gamma-Aminobutyric acid inhibits interleukin-1beta stimulated release of interleukin-6 from rat C6 glioma cells: In vitro evidence for a role of p38 Map kinase

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γ-AMINOBUTYRIC ACID INHIBITS INTERLEUKIN-1β STIMULATED RELEASE
OF INTERLEUKIN-6 FROM RAT C6 GLIOMA CELLS IN VITRO
EVIDENCE FOR A ROLE OF p38 MAP KINASE

by

Raghuram Royyuru
Bachelor of Pharmacy
Osmania University, India
2003

A thesis submitted in partial fulfillment
of the requirement for the

Master of Science Degree in Biochemistry
Department of Chemistry
College of Sciences

Graduate College
University of Nevada, Las Vegas
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Thesis Approval
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ABSTRACT

γ-Aminobutyric acid inhibits interleukin-1β stimulated release of interleukin-6 from rat C6 glioma cells in vitro: Evidence for role of p38 MAP Kinase

by

Raghuram Royyuru

Dr. Bryan L. Spangelo, Examination Committee Chair
Professor
University of Nevada, Las Vegas

The central nervous system (CNS) is composed of two cell types neurons and glial cells. Neurons mediate the function of sending and receiving signals in the brain. Glial cells provide nutrients and strength to the CNS. Glial cells are composed of microglia, oligodendroglia, and astrocytes. Activated microglia is the main source for the cytokine interleukin-1 (IL-1). Activated cytokines are elevated in Alzheimer’s disease (AD) (Tuppo, 2005), a neurodegenerative disorder. Cytokines mediate the inflammatory response, which is required for cells to carry out part of its normal functions. When inflammation is chronic it damages the cells and leads to the adverse effects like neuronal death which is a primary symptom for AD.

IL-1β stimulates IL-6 through by activation of a series of complexes, involving mitogen activated protein kinases (MAPK) and transcription factors. Increased IL-6 leads to neurodegeneration and finally leads to the death of the patient. IL-1β is activated by
cell stimuli like stress, oxidative damage and plaques, amyloid deposition and tumor activating proteins.

We investigated the pathway of IL-1β stimulated IL-6 release and the involvement of different MAPK's effectors. We used three different MAPK inhibitors SB203580 for p38, SP600125 for JNK, and PD98059 for ERK kinases. We described the role of γ-Aminobutyric acid (GABA) and GABA_B receptor in the inhibition of IL-1β signal transduction.

Bioassay results suggest that SB203580 inhibited IL-6 release while the other two MAPK inhibitors had no effect. It also suggested GABA inhibits the IL-1β stimulated release of IL-6. By Western analysis we found that IL-1β activated p38 MAPK time dependent fashion and we saw a maximum stimulation occurred at 15 min.

To elucidate the IL-1β signal transduction more clearly we investigated the role of the GABA receptor subclass. We investigated the role of GABA subclass receptors in IL-1β stimulation of IL-6 from C6 cells. We used muscimol (agonist for GABA_A) and Baclofen (agonist for GABA_B). Our result suggested that GABA_B receptor is involved in IL-1β stimulation of IL-6 release in C6 cells, while the GABA_A receptor agonist muscimol had no effect.

We investigated the role of SRIF in the activation of p38 MAPK by IL-1β and found that it enhanced the activation of p38. From the above results we described the IL-1β stimulated IL-6 release and the involvement of p38. GABA attenuates the stimulated release of IL-6 and suppressed p38 activation. Thus, GABA and its mimetics may be clinically effective as a treatment for AD.


