA – Enzyme-Linked Immunosorbent Assay
ERK – Extracellular-Related Kinase
FBS – Fetal Bovine Serum
G3PDH – glyceraldehydes-3-phosphate dehydrogenase
GABA – γ-Aminobutyric Acid
HEPES – 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HRP – Horseradish Peroxidase
IκB – Inhibitor of κB
IKK – IκB Kinase
IL – Interleukin
IL-1i – IL-1 Inhibitor
IL-1ra – IL-1 Receptor Antagonist
INF – Interferon
JNK – c-Jun-N-Terminal Kinase
kDa – Kilodalton
MAPK – Mitogen Activated Protein Kinase
NaCl – Sodium Chloride
NF-κB – Nuclear Factor-κB
NFT – Neurofibrillary Tangle
PAGE – Polyacrylamide Gel Electrophoresis
PBS – Phosphate-buffered Saline
PDL – Poly-D-Lysine
PHF – Paired Helical Filament
RPMI – Roswell Park Memorial Institute
SDS – Sodium Dodecyl Sulfate
TBST – Tris-Buffered Saline containing Tween
TI – Tumor Necrosis Factor-α and Interleukin-1β
TNF – Tumor Necrosis Factor
Tris – Tris-(hydroxymethyl)-amino methane
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CHAPTER 1

INTRODUCTION

The biological foundation of the central nervous system

In order to understand the effects of Alzheimer disease it is essential to discuss the biological foundations of the central nervous system. The central nervous system (CNS) consists of several cell types, including neurons responsible for information processing and transmission. Neurons are aided by supporting cells referred to glial cells. Nerve cells are secretory cells of varying morphologies capable of information transfer thus generating electrochemical impulses that travel the length reaching the synaptic cleft (Bowsher, 1988).

Nerve cells have the typical cellular features as well as other adaptations and sacrifices to suit their unique roles. For example, neuronal cells can be relatively large and long when compared to the morphology of other types of cells and form vast networks to undertake information transfer. This large size and length is essential in establishing communication networks with other cells in different parts of the body. Each neuron consists of the main cell body containing the nucleus and Nissl bodies which are large congregations of ribosomes (Bowsher, 1988), which are indicative of a higher than normal protein production.
Another notable adaptation of neuronal cells is that they are less tolerant to metabolic changes. For neurons, immediate cell death can occur if the oxygen supply and other substances are diminished which gives neurons the characteristics of a simple metabolic profile (Bowsher, 1988). Another adaptation is that neuronal cells consume energy at a higher rate than most other cells. To meet this energy demand mitochondria take up nearly 66% of the entire volume of a neuron (Bowsher, 1988). Yet another biological adaptation includes the inability to replicate. The cell must sacrifice replication because of its size and due branches that extend to varying distances and can range from a few millimeters to over a meter in length (Bowsher, 1988). These dendritic trees are essential in forming communication networks with other cells. In addition, considerable time and resources are used to establish these connections that can be lost if the cell were to undergo morphological changes during mitosis. These branching bodies that extend from the neuronal main cellular body are called dendrites and axons, which function to receive and transmit information, respectively.

Dendrites receive information transmitted from other cells and conduct this information towards the cell body and even to the axon or nerve fiber (Lawson, 2003). The ends of axons transmit and communicate to other cells (Lawson, 2003). Along these axons are microtubule (referred to as neurofilaments or neurotubules) networks which extend to the terminals where neurotransmission occurs (Bowsher, 1988). It is along these tubules that the phenomenon known as axonal transport occurs, which is the active movement of neurotransmitters
and/or their precursors along the axon in either direction (Bowsher, 1988). One can think of these filaments as a highway for substances to be shuttled to and from the axonal terminal, referred to as the bouton (button).

It is at the bouton where neurotransmission occurs through direct contact to another cell body or to the dendrites of another cell (Bowsher, 1988). Electron microscopy has shown that the bouton contain a large array of synaptic vesicles. A nervous impulse travels along the axon and arrives at the bouton, resulting in the release of neurotransmitters from the vesicles into the synaptic cleft (Bowsher, 1988).

Biochemical features of Alzheimer disease

Alzheimer disease (AD) is a debilitating disease that affects the CNS. It was first described by the German physician Alois Alzheimer in 1907 (Alzheimer, 1907) and despite the fact that researchers have had approximately one hundred years to study this disease the cause is still unknown (Griffin, 2006) and no cures have been developed. It is the most common form of dementia in the elderly. In the U.S. alone AD affects 4.5 million people and this is expected to increase to 13.2 million by 2050 (Herbert et al., 2003). In addition it has been estimated that 24.3 million people worldwide are affected by AD and this number is expected to double every 20 years as life expectancy increases (Ferri et al., 2005). On the biological level there are three features that define Alzheimer pathology. These are [1] senile plaque formation; [2] neurofibrillary tangles or paired helical filaments (PHF) first observed and characterized by Dr. Alzheimer in 1907; and
inflammation processes which contribute to the deposition of amyloid plaques that form senile plaques (Qiao et al., 2001).

The amyloid plaques (A\(\beta\)) originate from the processing of amyloid precursor protein (APP) (Kang et al., 1987). Though the exact function or purpose of APP or amyloid plaques is unknown, there is much data demonstrating potential roles for APP. APP has a structure similar to membrane bound proteins (Kang et al., 1987), indicative of some unknown function localized to cell membranes. Furthermore, APP is highly expressed in nerve terminals and may be essential for synaptic formation during brain development, neurogenesis and neuroregeneration (Loffler and Huber, 1982 Chen et al., 2006, and Selkoe et al., 1988). It is interesting to note that APP is up-regulated after head injury (Gentlemen et al., 1993) and that APP is also deposited in aged Down syndrome patients (trisomy-21); (Kang et al., 1987). Furthermore, it is also interesting to note that the APP gene is located on chromosome 21 (Selkoe et al., 1988) and that individuals affected with trisomy-21 have an earlier onset of brain growth arrest, brain atrophy and the development of AD (Wisniewski et al., 1985). Using trisomy-21 as a model, these observations suggests that up-regulation of APP expression and processing can lead to the progression of AD.

The formation of amyloid plaques occurs through the processing of APP fragments which are cleaved by a family of enzymes known as secretases. APP processing results in the formation of \(\beta\)-amyloid (A\(\beta\)) peptide fragments of \(\sim\) 39-43 amino acid residues in length (Buxbaum et al., 1998, Cintron et al., 1994 and Selkoe et al., 1996), each of which are found in amyloid plaque deposits in AD.
patients. In addition, missense mutations occur more frequently in the \( \beta \text{APP} \) gene of aged individuals resulting in the formation of extremely hydrophobic, amyloidogenic and insoluble fragments (Cintron et al., 1994) which can further exacerbate AD. These A\( \beta \) fragments are proposed to be the underlying force that ultimately initiates and drives AD development and progression (Hardy and Higgins 1992).

This idea that A\( \beta \) plaque formation is the causative force of AD has become known as the amyloid cascade hypothesis (Hardy and Higgins, 1992). This hypothesis is widely acknowledged by mainstream AD researchers but is not entirely accepted. For instance, it has been reported that there is a little or no correlation between A\( \beta \) plaque deposition and neurological dysfunction (Terry et al., 1999-2000 and Klein et al., 2001). Terry et al., argue that it is the loss of the synaptic terminals, rather than amyloid plaque deposition, which leads to the development and progression of dementia. Those who do not agree with the amyloid cascade hypothesis are few as there are over 5000 publications (Klein et al., 2001) that support the hypothesis.

