gamma-Aminobutyric acid inhibits cytokine-mediated synergistic induction of astrocytic interleukin-6 release: A mechanistic study

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γ-AMINOBUTYRIC ACID INHIBITS CYTOKINE-MEDIATED SYNERGISTIC
INDUCTION OF ASTROCYTIC INTERLEUKIN-6
RELEASE: A MECHANISTIC STUDY

by

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Bachelor of Arts, Chemistry
University of Nevada, Las Vegas
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γ-Aminobutyric Acid Inhibits Cytokine-Mediated Synergistic Induction of Astrocytic Interleukin-6 Release: A Mechanistic Study

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A paucity of γ-aminobutyric acid (GABA) levels is thought to encourage cytokine release in Alzheimer’s disease (AD). A mechanistic investigation into the GABA-mediated suppression of the synergistic release of interleukin (IL)-6 by IL-1β and tumor necrosis factor (TNF)-α is presented. Preliminary 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 are able to stimulate the phosphorylation of p38, however, no synergistic stimulation was observed. IL-1β or TNF-α is able to stimulate the degradation of the NF-κB inhibitor, IκB-α, with no change in IκB-β. Interestingly, TNF-α is able to accelerate IκB-α degradation in the presence of IL-1β. While GABA is unable to suppress IκB-α degradation and the phosphorylation of p38 by IL-1β and TNF-α, it is postulated that GABA may be able to inhibit IL-6 concentrations by lessening the rate of IκB-α degradation.
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ABBREVIATIONS

Aβ – β-Amyloid
AD – Alzheimer’s Disease
AMPA – α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AP – Activator Protein
APP – Amyloid Precursor Protein
APS – Ammonium Persulfate
ASCII – American Standard Code for Information Interchange
ATF – Activation Transcription Factor
BFGF – Basic Fibroblast Growth Factor
β-ME – 2-mercaptoethanol
BSA – Bovine Serum Albumin
cAMP – Cyclic Adenosine 3’-5’ Monophosphate
CNS – Central Nervous System
CRE – cAMP Response Element
CREB – cAMP Response Element Binding Protein
CSAID – Cytokine Synthesis Anti-Inflammatory Drug
CSF – Colony Stimulating Factor
D-MEM – Dulbecco’s Modified Eagle Medium
DMSO – Dimethyl Sulfoxide
EAA – Excitatory Amino Acid
ECL – Enhanced Chemiluminescence
EDTA – Ethylenediaminetetraacetic Acid
ELISA – Enzyme-Linked Immunosorbant Assay
EMSA – Electrophoretic Mobility Shift Assay
ERK – Extracellular-Related Kinase
FBS – Fetal Bovine Serum
GABA – γ-Aminobutyric Acid
GABAT – γ-Aminobutyric Acid Transaminase
GAD – Glutamic Acid Decarboxylase
GFAP – Glial Fibrillary Acidic Protein
HEPES – 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HK – High Potassium
HPLC – High Performance Liquid Chromatography
HRP – Horseradish Peroxidase
ICE – IL-1β Converting Enzyme
iGluR – Ionotropic Glutamate Receptors
IkB – Inhibitor of κB
IKK – IkB Kinase
TI – Tumor Necrosis Factor-α and Interleukin-1β
TNF – Tumor Necrosis Factor
TNFR – Tumor Necrosis Factor Receptor
TRAF – TNF Receptor-associated Factor
Tris – Tris-(hydroxymethyl)-amino methane
UBC – Ubiquitin-Conjugating Enzyme
UEV1A – Ubiquitin-Conjugating Enzyme E2 Variant
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CHAPTER 1

INTRODUCTION

Cellular Foundations of the Central Nervous System

In order to understand the biochemical basis of Alzheimer's disease (AD) the cellular foundations of the central nervous system (CNS) must be discussed. The CNS contains two distinct cell types: neurons and glia. Originally termed "nerve glue," glial cells are vital for the safeguarding of neuronal function. Neurons are necessary for information transfer, and are classified by the endogenous neurotransmitter products produced by each neuronal cell type. Glia are characterized by their function and include two general classes: microglia and macroglia. Macrogliia consist of oligodendrocytes and astrocytes. Oligodendrocytes produce myelin sheaths, which function to insulate the axon, except at the nodes of Ranvier. Astrocytes tend to surround areas close to blood vessels, and function to provide metabolic nutrients to their neuronal counterparts. Microglia are macrophages that originate from the mesoderm, and are found scattered throughout the brain and blood vessels. In response to traumatic injury, infection or inflammation, microglial cells become activated and release chemical messengers (i.e., cytokines) to localize and repair CNS damage.
Neurotransmission

The movement of information within the vast network within the CNS is accomplished through an ensemble of cells and events including neurons, action potentials, chemical and electrical synapses, neurotransmitters and postsynaptic receptor proteins. Presynaptic neurons receive unidirectional input signals, which cause the release of either excitatory or inhibitory neurotransmitters. Released neurotransmitters are able to bind to postsynaptic receptors on a receptive neuron, which subsequently modify the membrane potentials for either excitatory or inhibitory signal propagation.

As shown in Figure 1, synaptic clefts ranging in size from 50 to 300 Å separate presynaptic and postsynaptic terminals. The two most important structures of the presynaptic terminal are mitochondria and neurotransmitter vesicles. When an action potential reaches a presynaptic terminal, neurotransmitter vesicles undergo exocytosis to release their contents into the synaptic cleft. Once a neurotransmitter has entered the synaptic cleft, it is free to bind to a specific postsynaptic receptor protein, propagating information transfer between two neurons. It is noteworthy that glia do not contain an excitable membrane, and are devoid of the ability to transfer information.

Excitotoxicity

In 1986, Steven M. Rothman proposed the concept of excitotoxicity. Glu, the primary excitatory neurotransmitter, is toxic to neurons in ischemic stroke and traumatic brain injury models (Rothman and Oleny, 1986). This
Figure 1: Cytoarchitecture of pre- and post-synaptic neurons.
notion has been extended to suggest a role for excitotoxic insults in AD neuropathology, among many other neurodegenerative disorders (Lipton and Rosenberg, 1994). Glutamate binds to an array of postsynaptic receptors; however, the NMDA subclass of receptors has been particularly implicated in AD (Shimizu et al., 2001; Farber et al., 1998).

Postsynaptic receptors for Glu are divided functionally into two classes: metabotropic and ionotropic. Metabotropic Glu receptors (mGluR) are coupled to G-proteins, and stimulate adenylate cyclase or the metabolism of phosphoinositides. Ionotropic Glu receptors (iGluR) are ligand-gated ion channels selective for N-methyl-D-aspartate (NMDA), kainic acid (KA), or α-amino-3-hydroxyl-5-methylisoxazole-4-propionic acid (AMPA) (Figure 2). The NMDA receptor has a dual requirement for receptor activation with Glu being a primary ligand, and Gly or D-Ser needed as a co-agonist (Figure 36) (Wolosker et al., 1996).

Selective blockade of Glu-mediated NMDA receptor activation may be beneficial in the treatment AD (Bleich et al., 2003). Mitigating NMDA receptor activation is accomplished though an array of competitive and non-competitive receptor antagonists (Sonkusare et al., 2005). Of the many antagonists that have been tested, the low-to-moderate affinity uncompetitive antagonist memantine has shown great clinical promise in treating AD patients suffering from severe dementia (Reisberg et al., 2003) (Figure 2).
Figure 2: Structural projects of various neurochemicals that act at postsynaptic Glu receptors.
Glutamate as a Neurotransmitter

Glutamate is the primary excitatory amino acid (EAA) neurotransmitter within the CNS, and is involved in fast excitatory synaptic neurotransmission. Synthesis of Glu is accomplished through the transamination of the citric acid cycle metabolite α-ketoglutarate. Unlike other neurotransmission systems (e.g., acetylcholine), there is no enzymatic degradation pathway to remove Glu from the synaptic cleft.

Perisynaptic glia are able to regulate glutamatergic responses by their intrinsic synaptic removal activity. Glutamate released into the synaptic cleft is taken up by glial cells, where the neurotransmitter is subsequently converted into Gln using Gln synthetase. Glutamine is then released from the astrocyte and converted to Glu within the neuron, completing a single metabolic interconversion (Figure 3).

γ-Aminobutyric Acid as a Neurotransmitter

γ-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the CNS. Involved in fast inhibitory synaptic neurotransmission, GABA is largely responsible for mediating membrane hyperpolarization. Synthesis of GABA is primarily accomplished by the decarboxylation of Glu via glutamic acid decarboxylase (GAD). The metabolism and removal of GABA from the synaptic cleft is accomplished though the conversion of GABA to succinic semialdehyde using GABA-transaminase (GABAT), and the subsequent conversion to succinic
Figure 3: The glutamate-glutamine cycle.
acid by succinic semialdehyde dehydrogenase (SSDH). Succinic acid is further metabolized via the Krebs cycle.

Receptors for GABA are functionally subdivided into two classes: the ionotropic $\text{GABA}_A$ and the metabotropic $\text{GABA}_B$ receptors. The ionotropic $\text{GABA}_A$ receptor conducts $\text{Cl}^-$ ions and is a mediator of fast inhibitory postsynaptic action potentials. $\text{GABA}_A$ receptors are heteropentameric structures that are widely distributed within the mammalian CNS. In addition to GABA, selective activation of the $\text{GABA}_A$ receptor can be accomplished by muscimol or isoguvacine. Conversely, bicuculline is a competitive $\text{GABA}_A$ receptor antagonist (Figure 4).

In contrast to the $\text{GABA}_A$ receptor, the $\text{GABA}_B$ is a G-protein-coupled receptor that can be directly associated to ion channels though second messenger cascades. The actions of the $\text{GABA}_B$ receptor are mediated though slow inhibitory postsynaptic action potentials. Secondary effects upon $\text{GABA}_B$ receptor activation include decreasing $\text{Ca}^{2+}$ conductance, opening of $\text{K}^+$ channels and inhibition of adenylyl cyclase. Selective activation of the $\text{GABA}_B$ receptor is possible with R-$(+)$-baclofen. Antagonist molecules include phaclofen and CGP36742 (Figure 5).

Pathophysiological Hallmarks of Alzheimer's Disease

Alzheimer's disease is currently the most common form of dementia among the elderly population, affecting twelve million people worldwide (Herbert et al., 2003). Progressive and selective degeneration of the neural regions necessary
Figure 4: Structural projections of various neurochemicals that act at GABA_A receptors.
Figure 5: Structural projections of various neurochemicals that act at GABA$_B$ receptors.
for normal mental function is an invariant characteristic of AD. In 1907 Alois Alzheimer described the two major neuropathological lesions of AD: extracellular senile plaques and intracellular neurofibrillary tangles. To this day, pathologists still use these histological hallmarks to identify AD in post-mortem tissue. Twenty years later, it was discovered that the senile plaques first described by Alzheimer were aggregates of amyloid protein; furthermore, Kang and co-workers reported that the sources of these amyloid protein aggregates originate from amyloid precursor protein (APP) (Kang et al., 1987).

Amyloid precursor protein (APP) is encoded by a gene located on the short arm of chromosome 21. This membrane protein is highly expressed in nerve terminals and is important for synaptic terminal formation during development (Loffler and Huber, 1992; Selkoe et al., 1988). The exact function of APP in the adult brain is currently unknown; however, the role of APP is slowly starting to unfold. Currently, reports indicate that APP is selectively enriched in neurons (Lahiri and Ge, 2004), undergoes rapid axoplasmic transport (Koo et al., 1990) and is up-regulated in response to head injury (Gentleman et al., 1993). It is interesting to note that individuals with trisomy 21 (e.g., Down's syndrome) develop AD at an early onset (Wisniewski et al., 1985), suggesting that an overexpression of APP accelerates the decrease of mental stability and appearance of disease phenotype.

Amyloid precursor protein is enzymatically cleaved by the secretase family of enzymes to form β-amyloid (Aβ) peptide fragments (Buxbaum et al., 1998). It has been postulated that the cleavage of the hydrophobic portion of APP is a
two-step process, in which a β-secretase cleaves APP to form an amyloidogenic C-terminal fragment, which is subsequently cleaved by a γ-secretase to form Aβ peptides ranging in length from 40-43 amino acids (Buxbaum et al., 1998; Spires and Hyman, 2005; Selkoe, 1996; Chen and Tang, 2006). β-amyloid protein fragments are secreted as monomers (Iwatsubo et al., 1994), but Klein et al. have proposed that secreted oligomers and multimers of Aβ1-42 have the propensity to cause neurotoxicity (2001). Although the exact etiology of AD is currently unknown, John Hardy proposed that the causative driving force for AD is the formation and deposition of Aβ plaques. This lesion-centric hypothesis became known as the amyloid cascade hypothesis (Hardy and Higgins, 1992). The amyloid cascade hypothesis has provided a foundation in which to study AD pathogenesis. The hypothesis has not been fully proven, as Terry et al. have found a poor connection between the amyloid burden and the decline in mental fitness seen in AD (1999).