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CHAPTER 1

INTRODUCTION

Central Nervous System

The central nervous system (CNS) is made of two cell types namely neurons and glial cells. Neurons send and receive electro chemical signals and glia provide nutrition and support to neurons. Neurons are composed of a cell body, dendrites and axons. Dendrites receive signals and send signal to axon. Axonal terminations pass the signal to other neurons. Except for neurons from the hippocampus most neurons cannot grow after damage. In addition to supporting neurons, glia digest the parts of dead neurons. Glial cells include microglia, oligodendroglia and astrocytes. Microglia are the smallest of glial cells. Their main function is to protect brain from microorganisms. Oligodendrocytes function is to provide support to neurons and produce of myelin which insulates the axons of neurons. Astrocytes function is to provide support to neurons by providing nutrients and breakdown glucose to lactose and release lactate into the extracellular fluid surrounding neurons. Astrocytes also perform an action called phagocytosis by which they remove neuronal debris. Activated microglia is observed in neurodegenerative diseases such as Alzheimer’s disease (AD). AD is observed in 4.5 million people in United States. Elevated levels of cytokines are observed in AD (Tuppo, 2005). Cytokines are low molecular weight proteins which mediate and regulate immune response, inflammation, and apoptosis (Streit, 2004). Cytokines are produced by many cells but
predominantly by T cells and macrophages. Cytokines act at very low concentrations for a very short period of time.

Different types of cytokines include lymphokine cytokines which are made by lymphocytes, monokine cytokines which are made by monocytes, chemokine cytokines which control chemotactic activity and interleukin cytokines which act in a paracrine fashion in the immune system. Cytokines act via binding to their receptor and thereby subsequently stimulates the release of a second messenger that leads to a cellular response. Cytokines activate other cytokines and they may also act synergistically with other cytokines.

Interleukins and Their Signaling Pathways

Interleukins play a major role in cell differentiation and proliferation. Interleukins consists of interleukin-1 (IL-1) through interleukin (IL-12) (Carol, 1997). Other groups of cytokines such as interferon inhibit viral replication. Chemokines attract lymphocytes to the site of cellular damage. Interleukin-1 is the most important immune response modifying interleukin. IL-1 also induces expression of Interferon-γ (INF-γ). The IL-1s (α, andβ) are secreted primarily by macrophages but also from neutrophils, endothelial cells, smooth muscle cells, glial cells, astrocytes, B and T-cells, fibroblasts and keratinocytes. These cells express IL-1 upon cellular stimulation. Tumor necrosis factor-α (TNF-α) also acts like IL-1β (Carol, 1997). There are two distinct IL-1 (structure shown in figure 1) proteins IL-1α (17kDa) and IL-1β (17kDa) (Warren, 1990). Both forms are synthesized from a precursor of approximately 35 kDa. The mature proteins are produced by
proteolytic cleavage which is done by the enzyme interleukin-1β converting enzyme (ICE).

IL-1 binds to IL-1 receptor and this leads to the activation of interleukin receptor associated kinase -1 and 2 (IRAK-1 and IRAK-2) (Spangelo, 2004). This activates the recruitment of TRAF6 (TNF receptor associated factor 6). TRAF6 activates two pathways one leading to the activation of NF-kB and other to the activation of c-Jun pathway. TRAF and evolutionarily conserved signaling intermediate (ECSIT) in toll pathway leads to activation of c-Jun pathway by activating mitogen activated protein kinase (MAPK). TRAF associated with TAK1 binding protein/TGFβ activated kinase (TAB1/TAK1) leads to the activation of NF-kB pathway. Either of the pathways finally leads to the activation of transcription factors and alterations in the gene expression.

INF-α/β (induced by IL-1) binds to its receptor INF receptor-1 and activates the JAK/STAT pathway or Tyk2/STAT pathway. Phosphorylated STAT1 and STAT2 and activate p48. INF-γ also activates GAGA (GAF) factor a transcription factor by JAK/STAT pathway but do not activate p48 (Biron, 1998).

TNF activates TRAF2 which in turn activates the MAPK cascade finally activating NF-kB and thus produces a cell response. TNF-α and TNF-β (17 kDa, and 25 kDa respectively bind to common receptors (Vilcek, 1991). TNF-α and IL-1 share many pro-inflammatory properties. TNF-β shares properties of TNF-α by inducing apoptosis in tumor cells and virally infected cells.

IL-6 (structure in Figure 2) is a B-cell differentiating and T-cell activating cytokine and produced by macrophages fibroblasts, endothelial cells and activated T-helper cells (Akira, 1993). It is a 185 amino acid protein glycosylated at 73 and 172 positions. It is
synthesized as a precursor protein which is 212 amino acids. Amino acid sequence of precursor and mature IL-6 is given in the figure5. IL-1β and TNF-α stimulates IL-6 (Haddad, 2002). IL-6 enhances glucocorticoid expression. In addition to inflammation and hematopoiesis, IL-6 also induces neuronal differentiation and bone loss.