The second hallmark feature of AD is neurofibrillary tangles. Along the lengths of the axons are microtubule fibers forming the backbone of the axoplasmic transport system. These tubules serve as the internal support structures transporting nutrients, vesicles, mitochondria, and other substrates from the cell body to the ends of the axon and back (Griffin, 2006). A low molecular weight protein known as tau is an integral component of microtubules and can bind directly to the microtubule fiber promoting microtubule assembly.
In AD, tau can become hyper-phosphorylated allowing it to bind together and form tangles (Braak et al., 1994) which have become known as paired helical filaments (PHFs) or neurofibrillary tangles (NFTs). In addition, tau can impede axoplasmic transport by competitive binding with the motor protein kinesin (Ebneth et al., 1998, Stamer et al., 2002 and Trinczek et al., 1999). Another negative effect of tau hyper-phosphorylation is increased resistance to protein degradation (Poppek et al., 2006), blocking axoplasmic transport and stabilizing PHFs.

The third feature of AD affected tissues is neuroinflammation. The microglia which support neurons perceive amyloid plaques as foreign inclusions and initiate a inflammatory response to remove these depositions by phagocytosis (Dewitt et al., 1998). Initiation of the inflammatory response causes activation of glial cells resulting in the release of pro-inflammatory cytokines such as IL-1β and S100β, both of which have been associated with senile plaque formation (Griffin, 2006). In addition, the release of inflammatory cytokines such as IL-1β can up-regulate the expression and processing of APP leading to the deposition of additional Aβ fragments (Goldgaber et al., 1989 and Buxbaum et al., 1992). As more APP is processed and Aβ deposition increases, the microglia cells eventually become overburdened in an attempt to remove plaques Storing these fragments within the glia cell forces the cell to undergo lysis releasing the Aβ fragments that had been removed (Nagele et al., 2004). This system may self propagate and amplify itself promoting the development and progression of AD through an inflammatory response.
IL-1β can lead to the phosphorylation of tau, which demonstrates a possible relationship between IL-1β, tau and the formation of neurofibrillary tangles. Li and colleagues have reported that an increase in IL-1β correlated with the increased phosphorylation of tau, which in turn decreased synaptophysin synthesis (Li et al., 2003). They also reported that this effect can be blocked using the p38 inhibitor SB203580. They concluded that IL-1β signaling is mediated through p38 and is involved in tau phosphorylation, potentially leading to neurofibrillary tangles (Figure 1).

Mitogen-activated protein kinases

The MAPK superfamily of kinases plays a major role in cellular signaling of many different cellular processes. The MAPK family includes p38, extracellular-signal regulating kinase (ERK) and c-Jun N-terminal kinase (JNK). As shown earlier, there are data supporting p38 in pro-inflammatory and stress responses (Xia et al., 1995). Indirect evidence suggests that JNK may also play a role in inflammation (Ip and Davis 1998 and Eynott et al., 2003). In contrast ERK has an opposing role to that of JNK and p38 as it has been implicated in proliferation and cell survival (Xia et al., 1995 and Rescigno et al., 1998).

Increased activities of p38 and JNK have been implicated in a rodent model of AD (Savage et al., 2002) and post mortem analysis of human AD affected brain tissues show activated c-Jun in neurofibrillary tangles implying a role for JNK in AD (Pearson et al., 2006). It is interesting to note that Savage and colleagues reported that the pro-inflammatory and stress responsive kinases p38
and JNK were activated in a mouse model of AD whereas the developmental and cell survival responsive ERK was not. From these data one can see further evidence of the link between inflammation and AD and the potential involvement of JNK. One could also argue that it is a combination of the pro-inflammatory and stress response of p38 and JNK, respectively, in combination with the breakdown of cell survival signaling of ERK that may allow the development and progression of AD.

Cytokines and their biological effects

Cytokines are proteins that play roles in differentiation, proliferation, inflammation, apoptosis, and induction of various proteins. Some cytokines include interleukins (IL) and tumor necrosis factors (TNF) which carry out a broad range of different functions in the cell. There are 33 known types of interleukins designated interleukins, 1-33. Interleukin-1β (IL-1β) is an important signaling molecule in the pro-inflammatory response and has even been linked to AD (Griffin, 2006).

The IL-1 subfamily of cytokines consists of three members: IL-1α, IL-1β and their negative feedback inhibitor IL-1 inhibitor (IL-1i) which was later renamed to IL-1 receptor antagonist (IL-1Ra) (Dinarello, 1994). The precursor of IL-1 is a 35-kilodalton (kDa) inactive peptide that must be processed to a 17 kDa active polypeptide (Hazuda et al., 1990), which can be further altered via glycosylation. This biologically active form binds to an IL-1 cell receptor initiating a pro-inflammatory response which activates the MAPK p38 and the transcription
factor NF-κB (O’Niell, 2000). It is interesting to note that both p38 and NF-κB have been associated with inflammatory response leading to the production and secretion of IL-6 (Craig et al., 2000).

Another inflammatory cytokine which seems to play a role in AD is a family of cytokines collectively referred to as tumor necrosis factors (TNF). The first TNF was originally detected and observed over thirty years ago as a cell free cytotoxic substance that induced apoptosis in tumor cells (Granger et al., 1969). Probably the most characterized member of this family is TNF-α, first isolated by Carswell and colleagues in 1975. In addition to inducing apoptosis, TNFs have been linked to inflammation, host defenses, autoimmunity and other immunological responses (Locksley et al., 2001). Although TNF triggers beneficial immunological responses, some of its effects are injurious to the host organism. These detrimental effects include septic shock, cachexia and disease symptoms (Tracey and Cerami 1994).

One can postulate that IL-1β and TNF-α may initiate an inflammatory response through the activation of p38 and NF-κB, resulting in the production and release of IL-6. Inflammation contributes to the processing and release of Aβ fragments (Goldgaber et al., 1989 and Buxbaum et al., 1992). Li and colleagues reported that hyperphosphorylation is caused by p38. As for NF-κB, it is proposed by O’Niell and colleagues that IL-1β activates p38, which in turn activates NF-κB resulting in IL-6 transcription. It has also been demonstrated that activation of NF-κB can be reversed or inhibited by p38 inhibitor SKF (Kaur et al., 2003). These data provides evidence that p38 inhibitors and potentially
anti-inflammatory agents may reverse or inhibit inflammation which in turn may alleviate some effects of AD.

Nuclear Factor-κB

Nuclear Factor-κB (NF-κB) is a transcription factor related to the Rel family proteins (Ghosh et al., 1998) and exists in virtually all cell types. It is found in the cytoplasm bound to an intrinsic inhibitor κB (IκB) which prevents it from migrating to the nucleus of the cell. The activity of NF-κB can be summed up in three steps: [a] the inhibitor is phosphorylated by IκB kinases (IKK); [b] ubiquination marks it for degradation by proteosomes; and [c] released NF-κB translocates to the nucleus and binds to the κB binding sites located in the promoters and enhancers of many genes (Ghosh et al., 1998 and Abraham 2000). Some genes that become activated include IL-1, IL-6, TNF-α, lymphotoxins and Interferons (IFN). Furthermore these molecules can activate NF-κB (Kopp & Ghosh 1999) allowing them to prime the inflammatory response or further exacerbate it. The nature of the genes that are activated suggests that NF-κB is responsible for initiating inflammation and plays a major role in immune response (Kopp & Ghosh 1999).

The IκB family of NF-κB inhibitors include IκB-α, IκB-β, IκB-ε and IκB-γ (Ghosh et al., 1998). Though inhibitors, their effects vary depending on the source of induction. For example, the first inhibitor to be characterized and cloned was IκB-α (Davis et al., 1991), which regulates NF-κB through a rapid transient induction activity that also regulates itself in a negative feedback loop.
fashion where activated NF-κB causes the up-regulation of IκB-α transcription resulting in a bi-phasic activation period (Ghosh et al., 1998).