In addition to Aβ plaques, AD neuropathology includes neurofibrillary tangles of the low molecular weight microtubule associated protein tau (Aliva, 2006). Essential for the maintenance of the axoplasmic transport system, tau is needed to mobilize essential nutrients within the neuron. Tau is normally bound to tubulin, thus promoting microtubule assembly. In AD, however, tau is dissociated from tubulin and forms paired helical filaments (PHF), which promote the formation of neurofibrillay tangles. Furthermore, tau PHF are hyperphosphorylated at more than 25 sites proximal to the tubulin binding domain. Hyperphosphorylated tau is subsequently more resistant to proteolysis,
and as a result is able to form aggregates that interfere with axoplasmic transport which culminate in neuronal attrition.

Dysfunctional neurotransmitter systems are another consequence of the catastrophic dementia within AD (Bell et al., 2005). These include glutamatergic, GABAergic, somatostatinergic, cholinergic, and acetylcholinergic neurotransmission systems. Perturbations to these systems include excess synaptic release, decreased presence and/or an increased rate of synaptic removal. The deterioration of certain neurotransmitters (n.b., GABA, and somatostatin) lead to widespread detrimental consequences. The benefit of replacing these endogeneous neurotransmitters in AD is demonstrated by the administration of the somatostatin (SRIF) analog octreotide, which enhances the memory of AD patients in story recall (Craft et al., 1999). Additionally, GABA and other GABA$_A$ receptor agonists were reported to alleviate A$\beta$-mediated cell death in rat cortical and hippocampal neurons in vitro (Lee et al., 2005; Paula-Lima et al., 2005; Louzada et al., 2004).

**The Interleukin Family of Cytokines**

Cytokines are low-molecular weight secreted proteins that modulate many cellular processes. The interleukin (IL) family of cytokines is comprised of 18 different proteins (interleukins 1-18), tumor necrosis factor (TNF), colony stimulating factor (CSF), transforming growth factor (TGF), interferons (INF), and many other growth factors.
The IL-1 subfamily of cytokines is comprised of three members: IL-1α, IL-1β and their intrinsic negative feedback regulator, IL-1 receptor antagonist (IL-1ra). Precursor IL-1β is produced as an inactive 31-kDa polypeptide and is processed by the IL-1β converting enzyme (ICE) to its mature, non-glycosylated 17-kDa construct. The IL-1ra is present both as a secreted and intracellular isoform. Both isoforms prevent IL-1β/IL-1R₁ receptor ligation. Interleukin-1β is a pleiotropic, pro-inflammatory cytokine with a sophisticated bidirectional communication property enabling immune and CNS integration. It is widely accepted that IL-1β is one of the many mediators of the insidious neuroinflammatory state present in AD.

Numerous reports indicate that the cytokine IL-6 may serve as both an anti- (D'Arcangelo et al., 2000; Peng et al., 2005) or pro-inflammatory (Qiu and Groul, 2003) mediator in AD. Despite the enigmatic nature of this cytokine, past work (Spangelo et al., 2004; Zumwalt et al., 1999), and the work presented here show that IL-1β is able to drive a p38-mediated signaling cascade culminating in release of IL-6 in C6 glioma cells in vitro. Within the context of the studies presented, IL-6 is regarded as a pro-inflammatory cytokine. Soluble IL-6 is a 21-26-kDa glycosylated protein important in B-cell differentiation and T-cell activation.

Tumor necrosis factor-α is a 17.3 kDa non-glycosylated cytokine that induces necrosis or cytotoxicity in select tumor cell lines. Tumor necrosis factor-α is up-regulated in AD along with IL-1β and may be able to further exacerbate the neuroinflammatory state seen in the disorder.
Interleukin-1β Signal Transduction

Interleukin-1β signaling is initiated upon its binding to the IL-1β type I receptor (IL-1R₁) (Akira and Takeda, 2004). A type II receptor functions as a decoy receptor for IL-1β as this receptor is devoid of transducing a signal upon ligation. Binding of IL-1β to the type I receptor initiates the formation of complex 1, which includes IL-1R₁ and the IL-1 receptor accessory protein (IL-1 RAcP) (Figure 6). The heterotrimeric complex induces binding of the myeloid differentiation primary-response protein 88 (MyD88) to the membrane-bound receptor complex, which then recruits the IL-1 receptor associating kinase (IRAK)-4 thereby allowing binding of IRAK-1. The binding of IRAK1 to the MyD88-IRAK-4 complex activates, via phosphorylation, IRAK-1. Subsequent phosphorylation of this Ser/Thr kinase results in the binding of TRAF (TNF receptor-associated factor)-6 to the IRAK-1-MyD88-IRAK-4 complex. The binding of TRAF-6 to the phosphorylated IRAK-1 protein results in the disengagement of the TRAF-6/phosphorylated-IRAK-1 species from the membrane bound receptor. Binding of the newly freed TRAF-6-IRAK-1 complex to another initially membrane bound protein complex of TAK-1 binding protein (TAB)-1, TAB-2 and TGF-β-activated kinase (TAK)-1 induces the phosphorylation of TAK-1. The phosphorylation of TAK-1 results in the degradation of IRAK-1; consequently, the TRAF-6-TAK-1 protein complex is released from the plasma membrane.

The cytosolic TRAF-6-TAK-1-TAB-1-TAB-2 complex leads to the activation of ubiquitin-conjugating enzyme 13 (UBC13) and ubiquitin-conjugating enzyme variant 1 (UEV1A), which results in the ubiquitination degradation of TRAF-6 in a
proteasome-independent fashion (Akira, 2003). The degradation of TRAF-6 allows for the fully active TAK-1 protein to selectively activate various signaling modules that culminate in the activation of numerous genes through transcription factors such as activator protein (AP)-1 and nuclear factor-κB (NF-κB).

The active TAK-1 protein — commonly referred to as the mitogen-activated protein kinase kinase kinase (MAPKKK), is a branch point kinase that is able to bifurcate IL-1β signal transduction into the mitogen-activated protein kinase (MAPK) signaling modules, as well as the activation of NF-κB. Activation of the MKK3/MKK6 subclass of mitogen-activated protein kinase kinases (MAPKK) by TAK-1 subsequently leads to an activation of p38 and activation transcription factor (ATF)-2. On the other hand, TAK-1 is also able to phosphorylate MKK4/MKK7, which causes the activation of c-jun-N-terminal kinase (JNK) and c-Jun. Activation of both of these terminal kinase targets is able to induce AP-1 controlled genes.

Mitogen-Activated Protein Kinase Signaling Modules

The MAPK superfamily is central to many different intracellular signaling cascades. To date, three different groups of MAPKs have been cloned, including extracellular-related protein kinase (ERK), JNK, and p38 reactivating kinase (p38). Each of these kinases is activated by a dual phosphorylation by their respective upstream MAPKK, on a Thr-Xaa-Tyr (TXY) motif. The three amino acid activation sequence differs for each kinase: ERK (Thr-Glu-Tyr), JNK (Thr-
Figure 6: Intracellular initiation of IL-1 signaling.
Pro-Tyr) and p38 (Thr-Gly-Tyr). Upon activation, each of these MAPKs phosphorylates a variety of downstream targets to regulate many physiological processes within the cell. The best-known downstream targets for each MAPK are Elk-1, c-Jun and ATF-2 for ERK, JNK and p38, respectively.

Increased activities of p38 and JNK have been observed in numerous rodent models of AD (Savage et al., 2002), as well as in post-mortem brain tissue from confirmed AD patients (Pearson et al., 2006). The malicious activity of p38 is implicated in the hyperphosphorylation of the microtubule associated protein, tau (Hensley et al., 1999; Zhu et al., 2000), and in the release of pro-inflammatory cytokines (n.b., IL-1β and IL-6), which further orchestrate the inflammatory state in AD. Culbert et al. have reported that microglia isolated from MAPKAP kinase (MK)-2 (a known downstream target of p38 MAPK) deficient mice (MK2^{-/-}) have an impaired release of pro-inflammatory mediators (compared to MK2^{+/+} mice) when stimulated with Aβ 1-42, further substantiating the role of p38 MAPK in AD (2006). In addition, phorbol-ester stimulated APP secretion is sensitive to the methoxyflavone inhibitor of ERK, PD98059 (Dessdouits-Magnen et al., 1998) (Figure 7). Therapeutic interventions that selectively impinge on the aberrant signaling systems in the inflammatory milieu were developed in the 1980s, and were termed cytokine-synthesis anti-inflammatory drug (CSAID). It was later found that CSAIDs target aberrant p38 MAPK signaling.
Figure 7: Structural projection of the methoxyflavone inhibitor of ERK – PD98059.
Nuclear Factor-κB

Belonging to the Rel protein family, the mammalian NF-κB transcription factors are a ubiquitously expressed group of proteins that currently consist of p65 (RelA), RelB/p100, c-Rel, NF-κB1 (p50), and NF-κB2 (p52). Nuclear factor-κB is responsible for the coordinate control of the transcription of a battery of genes that participate in various pro- and anti-inflammatory roles including IL-1β, IL-6 and TNF-α. The regulation of these Rel proteins is accomplished though the combined efforts of [a] subcellular localization within the cytosol, and [b] their association with inhibitory proteins such as inhibitor of κB (IκB) and p100. Currently, the IκB family consists of the three protein isoforms IκB-α, IκB-β and IκB-ε, which function to mask the nuclear localization signal as well as the DNA binding domain of the "canonical" NF-κB p65-p50 heterodimer. The activation of IκB kinase (IKK) leads to the phosphorylation, with consequent degradation, of IκB. Two different mechanisms exist for the activation of IKK. In the first, MAPK/ERK kinases such as the nuclear factor κB inducing kinase (NIK), nuclear-factor κB activating kinase (NAK), TAK-1, mitogen activated protein kinase/ERK kinase kinase (MEKK)-1, or MEKK-3 stimulate IKK activity within the cytoplasm. A second mechanism postulates that IKK is recruited to the cell membrane where the phosphorylation and activation of IKK can take place. Once IKK has been activated, two putative NF-κB activation pathways exist to activate gene transcription. These pathways are referred to as the canonical and non-canonical pathways. These pathways are activated in response to different types of stimuli.
and also differ in activation kinetics with the quicker canonical pathway more prevalent within neuroinflammatory signaling.

Within the canonical pathway, cytokines or cellular stresses are able to activate a respective MAPKKK, leading to a phosphorylation with subsequent activation of $\text{I}_{\kappa B}$ kinase ($\text{IKK}$)-$\beta$ which is then able to phosphorylate $\text{I}_{\kappa B}$ at distinct Ser residues (32 and 36 for $\text{I}_{\kappa B}$-$\alpha$; 19 and 23 for $\text{I}_{\kappa B}$-$\beta$; 18 and 22 for $\text{I}_{\kappa B}$-$\epsilon$). Phosphorylated $\text{I}_{\kappa B}$ becomes a substrate for the $E3^{\kappa B}$ ubiquitin ligase leading to the polyubiquitination with subsequent degradation of $\text{I}_{\kappa B}$ by the 26S proteosme. In the non-canonical pathway, cognate ligands for numerous receptors such as B-cell-activating factor receptor, CD40, or the lymphotoxin $\beta$ receptor, stimulate the activity of NIK leading to the activation of $\text{IKK}\alpha$. Active $\text{IKK}\alpha$ phosphorylates the p100 portion of the RelB/p100 complex, leading to the ubiquitination with subsequent processing of p100 to p52. In both cases, the nuclear translocation of the free RelB-p52 or p65-p50 heterodimers allows for gene transcription upon binding to the NF-$\kappa B$ consensus sequence $5'-\text{GGG ATT TTC CC-3'}$.

**Neuroinflammation**

Microglia of the CNS perceive amyloid plaques as foreign inclusions and initiate an inflammatory response to attack and remove the insoluble deposits. Initiation of the inflammatory response results in gliosis and activated microglia, causing release of inflammatory cytokines such as IL-1$\beta$ and IL-6 (Griffin, 2006). Furthermore, CNS levels of IL-1$\beta$ and IL-6 increase in the early stages of AD and
correlate closely with the severity of the disorder. The amplified release of these neuroinflammatory mediators adds a cytokine component to the previously described amyloid cascade hypothesis. The additive result of amplified cytokine release with the amyloid cascade hypothesis conceivably initiates a destructive and recursive cascade of events further exacerbating plaque formation and neurodegeneration.