IL-6 signals through a receptor consisting of an α subunit (which is ligand specific) and gp130 subunit (which shared in common with other cytokines) (Hirano, 1992). These subunits lead to activation of JAK kinase and ras mediated signaling. JAK kinase activates STAT transcription factors. (Bode, 2003). The ras mediated pathway acts through Src homolog 2 containing domain (shc), Growth factor receptor-bound protein 2 (Grb-2) and Son of sevenless homolog 1(Sos-1) upstream and MAP kinase downstream. Finally, activation of transcription factors such as AP-1 and Serum response element (SRE) leads to gene transcription (Bode, 2003, Heinrich, 1998).

**Mitogen Activated Protein Kinase**

MAPK pathways transduce a large variety of external signals which lead to internal responses like cell differentiation, inflammation and apoptosis. The MAPK family yields signal in the form of phosphorylation. MAPK kinase kinase which activates MAP kinase kinase activates MAP kinase leading to a biological response. There are three major groups of MAPK which include p38 MAPK family, extracellular regulating kinase family (ERK) and c-Jun NH₂ terminal kinase family (JNK) (Tanoue, 2002). The p38 family includes four isoforms (p38α, p38β, p38γ, and p38δ) (Dong, 2002), while the ERK family consists of two isoforms, ERK1 and ERK2. JNK consists of three isoforms which include JNK1, JNK2, and JNK3 (Chang, 2001).
Each MAPK family has unique activators, substrates and inactivators. p38 members are activated by stress stimuli and by different cytokines. p38 kinase is required for regulation of apoptosis, cell cycle arrest, cytokine production and inflammation. ERK kinases are activated by cytokines and growth factors and regulate mitogenic and anti-apoptotic signals. JNK family is activated by stress stimuli and growth factors and regulates cell cycle arrest and progression and cytokine production.

For a MAP kinase to be activated it should be phosphorylated at two positions which includes Thr and Tyr residues. Such dual phosphorylation groups are located in the activation loops of a MAP kinase family. For p38 it is Thr-Gly-Tyr motif, for ERK it is Thr-Glu-Tyr motif and for JUN it is Thr-Pro-Tyr motif. MEK activates MAP kinases by phosphorylating Ser, Thr and Tyr residues (Platanias, 2003). MKK3, MKK4, and MKK6 activate p38. MKK1 and MKK2 activate ERK, and MKK4 and MKK7 activates JNK family of kinases (Dong, 2002). MKK6 activate all the 3 isoforms of p38 while MKK3 activates only p38α and p38γ. Activation of MAPKKK or MAPKK occurs downstream of G-protein activation which is regulated by Guanine Exchange Factor (GEF) (Platanias, 2003). Phosphorylation of MAPK is important for may biological responses such as phosphorylation of transcriptional factors, nuclear chromatin remodeling, immediate gene expression, cytokine production, cell cycle progression and apoptosis. MAPK signaling cascade is shown in Figure 3.

γ-Aminobutyric acid (GABA)

GABA is the primary inhibitory neurotransmitter in the adult CNS and is present in most of the neurons and produces inhibitory and hyperpolarizing effects on neurons.
Hyperpolarizing effects are due to the excessive influx of chloride ion into cells. Thus by creating differences in the electrochemical gradient it inhibits the transfer of messages from one neuron to other. GABA is converted from glutamic acid by the action glutamic acid decarboxylase (GAD) (Guin, 2003). Following the release of GABA it can be taken up by either astrocytes or neurons (Semyanov, 2004). GABA is then degraded with in cells and synaptic cleft by GABA transaminase. Hypoactivity of GABA leads to epilepsy, anxiety, stress, and sleep disorders (Guin, 2003). GABA is composed of two receptors, GABA\(_A\) and GABA\(_B\). GABA\(_A\) is an ionotropic receptor and GABA\(_B\) is a metabotropic receptor.