Where IκB-α degradation leads to transient activation of NF-κB, degradation of IκB-β leads to a persistent activation of NF-κB. First purified, cloned and characterized in 1995 its activity is much different than that of IκB-α. The inhibitory effect of IκB-α relies on activated NF-κB to promote the transcription of additional IκB-α, where as activated NF-κB does not promote IκB-β transcription. The lack of this autoregulatory feedback loop leads to the persistent activation of NF-κB (Thompson et al., 1995). One can speculate that the chronic inflammation associated with AD may be a result of the persistent activation of NF-κB rather than IκB-β.

The last two members, IκB-ε and IκB-γ are the more recently discovered members. Currently, IκB-ε and its functional relationship with NF-κB is still unknown (Spiecker et al., 2000). All members of this family of inhibitors have nuclear import and export functions and it has been postulated that each member possesses this ability to varying degrees (Lee and Hannink 2002). There is evidence suggesting that IκB-ε is less efficient in nuclear importing but is very effective in NF-κB export from the nucleus (Lee and Hannink 2002, Tam and Sen 2001). Although nucleocytoplasmic shuttling abilities of IκB-ε has not been examined in detail (Lee and Hannink 2002), this proposed function is gaining in popularity. The last member, IκB-γ has been better characterized than IκB-ε. There is much experimental data suggesting that IκB-γ inhibits NF-κB by
attaching directly to nucleotides and thus preventing NF-κB from binding to DNA (Bell et al., 1996). In addition, one can measure NF-κB activation indirectly by measuring the degradation of the different IkB inhibitors.

γ-Aminobutyric acid an inhibitory neurotransmitter

γ-Aminobutyric Acid (GABA) is probably one of the most important neurotransmitter in the CNS (Figure 2). It is capable of mediating membrane hyperpolarization or depolarization and is believed to be responsible for developmental neuronal migration (Han et al., 2002) and rapidly inhibits synaptic transmission (Kolaj et al., 2004 and Otis and Mody 1992). It has even been shown that a GABA can inhibit the proinflammatory responses in T-cells (Tian et al., 2004) and inhibit IL-1β induced IL-6 release (Spangelo et al., 2004). Interestingly, individuals affected by AD have lower levels of GABA (Bareggi et al., 1982); however, these findings are in constant debate as there are many reports that support strong, little or no correlations of AD and GABA levels.

Receptors of GABA are functionally subdivided as GABA_A or GABA_B receptors. The ionotropic GABA_A receptor conducts chloride ions (Olsen and Tobin 1990) and mediates fast inhibitory responses (Banks et al., 2002). GABA_A receptors are hetero-oligomeric protein structures that are composed of pentamers each approximately 50 kDa (Olsen and Tobin 1990). In addition, it is possible to selectively activate the GABA_A receptor through the use of muscimol (Figure 3; Murashita el al., 2007) or isoguvacine oxide (Frolund et al., 1995).
In contrast to the ionotropic GABA\textsubscript{A} receptor the GABA\textsubscript{B} is a metabotropic G protein coupled receptor (Kerr and Ong 2001). GABA\textsubscript{A} illicits fast inhibitory responses, GABA\textsubscript{B} is responsible for slower inhibitory responses (Tamas et al., 2003 and Federici et al., 2005). Activation of GABA\textsubscript{B} receptors results in the reduction of neurotransmission at excitatory and inhibitory synapses through an increase in potassium conductance or a decrease in calcium currents (Kerr and Ong 2001). One can selectively activate the GABA\textsubscript{B} receptor with the use of the drug baclofen, structure shown in (Figure 3; Bartoletti et al., 2004).

Hypothesis Statement

Previous unpublished results demonstrated that GABA inhibits the release of IL-6 due to TNF-\(\alpha\) and IL-1\(\beta\) and these results were latter confirmed (Figure 4). In addition, our preliminary results suggest that GABA inhibits IL-6 release and may not be through a decrease in p38 activation or an inhibition of NF-\(\kappa\)B activation. In respect to the role played by inflammatory cytokines in AD, the work presented in this thesis come from three separate but related hypothesis statements. Hypothesis I: the synergistic effect of TNF-\(\alpha\) and IL-1\(\beta\) arises from an increase in IL-6 transcription. Hypothesis II: GABA, affects p38 or NF-\(\kappa\)B activation. Hypothesis III: GABA may also inhibit the release of the pro-inflammatory cytokine IL-6 by preventing its transcription induced by TNF-\(\alpha\) and IL-1\(\beta\).
CHAPTER 2

MATERIALS AND METHODS

Chemicals, reagents and equipment

γ-Amino butyric acid (GABA), glycine, sodium chloride (NaCl), potassium chloride, 4-(2-hydroxyethyl)-monosodium salt (HEPES), sodium dodecyl sulphate (SDS), tris-(hydroxymethyl)-amino-methane (Tris), tris-(hydroxymethyl)-amino-methane hydrochloride, Tween-20, dimethyl sulfoxide (DMSO), 2-mercaptoethanol (β-ME), muscimol, baclofen and bromophenol blue were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest grade possible. High-molecular weight poly-D-lysine (PDL) hydrobromide was obtained from BD Biosciences (Bedford, MA). The pre-stained protein ladder was obtained from Bio-Rad (Hercules, CA). Roswell Park Memorial Institute (RPMI)-1640, PSN antibiotic mix, trypsin- ethylenediaminetetraacetic acid (EDTA), heat-inactivated fetal bovine serum (FBS), phosphate-buffered saline (PBS pH 7.2), PureLink Micro-to-Midi® total RNA purification system, SuperScript II reverse transcriptase and trypan blue were obtained from Invitrogen (Carlsbad, CA). Methanol was obtained from EMD Chemicals (Gibbstown, NJ). Bovine serum albumin (BSA) was obtained from Gemini Bioproducts (Woodland, CA). Recombinant rat IL-1β, TNF-α and rabbit anti-rat IL-6 antibody were obtained
from PeproTech Inc. (Rocky Hill, NJ). Rabbit anti-rat IκB-α, IκB-β, phosphorylated p38 (T180/Y182), total p38, total JNK, mouse anti-rat phosphorylated JNK (T183/Y185), rabbit anti-rat IL-6 antibody and the biotinylated protein ladder was obtained from Cell Signaling (Danvers, MA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from KPL (Gaithersburg, MD). Bay 11-7082, were obtained from EMD Biosciences (La Jolla, CA). The rat C6 Glioma cell lines were from the American Tissue Type Culture Collection (Rockville, MD). Rat IL-6 sandwich enzyme-linked immunosorbent assay (ELISA) kits, M-PER®, precasted 12% SDS- polyacrylamide gel electrophoresis (PAGE) gels, protease and phosphatase inhibitor cocktails, and the microBCA® protein assay kit were all obtained from Pierce Biotechnology (Rockford, IL). Hoefer SE 260 mini-vertical gel electrophoresis unit, EPS 2A200 power supply, Hoefer TE 22 tank transfer unit, MultiTemp III thermostatic circulator, Enhanced Chemiluminescence (ECL)-Plus® detection reagent was obtained from Amersham (Piscataway, NJ). Schleicher & Schuell Optitran® brand supported 0.45 μm nitrocellulose membranes were obtained from ISC Bioexpress (Kaysville, UT). Rabbit anti-rat IL-6 antibody was purchased from Cell Sciences (Canton, MA). All small molecule inhibitors dissolved in sterile solvent, aliquoted and stored at -20 °C until use. The final concentrations of DMSO never exceeded 0.1% of the final solution.
C6 glioma cell culture

Rat C6 glioma cells were maintained in continuous culture in a humidified atmosphere containing 95% air and 5% CO₂ in complete medium (RPMI-1640/phenol red, medium containing 10% heat-inactivated FBS, 25 mM HEPES pH 7.4, and PSN antibiotic mix). Cells were grown either in 25 cm² or 75 cm² area flasks. After 3-4 days in culture, cells were removed from the tissue culture flasks with 2 mL (for the 25 cm² area flask) or 6 mL (for 75 cm² area flask) of 0.25% trypsin/ 0.05% EDTA in Hanks-buffered salt solution. Trypsin was inactivated upon the addition of 8-24 mL of complete medium, cells were centrifuged, pelleted, supernatant removed, and the pelleted cells resuspended in complete medium. Cell suspension densities and cellular viability were determined via trypan blue exclusion. The cells were then placed (passed) back into continuous culture (1.25 x 10⁶ cells/25 cm²-area flask, BD Falcon) or were seeded into tissue culture plastic-ware at the plating densities noted for experiments. In all experiments presented, C6 glioma cells were used between passages 5-35.