Pro-inflammatory cytokines encourage the development of Aβ and tau lesions in AD. Yang et al. reported that hippocampal neurons treated with IL-1β respond with a two-fold increase in the AP-1-driven APP promoter reporter gene activity, suggesting that IL-1β could contribute to AD by up-regulating APP synthesis (1998). Interleukin-1β mediates tau phosphorylation with a concomitant decrease in synaptophysin expression in cortical neuron/microglial co-cultures; interestingly, the phosphorylation of tau is sensitive to the p38 inhibitor SB203580 (Li et al., 2003). These data suggest that IL-1β stimulates p38-mediated phosphorylation of tau. In addition, TNF-α, IL-1β and INF-γ stimulate the γ-secretase-mediated cleavage of APP in vitro, a result that is sensitive to the anthrapyrazolone inhibitor of JNK, SP600125 (Liao et al., 2004) (Figure 8).

Interleukin-1β is also implicated in the regulation of acetylcholinesterase, a postsynaptic enzyme responsible for terminating acetylcholine synaptic transmission though neurotransmitter hydrolysis. Li et al. have reported that IL-1β enhances neuronal acetylcholinesterase activity, mRNA, and protein levels in vitro and in vivo (2000). Interestingly, inhibition of acetylcholinesterase activity is a long-standing drug target for AD. A link between the formation of Aβ and
Figure 8: Structural projection of the anthrapyrazolone inhibitor of JNK – SP600125.
acetlycholinesterse is further substantiated by the findings that acetylchoinesterase is able to regulate the processing of APP (Mori et al., 1995).

Hypothesis Statement

In light of the detrimental role played by pro-inflammatory cytokines in AD, the work presented in this thesis stems from two separate but related hypothesis statements. The first states that IL-1β stimulates glutamatergic neurotransmission (as monitored by the release of Glu), and exacerbates excitotoxic insults mediated by Glu in rat B35 neuroblastoma cells. The second hypothesis states that the inhibitory neurotransmitter GABA will selectively antagonize p38 MAPK activity during the IL-1β-stimulated release of IL-6 from rat C6 glioma cells.
CHAPTER 2

MATERIALS AND METHODS

Chemicals and Reagents

Ortho-phthaldehyde (OPA), boric acid, L-Glu, L-Gly, D-glucose, monobasic sodium phosphate, \( \gamma \)-amino butyric acid (GABA), sodium chloride, potassium chloride, calcium 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), methylthiazolyldiphenyl-tetrazolium bromide (MTT), 2-mercaptoethanol (\( \beta \)-ME), bromophenol blue and ammonium persulfate (APS) 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). Acrylamide (30\%, 37.5:1\% bis), and the pre-stained protein ladder were obtained from Bio-Rad (Hercules, CA). Dulbecco’s Modified Eagle Medium (D-MEM), 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), \( N,N,N',N' \)-tetramethylethlenediamine (TEMED) and trypan blue were obtained from Invitrogen (Carlsbad, CA). Fetal Clone-1 was obtained from HyClone (Logan,
Sodium hydroxide, isopropanol and methanol were obtained from EMD Chemicals (Gibbstown, NJ). Bovine serum albumin (BSA) was obtained from Gemini Bioproducts (Woodland, CA). Recombinant rat IL-1β, and TNF-α were obtained from PeproTech Inc. (Rocky Hill, NJ). Recombinant murine (rm) IL-6 was obtained from RD Systems (Minneapolis, MN). Rabbit anti-rat IκB-α, IκB-β, phosphorylated p38 (T180/Y182), total p38, total JNK, mouse anti-rat phosphorylated JNK (T183/Y185), and the biotinylated protein ladder was obtained from Cell Signaling (Danvers, MA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase conjugated secondary antibodies were obtained from KPL (Gaithersburg, MD). Staurosporine (STS), anisomycin, SB203580, SB202190, Bay 11-7082, 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (NAI), SP600125, and PD98059 were obtained from EMD Biosciences (La Jolla, CA). The rat B35 Neuroblastoma and 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). Enhanced Chemiluminescence (ECL)-Plus® detection reagent was obtained from Amersham (Piscataway, NJ). Schleicher & Schuell Optitran® brand supported 0.45μm nitrocellulose membranes were obtained from ISC Bioexpress (Kaysville, UT). All small molecule inhibitors were initially dissolved in sterile DMSO.
aliquoted and stored at -20 °C until use (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). After 3-4 days in culture, cells were removed from the tissue culture flasks with 2 mL of 0.25% trypsin/0.05% EDTA in Hanks-buffered salt solution. The trypsin was inactivated upon the addition of 8 mL of complete medium, the cells were subsequently pelleted, the supernatant removed, and the pelleted cells resuspended in complete medium. Cell suspension densities were determined via coulter particle counter, and cellular viability was routinely assessed via trypan blue exclusion. The cells were then placed (passed) back into continuous culture (1 x 10⁶ cells/25 cm²-area flask, BD Falcon) or were seeded into tissue culture plasticware at the plating densities noted for experiments. In all experiments presented, C6 glioma cells were used between passages 5-50.

B35 Neuroblastoma Cell Culture

Rat B35 neuroblastoma cells were maintained in continuous culture in a humidified atmosphere containing 95% air and 5% CO₂ in complete medium (D-MEM/phenol red medium containing 10% heat-inactivated FBS, 25 mM HEPES
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Associated Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB203580</td>
<td>Direct inhibitor of p38 activity</td>
</tr>
<tr>
<td>SB202190</td>
<td>Direct inhibitor of p38 activity</td>
</tr>
<tr>
<td>PD98059</td>
<td>Direct inhibitor of ERK activity</td>
</tr>
<tr>
<td>SP600125</td>
<td>Direct inhibitor of JNK activity</td>
</tr>
<tr>
<td>Bay 11-7082</td>
<td>Prevents the phosphorylation of IκB-α</td>
</tr>
<tr>
<td>NAI</td>
<td>NF-κB activation inhibitor</td>
</tr>
<tr>
<td>STS</td>
<td>Protein kinase inhibitor and inducer of cytotoxicity</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>Stimulates phosphorylation of p38</td>
</tr>
</tbody>
</table>

Table 1: Pharmacological inhibitors used in this study and their associated effects.
<table>
<thead>
<tr>
<th>Entry</th>
<th>Inhibitor</th>
<th>Net Mitochondrial Activity (percent control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>100.00 ± 1.91</td>
</tr>
<tr>
<td>2</td>
<td>10 μM SB203580</td>
<td>102.13 ± 3.71</td>
</tr>
<tr>
<td>3</td>
<td>10 μM SB202190</td>
<td>95.13 ± 1.80</td>
</tr>
<tr>
<td>4</td>
<td>10 μM SP600125</td>
<td>27.89 ± 0.65</td>
</tr>
<tr>
<td>5</td>
<td>10 μM PD98059</td>
<td>93.68 ± 5.60</td>
</tr>
<tr>
<td>6</td>
<td>0.7 nM STS</td>
<td>101.33 ± 0.47</td>
</tr>
<tr>
<td>7</td>
<td>7 nM STS</td>
<td>100.62 ± 0.96</td>
</tr>
<tr>
<td>8</td>
<td>25 μM Bay 11-7082</td>
<td>1.50 ± 0.38</td>
</tr>
<tr>
<td>9</td>
<td>11 nM NAI</td>
<td>96.24 ± 5.13</td>
</tr>
<tr>
<td>10</td>
<td>1 mM GABA</td>
<td>101.46 ± 2.98</td>
</tr>
<tr>
<td>11</td>
<td>Control</td>
<td>100.00 ± 3.57</td>
</tr>
<tr>
<td>12</td>
<td>10 μM Bay 11-7082</td>
<td>98.49 ± 1.69</td>
</tr>
<tr>
<td>13</td>
<td>25 μM Bay 11-7082</td>
<td>103.70 ± 6.84</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Entry</th>
<th>Inhibitor</th>
<th>Viability (percent viable)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>Control</td>
<td>96.08 ± 6.71</td>
</tr>
<tr>
<td>15</td>
<td>10 μM SP600125</td>
<td>84.87 ± 9.30</td>
</tr>
</tbody>
</table>

Table 2: Viability of C6 glioma cells following a treatment with various pharmacological inhibitors. Entries 1-15 represent the net mitochondrial activity (as monitored via the metabolism of MTT) of C6 cells that have been treated for 24 hours (entries 1-10) or for 15 minutes (entries 11-13). Entries 14 and 15 represent the cell viability of C6 glioma cells treated with SP600125 for 20 hours as quantified via trypan blue exclusion. In all treatments, C6 glioma cells were treated with either a vehicle control (serum-free RPMI-1640), or the inhibitor indicated. The data are presented as the mean ± s.e.m. of triplicate observations obtained from a single representative experiment.
pH 7.4, and PSN antibiotic mix). After 3-4 days in culture, cells were removed from the tissue culture flasks with 2 mL of 0.25% trypsin/0.05% EDTA in Hanks-buffered salt solution. The trypsin was inactivated upon the addition of 8 mL of complete medium, the cells were subsequently pelleted, the supernatant removed, and the pelleted cells resuspended in complete medium. Cell suspension densities were determined via coulter particle counter, and cellular viability was routinely assessed via trypan blue exclusion. The cells were then placed (passed) back into continuous culture (2 x 10^6 cells/25 cm^2-area flask, BD Falcon) or were seeded into PDL-coated tissue culture plasticware at the plating densities noted for experiments. In all experiments presented, B35 neuroblastoma cells were used between passages 5-35.

7TD1 Hybridoma Cell Culture

The 7TD1 hybridoma 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 5% fetal clone I, 50 μM β-ME, 5 pg/mL rmIL-6 and PSN antibiotic mix). After 3-4 days in culture, cells were removed from the tissue culture flasks by trituration, and subsequently pelleted, the supernatant removed, and the pelleted cells resuspended in complete medium. Cell suspension densities determined via coulter particle counter, and cellular viability was routinely assessed via trypan blue exclusion. The cells were then placed (passed) back into continuous culture (1 x 10^6 cells/25 cm^2-
area flask, BD Falcon) or were seeded (4 x 10^3 cells/well) into tissue culture plates for IL-6 release studies using the 7TD1 bioassay.

**7TD1-Bioassay for IL-6**

Rat C6 glioma cells were plated (125 x 10^3 cells/well in a 96-well plate) in complete medium and allowed 24 h for attachment to the tissue culture plastic. Following the attachment period, the cells were washed with serum-free RPMI-1640 (2 x 200 µL) and incubated for the indicated time periods with the vehicle (sfRPMI-1640) in the presence or absence of IL-1β and other agents for 20 h. Post-stimulation, the conditioned medium was removed and either stored at 4 °C or assayed for IL-6 using the 7TD1-bioassay (Spangelo et al., 2004).

During the 7TD1-bioassay, 20 µL of conditioned medium were added to a 96-well plate that contained 180 µL of complete medium in the presence of 4 x 10^3 7TD1 cells and left to incubate in a humidified atmosphere containing 95% air and 5% CO₂ for 3-4 days. Post incubation, the MTT cell viability assay was performed to quantify the growth of the 7TD1 cells during the incubation period. The range of detection (ROD) for the 7TD1-bioassay was between 0.3125 – 320 pg/mL.

**Glutamate Release Experiments**

Rat B35 neuroblastoma cells were seeded (1.5 x 10^6 cells/well in a PDL-coated 12-well plate) in complete medium, and allowed 24 h for attachment to tissue culture plastic. Following the attachment period, cells were washed with
an amino-acid free modified HEPES-buffered Krebs Ringer solution (2 x 1 mL) (KRH) (130 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.8 mM CaCl_2, 10 mM glucose, 1% BSA, 10 mM NaHCO_3 and 25 mM HEPES, pH 7.4), and incubated for the indicated time periods with the vehicle (KRH) or in the presence of IL-1β. A high K^+ (HK) containing KRH solution was prepared with 85 mM NaCl, 50 mM KCl, 1.2 mM NaH_2PO_4, 1.8 mM CaCl_2, 10 mM glucose, 1% BSA, 10 mM NaHCO_3 and 25 mM HEPES pH 7.4.

For n minute collection periods, B35 cells were incubated for n minute(s) in KRH. Post incubation, the condition medium was removed, and replaced with fresh KRH, and incubated for a second n minute incubation period. After the second n minute period, the conditioned medium was collected, and replaced with HK in the presence or absence of IL-1β and incubated for another n minute(s). At the end of the third n minute stimulation period the conditioned medium was removed, and replaced with KRH for a fourth n minute incubation period. Post-incubation, the conditioned medium was removed and replaced with fresh KRH for a fifth and final n minute incubation period. After the fifth incubation period, all 5 samples were filtered (0.22 µm), and stored at -80 °C for analysis of Glu using HPLC.