GABAergic neurotransmission can be divided into two components; fast and slow. When GABA bind to GABA\(_A\) receptor it a fast response and occurs in a time period of one milli second, whereas GABA binding to GABA\(_B\) receptor is a slow response and requires one second (Guin, 2003). When GABA binds to GABA\(_A\) receptor it acts through ion ligand gated chloride channels. When GABA bind to GABA\(_B\) receptor it acts through G-protein coupled receptors and a second messenger leading to opening of near by potassium channels. GABA\(_A\) receptor is a heteromeric pentamer. Currently, 20 GABA\(_A\) receptor subunits have been identified, including six \(\alpha\), four \(\beta\), three \(\gamma\), one of \(\delta, \epsilon, \pi, \theta\) and three \(\rho\) subunits (Lambert, 2003; Steiger, 2004). The \(\alpha\) and \(\beta\) subunit play an important role in GABA\(_A\)/muscimol affinity (structure of muscimol is shown in figure 5).

GABA\(_A\) receptor is most prevalent in mammalian brain. There are three binding sites on GABA\(_A\) receptors, including the GABA site, second the benzodiazepine site, and the barbiturate site. Pharmacologically, muscimol is considered to be an agonist of GABA\(_A\).
receptor. Benzodiazepines increase GABA binding to GABA<sub>A</sub> receptor and thereby increase chloride influx.

GABA<sub>B</sub> receptor is a heterodimer composed of GABA<sub>B</sub> R1 (GABA<sub>B</sub> receptor 1) and GABA R2 (GABA receptor 2) (Bettler, 2004). The highest concentration of GABA<sub>B</sub> receptor is in interpenduncular nuclei and cerebellum (Bowery NG, 2002). Baclofen (structure shown in figure 6) is considered an agonist to GABA<sub>B</sub> receptor. Occupancy of this receptor by baclofen results in muscle relaxation.

**Somatostatin**

Somatostatin (SRIF) has neuromodulatory effects in CNS. Somatostatin inhibits the IL-1β stimulated release of IL-6. It is present in hypothalamus, pancreas, GI track, and in the cerebral cortex. In CNS it acts as neurotransmitters its function include inhibition of growth hormone, insulin, ACTH and glucagon and as an anti-inflammatory factor. Two forms SRIF are synthesized one corresponding to SRIF-14 (14 amino acids) and other SRIF-28 (28 amino acids). Both forms of SRIF are formed by proteolytic cleavage of prosomatostatin (10.2 kDa and 92 amino acids), which is produced by preprosomatostatin (12.7 kDa and 116 amino acids).

SRIF has five different receptors namely SRIF<sub>1-5</sub>. Each receptor is G-protein coupled receptors and these interactions with G-proteins leads to several effects in the cell including inhibition of adenyl cyclase, and calcium channel activity, stimulation of potassium channel and tyrosine phosphate activity, and regulation of intracellular pH. SRIF acts by both paracrine and autocrine actions. Interestingly, in AD the levels of SRIF are decreased in cortex of brain. (Foy, 1994)
Cytokines and Inflammatory Disease

Inflammation is an immune response which requires coordination between different cell types including macrophages and lymphocytes. Inflammation is a process of protective response to a infection or trauma. Inflammation is characterized by increased blood flow and vascular permeability along with accumulation of fluids, leukocytes and cytokines. Inflammation can be protective but when inflammation is chronic cellular damage can leads to the adverse effects including neuronal death and neurodegeneration. Cytokines such as IL-1, IL-6 and TNF control the inflammatory response. Elevated levels of cytokines are observed in neuronal disorders such as AD.

IL-1 and TNF cause fever through alteration of body temperature set by the hypothalamus (Warren, 1990). IL-2 acts a key moderator for T-cell proliferation and activation. IL-3 and IL-5 stimulate proliferation (Arai, 1991) and IL-10 and IL-4 activates B cell responses via T helper cell snb population (Carol, 1997). IL-6 stimulates differentiation of bone marrow stem cells and secretion of antibody proteins from plasma cells. IL-7 stimulates differentiation of stem cells into progenitor B and T cells (Goodwin, 1998). Finally, IL-8 stimulates chemotaxis (Miller, 1992). Excessive production of cytokines, especially IL-1β, is seen in the pathology of AD.