Cytokine quantification via ELISA

Rat C6 glioma cells were plated (1.0 x 10⁶/well) on 24 well plates using poly-D-lysine (PDL) in complete medium and allowed to incubate 24 h to ensure attachment. For the experiment, cells were washed twice with 2 mL serum-free RPMI 1640 and incubated in serum-free RPMI-1640 in the absence or presence of agents for the times and doses indicated for the experiments. IL-6 release was
then assayed via rat IL-6 ELISA (Pierce Biotechnology) according to the manufacturers' instructions.

Collection of cellular protein lysates

Rat C6 glioma cells were plated (3.0 x 10^6/dish) on PDL-coated 35 x 10 mm dishes in complete medium, and allowed 24 h to attach. Following the attachment period, the cells were washed twice with 2 mL serum-free RPMI-1640 and incubated in serum-free RPMI-1640 in the absence or presence of stimulation agents for the times and doses indicated for the experiments. After treatments cells were washed twice in 2 mL ice cold PBS, and scraped in 200 μL of M-PER® (Pierce) containing both phosphatase and protease inhibitors (each from Pierce). Protein-containing solutions were kept on ice and vortexed in 10 minute intervals for a total time of 30 minutes on ice. Lysates were then centrifuged at 14,000 x g for 10 min at 4 °C and 175 μL of the supernatant was recovered. Protein concentrations were determined with Micro-BCA protein assay kit (Pierce) using BSA as a standard and accordance to manufacturers' instructions. Protein lysates were diluted by 20% using a 2X SDS-PAGE loading buffer (10 % w/v SDS, 2 % v/v glycerol, 0.1 % w/v bromophenol blue, 0.5 M Tris pH 6.8, and 5% v/v β-ME), and boiled for 3 min. Protein lysates were subsequently separated by SDS-PAGE Western blot analysis and samples were stored at -80 °C for future analysis.
RNA extraction and semi-quantitative RT-PCR

Rat C6 glioma cells were plated (3.0 x 10^6/dish) on PDL-coated 35 x 10 mm dishes in complete medium, and allowed 24 h to attach. Following the attachment period, the cells were washed twice with 2 mL serum-free RPMI-1640 and incubated in serum-free RPMI-1640 in the absence or presence of stimulation agents for the times and doses indicated for the experiments. After treatments cells were washed twice in 2 mL ice cold PBS. Total RNA was then extracted using the Invitrogen PureLink total RNA purification system.

To detect the presence of mRNA encoding IL-6 and glyceraldehydes-3-phosphate dehydrogenase (G3PDH), semi-quantitative RT-PCR analyses were performed as described by Elsawa et al., 2004 and Bost et al., 1995. One µg of total RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen), and a portion of the total cDNA was amplified by PCR using 94°C denaturation, 55°C annealing and 72°C extension temperatures, for 27 cycles with the first four cycles having extended times. Positive and negative strand primers used for the amplification of each mRNA species were as follows; G3PDH, GGAGCCAAACGGGTCATCATCTC and ATGCCTCTTCACCACCTTCTTG; IL-6, AGAGTTGTGCAATGGCAATTC and CCTTCTGTGACTCTAAAATTCTCCA. Amplified products were electrophoresed on ethidium bromide stained gels and visualized under UV illumination. Analysis of cDNA samples were corrected for expression of G3PDH.
SDS-PAGE Western blot analysis

Protein lysates were separated on a SDS-PAGE gel. Poly-acrylamide gels were purchased precasted (Pierce). The gels were subsequently transferred to a Hoffer SE 250 Minivertical Gel Electrophoresis Unit (Amersham) filled with Tris-HEPES (0.1 M HEPES, 0.1 M Tris, 0.1% w/v SDS) running buffer. Equal amounts of protein (5 – 25 µg) were loaded into each lane of the precast gel. Biotinylated and prestained protein ladders were also loaded onto the stacking gel to determine molecular weight mobility and transfer efficiency, respectively. The proteins were electrophoretically separated at 4 °C, using a constant voltage of 100 V. Following electrophoresis, the proteins were transferred (Hoefer TE22 Mighty Small TransPhor Tank, Amersham) to 0.45 µm nitrocellulose membranes at 4 °C, using constant amperage of 360 mA, for 90 min in Western transfer buffer (25 mM Tris, 192 mM Glycine, and 20% v/v methanol, note: pH was not adjusted).

Following the transfer period, protein-containing membranes were washed once for 5 minutes in tris-buffered saline containing tween (TBST: 50 mM Tris pH 7.4, 150 mM NaCl, and 0.1% v/v Tween-20) for 5 min. After the washing period the membranes were blocked with TBST containing 5 % nonfat milk (Nestlé Carnation) for 1 hour, then washed three times in 5 minutes intervals in TBST. The membrane was incubated 12 hours with primary antibodies at the dilutions indicated by manufacturer's instructions at 4°C. After the antibody incubation period, the antibody solution was removed and the membranes washed in TBST three times for 5 minute intervals. Membranes were then incubated with a
secondary antibody conjugated to a horseradish peroxidase (HRP) and anti-biotin. Both secondary and anti-biotin antibodies were diluted using the manufactures recommendation, of 1:5000 and 1:1000, respectively. All antibodies were diluted in TBST containing 0.1% BSA (Antibody Dilution Buffer). The incubation period for secondary antibody and anti-biotin of 1 hour, afterwards both were removed and washed in TBST three times for 5 minute intervals. Proteins were visualized on the Typhoon multipurpose imager using the ECL-plus® detection reagent according to the manufacturer’s specifications (Amersham).
CHAPTER 3

RESULTS

Time dependent release of IL-6 induced by TNF-α and IL-1β

We characterized the effect of TNF-α and IL-1β on IL-6 release in a time dependent manner using an ELISA format. For groups treated with both cytokines we determined that at 4 hours no detectable amounts of IL-6 were release. Furthermore, we observed a time dependent release of IL-6 starting from 6-12 hours with an even greater amount of IL-6 release occurring at 24hours. (Figure 5).

GABA inhibition of the synergistic release of IL-6 induced by TNF-α and IL-1β

We characterized the effect GABA on TNF-α and IL-1β induced IL-6 release in a time dependent manner using an ELISA format. For groups treated with both cytokines we observed a time dependent release of IL-6 starting from 6 hours onward. Furthermore, we observed that GABA inhibits the release of IL-6 at all time points. (Figure 6).
Effects of GABA, muscimol, baclofen, TNF-α and IL-1β on IL-6 release.

We have previously reported that GABA can inhibit the release of IL-6 induced by IL-1β treatment (Spangelo et al., 2004) using the 7TD-1 bioassay. We decided to re-explore these observations using an ELISA format to examine the effects of GABA, muscimol, baclofen, TNF-α and IL-1β on IL-6 Release (Figure 7). In addition GABA, muscimol and baclofen were able to inhibit IL-6 release caused by the synergistic stimulation of both TNF-α and IL-1β combined.