Glutamate concentrations were quantitated reversed-phase high performance liquid chromatography (HPLC). Two Waters 501 pumps were equipped with a 0.46 x 50 mm C18 column (Econosphere®, Alltech). Column effluent [sodium phosphate (0.1 M, pH 6.0) and methanol] was monitored using a Gilson fluorescence detector using excitation and emission wavelength range filters of
305 – 395 and 430 – 470 nm, respectively. Data was collected using the SR1 data collection system with the Peak Simple software package. Chromatograms were reproduced (by plotting fluorescence intensity against retention time) using Sigma Plot® from the raw data obtained from Peak Simple in the American Standard Code for Information Interchange (ASCII) format.

HPLC Analysis of Glutamate

The column was first equilibrated for 15 min by pumping the initial mobile phase (0.1 M NaH$_2$PO$_4$ pH 6.0 and 70% methanol) through the column at a flow rate of 1.0 mL/min. Following the equilibration period, the mobile phase was adjusted to 35% methanol where sample injections then took place. All the separations used with this column were carried out at a flow rate of 1.0 mL/min. Following each separation, the column was allowed to equilibrate for 5 minutes in preparation for the injection of another sample.

After a sample was injected, the eluant composition was maintained at 35% methanol for 0.5 min. At 0.5 min, a 3.5 min gradient to 70% methanol was followed by a 1.5 min gradient to 35% methanol.

Synthesis of OPA Amino Acid Derivatives

The derivatization reagent was prepared by dissolving OPA (0.270 g, 2.01 mmol) in 5 mL of methanol. Sodium borate buffer (0.4 M, pH 9.5) (40 mL) and β-ME (50 μL) were then added, and the solution volume was adjusted to 50 mL using sodium borate buffer. Roth reported that there is roughly a 500-fold
decrease in the fluorescence intensity of OPA-derivatized amino acids lacking
the mercaptan substituent (1971). To avoid the loss in fluorescence intensity due
to evaporation of β-ME, the derivatization reagent was prepared fresh each day.
An aliquot of Glu standard was mixed with 0.5 mL of the derivatization reagent
and allowed to react for 2 min at room temperature according to Scheme 1. After
2 min, a 10 μL aliquot was injected onto the HPLC.

MTT Assay for Mitochondrial Viability

Rat B35 neuroblastoma or C6 glioma cells were plated (125 x 10^3 cells/well in
a PDL-coated 96-well plate) in complete medium, and allowed 24 h for
attachment to the tissue culture plastic. Following the attachment period, the
cells were washed (2 x 200 μL) with serum-free medium, and incubated in the
absence or presence of stimulation agents for the times and doses indicated.
Post-exposure, net mitochondrial viability was assessed via MTT metabolism
(Carmichael et al., 1987). In this protocol, 20 μL of a 5 mg/mL MTT solution in
phosphate-buffered saline (PBS) was added to each well, and incubated at 37 °C
for 4 h. Post incubation, 150 μL of solution was removed from each well, and
replaced with 150 μL of a MTT crystal solubilization solution (40 mM HCl in
isopropanol). Purple formazan crystals (Scheme 2) were quantified after an
overnight incubation in darkness using a microelisa spectrophotometer set with
test and reference wavelengths of 570 and 630 nm, respectively. While other
factors may contribute to changes in mitochondrial activity (i.e., changes that
could present a false positive in regard to cytotoxicity), a previous report
Scheme 1: The formation of fluorogenic OPA-derived 1-(2-hydroxylethyl)thio-N-substituted-isoindoles.
indicates that mitochondrial activity, as assessed via MTT metabolism, provides similar results to that of a clonogenic survival (Carmichael et al., 1987).

Cytokine Quantification via ELISA

Rat C6 glioma cells were plated (125 x 10^3/well in a PDL-coated 96-well plate) in complete medium and allowed 24 h for attachment to the tissue culture plastic. Following the attachment period, the cells were washed (2 x 200 μL) with 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. Post-stimulation, cytokine concentrations were assessed via ELISA (Endogen) according to the manufacturer's specifications. The ROD for the commercially available ELISA was 31 – 2000 pg/mL.

Collection of Cellular Protein Lysates

Rat C6 glioma cells were seeded (2.0 x 10^6/dish in a PDL-coated 35 x 10 mm dish) in complete medium, and allowed 24 h for attachment to the tissue culture plastic. Following the attachment period, the cells were washed (2 x 2 mL) with 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. Post-exposure, cells were washed in 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 every 10 min for a total time of no longer than 30 min. Lysates were clarified through centrifugation.
Scheme 2: The conversion of the MTT substrate to the respective formazan product by active mitochondria.
at 14,000 x g for 10 min at 4 °C. Protein determinations were done via the bicinchoninic acid method (Pierce) using BSA as a standard. 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% w/v β-ME), and boiled for 5 min. Protein lysates were subsequently separated by SDS-PAGE, or stored at -80 °C for future analysis.

**SDS-PAGE/Western Analysis**

Clarified protein lysates were separated on a SDS-PAGE gel. Polyacrylamide gels were either purchased precasted (Pierce) or formed in a Hoffer SE-245 gel caster (Amersham) using a 5% stacking gel (3.375 mL H2O, 625 μL 1M Tris pH 6.8, 50 μL 10% w/v SDS, 825 μL 30% w/v 37.4:1 bis-acrylamide, 50 μL 10% w/v APS and 5 μL TEMED) and a 12% resolving gel (3.3 mL H2O, 2.5 mL 1.5 M Tris pH 8.8, 100 μL 10% w/v SDS, 4 mL 30% w/v 37.5:1 bis-acrylamide, 100 μL 10% w/v APS, and 4 μL TEMED). The gels were subsequently transferred to a Hoffer SE 250 Minivertical Gel Electrophoresis Unit (Amersham) filled with SDS-PAGE Tris-Gly (25 mM Tris, 192 mM Gly, and 0.1% w/v SDS, pH ~ 8.8, note: pH was not adjusted) or Tris-HEPES (0.1 M HEPES, 0.1 M Tris, 0.1% w/v SDS) (for precasted gels) running buffer, where equal amounts of protein (usually 10 – 25 μg) were loaded into each lane in the stacking 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 70 V (100 V for precasted gels) until the bromophenol blue dye front was 1 cm from the bottom of the resolving gel. Both gels produced identical results, and were used interchangeably.

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 375 mA, for 90 min in western transfer buffer (25 mM Tris, 192 mM Gly, and 20% v/v methanol, pH ~ 8.8, note: pH was not adjusted).

Following the transfer period, protein-containing membranes were [a] washed (1 x 5 min) 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 at 22 °C, [b] blocked with TBST containing 5 % nonfat milk (Nestlé Carnation) for 1 h at 22 °C, [c] washed (3 x 5 min) in TBST at 22 °C, [d] exposed overnight to primary antibodies at the dilutions indicated within the text in TBST containing 0.1% BSA at 4°C, [e] washed in TBST (3 x 5 min), [f] exposed to a horseradish peroxidase (HRP)-conjugated secondary (1:5000), and anti-biotin (1:1000) antibodies in TBST containing 0.1% BSA at 22 °C and [g] washed in TBST (3 x 5 min) at 22 °C. Proteins were visualized on the Typhoon multipurpose imager using the ECL-plus® detection reagent according to the manufacturer’s specifications (Amersham).
CHAPTER 3

RESULTS

HPLC Analysis of Amino Acids

The separation of fluorogenic OPA-derived 1-(2-hydroxylethyl)thio- N-substituted-isoindoles has been previously accomplished via reversed-phase HPLC within a variety of matrices (Yang et al., 1999; Tcherkas and Denisenko, 2001; Lindroth and Mopper, 1979). The sensitivity of this method allowed for the analytical determination of amino acids released from the rat B35 neuroblastoma cell line. Conditioned KRH medium was obtained as outlined in the Materials and Methods section, derivatized, and separated via HPLC using the solvent program previously described. A sample chromatogram of the basal release of amino acids into KRH medium conditioned for 1 min by rat B35 neuroblastoma cells is shown in Figure 9. Glutamate was resolved at 2.1 min with a fluorescence intensity of 4.28 arbitrary units.
Rat B35 cells were plated (1.0 x 10⁶ cells/well in a PDL-coated 12-well plate), and incubated in KRH for 1 min. Conditioned medium was collected and filtered when an aliquot was injected onto HPLC. The arrow indicates the elution of Glu from the column. Run specifics: flow rate 1.0 mL/min, injection volume: 10 μL. The data are presented as observations obtained from a single representative experiment.
Standard Curve of the HPLC Analysis of Glutamate

A standard curve for the HPLC analysis of amino acids was generated by injecting 10 µL of Glu standard. The equation for the best fit line between the data points was obtained by performing linear regression on the data set generated when the maximum relative intensity of each peak was graphed against known concentrations of Glu. A representative standard curve is shown in Figure 10A, for the range of 0.01 – 10 µM Glu, with a correlation coefficient ($r^2$) > 0.99. Optimum resolution of Glu was achieved with an average retention time ($R_t$) of 1.976 min, using a flow rate of 1 mL/min. A gradient elution of Glu occurred at roughly 50% methanol (Figure 10B).

Prior to each amino acid analysis, a 5 µM standard of Glu was injected, to monitor the drift in the column. A new calibration plot was generated if the known standard differed by more than 5% (with respect to both retention time and fluorescence intensity) compared to the 5 µM intensity on the previous standard curve.

Stimulated Release of Glutamate from B35 Cells

Neurotransmission within the CNS is controlled by a plethora of mechanisms involving the intricate interaction of different factors and stimuli. In order to validate the HPLC quantification of amino acids, rat B35 neuroblastoma cells were incubated under depolarizing conditions using a KRH solution adjusted to contain 50 mM $K^+$ (Numakawa et al., 2002). When B35 cells were incubated for
Figure 10: Standard curve for the HPLC analysis of Glu. Increasing concentrations of Glu standard (0.01 – 10 µM) in KRH were made. Subsequently, 10 µL of each standard were injected onto the HPLC. The best-fit line (panel A) was obtained via linear regression of the maximum peak height versus each respective standard concentration. Panel B is a representative elution of a Glu peak (5 µM) superimposed over the solvent program for the first 5 min. Run specifics: average Rt = 1.976, flow rate 1 mL/min, effluent composition B = methanol. Equation of the best fit line: y = 402.61x + 0.1695; r² = 0.9957. The data are presented as observations obtained from a single representative experiment.
1, 3, or 5 minute(s) in 50 mM K⁺ (HK), there was no apparent release of Glu as indicated through HPLC (a representative figure illustrating these observations is shown in Figure 11A. Similarly, 85 mM and 130 mM K⁺ also failed to depolarize B35 cells (data not shown). Numakawa et al. reported that basic fibroblast growth factor (BFGF) simulates the release of Glu from cultured rat cortical neurons (2002). The pro-inflammatory cytokine IL-1β was tested for its ability to stimulate release of the excitatory neurotransmitter Glu from B35 cells. When B35 cells were incubated with 25 ng/mL IL-1β, a concentration that is successful for IL-6 release by C6 cells (Spangelo et al., 2004), for 1, 3, or 5 minute(s) there was no apparent release of Glu as monitored via HPLC (Figure 11B). Due to the fact that HK and IL-1β could synergistically stimulate Glu release (Numakawa et al., 2002) from B35 cells, B35 cells were incubated in the presence of 50 mM K⁺ and 25 ng/mL IL-1β for 1, 3, or 5 minute(s). As shown in Figure 11C, the combined presence of these two agents once again failed to stimulate release of Glu from this cell line.

**Staurosporine-Mediated Apoptosis**

The alkaloid toxin staurosporine (STS: Figure 12B) is known to be a positive stimulator of apoptosis (Couldwell et al., 1994) and an effective non-specific inhibitor of protein kinase C (PKC) (Friedman LM et al., 1997; Rüegg et al., 1989) at micromolar and nanomolar concentrations, respectively. In preliminary trials, STS’s ability to compromise cell viability was assessed via the mitochondrial
Figure 11: Stimulated release of Glu from B35 cells. Rat B35 cells were plated (1 x 10^6 cells/well in a PDL-coated 12-well plate) and treated for various time periods [(▲) 1 min], (▼) 3 min, (♦) 5 min]. Post treatment, Glu content in conditioned medium was determined via HPLC analysis. KRH represents a treatment group where B35 neuroblastoma cells were treated with the KRH vehicle alone; a (+) indicates that B35 neuroblastoma cells were treated with 50 mM K^+ (K, panel A), 25 ng/mL IL-1β (IL, panel B) or both 50 mM K^+ and 25 ng/mL IL-1β (K & IL, panel C). Each hatch mark on the abscissa represents a Glu-collection time period in the presence of the noted solution. The data are presented as a single representative experiment.
metabolism of MTT in the B35 neuroblastoma cell line. Compared with the vehicle control (KRH), increasing STS concentrations were able to produce dose-dependent reductions in net mitochondrial activity ($IC_{50} = 0.441 \ \mu M$) as determined via the metabolism of MTT (Figure 12A). For example, 0.2 $\mu M$ STS caused a modest 2% decrease in cell viability. However, when 1 $\mu M$ STS was used, a 98% decrease in cell viability was seen in comparison to the control.