TNF-α and IL-1β Effects on p38, IκB-α and IκB-β in an extended time course

In order to determine how TNF-α and IL-1β synergy occurs it was necessary to characterize the effects of TNF-α and IL-1β on p38, IκB-α and IκB-β. In the case of p38 we see that the combination of both cytokines caused the activation of p38 at 5 minutes with maximum activation occurring at 15 minutes followed by a return to basal activity at 30 minutes (Figure 8). In the case of the NF-κB inhibitors IκB-α and IκB-β we observe IκB-α degradation at 5 minutes with maximum degradation at 15 minutes and a return to basal levels after approximately 30 minutes (Figure 8). As for IκB-β we observed no degradation for any treatments or time points.
Effects of split number on cellular responses

During our research we observed that stimulation agents had dissimilar effects on cells from different split numbers. We determined that the older cells which were split in excess of 38 times became less responsive to treatments than that of the newer lower split numbers. We have established that TNF-α and IL-1β have an effect on IκB-α degradation as early as 5 minutes followed by maximum degradation at 15 minutes. Experiments carried out together involving cells of a higher split number and lower split demonstrated that the higher split becomes less responsive at 5 and 15 minutes than that of the lower split (Figure 9).

Effects of TNF-α and IL-1β on p38, IκB-α and IκB-β

at 2-10 minutes

During our studies we hypothesized that the release of IL-6 induced by both cytokines in combination may be a result of an enhanced effect on p38 activation or IκB-α degradation. We first investigated two time points at 2 and 5 minutes and did not observe any enhanced effect on p38 activation or IκB-α degradation at 2 minutes (Figure 10). At 5 minutes both cytokines combined appeared to have a stronger effect on IκB-α degradation than either single cytokine treatments alone. Additional time points at 7.5 and 10 minutes were further analyzed (Figure 11). At 7.5 minutes there is more IκB-α degradation when treated with both cytokines and at 10 minutes we see considerably more
degradation of IkB-α when treated with both cytokines. There appeared to be no enhanced effect on p38 activation at any time points.

**Effects of muscimol, baclofen, TNF-α and IL-1β on p38, IkB-α and IkB-β**

With the effects of muscimol, baclofen, TNF-α and IL-1β on IL-6 characterized in an ELISA format we then explored the effects each had on p38, IkB-α and IkB-β using Western blots (Figure 12). At the maximum doses of 100 ng/mL for TNF-α and 50 ng/mL IL-1β we observed activation of p38 and degradation of IkB-α but not IkB-β when treated with cytokines alone or in combination. In addition, muscimol and baclofen did not reverse the effects of the cytokines when used in combination.

**Effects of TNF-α, IL-1β, BAY 11-7082 and GABA on IkB-α degradation**

We determined the effects TNF-α, IL-1β, Bay 11-7082 and GABA on IkB-α degradation via Western blot. Bay 11-7082 (Figure 13) is an inhibitor of NF-κB and prevents the phosphorylation of inhibitory protein inhibitor-κB-β (IkB-β) bound to NF-κB (Ohkita et al., 2002), in addition it can also inhibit IkB-β expression (Lappas et al., 2005). We observed a considerable increase in degradation IkB-α when cells were treated with 100 ng/mL TNF-α, 50 ng/mL IL-1β alone and with both cytokines combined (Figure 14) which was reversed by
Bay 11-7082. In addition the inhibitory peptide GABA had no effect in reversing this degradation.

**Effects of low concentrations of TNF-α and IL-1β on p38, IκB-α and IκB-β**

We previously characterized the effects TNF-α and IL-1β have on p38, IκB-α and IκB-β (data not shown). Although both cytokines are effective at higher concentrations it was necessary to characterize the effects of these cytokines at lower concentrations to determine a minimum effective concentration. We first analyzed 1 ng/mL TNF-α at three different concentrations of IL-1β that included 0.5, 1 and 5 ng/mL. For the TNF-α treatment alone we observed little or no effect on p38 activation and virtually no effect on IκB-α or IκB-β degradation (**Figure 15**). We observed little or no effect on p38 activation, IκB-α or IκB-β degradation when treated with IL-1β at 0.5 ng/mL. At 1 ng/mL we observed a weak activation of p38 when compared to the 0.5 ng/mL treatment and an increased activation when treated with 5 ng/mL of both cytokines. This p38 activation coincided with a strong degradation of IκB-α. In addition there was no effect on IκB-β degradation for any treatment groups.

**Effects of low concentrations of TNF-α, IL-1β and GABA on IκB-α and IκB-β**

With the effects of low concentrations cytokines characterized we opted to increase these concentrations of TNF-α and IL-1β in an attempt to clarify a more
observable effect on \( \text{i} \text{kB-\( \alpha \)} \) and \( \text{i} \text{kB-\( \beta \)} \), for this we used concentrations of 2, 5 and 10 ng/mL for both TNF-\( \alpha \) and IL-1\( \beta \). Consistent with previous findings the co-treatments of both cytokines caused the degradation of the NF-\( \kappa \)B Inhibitors \( \text{i} \text{kB-\( \alpha \)} \) but not \( \text{i} \text{kB-\( \beta \)} \). Furthermore, 1mM GABA did not reverse this effect (Figure 16).

Effects of TNF-\( \alpha \), IL-1\( \beta \) and GABA dose responses on \( \text{i} \text{kB-\( \alpha \)} \) and \( \text{i} \text{kB-\( \beta \)} \)

With low cytokines concentrations characterized we decided to use 2 ng/mL as our low effective concentration. Furthermore, in conjunction with the cytokine co-treatments we characterized a GABA dose response ranging in concentration from 0.01 to 10 mM. As expected, co-treatments of both cytokines caused the degradation of the NF-\( \kappa \)B inhibitors \( \text{i} \text{kB-\( \alpha \)} \) but not \( \text{i} \text{kB-\( \beta \)} \). In addition co-treatments caused the activation of p38 (Figure 17). Furthermore, GABA treatments did not reverse the effects of the cytokines.

Effects of TNF-\( \alpha \), IL-1\( \beta \) and GABA on IL-6 transcription

Next, we evaluated the effects of TNF-\( \alpha \), IL-1\( \beta \) and GABA on IL-6 transcription. TNF-\( \alpha \) and IL-1\( \beta \) caused an increase in IL-6 transcription (Figure 18), in addition both cytokines combined induced a large increase in IL-6 transcription. Interestingly, when cytokines and GABA are combined, GABA did not inhibit IL-6 transcription induced by both cytokines. Relative expression levels were measured verifying visual observations (Figure 19).
Evaluation of total cellular content of IL-6

We have demonstrated that we can measure IL-6 release through the use of ELISA. We explored the idea of assaying total cellular IL-6 content via Western blot. We evaluated three different rabbit anti-rat IL-6 antibodies purchased from Pierce Biotechnology, Peprotech and Cell Sciences (Figure 20). The antibody purchased from Cell Sciences demonstrated more specific binding to IL-6 allowing us to better distinguish IL-6. In addition when 25 μg of protein were loaded into each well the bands were clearly more intense than that of the other antibodies. With these results further IL-6 Western analysis was done using the Cell Sciences antibody and based on the intensity of the bands a lower amount of protein was analyzed. We analyzed 10 μg of protein separated on Western blot to determine the effects of GABA and cytokines (Figure 21). We observed a more intense IL-6 band for the TNF-α and IL-1β combined treatment indicating a large amount of IL-6. This accumulation of IL-6 was reversed with the use of GABA.
CHAPTER 4

DISCUSSION

GABA inhibition of the synergistic release
of IL-6 by TNF-α and IL-1β

Our lab has previously established that TNF-α and IL-1β combined induce a synergistic effect on IL-6 release (data not shown). To evaluate the roles of GABA and agonists on IL-6 release, it was necessary to characterize IL-6 release induced by these two cytokines in a time dependent manner (Figure 5). After 4 hours of treatment no detectable amounts of IL-6 was released. Detectable levels of IL-6 occurred at approximately 6 hours, continuing after 8 hours with maximum accumulation of IL-6 after 24 hours.

We have previously reported that GABA can inhibit the release of IL-6 induced by IL-1β treatment (Spangelo et al., 2004) using the 7TD-1 bioassay. These observations were re-explored in an ELISA format from 6-12 hours (Figure 6). We found that GABA can inhibit IL-6 release at all time points supporting our previous findings. We then examined the effects of GABA muscimol, baclofen TNF-α and IL-1β on IL-6 release and found that GABA, muscimol and baclofen were all able to inhibit the synergistic stimulation of IL-6 release induced by TNF-α and IL-1β (Figure 7). This preliminary data suggests
that GABA inhibition of IL-6 can use either GABA receptor subtypes for signaling. We determined through a literature search that 100 μM of baclofen is the standard concentration used, whereas we used 500 μM. We will reevaluate these results using the recommended dose of 100 μM baclofen and we predict that GABA and muscimol but not baclofen will inhibit IL-6 release caused by T1 synergy.

Mechanism of the TNF-α and IL-1β synergistic stimulation of IL-6 release

Our first hypothesis states that the synergistic effect of TNF-α and IL-1β on IL-6 release is caused by an increase in IL-6 transcription. In order to test this we felt it was necessary to first characterize the kinetics of TNF-α and IL-1β on p38 activation and IκB degradation. We see that both cytokines used together activated p38 at approximately 5 minutes reaching maximum activation at 15 minutes followed by a return to basal activity after 30 minutes (Figure 8). Coincidentally there is considerable degradation in IκB-α starting at approximately 5 minutes with maximum degradation occurring at 15 minutes and a return to basal activity at 30 minutes. Consistent with previous experiments we observed no changes in IκB-β degradation.

During our research we observed that stimulatory agents were having dissimilar effects on cell lines of different split numbers. It appeared that the higher split numbers starting at approximately 38 become less responsive to our treatments. We tested this observation on the two different split lines with
cytokines alone and in combination and we determined that the high split numbers become less responsive. We have established that cytokine treatments induce IκB-α degradation as early as 5 minutes followed by maximum degradation at 15 minutes. Experiments carried out together involving the two different split numbers demonstrated that the higher split number did not respond to our treatments at either 5 or 15 minutes (Figure 9). From these observations we determined that cell cultures of approximately 38 splits were to be discarded and fresh cell cultures grown to replace them. After these observations all subsequent experiments were done with cell splits of 5-35.

In regards to the previous kinetics study we postulated that the synergistic effect of IL-6 transcription and subsequent release may be a result of an accelerated effect on p38 activation and/or NF-κB activation. We have established that p38 activation and IκB-α degradation occur as early as 5 minutes when cytokines are used alone. We predicted that if there is an accelerated effect when both cytokines are used together we would observe activation of p38 or IκB-α degradation at earlier time points or a stronger activation. We decided the first time points to be examined would be 2 and 5 minutes (Figure 10). For the 2 minute time point we did not observe any effect on p38 activation or IκB-α degradation. However, when both cytokines are combined there appears to be an increase in p38 activation and IκB-α degradation at 5 minutes when compared to cytokines alone. From this observation we then decided to analyze the effects of both cytokines at further time points of 7.5 and 10 minutes (Figure 11). Consistent with our previous findings, groups treated
with cytokines alone had an increase in activated p38 and IκB-α degradation. When compared to groups treated with both cytokines, we observed a stronger activation of p38 and degradation of IκB-α for all time points. From this data presented we propose that the synergistic induction of IL-6 release caused by the combined effects of TNF-α and IL-1β is a result of an accelerated effect on p38 and NF-κB activation.

**Effects of GABA on the inhibition of IL-6 release induced by TNF-α and IL-1β**

With guidance from our ELISA data we then explored the effects of each cytokines alone, together and in combination with muscimol or baclofen. This was done to determine how each affected p38 activation, IκB-α and IκB-β degradation in an attempt to determine if the signaling goes through one of these modes. At the maximum doses of 100 ng/mL for TNF-α and 50 ng/mL IL-1β we observed activation of p38 and degradation of IκB-α but not IκB-β when treated with cytokines alone or in combination (Figure 12). In addition, muscimol and baclofen did not reverse the effects of the cytokines when used in combination.

Our previous findings have shown that Bay 11-7082 can inhibit the synergistically stimulated release of IL-6 (data not shown). With this in mind we used this NF-κB inhibitor as a positive control for GABA. We observed a considerable increase in degradation IκB-α when 100 ng/mL TNF-α, 50 ng/mL IL-1β and with both cytokines combined (Figure 14). In addition the NF-κB inhibitor Bay 11-7082 effectively inhibited the degradation of IκB-α but GABA had
no effect in reversing this degradation. From our ELISA data and Western data we concluded that GABA, muscimol and baclofen had no inhibitory effect on p38 activation or IκB degradation.

Using maximum doses of cytokines, GABA and agonists resulted in no observable effects on p38 activation and IκB degradation. We postulated that the effects of GABA and agonists may be subtle and thus masked by a high effective concentration of both cytokines. Therefore, we designed a series of experiments to determine the minimum effective concentration of these cytokines that induces a minimal response to p38 activation and IκB degradation. Once a concentration of minimal effect was determined we would then use cytokines at this low concentration treat and co-treat with the maximal dose of GABA in an attempt to induce a stronger response from GABA that should not be masked by the cytokines at minimal doses.

To determine the minimal effective dose we started at 1 ng/mL TNF-α and compared this to three different doses of IL-1β that included 0.5, 1 and 5 ng/mL (Figure 15). For the TNF-α treatments we observed little or no effect on p38 activation and virtually no effect on IκB-α or IκB-β degradation. Treatment with IL-1β of 0.5 ng/mL the effects were similar to TNF-α and there was little effect on p38 activation and no degradation on either IκB. At 1 ng/mL we observed a stronger activation of p38 when compared to the 0.5 ng/mL and no degradation in IκB-α and no change in IκB-β. When treated 5 ng/mL IL-1β we observed stronger activation of p38 and degradation of IκB-α than those compared to the other doses and 1 ng/mL TNF-α. When combined with 1 ng/mL TNF-α we find
that TNF-α and 0.5 ng/mL IL-1β had little or no effect on p38 activation and virtually no effect on degradation on either IkB. When treated with 1 ng/mL TNF-α and 1 ng/mL IL-1β we find a weak activation of p38 and some degradation in IkB-α and this effect is even more obvious when 1 ng/mL TNF-α is combined with 5 ng/mL IL-1β.

With a minimum effective dose determined for both cytokines we decided to further explore the effects of minimal concentrations. We decided to analyze the effects of equal amounts of cytokines and analyzed the effects of 2, 5 and 10 ng/mL (Figure 16). For these treatments we observed degradation of IkB-α but no change in IkB-β degradation. However, the effects were weaker for the 2 and 5 ng/mL treatment groups when compared to the 10 ng/mL group. From these results we decided to use 2 ng/mL concentrations combined with GABA. The concentrations of GABA for this treatment included 0.01, 0.1, 1 and 10 mM. We observed no effect in p38 activation or IkB-β degradation from any treatment groups for cytokines alone or in combination with GABA (Figure 17). With these results it has been demonstrated that GABA did not inhibit p38 activation or IkB-α degradation at both low and high doses. All the results presented to this point effectively refuted our second hypothesis that GABA may have an effect on p38 and NF-κB activation.