Glutamate-Mediated Cytotoxicity

The finding that B35 cells are capable of responding to cytotoxic stimuli prompted the investigation of the excitotoxic effects of the primary excitatory neurotransmitter, Glu. Reductions in the mitochondrial activity of B35 cells were dependent on Glu concentrations and initial cell densities (Figure 13). A maximal suppression of net mitochondrial activity (~70%) was achieved at 10 mM Glu using a cell density of 15 x 10^3 cells/well (Figure 13A). A 25% reduction in activity was seen using both 20 x 10^3 and 40 x 10^3 cells/well, at 10 mM Glu (Figure 13). Conversely, the sensitivity of 5 x 10^3 cells/well was greatest at 5 mM Glu (Figure 13B).

The cytotoxic effects of Glu could not be substantiated in a new line of B35 neuroblastoma cells. As shown in Figure 14B, concentrations that were proven to be successful in previous studies (i.e., Figure 13), were unable to cause a reduction in mitochondrial activity, using a low and high passage number (9 and 20, respectively). In addition, B35 cells were not sensitized by overnight

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Figure 12: Staurosporine-mediated apoptosis in B35 cells. Rat B35 cells (100 x 10^3 cells/well in a PDL-coated 96-well plate) were treated with vehicle control (KRH) or increasing concentrations of STS for 24 hours. Post-incubation, net mitochondrial activity was assessed via the metabolism of MTT. (B) Structural projection of STS. The data are presented as mean ± s.e.m. of quadruplicate observations obtained from a single representative experiment.
incubations in a reduced serum environment (0, 5, or 10 % FBS) followed by a 
24-hour treatment with Glu (Figure 14A). The inability of B35 cells to respond as 
postulated in Hypothesis 1 prompted an investigation into Hypothesis 2.

Standard Curve Comparison Between the 7TD1-Bioassay and the Rat IL-6 
ELISA

In all previous reports from the Spangelo laboratory, the 7TD1-bioassay has 
been used in the quantification of IL-6 released from C6 glioma cells and other 
cell types (Zumwalt et al., 1999; Spangelo et al., 2004; Spangelo and Jarvis, 
1996). Although highly sensitive for the determination of IL-6, the 7TD1-bioassay 
has intrinsic limitations that limit the use of this quantification method. The most 
prominent of these are the shape and restricted region (i.e., the “linear” portion till 
~80 pg/mL) of the standard curve generated by the 7TD1-bioassay (Figure 15). 
Other limitations include the fact that stimulation agents must be properly chosen 
such that they will not interfere with the viability of both the C6 glioma and 7TD1 
hybridoma cell lines. To circumvent the intrinsic limitations of the 7TD1-
bioassay, the work presented here made use of commercially available ELISA 
kits that were specific for rat IL-6 (i.e., mouse IL-6 is not detected). When a 
comparison is made between the standard curves generated by the 7TD1-
bioassay and ELISA kits, it becomes evident that although the bioassay has 
roughly a 100-fold lower ROD (0.3125 – 320 pg/mL), the ELISA is able to 
quantify more concentrated samples with a ROD of 31 – 2000 pg/mL.
Figure 13: Glutamate-mediated cytotoxicity in B35 cells. Rat B35 cells were plated at differing densities as shown and treated with a vehicle control (KRH) or increasing concentrations of Glu for 24 hours. Post-incubation, net mitochondrial activity was assessed via the metabolism of MTT. Passage numbers: 29 and 30 for panels A and B, respectively. Data presented are the mean ± s.e.m. of quadruplicate observations obtained from two separate experiments.
Figure 14: Effects of serum and passage number on Glu-mediated cytotoxicity in B35 cells. Rat B35 cells were plated (100 x 10^3 cells/well in a PDL-coated 96-well plate) in varying serum conditions (A) or complete medium (B) and treated with a vehicle control (KRH) or increasing concentrations of Glu for 24 hours. Post incubation, net mitochondrial activity was assessed via the metabolism of MTT. The data are presented as mean ± s.e.m. of quadruplicate observations obtained from a single representative experiment.
Figure 15: Comparison between the standard curves from the 7TD1-bioassay and the rat IL-6 ELISA. (▲) $y = (5.349 \times 10^{-4})x + 0.1040$; $r^2 = 0.998$; ROD 31 – 2000 pg/mL (▼) $y = 0.1809 \ln (x) + 0.1375$; $r^2 = 0.972$; ROD 0.3125 – 320 pg/mL. The data are presented as mean ± s.e.m. of triplicate observations obtained from a single representative experiment. The error bars (n = 3 for both the 7TD1-bioassay and ELISA) have been omitted to increase figure clarity.
ELISA Quantification of Rat IL-6: Standard Curve and the Effects of Serum

To determine the effect of a carrier protein in the ELISA as indicated in the manufacturer’s protocol (Pierce product number, ER2IL6), a standard curve of recombinant rat IL-6 was generated using either serum-free RPMI-1640 (0% FBS) or RPMI-1640 supplemented with 5% FBS (5% FBS). Immunoreactive IL-6 was analyzed via ELISA, where the best fit line between the absorbance and respective IL-6 concentrations was generated. As shown in Figure 16, the presence of 5% FBS had no effect on the standard curve. All ELISA experiments were performed for IL-6 in the absence of FBS.

The IL-1β-Stimulated Release of IL-6: Effects of Serum

The presence of 5% FBS did not have an effect on the immunoreactivity of IL-6 within the ELISA. Interestingly, the presence of 5% FBS did effect the ability of IL-1β to stimulate the release of IL-6 from C6 glioma cells. As shown in Figure 17, the presence of 5% FBS led to a three-fold reduction (121.98 ± 18.34 pg/mL) compared to 0% FBS (343.21 ± 26.64 pg/mL) in the IL-1β-stimulated release of IL-6. All standard curves for ELISA quantifications were done in the absence of serum.
Figure 16: Effects of serum on the standard curve for the rat IL-6 ELISA. A standard curve was generated using either serum-free RPMI-1640, or RPMI-1640 containing 5% FBS and quantitated using the Pierce rat IL-6 ELISA according to the manufacturer's specifications. Regression equations, and correlation coefficients (▲) \( y = (5.349 \times 10^{-4})x + 0.1040; r^2 = 0.999 \) (▼) \( y = (5.998 \times 10^{-4})x + 0.0755; r^2 = 0.997 \). The data are presented as mean ± s.e.m. of triplicate observations obtained from a single representative experiment.
Figure 17: Effects of serum on the IL-1β-stimulated release of IL-6. Rat C6 cells (125 x 10^3 cells/well in a 96-well plate) were treated with a vehicle control (RPMI-1640 containing either 0% or 5% FBS) or 50 ng/mL IL-1β 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 that the concentration of IL-6 could not be detected by ELISA. Data presented are the mean ± s.e.m. of quadruplicate observations of a single experiment.
Synergistic Release of IL-6 by IL-1β and TNF-α

Zumwalt et al. reported that TNF-α and IL-1β (Tl) are able to synergistically stimulate the release of IL-6 from rat C6 glioma cells (1999). The quantification of IL-6 from this prior study was done using the 7TD1-bioassay, which is prone to artifact. Due to the nature of the 7TD1-bioassay, the synergistic effect of Tl was revisited using ELISA quantification of rat IL-6. As shown in Figure 18, 50 ng/mL IL-1β and 100 ng/mL TNF-α produced a striking 20-fold increase in the release of IL-6 (2327.82 ± 159.95 pg/mL) compared to 50 ng/mL IL-1β alone (119.53 ± 8.05 pg/mL). Interestingly, 100 ng/mL TNF-α had only a marginal effect on the release of IL-6 (58.07 ± 4.21 pg/mL) when compared to control (32.95 ± 18.05 pg/mL). These data are in accord with the previous observations of Zumwalt et al. (1999).

Synergistic Release of IL-6 by IL-1β and TNF-α: MAPK Contribution

Spangelo et al. have previously reported that p38 is essential for the IL-1β-stimulated release of IL-6 (2004). To ascertain the roles of p38, JNK and ERK in the synergistic release of IL-6 by Tl, C6 glioma cells were pretreated with 10 μM SB203580, SB202190, PD98059, or SP600125 followed by a co-treatment with each inhibitor and 50 ng/mL IL-1β and 100 ng/mL TNF-α. As shown in Figure 19A, inhibition of p38 activity using SB203580 or its hydroxyl-phenyl analogue SB202190 (Figure 19C) completely blocked the release of IL-6 by Tl. For example, when SB203580 was used, an IL-6 concentration of 64.07 ± 5.88 pg/mL was observed in comparison to Tl alone.

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Figure 18: 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) in complete medium and treated with a vehicle control (serum-free RPMI-1640), 50 ng/mL IL-1β, 100 ng/mL TNF-α, or both TNF-α, and IL-1β for 20 hours. Post-stimulation, conditioned medium was removed and assayed for IL-6 using Pierce ELISA according to the manufacturer's specifications. The data are presented as mean ± s.e.m. of quadruplicate observations obtained from a single representative experiment.
Furthermore, SB202190 (100.22 ± 8.00 pg/mL) caused an impressive 94% suppression of IL-6 when compared to Tl (1541.22 ± 242.48 pg/mL). Inhibition of JNK activity using SP600125 (Figure 19D), was unable to provide a similar substantial suppression of IL-6 release (759.95 ± 68.36 pg/mL versus Tl alone, 1541.22 ± 242.48 pg/mL). Lastly, inhibition of ERK activity using PD98059, (Figure 19B) also was unable to suppress the release of IL-6 by Tl (709.89 ± 42.28 pg/mL versus Tl alone, 973.78 ± 54.58 pg/mL). These data substantiate the role of p38 in the synergistic release of IL-6 by Tl.

GABA Inhibition of the Synergistic Release of IL-6 by IL-1β and TNF-α

The GABA-mediated suppression of the IL-1β-driven release of IL-6 has been reported (Spangelo et al., 2004). To determine if GABA has the propensity to inhibit the synergistic release of IL-6 by Tl, C6 cells were pretreated for 1 h with 1 mM GABA followed by a 20 h co-treatment of 1 mM GABA, 50 ng/mL IL-1β and 100 ng/mL TNF-α. As shown in Figure 20, GABA was able to produce a 2.5-fold suppression of the synergistic release of IL-6 release by 100 ng/mL TNF-α and 50 ng/mL IL-1β in C6 cells. In the presence of Tl, an IL-6 concentration of 973.78 ± 54.58 pg/mL was measured. When C6 cells were pretreated for 1-hour with 1 mM GABA, and co-treated with GABA, TNF-α and IL-1β, for 20 hours IL-6 concentrations were suppressed by 67% (382.85 ± 30.90 pg/mL).
Figure 19: MAPK contribution to 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 10 μM SB203580 (A), PD98059 (B), SB202190 (C), SP600125 (D), or for 1 hour and co-treated with 10 μM SB203580, SB202190, SP600125, or PD98059, 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. (E) Structural projections of SB203580 and SB202190. A (+) indicates that C6 glioma cells were treated with IL-1β, TNF-α, and the inhibitor; a (-) indicates that cells were treated with the inhibitor alone. The data are presented as mean ± s.e.m. of quadruplicate observations obtained from a single representative experiment.
Figure 20: GABA inhibition of the synergistic release of IL-6 by IL-1β and TNF-α. Rat C6 cells were plated (125 x 10³ cells/well in a PDL-coated 96-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 that C6 glioma cells were treated with IL-1β, TNF-α, and the inhibitor; a (-) indicates that cells were treated with the inhibitor alone. The data are presented as mean ± s.e.m. of quadruplicate observations obtained from a single representative experiment.
Synergistic Release of IL-6 by IL-1β and TNF-α: Role of PKA

To determine the role of protein kinase A (PKA) in the synergistic release of IL-6 release, rat C6 glioma cells were pretreated with 7 nM STS for 1 hour, and co-treated with 7 nM STS, 50 ng/mL IL-1β and 100 ng/mL TNF-α for 20 hours. Post stimulation, conditioned medium was collected and assayed for IL-6 content via the rat IL-6 ELISA. As shown in Figure 21, 7 nM STS modestly reduced the synergistic release of IL-6 by TI. These data suggest that cellular signaling through PKA is not imperative for the synergistic release of IL-6 by TI.