With one hypothesis refuted we then analyzed the effects of IL-6 transcription which is the basis of our other two hypotheses. We observed that treatment with 100 ng/mL TNF-α or 50 ng/mL IL-1β resulted in increase of IL-6 transcription (Figure 18 and 19). When both cytokines used in combination a massive

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induction of IL-6 occurs, this induction was not simply an additive effect of either cytokine but several times greater (synergistic). This result provides evidence that supports our first hypothesis that states the synergistic effect of TNF-α and IL-1β may act on IL-6 transcription. We have demonstrated that when both cytokines are used in combination there is an accelerated effect on p38 activation and IκB-α degradation. Combined with this result we propose that this synergistic effect is a result of the combined cytokines having accelerated effects on p38 activation and NF-κB activation ultimately resulting in an accelerated increase in IL-6 transcription. Furthermore, we hypothesized that GABA may inhibit this synergistic effect by inhibiting IL-6 transcription. If true, we predict that we would see a decrease in IL-6 transcripts when treated with GABA. However, we observed that GABA did not inhibit IL-6 transcription caused by both cytokines combined. This is in opposition to our prediction and suggests that GABA contributes to an increase of IL-6 or even stabilizes the transcript. We argue that there is an accumulation in IL-6 transcript in the GABA treated groups because GABA may prevent the translation of IL-6.

From the mRNA data we hypothesized that GABA may be preventing the translation of IL-6. With this in mind we predicted that if GABA prevents IL-6 translation we will see a decrease in IL-6 content in GABA treated groups when analyzed via Western blot. In order to test this we first had to determine if a Western format is feasible for IL-6 analysis. For this experiment C6 cells were plated, no stimulation agents were used and protein extracted (Figure 20). We analyzed these blots with an anti-rat IL-6 antibody (1:1000 dilution) purchased
from Pierce Biotechnology, Peprotech and Cell Sciences. For all antibodies we were able to detect IL-6 and also observed non-specific binding at other weight ranges. The antibodies purchased from Pierce and Peprotech were very similar, the antibody purchased from Cell Sciences gave us the best results. We determined an effective detection by two categories [a] a limit on non-specific binding and [b] the intensity of the bands/signal at the 28 kDa range where functional IL-6 should be isolated. With the Cell Sciences antibody there was more specific binding and a stronger signal detected at the 28 kDa range. Also we concluded that the band was too intense and may be indicative of protein overloading. Therefore for future IL-6 Westerns we lowered the amount of protein to load from 25 µg to 10 µg. With these results we concluded that it is possible to measure IL-6 effectively in a Western format and that the Cell Sciences antibody proved to be the ideal reagent.

We then analyzed the effect of GABA and cytokines on IL-6 content (Figure 21). We observed a large accumulation of IL-6 for the TNF-α and IL-1β combined treatments and when these two were combined with GABA less IL-6 was detected. This result combined with the mRNA data suggests that GABA inhibition of IL-6 release may act through the prevention of IL-6 translation. This possibility will be explored in future endeavors by our lab. From these results we ruled out our third hypothesis that GABA inhibits IL-6 transcription but determined that GABA appears to inhibit IL-6 translation. With the information gathered for this thesis we have proposed a potential GABA inhibitory pathway shown in Figure 22.
A potential model for GABA inhibition of IL-6 translation may be through the activation of 4E-BP1 also known as PHAS 1. In eukaryotes, mRNA posses a 5′ "cap" structure which is recognized by the cap binding protein eIF4E allowing the first step in ribosome scanning (Martin et al., 2000). Different stimuli can cause the phosphorylation of eIF4E, which activates this initiation factor resulting in an increase in protein synthesis (Sonenberg 1996). In addition the activation of eIF4E enhances its affinity to bind to mRNA (Minich et al., 1994). 4E-BP1 is a cytoplasmic inhibitor which maintains eIF4E in an inactive form (Martin et al., 2000). Phosphorylation of 4E-BP1 causes it to release eIF4E allowing the proper formation of a functional eIF4E complex that allows efficient binging and proper positioning of the 40 S ribosomal subunit on mRNA (Lawrence and Abraham 1997).

GABA may be preventing 4E-BP1 from releasing eIF4E, preventing the ribosomal complex from forming in turn inhibiting translation. Interestingly, a functional assay for 4E-BP-eIF4E binding was developed by Gingras and colleagues from which we could test the effects of GABA. Using this method we predict that TNF-α and IL-1β combined treatments will result in the dissociation of 4E-BP-eIF4E complex which will be inhibited by GABA.

Polysome fractionation is another potential method to assay for translational inhibition in a method similar to that described by Koritzinsky and colleagues. This method involves the separation of cell lysates on sucrose gradients then RNA levels are measured as a function of gradient depth. Efficient translation of mRNA requires the association with many ribosomes (polysomes) and therefore
would sediment deep in the sucrose gradients (Koritzinsky et al., 2005). We hypothesize that GABA may inhibit translation by preventing the formation of the polysomes. We predict that TNF-α and IL-1β combined treatments will result in polysomal bands deep in the sucrose gradients. Furthermore, we predict that this banding deep in the sucrose gradient will not be present in the GABA treated groups.

In summary, from the results presented in this thesis we have evidence suggesting that the synergistic effect of TNF-α and IL-1β is a result of an enhanced effect on p38 and NF-κB activation resulting in enhanced IL-6 transcription. Furthermore, GABA may prevent IL-6 translation based on the following three observations, [a] GABA did not prevent the activation of p38 and or NF-κB, [b] GABA did not reduce the transcription of IL-6 and [c] GABA treated groups had less total cellular IL-6. From this data we postulate that this decrease of IL-6 protein is a result of GABA inhibiting translation possibly by blocking IL-6 transcript release from the nucleus or by inhibiting translation directly.
Figure 1: Representation demonstrating that neuroinflammation and beta amyloid synergy is essential to AD progression.
Figure 2: Structural projection for the inhibitory neurotransmitter GABA.
Figure 3: Structural projections of GABA receptor agonists. Muscimol acts on GABA\textsubscript{A} receptors and baclofen on GABA\textsubscript{B} receptors.
Figure 4: GABA inhibition of the synergistic release of IL-6 by IL-1β and TNF-α. Rat C6 cells were plated (1.0 x 10^6 cells/well in a PDL-coated 24-well plate) and were pretreated with 1 mM GABA for 1 hour and co-treated with 1 mM GABA, 50 ng/mL IL-1β and 100 ng/mL TNF-α for 20 hours. Post stimulation, conditioned medium was removed and assayed for IL-6 using Pierce ELISA according to the manufacturer's specifications. A (+) indicates C6 glioma cells were treated as; con = no stimulation agents T = TNF-α, I = IL-1β, G = GABA. Data presented in this figure is a confirmation of previous unpublished results. The data are presented as mean ± s.e.m. of triplicates observations obtained from a single representative experiment.
Figure 5: Time dependent release of IL-6 by IL-1β and TNF-α. Rat C6 cells were plated (1.0 x 10⁶ cells/well in a PDL-coated 24-well plate) treated with 50 ng/mL IL-1β and 100 ng/mL TNF-α alone or in combination for 4, 6, 8, 12 and 24 hours. Post stimulation, conditioned medium was removed and assayed for IL-6 using Pierce ELISA according to the manufacturer’s specifications. C6 glioma cells were treated as; C = no stimulation agents TI = TNF-α and IL-1 combined with times indicated by hrs = hours. The data are presented as mean ± s.e.m. of triplicates observations obtained from a single representative experiment.
Figure 6: GABA inhibition of the synergistic release of IL-6 by IL-1β and TNF-α. Rat C6 cells were plated (125 x 10^3 cells/well in a PDL-coated 96-well plate) and were pretreated with 1 mM GABA alone for 1 hour and co-treated with 50 ng/mL IL-1β and 100 ng/mL TNF-α for 4-12 hours. Post stimulation, conditioned medium was removed and assayed for IL-6 using Pierce ELISA according to the manufacturer's specifications. T = TNF-α, I = IL-1β and G = GABA. The data are presented as mean ± s.e.m. of triplicate observations obtained from a single representative experiment.
Figure 7: GABA inhibition of the synergistic release of IL-6 by IL-1β and TNF-α. Rat C6 cells were plated (125 x 10^3 cells/well in a PDL-coated 96-well plate) and were pretreated with inhibitors (1 mM GABA, 100 µM muscimol and 500 µM baclofen) alone for 1 hour and co-treated with inhibitors alone, 50 ng/mL IL-1β and 100 ng/mL TNF-α for 20 hours. Post stimulation, conditioned medium was removed and assayed for IL-6 using Pierce ELISA according to the manufacturer’s specifications. T = TNF-α, I = IL-1β, G = GABA, M = muscimol and B = baclofen. The data are presented as mean ± s.e.m. of triplicate observations obtained from a single representative experiment.
Figure 8: The extended time course and effects of TNF-α and IL-1β on p38 activation and degradation of IκB-α and IκB-β. Rat C6 cells (3 x 10⁶ cells/dish in a 35 x 10 mm-dish) were treated 50 ng/mL IL-1β and 100 ng/mL TNF-α for 0, 5, 15, 30, 45, 60, 90 and 120 minutes. Post stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, IκB-β, phosphorylated or total p38 protein (1:1000 for all). The data are presented as the observations obtained from a single experiment.
Figure 9: The effects of TNF-α and IL-1β degradation of IκB-α and IκB-β for high and low cell splits. Rat C6 cells (3 x 10⁶ cells/dish in a 35 x 10 mm-dish) were treated 50 ng/mL IL-1β and 100 ng/mL TNF-α for 0, 5, 15, 30, 45, 60, 90 and 120 minutes. Post-stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, IκB-β, phosphorylated or total p38 protein (1:1000 for all). The data are presented as the observations obtained from a single experiment.
Figure 10: The effects of TNF-α and IL-1β on p38 activation and degradation of IκB-α and IκB-β at 2 and 5 minute time points. Rat C6 cells (3 x 10^6 cells/dish in a 35 x 10 mm-dish) were treated 50 ng/mL IL-1β and 100 ng/mL TNF-α for 2 and 5 minutes. Post stimulation, cellular protein was extracted, and 25 µg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, IκB-β, phosphorylated or total p38 protein (1:1000 for all). The data are presented as the observations obtained from a single experiment.
Figure 11: The effects of TNF-α and IL-1β on p38 activation and degradation of IκB-α and IκB-β at 5, 7.5 and 10 minute time points. Rat C6 cells (3 x 10^6 cells/dish in a 35 x 10 mm-dish) were treated 50 ng/mL IL-1β and 100 ng/mL TNF-α for 5, 7.5 and 10 minutes. Post stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, IκB-β, phosphorylated or total p38 protein (1:1000 for all). The data are presented as the observations obtained from a single experiment.
Figure 12: The effects of muscimol and baclofen on p38, IκB-α and IκB-β. Rat C6 cells (3 x 10^6 cells/dish in a 35 x 10 mm-dish) were pre-treated with 100 μM muscimol or 100 μM baclofen for 1 hour, and co-treated with either 100 μM muscimol or 100 μM baclofen, 50 ng/mL IL-1β and 100 ng/mL TNF-α for 15 min. Post stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, IκB-β, phosphorylated or total p38 protein (1:1000 for all). The data are presented as the observations obtained from a single experiment.
Figure 13: Structural projections of BAY 11-7082 which inhibits NF-kappaB by preventing the phosphorylation of IxB-β bound to NF-κB.
Figure 14: The effect of Bay 11-7082 on the degradation of IκB-α. Rat C6 cells (3 × 10^6 cells/dish in a 35 x 10 mm-dish) were pre-treated with 25 μM Bay 11-7082 for 1 hour, and co-treated with 25 μM Bay 11-7082, 50 ng/mL IL-1β and 100 ng/mL TNF-α for 15 min. Post stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB protein (1:1000). The data are presented as the observations obtained from a single experiment.
Figure 15: The effects of low concentrations on cytokine on p38 activation and the degradation of IκB-α and IκB-β. Rat C6 cells (3 x 10^6 cells/dish in a 35 x 10 mm-dish) were treated 0.5, 1 and 5 ng/mL IL-1β and 1 ng/mL TNF-α for 15 min. Post stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, IκB-β, phosphorylated or total p38 protein (1:1000 for all). The data are presented as the observations obtained from a single experiment.

<table>
<thead>
<tr>
<th></th>
<th>TNF (1 ng/mL)</th>
<th>IL-1 (0.5 ng/mL)</th>
<th>IL-1 (1 ng/ml)</th>
<th>IL-1 (5 ng/mL)</th>
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<tr>
<td>IκB-α</td>
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<td>p38</td>
<td>-</td>
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Figure 16: The effects of low concentrations of cytokine on p38 activation and the degradation of IκB-α and IκB-β. Rat C6 cells (3 x 10⁶ cells/dish in a 35 x 10 mm-dish) were treated 2, 5 and 10 ng/mL IL-1β and TNF-α for 15 min. Post stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α and IκB (1:1000 for both). The data are presented as the observations obtained from a single experiment.
Figure 17: The effects of low concentrations of cytokine and GABA on p38 activation and the degradation of IκB-α and IκB-β. Rat C6 cells (3 x 10^6 cells/dish in a 35 x 10 mm-dish) were pre-treated with 0.01, 0.1, 1 and 10 mM GABA for 1 hour, and co-treated with either 0.01, 0.1, 1 and 10 mM GABA, 2 ng/mL IL-1β and 2 ng/mL TNF-α for 15 min. Post stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, IκB-β, phosphorylated or total p38 protein (1:1000 for all). The data are presented as the observations obtained from a single experiment.
Figure 18: Relative expression of IL-6 transcript in cells treated with GABA, TNF-α and IL-1β. Rat C6 cells (3 x 10^6 cells/dish in a 35 x 10 mm-dish) were pre-treated with 1 mM GABA for 1 hour, and co-treated with either 1 mM GABA, 50 ng/mL IL-1β and 100 ng/mL TNF-α for 4 hours. Post stimulation, cellular RNA was extracted, and 1 µg of total RNA extract were subsequently separated and analyzed via semi-quantitative RT-PCR. The data are presented as the observations obtained from a single experiment.
Figure 19: Relative expression of IL-6 transcript in cells treated with GABA, TNF-α and IL-1β. Rat C6 cells (3 x 10⁶ cells/dish in a 35 x 10 mm-dish) were pre-treated with 1 mM GABA for 1 hour, and co-treated with either 1 mM GABA, 50 ng/mL IL-1β and 100 ng/mL TNF-α for 4 hours. Post stimulation, cellular RNA was extracted, and 1 µg of total RNA extract were subsequently separated and analyzed via semi-quantitative RT-PCR for IL-6 transcript relative expression levels determined. The data are presented as the observations obtained from four experiments.
Figure 20: Relative effectiveness of IL-6 antibody from Pierce Biotechnology, Peprotech and Cell Sciences. Rat C6 cells (3 x 10^6 cells/dish in a 35 x 10 mm-dish). Cellular protein was extracted, and 25 µg of total protein were subsequently separated via SDS-PAGE followed by Western analysis using antibodies from each manufacturer (1:1000 for all). The data are presented as the observations obtained from a single experiment.
Figure 21: IL-6 content of TNF-α and IL-1β on p38 activation and degradation of IκB-α and IκB-β. Rat C6 cells (3 x 10^6 cells/dish in a 35 x 10 mm-dish) were treated 50 ng/mL IL-1β, 100 ng/mL TNF-α and 1 mM GABA alone or in combination 4 hours. Post stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, IκB-β, phosphorylated or total p38 protein (1:1000 for all). The data are presented as the observations obtained from a single experiment.

<table>
<thead>
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<th>TNF (100 ng/mL)</th>
<th>IL-1 (50 ng/mL)</th>
<th>GABA (1 mM)</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>- + - + - + - + +</td>
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Figure 22: Proposed mechanism of GABA inhibition of IL-6 release caused by the synergistic induction of TNF-α and IL-1β.
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