Synergistic Release of IL-6 by IL-1β and TNF-α: Role of PKC

The data presented by Zumwalt et al., suggest that PKC could be an indispensable kinase in the synergistic release of IL-6 by TI (1999). To verify the role of PKC in the synergistic release of IL-6 by TI, C6 cells were pretreated with 0.7 nM STS – a concentration that selectively inhibits PKC isoforms (Rüegg et al., 1989). As shown in Figure 22, STS caused a 3.5-fold suppression of the synergistic release of IL-6 by TI (i.e., the synergistic release was reduced from $973.78 \pm 54.58 \text{ pg/mL}$ to $281.14 \pm 25.05 \text{ pg/mL}$ of IL-6).
Figure 21: PKA contribution to 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 7 nM STS for 1 hour and co-treated with 7 nM STS, 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 that C6 glioma cells were treated with IL-1β, TNF-α, and the inhibitor; a (-) indicates that cells were treated with the inhibitor alone. The data are presented as mean ± s.e.m. of quadruplicate observations obtained from a single representative experiment.
Figure 22: PKC contribution to 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 0.7 nM STS for 1 hour and co-treated with 0.7 nM STS, 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 that C6 glioma cells were treated with IL-1β, TNF-α, and the inhibitor; a (-) indicates that cells were treated with the inhibitor alone. The data are presented as mean ± s.e.m. of quadruplicate observations obtained from a single representative experiment.
Synergistic Release of IL-6 by IL-1β and TNF-α: Role NF-κB

Previous reports indicate that the NF-κB transcription factor is a key mediator in the induction of the IL-6 gene by both IL-1β and TNF-α (Berghe et al., 2000). To illustrate the importance of this transcription factor in the synergistic release of IL-6 by Tl, C6 cells were treated with two different inhibitors of NF-κB transcriptional activation: NAI and Bay 11-7082. Cells were pretreated for 1 hour with 25 μM Bay 11-7082 or 11 nM NAI (concentrations that were successful for the inhibition of transcriptional activation of NF-κB in C6 glioma and Jurkat cells, respectively, Davis et al., 2005; Tobe et al., 2003) and co-treated with either Bay 11-7082 or NAI, 50 ng/mL IL-1β and 100 ng/mL TNF-α. As shown in Figure 23, only Bay 11-7082 was able to suppress the synergistic release of IL-6 by Tl. For example, a 95% suppression of IL-6 release was observed using Bay 11-7082 (82.63 ± 9.78 pg/mL versus 1541.22 ± 242.48 pg/mL for Tl). Conversely, no suppression was observed when NAI was used (1144.40 ± 200.97 pg/mL versus 1541.22 ± 242.48 pg/mL for Tl). Taken together, these data suggest that [a] although both of these compounds are thought to inhibit NF-κB transcriptional activity, their mechanisms of action differ among cell types and [b] NF-κB transcriptional activity is essential for the synergistic release of IL-6 by Tl.
Figure 23: NF-κB contribution to 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 25 μM Bay 11-7082 (panel A) or 11 nM NAI (panel B) for 1 hour and co-treated with either 25 μM Bay 11-7082 or 11 nM NAI, 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. (C) Structural projections of Bay 11-7082 and NAI. A (+) indicates that C6 glioma cells were treated with IL-1β, TNF-α, and the inhibitor; a (-) indicates that cells were treated with the inhibitor alone. The data are presented as mean ± s.e.m. of quadruplicate observations obtained from a single experiment.
Time- and Dose-Dependent Accrual of Phosphorylated p38: Anisomycin

The protein translation inhibitor anisomycin was initially isolated from strains of the bacteria Streptomyces (Sobin, and Tanner, 1954). The alkaloid is reportedly a selective and potent stimulator of JNK and p38 phosphorylation in vitro (Kyrikis et al., 1994; Hazzalin et al., 1998). The ability of anisomycin to stimulate the phosphorylation of p38 in C6 glioma cells was assessed via SDS-PAGE followed by Western analysis. As presented in Figure 24A, anisomycin (15 μg/mL) produced a time-dependent accrual of cytoplasmic phosphorylated p38 MAPK beginning at 5 minutes (an effect that was maintained through 90 minutes). Dose-dependent accumulation of phosphorylated p38 MAPK was maximal between 5 – 15 μg/mL; additionally, higher concentrations of anisomycin (e.g., 25 or 30 μg/mL) were less efficacious in stimulating p38 phosphorylation (Figure 24B). In all experiments, anisomycin did not alter total p38 protein levels (Figure 24). These data validate our ability to detect phosphorylated p38 via Western analysis.

Time- and Dose-Dependent Accrual of Phosphorylated p38: IL-1β

Previously, SB203580 (structure shown in Figure 19) was shown to inhibit the IL-1β-stimulated release of IL-6 in the rat C6 glioma cell line. Those data strongly support the role of the p38 MAPK signaling module in IL-1β-stimulated release of IL-6 (Spangelo et al., 2004). The following studies were conducted in order to elucidate the mechanisms used in the IL-1β-stimulated release of IL-6. The efficacy of IL-1β to stimulate phosphorylation of p38 was assessed via SDS-
Figure 24: Time- and dose-dependent accrual of phosphorylated p38 by anisomycin. Rat C6 cells (3 x 10⁵ cells/dish in a PDL-coated 35 x 10 mm-dish) were treated with vehicle control (serum free RPMI-1640) for increasing periods of time (A) with 15 μg/mL anisomycin or increasing concentrations of anisomycin for 60 min (B). Post stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for phosphorylated or total p38 protein (1:1000 for both). (C) Structural projection of anisomycin. The data are presented as the observations obtained from a single representative experiment.
PAGE followed by Western analysis. As shown in Figure 25A, IL-1β (50 ng/mL) produced a biphasic time-dependent accumulation of phosphorylated p38 detectable at 5 minutes and maximal at 15 minutes. Interleukin-1β dose-dependently stimulated the phosphorylation of p38 from 5 – 100 ng/mL (Figure 25B). In all experiments, IL-1β was unable to alter the levels of total p38 protein levels. These data support the results reported by Spangelo et al., indicating that p38 MAPK is an essential component of IL-1 signaling (2004).

Phosphorylation of MAPKs by IL-1β

Pharmacological data obtained previously indicate that JNK and ERK are not essential for the IL-1β-stimulated release of IL-6. To further assess the ability of IL-1β to stimulate other MAPKs, C6 cells were treated with 50 ng/mL IL-1β for 15 minutes, and probed for MAPK (p38, JNK or ERK) phosphorylation. Cellular protein was extracted and separated via 12% SDS-PAGE followed by Western analysis. As shown in Figure 26, 50 ng/mL IL-1β induced the accrual of the phosphorylated forms of p38, and JNK 1/2, but not ERK 1/2. In all treatments, 50 ng/mL IL-1β were unable to alter the total levels of p38, JNK or ERK. Taken together, these data suggest a role for p38 and JNK in IL-1β-mediated signal transduction.
Figure 25: Time- and dose-dependent accrual of phosphorylated p38 by IL-1β. Rat C6 cells (3 x 10⁶ cells/dish in a PDL-coated 35 x 10 mm-dish) were treated with vehicle control (serum free RPMI-1640) for increasing periods of time (A) with 50 ng/mL IL-1β or increasing concentrations IL-1β for 15 min (B). Post stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for phosphorylated or total p38 protein (1:1000 for both). The data are presented as the observations obtained from a single representative experiment.
Figure 26: Accrual of phosphorylated MAPks by IL-1β: Rat C6 cells (3 x 10^6 cells/dish in a PDL-coated 35 x 10 mm-dish) were treated with vehicle control (serum free RPMI-1640) for increasing periods of time with 50 ng/mL IL-1β or increasing concentrations IL-1β 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 phosphorylated or total p38, JNK, or ERK proteins (1:1000 for all). The data are presented as the observations obtained from a single representative experiment.
Time and Dose-Dependent Acquisition of Phosphorylated p38: TNF-α

In light of the striking synergistic release of IL-6 seen by IL-1β and TNF-α, a possible mechanistic rationale was sought. The efficacy of TNF-α to stimulate phosphorylation of p38 was assessed via SDS-PAGE followed by Western analysis. As shown in Figure 27A, TNF-α (50 ng/mL) caused a biphasic-time-dependent phosphorylation of p38 with maximal immunoreactivity seen at 15 minutes. Tumor necrosis factor-α also stimulated an increase in cytoplasmic phosphorylated p38 beginning at 5 ng/mL with no distinct change in the phosphorylation pattern up to 200 ng/mL (Figure 27B). In all experiments, TNF-α did not alter the levels of total p38 protein. Taken together, these data suggest that TNF-α is able to stimulate the phosphorylation of p38.

Effect of IL-1β and TNF-α co-culture on MAPK phosphorylation

To ascertain the signaling mechanism involved in the synergistic release of IL-6, rat C6 cells were treated with 50 ng/mL IL-1β, 100 ng/mL TNF-α, or 50 ng/mL IL-1β and 100 ng/mL TNF-α for 15 minutes, and examined for phosphorylation of p38, JNK and ERK. Post-stimulation, cellular protein was extracted, separated by SDS-PAGE followed by Western analysis for phosphorylated or total MAPK. As shown in Figure 28, no additional increases in phosphorylated p38 were seen in C6 cells treated with 50 ng/mL IL-1β and 100 ng/mL TNF-α. Additionally, similar results were noted for the phosphorylation of JNK. Similar to the study presented in Figure 26, ERK is not sensitive to stimulations by IL-1β, TNF-α or to the combined treatment of IL-1β and TNF-α.
Figure 27: Time- and dose-dependent accrual of phosphorylated p38 by TNF-α. Rat C6 cells (3 x 10⁶ cells/dish in a 35 x 10 mm-dish) were treated with vehicle control (serum free RPMI-1640) for increasing periods of time (A) with 50 ng/mL TNF-α or increasing concentrations of TNF-α for 15 min (B). Post stimulation, cellular protein was extracted, and 25 μg of total protein were subsequently separated via SDS-PAGE followed by Western analysis for phosphorylated or total p38 protein (1:1000 for both). The data are presented as the observations obtained from a single representative experiment.
In all treatments, stimulation by IL-1β and TNF-α did not cause a change in total levels of MAPK protein.

Degradation of IκB-α by IL-1β

In an attempt to clarify the mechanism of the synergistic release of IL-6 by IL-1β and TNF-α, the role of the NF-κB activation was investigated. Since the activity of NF-κB can be assessed though the degradation of its intrinsic inhibitor IκB, C6 cells were treated with 50 ng/mL IL-1β for 5, 15, 30, 60, or 90 minutes. Post stimulation, cytosolic protein was extracted and assayed for the degradation of IκB-α via SDS-PAGE followed by Western analysis. As shown in Figure 29, 50 ng/mL IL-1β caused a rapid and transient decrease in IκB-α immunoreactivity at 15 and 30 minutes which coincided with the maximal accrual of phosphorylated p38 (Figure 29).

Degradation of IκB-α by TNF-α

The degradation of the intrinsic inhibitor IκB in C6 cells treated with 50 ng/mL TNF-α for 5 – 90 minutes was also determined. Cytosolic protein was extracted and assayed for the presence of IκB-α via SDS-PAGE followed by Western analysis. As shown in Figure 30, 50 ng/mL TNF-α caused a rapid and transient decrease in IκB-α at 15 and 30 minutes, which strongly coincided with the maximal accrual of phosphorylated p38 MAPK.
Figure 28: Accrual of phosphorylated MAPKs by IL-1β and TNF-α. Rat C6 cells (3 x 10^6 cells/dish in a PDL-coated 35 x 10 mm-dish) were treated with vehicle control (serum free RPMI-1640), 50 ng/mL IL-1β, 100 ng/mL TNF-α or both IL-1β and TNF-α for 15 min. Post stimulation, cellular protein was extracted and 25 μg (10 μg for ERK) of total protein was subsequently separated via SDS-PAGE followed by Western Analysis for phosphorylated or total p38, JNK, or ERK proteins (1:1000 for all). The data are presented as the observations obtained from a single representative experiment.
Figure 29: Time-dependent degradation of IκB-α by IL-1β. Rat C6 cells (3 x 10⁵ cells/dish in a PDL-coated 35 x 10 mm-dish) were treated with vehicle control (serum free RPMI-1640) or increasing periods of time with 50 ng/mL IL-1β. Post stimulation, cellular protein was extracted, and 25 µg of total protein was 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 representative experiment.
Figure 30: Time-dependent degradation of IkB-α by TNF-α. Rat C6 cells (3 x 10^5 cells/dish in a 35 x 10 mm-dish) were treated for increasing periods of time with 50 ng/mL TNF-α. Post stimulation, cellular protein was extracted, and 25 μg of total protein was subsequently separated via SDS-PAGE followed by Western Analysis for IkB-α, IkB-β, phosphorylated or total p38 protein (1:1000 for all). The data are presented as the observations obtained from a single representative experiment.
Degradation of IκB-α: Role of p38 and GABA

Although activation of NF-κB and MAPKs are postulated to be parallel events, Jang et al., have reported that the p38 inhibitor SB203580 can subdue Aβ-stimulated binding of NF-κB to its cognate sequence as assessed by the electrophoretic mobility shift assay (EMSA) in the PC12 rat pheochromocytoma cell line (2005). These data indicate that p38 is indispensable for NF-κB transcriptional activity in neurons. To elucidate the role of p38 in the degradation of IκB-α, C6 glioma cells were treated with 10 μM SB203580 for 1 hour and stimulated with 50 ng/mL IL-1β and 100 ng/mL TNF-α for 15 min. Post stimulation, cellular protein was extracted and separated using SDS-PAGE followed by Western analysis. As shown in Figure 31, inhibition of p38 activity did not suppress degradation of IκB-α. Furthermore, a 1 hour pre-treatment with 1 mM GABA followed by a 15 minute co-treatment with 1 mM GABA, 50 ng/mL IL-1β and 100 ng/mL TNF-α was also unable to suppress the degradation of IκB-α. Taken together, these data suggest that glial p38 does not mediate NF-κB activation. Additionally the GABA-mediated suppression of synergistic astrocytic IL-6 release may not involve NF-κB activation. No stimulation was able to alter total levels of p38 protein.

Degradation of IκB-α: Effects of Lower TNF-α Concentrations

In all studies presented, C6 cells were treated with 50 ng/mL IL-1β and 100 ng/mL TNF-α. The data shown in Figure 27, indicate that 5 ng/mL TNF-α stimulates the phosphorylation of p38, with no further increases in
Figure 31: The role of p38 in the degradation of IκB-α. Rat C6 cells (3 x 10^6 cells/dish in a 35 x 10 mm-dish) were pre-treated with 10 μM SB203580 or 1 mM GABA for 1 hour, and co-treated with either 10 μM SB203580 or 1 mM GABA, 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). A (+) represents a treatment group where C6 glioma cells were treated with both IL-1β and TNF-α; a (-) indicates that C6 glioma cells were treated with the inhibitor alone. The data are presented as the observations obtained from a single experiment.
phosphorylated p38 immunoreactivity at higher doses (i.e., 200 ng/mL TNF-α). We postulate that treatment of C6 glioma cells with 50 ng/mL IL-1β and lower concentrations of TNF-α may allow for a synergistic stimulation of p38 phosphorylation and a concurrent degradation of IκB-α. As shown in Figure 32, IκB-α degradation was accelerated by either 5 or 50 ng/mL TNF-α in the presence of 50 ng/mL IL-1β (i.e., compare T1 treatments to IL-1β or TNF-α alone). Taken together, these data indicate that the phosphorylation of p38 and the degradation of IκB-α can be further stimulated by decreasing TNF-α concentrations in the presence of 50 ng/mL IL-1β.

Degradation of IκB-α: Roles of PKA and PKC

The ability of 0.7 nM STS to inhibit the synergistic release of IL-6 by T1 (Figure 22) prompted the investigation of the ability of this treatment to inhibit the degradation of either IκB-α or phosphorylation of p38. As shown in Figure 33, the pretreatment of C6 cells with 0.7 nM STS (IC_{50} for PKC inhibition) subsequent stimulation with 50 ng/mL IL-1β and 100 ng/mL TNF-α did not inhibit the degradation of IκB-α or phosphorylation of p38. Additionally, treatment of C6 cells with 7 nM STS (IC_{50} for PKA inhibition) resulted in no change in the degradation and phosphorylation patterns of IκB-α and p38, respectively.
Figure 32: Effects of lower TNF-α concentration on p38 phosphorylation and the degradation of IκB-α. Rat C6 cells (3 x 10^6 cells/dish in a PDL-coated 35 x 10 mm-dish) were pretreated with either 5 ng/mL or 50 ng/mL TNF-α in the absence or presence of 50 ng/mL IL-1β for 15 min. Post-stimulation, cellular protein was extracted, and 25 µg of total protein was subsequently separated via SDS-PAGE followed by Western analysis for IκB-α, phosphorylated or total p38 protein (1:1000 for all). A (+) represents a treatment group where C6 glioma cells were treated with both IL-1β and TNF-α; a (-) indicates that C6 glioma cells were treated with the TNF-α alone. The data are presented as the observations obtained from a single experiment.
Figure 33: The roles of PKA and PKC in the degradation of IκB-α. Rat C6 cells (3 x 10^6 cells/dish in a PDL-coated 35 x 10 mm-dish) were pre-treated with 7 nM or 0.7 nM mM STS for 1 hour, and co-treated with either 7 nM or 0.7 nM STS, 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 was subsequently separated via SDS-PAGE followed by Western analysis for IκB-α phosphorylated or total p38 protein (1:1000 for all). A (+) represents a treatment group where C6 glioma cells were treated with both IL-1β and TNF-α; a (-) indicates that C6 glioma cells were treated with the inhibitor alone. The data are presented as the observations obtained from a single experiment.
Inhibition of NF-κB and Degradation of IkB-α

The data presented thus far strongly suggest a role for NF-κB in the synergistic release of IL-6 by Tl. To further ascertain a mechanistic rationale for this release of IL-6, inhibitors of NF-κB activation were used to monitor the degradation of IkB-α. As shown in Figure 34, a 1 hour pretreatment for with 11 nM NAI (Tobe et al., 2003) followed by a 15 minute co-treatment with 11 nM NAI, 50 ng/mL IL-1β and 100 ng/mL TNF-α was unable to suppress the degradation of IkB-α as indicated though SDS-PAGE followed by Western analysis. Conversely, a 1 hour pretreatment with 25 μM Bay 11-7082 followed by a 15 min co-treatment with 25 μM Bay 11-7082, 50 ng/mL IL-1β and 100 ng/mL TNF-α was able to suppress the degradation of IkB-α and the phosphorylation of p38. These data are consistent with the ELISA release data presented in Figure 23, and indicate the mechanism of action for these two inhibitors are completely different from each other.

Dose-Dependent Inhibition of IkB-α Degradation by Bay 11-7082

To determine the dose-dependent inhibition of IkB-α degradation by Bay 11-7082, in the presence of IL-1β and TNF-α, C6 glioma cells were co-treated with either 10 or 25 μM Bay 11-7082, 50 ng/mL IL-1β and 100 ng/mL TNF-α for 15 minutes. As indicated though SDS-PAGE followed by Western analysis (Figure 35), 25 μM but not 10 μM Bay 11-7082 was able to preserve IkB-α protein within the cells in the presence of Tl. Additionally, 25 μM but not 10 μM Bay 11-7082
was able to produce a modest reduction of phosphorylated p38 immunoreactivity in the presence of Tl. As expected, no changes in total p38 or IκB-β proteins were observed over all treatments. These data suggest that the abilities of Bay 11-7082 to prevent the release of IL-6 and the degradation of IκB-α in the presence of Tl is dependent on the concentration of the small molecule inhibitor.
Figure 34: Inhibitors of NF-κB and degradation of IκB-α. Rat C6 cells (3 x 10⁶ cells/dish in a PDL-coated 35 x 10 mm-dish) were pretreated with either 11 nM NAI, 25 μM Bay 11-7082 or 10 μM SB202190 for 1 hour, and co-treated with either NAI, Bay 11-7082 or SB202190, 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 phosphorylated or total p38 protein (1:1000 for all). A (+) represents a treatment group where C6 glioma cells were treated with both IL-1β and TNF-α; a (-) indicates that C6 glioma cells were treated with the inhibitor alone. The data are presented as the observations obtained from a single experiment.
Figure 35: Dose-dependent degradation of IkB-α by Bay 11-7082. Rat C6 cells (3 x 10^6 cells/dish in a PDL-coated 35 x 10 mm-dish) were treated with 50 ng/mL IL-1β and 100 ng/mL TNF-α in the presence or absence of 10 μM or 25 μM Bay 11-7082 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 IkB-α, IkB-β, phosphorylated or total p38 protein (1:1000 for all). A (+) represents a treatment group where C6 glioma cells were treated with the inhibitor, IL-1β and TNF-α; a (-) indicates that C6 glioma cells were treated with the inhibitor alone. The data are presented as the observations obtained from a single experiment.
DISCUSSION

Glutamate-Mediated Excitotoxicity in the B35 Neuroblastoma Cell Line

The aberrant release and subsequent metabolism of the primary excitatory neurotransmitter Glu has been implicated in AD, epilepsy and ischemia (Obrenovitch et al., 2000). Superfluous concentrations of perisynaptic Glu are reported to encourage excessive NMDA receptor activation, leading to an increased $\text{Ca}^{2+}$ influx into the cell and a dissemination of excitotoxic events (Figure 36). The use of memantine (Figure 2), a low to moderate non-competitive inhibitor of NMDA receptor activation by Glu, has shown clinical promise in treating AD patients with severe dementia. There are accumulating amounts of evidence indicating that pro-inflammatory cytokines (e.g., IL-1$\beta$, TNF-$\alpha$ and IL-6) have the propensity to further exacerbate the already compromised aspects of glutamatergic neurotransmission, NMDA receptor activation and metabolism in AD (Hu et al., 2000; Viviani et al., 2003; Floden et al., 2005). Much effort has been made to elucidate the mechanisms in which pro-inflammatory cytokines and/or signal transduction pathways affect aspects of glutamatergic neurotransmission, receptor activation and metabolism (Qiu and Gruol, 2003; Wu...
Figure 36: Consequences associated with prolonged NMDA receptor activation by Glu.
et al., 2004; Takahashi et al., 2003; Chaparro-Huerta et al., 2005; Pelidou et al., 2002).

The rat B35 neuroblastoma cell line was chosen as a model cell line in which to study the signal transduction mechanisms involved in glutamatergic neurotransmission and subsequent NMDA receptor activation. Numakawa et al. demonstrated that primary rat cortical neurons release Glu when stimulated with either BFGF and/or 50 mM K⁺ (2000). In an attempt to ascertain the role IL-1β could have on the release of Glu, HPLC determination of amino acids (n.b., Glu) was implemented. As presented in Figure 10 and 11, the assay for the quantification of Glu was properly developed; however, much to our disappointment, B35 cells were unresponsive to either depolarizing concentrations of K⁺ and/or IL-1β (Figure 11). The fact that GABA is a major cellular product may explain the unresponsiveness of B35 cells to the aforementioned stimulus (Schubert et al., 1974). While it could be true that GABA could be released under the experimental conditions, the size of the column (5 cm) and solvent program was optimized for Glu and not GABA.

The finding that STS is able to cause a dose-dependent decrease of cell viability (Figure 12) provided a foundation on which to further elucidate the role of pro-inflammatory cytokines in Glu-mediated excitotoxicity. In two separate studies (i.e., passage numbers 29 and 30), Glu was able to induce a dose-dependent decrease in cell viability (Figure 13). Again to our disappointment, new lines of B35 cells were completely unresponsive to the same concentrations of Glu at a low and high passage number (9 and 20), and to an overnight
incubation in reduced serum conditions (Figure 14). Two previous reports indicate that the all-trans-retinoic acid (RA)-induced differentiation of the human SK-N-SH neuroblastoma and NT2 teratocarcinoma cell lines produce functional NMDA receptors capable of mediating excessive Glu stimulation though NMDA receptors (Pizzi et al., 2002; Younkin et al., 1993). Although not reported in the primary literature, it is plausible that RA-induced differentiation of the rat B35 neuroblastoma cell line may cause the expression of functional NMDA receptors. In summary, the inability of the rat B35 neuroblastoma cell line to respond to pro-inflammatory or excitotoxic stimulus may be due to an intrinsic characteristic of the cell line (i.e., GABAergic neuron unable to express functional NMDA receptors).

Synergistic Release of IL-6 by IL-1β and TNF-α

Blockade of the increased production of pro-inflammatory cytokines in AD may lead to novel modalities to treat this catastrophic disorder (Ranaivo et al., 2006; Allan and Pinteaux, 2003; Braddock and Quinn, 2004). Elucidation of the signal transduction pathways used by IL-1β may identify potential targets to which pharmacological inhibitors may be used to break the recursive cascade present in the amyloid cascade hypothesis. A core focus of the Spangelo laboratory has been to ascertain the mechanism of the IL-1β-stimulated release of IL-6 in the rat C6 glioma cell line. Previous reports from the laboratory indicate that the IL-1β-stimulated release of IL-6 is sensitive to cyclic adenosine 3'-5' monophosphate (cAMP), catecholamines, TNF-α, SRIF, and the primary inhibitory neurotransmitter GABA (Zumwalt et al., 1999; Spangelo et al., 2004).
These data suggest that the release of IL-6 is controlled by a multitude of factors. The ability of TNF-α to synergistically stimulate the IL-1β-stimulated release of IL-6 presents an interesting discovery because increased levels of TNF-α have been seen in AD (Dickson et al., 1993). Additionally, the dysfunction of numerous neurotransmitter systems (i.e., GABAergic) may provide insight into the reasons why elevated levels of pro-inflammatory cytokines exist in AD neuropathology (Bell et al., 2005). The studies presented within this work attempt to provide a mechanistic rationale for both the synergistic release of IL-6 by Tl (Figure 18) as well as the GABA-mediated inhibition of this effect (Figure 20).

Spangelo et al. have suggested that the p38 (1996) and PKC signaling modules (2004) are both essential for the release of IL-6 by IL-1β or its putative second messenger lysophosphatidylcholine (LPC) in the C6 glioma cell line and in primary rat anterior pituitary cells, respectively. The relative importance of these kinases (i.e., p38 and PKC) is in accord with existing reports suggesting that increased activities of both of these kinases are present during AD. Inhibition of p38 with SB203580, or its hydroxy-phenyl analog, SB202190, blocked the synergistic release of IL-6 from the C6 glioma cell line (Figure 19A, and B); moreover, inhibition with the broad range PKC inhibitor, STS, also effectively suppressed immunoreactive IL-6 release caused by Tl (Figure 22).

The promoter region of the rat IL-6 gene contains AP-1, cAMP response element (CRE) and NF-κB binding sites (Li et al, 2004) (Table 3). The presences of these binding sites provide pertinent information with which to elucidate the
mechanistic basis for the synergistic release of IL-6 by IL-1β and TNF-α. Nuclear factor-κB has been implicated in the production of IL-6 by T lymphocytes (Sparacio et al., 1992); furthermore, Moynagh et al. reported that both IL-1α and IL-1β are able to activate NF-κB in C6 glioma cells (1993). Pradeep and Kuttan reported that piperine treatment of B16F-10 melanoma cells resulted in a decreased expression of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and others through a decreased nuclear translocation of transcription factors such as cAMP response element binding protein (CREB) and NF-κB (2004). While many other reports exist, the abilities of IL-1β and TNF-α to increase NF-κB transcriptional activation provide a foundation in which to study the synergistic release of IL-6 in C6 glioma cells.

Activation of the "canonical" p50-p65 NF-κB heterodimeric transcription factor has been reported to be dependent (Jang et al., 2005) and independent (Fiebich et al., 2000; Wu et al., 2004) of p38 activation. Additionally, Jang and Surh have reported that AP-1-mediated transcription is dependent on p38 activation (2005B). Although these reports are based on different cell types (PC12, SK-N-SH and primary rat astrocytes), it is plausible that activation of p38 by IL-1β and/or TNF-α leads to a concomitant increase in NF-κB activation in the C6 glioma cell line. The abilities of IL-1β and/or TNF-α to stimulate the phosphorylation of p38 was investigated; however, while each of these cytokines alone are able to stimulate phosphorylation of p38, the combined presence of these cytokines were unable to cause reproducible synergistic increases in p38.

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<table>
<thead>
<tr>
<th>Binding Site</th>
<th>Consensus Sequence</th>
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<tr>
<td>AP-1</td>
<td>5'-AGTCA-3'</td>
</tr>
<tr>
<td>CRE</td>
<td>5'-CGTCA-3'</td>
</tr>
<tr>
<td>NF-κB</td>
<td>5'-GGGATTTTCCC-3'</td>
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Table 3: Transcription factor binding sites present in the rat IL-6 gene.
phosphorylation (Figure 28). Although NF-κB activation can be determined by the separation of nuclear protein via SDS-PAGE followed by Western analysis, the nuclear translocation of NF-κB can also be monitored by the degradation of its inhibitory factor, IκB. Three isoforms of IκB currently exists (α, β and ε) and prior publications indicate that IκB-α is sufficient for monitoring the liberation of the free NF-κB p50-p65 heterodimer. As shown in Figures 31 and 34, blockade of p38 activity using SB203580 or its hydroxyl-phenyl analog, SB202190 were unable to suppress the degradation of IκB-α when treated with IL-1β and TNF-α. These data indicate that [a] SB203580 and SB202190 do not inhibit phosphorylation of p38 itself, but rather prevent this kinase from activating a downstream target and [b] inhibition of p38 activity does not result in the reversal of IκB-α degradation by IL-1β and/or TNF-α. In contrast to Jang et al. who reported SB203580-mediated suppression of the Aβ-mediated binding of AP-1 and NF-κB to a labeled oligonucleotide probe for AP-1 and NF-κB in the pro-neuronal-like PC12 pheochromocytoma cell line, the data collected in the C6 glioma cell line suggest that p38 is dispensable for NF-κB activity as monitored by IκB-α degradation (2005; 2005B). The discrepancy seen between Jang et al. (2005B) and the data presented in this study are supported by the observations of Srinivasan et al. who demonstrate that IL-1β is able to increase binding of NF-κB to a labeled oligonucleotide probe for NF-κB in primary rat astrocytes, but not in hippocampal neurons (2004).
The synergistic release of IL-6 by Tl was also sensitive to non-specific inhibition of PKC, but not PKA by using 0.7 and 7 nM STS, respectively (Figure 22). Consistent with the lack of inhibition of p38 phosphorylation, 0.7 nM STS was unable to prevent the degradation of IκB-α in the C6 glioma cell line (Figure 33). Inhibition of PKC using bisindolylmaleimide was shown to prevent the IL-1β-stimulated phosphorylation of ERK in primary murine astrocytes suggesting that PKC is essential for ERK activity (Molina-Holgado et al., 2000). As presented in Figure 33, inhibition of PKC with 0.7 nM STS was unable to mitigate the phosphorylation of p38 by Tl. Taken together, these data suggest that PKC and p38 do not cooperate with respect to IL-6 release by Tl.

Human Aβ pentapeptides (either 31-35 or 34-39) are reportedly neurotoxic to GABAergic neurons isolated from primary rat forebrain cultures (Pakaski et al., 1998). Furthermore, Samland et al. have reported that transgenic mice expressing IL-6 from the glial fibrillary acidic protein (GFAP) promoter show a decrease in immunostaining for GABAergic neurons beginning at 2-months of age, suggesting that IL-6 is detrimental to GABAergic neuron viability (2003). These results suggest that levels of the primary inhibitory neurotransmitter GABA are decreased in AD. The exogenous administration of inhibitory neurotransmitters could be of a therapeutic benefit in AD. Craft et al. have reported that administration of the SRIF (inhibitory neuropeptide) analog octreotide, enhances the memory of AD patients in story recall (1999). Spangelo et al. have reported that both SRIF and GABA are able to attenuate the IL-1β-stimulated release of IL-6 from C6 glioma cells (2004). As shown in Figure 20,
GABA is also able to inhibit the synergistic release of IL-6 by Tl. In contrast to our preliminary studies suggesting that GABA suppression of IL-1β-stimulated IL-6 release is mediated by a reduction in p38 phosphorylation (data not shown), the data presented in Figure 31 show no GABAergic effect on phosphorylated p38. GABA was also unable to suppress the IL-1β-stimulated phosphorylation of p38 in the C6 glioma cell line in the presence of Tl. These results contradict those of Simi et al., who observed that chlomethiazole (a GABA<sub>A</sub> receptor agonist) is able to produce a neuroprotective effect in lipopolysaccharide (LPS)-stimulated primary cortical glial cultures through a decrease in p38 phosphorylation (2000).

The individual signal transduction pathways used by IL-1β and TNF-α converge upon the activation of NF-κB (Akama and Van Eldik, 2000). The data presented in Figure 32 indicate that lowering TNF-α concentrations may allow for a synergistic stimulation of p38 phosphorylation. More importantly, Figure 32 shows that the degradation of IκB-α can be greatly accelerated by the co-treatment of IL-1β and TNF-α. Bourke et al., have reported that signaling by IL-1β to NFκB is mediated though the long-term degradation of IκB-β instead of the transient degradation of IκB-α (2000). Interestingly, the data obtained from this study indicate that IL-1β or TNF-α is unable to stimulate the degradation of IκB-β. Taken together these findings strengthen the suggestion that the synergistic release of IL-6 by Tl could be dependent upon the accelerated degradation of IκB-α. The ability of Bay 11-7082 to prevent both the degradation of IκB-α (Figure 34) and the synergistic release of IL-6 (Figure 23) may provide clues into
the mechanistic action of GABA. GABA's inability to lessen p38 phosphorylation while inhibiting the release of IL-6, suggests that GABA's ability to inhibit the synergistic release of IL-6 could be through a lessened rate of degradation of \( \kappa B-\alpha \). While one may suggest that GABA could prevent the degradation of \( \kappa B-\alpha \) altogether, the data presented rule against this mechanism. Juxtaposition of the ELISA release data obtained by GABA (Figure 20) and Bay 11-7082 (Figure 23) with the data illustrating the effects of these two agents on \( \kappa B-\alpha \) degradation (Figures 31 and 34, respectively) clearly indicate that even though GABA is able to provide a ~60% suppression of IL-6 release, GABA is unable to prevent \( \kappa B-\alpha \) degradation. In sharp contrast to GABA, 25 \( \mu \)M Bay 11-7082 is able to produce an unsurpassed ~95% suppression of IL-6 release (Figure 23) and a complete prevention of \( \kappa B-\alpha \) degradation (Figures 34 and 35). We suggest that GABA's ability to suppress IL-6 release maybe mediated by a decreased rate of \( \kappa B-\alpha \) degradation. Phosphorylation of \( \kappa B \) initiates the process for degradation. We will investigate the effects of GABA on the phosphorylation of this protein in the presence of a proteasomal inhibitor such as pyrrolidine dithiocarbamate (PDTC) or MG132. We will also conduct a kinetic study (using SDS-PAGE followed by Western analysis) of GABA's ability to lessen the rate of \( \kappa B-\alpha \) degradation.

The work presented made use of the rat C6 glioma cell line, while there are many advantages to using an immortalized cell line (i.e., rapid doubling time and no animal surgery procedures), the generation and use of rat primary type I astrocytes would allow for a more physiologically relevant platform (i.e., no aberrant growth and/or signaling mechanisms) in which this mechanistic study

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can take place. We are currently working to establish primary cultures of rat type I astrocytes such that select experiments presented within this work can be replicated using a non-immortalized cell type.

Occupancy of the IL-1-R1 and TNF receptor (TNFR) by IL-1β and TNF-α, respectively, stimulates the activity of the MAPKKK branch point kinase (Figure 37). Activation of MAPKKK leads to a signal bifurcation into the p38 and NF-κB signaling modules culminating in the synergistic release of IL-6 from C6 glioma cells. The use of SB203580 or SB202190 is able abrogate the synergistic release of IL-6 without an inhibition of p38 phosphorylation (Figures 31 and 34). Similarly, Bay 11-7082 is able inhibit the release of IL-6 (Figure 23) while preventing IκB-α degradation in the presence of Tl (Figures 34 and 35). GABA is postulated to diminish the rate of IκB-α degradation leading to the ~60% suppression of IL-6 release synergistically driven by Tl. The finding that both SB203580 and SB202190 is unable to prevent IκB-α degradation while inhibiting the release of IL-6 suggest that NF-κB activation is essential but not sufficient for the synergistic release of IL-6 by Tl.

Although both of the stated hypotheses were disproved, the data presented within this work suggest that the mechanistic basis for the synergistic release of IL-6 by Tl lies within the ability of these cytokines to synergistically enhance the rate of degradation of IκB-α leading to increased NF-κB activity. Furthermore, GABA's ability to antagonize the synergistic release of IL-6 by Tl is postulated to be through a suppressed rate of IκB-α degradation. Verification of these
mechanisms could lead to novel treatments able combat this catastrophic neurodegenerative disorder that is AD.
Figure 37: Postulated signaling scheme for the synergistic release of IL-6 by IL-1β and TNF-α in C6 glioma cells.
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