Activated microglia stimulates the production IL-1 (Kim, 2005). AD patients have a six fold increase in plaque deposition compared to the unaffected patients. Plaque formation is dependent on expression of β-amyloid protein. Amyloid precursor protein (APP) is a precursor protein for the formation of β-amyloid protein (Bernhardi, 2004). IL-1 helps in up regulation of APP.
Current evidence suggests that GABA and SRIF down regulate the IL-1β stimulation of IL-6 by inhibiting one or more MAP kinases. CNS patients suffering from AD present with a degradation of somatostaticergic receptors while the GABAergic interruption is unhalted.

Hypothesis Statement

Interleukin-6 (IL-6) is a 212 amino acid cytokine synthesized by various types of cells. It plays an important role in the immune system and the CNS. Elevated levels of IL-6 are observed in AD which is a progressive brain disorder where gradually brain cells get destroyed and leads to a progressive decline in mental function. Previous studies suggest that IL-1β dose dependently stimulates the release of IL-6. In this study, we are investigating the role of p38 MAPK, GABA, GABA<sub>A</sub>, GABA<sub>B</sub>, and PD98059 an ERK inhibitor in C6 glioma cells. Previous studies suggest inhibitor of p38 MAPK SB203580 (structure shown in figure 7) inhibits the IL-1β stimulated release of IL-6 while an JNK inhibitor SP600125 had no effect (Spangelo, 2004). We hypothesize that p38 MAPK will be stimulated in a dose and time dependent manner with IL-1β. From the results we hypothesized that GABA attenuates the IL-1β stimulated p38 MAPK in C6 cells and further GABA<sub>B</sub> will be involved in the attenuation mechanism of stimulated IL-6 release. We further hypothesize that, PD98059 (structure shown in figure 8) will not have any effect in the stimulated release of IL-6 and thus the role of ERK (and from previous studies JNK MAPK) can be discounted. Our results provide a better understanding the inflammation mechanism underlying AD.
CHAPTER 2

MATERIALS AND METHODS

Chemicals and reagents

The 7TD1 hybridoma cell line was obtained from Dr. J. Van Snick, Ludwig Institute (Brussels, Belgium). The C6 glioma cell line was obtained from the American Tissue Type Culture Collection (Rockville, MD). Recombinant rat interleukin-1β (IL-1β) was obtained from PeproTech Inc. (Rocky Hill, NJ). Recombinant murine interleukin-6 (IL-6) was obtained from R & D Systems (Minneapolis, MN). GABA (γ-amino-n-butyric acid), recombinant somatostatin-14 (SRIF-14), recombinant somatostatin-28 (SRIF-28), isopropanol (reagent grade), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), antibiotic mix, were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI-1640, fetal bovine serum (FBS), horse serum (HS), and trypsin were obtained from Gibco (Grand Island, NY). Fetal calf serum (FCI) was obtained from Hyclone (Logan, UT). Trypan blue were obtained from Fisher Scientific (Santa Clara, CA). Primary Antibodies was obtained from Cell Signaling Technology (Beverly, MA). Secondary Antibodies was obtained from KPL incorporation (Gaithersburg, CT).

C6 glioma cell culture

The C6 glioma cells were maintained in continuous culture in a humidified atmosphere of 5% CO₂-95% air at 37 °C in RPMI-1640 medium supplemented with 10%
FBS and PSN antibiotic mix. After 3 days in culture the C6 cells were removed from the tissue culture flask using 4mL of 0.25% trypsin. Trypsin was deactivated by adding 6mL of C6 culture media. The C6 cells were centrifuged at 400 rpm for 5 minutes, trypsin was removed, and the cells were resuspended in the C6 culture medium. The C6 cells were counted using a hemacytometer and were placed either back into continuous culture at 1.0 x 10^5 cells per 25 cm^2 flask, or were plated into a 96-well tissue culture plates at 100 x 10^3 cells/well with C6 medium for 7TD1 bioassay.

C6 cells were allowed to adhere to the plates for 24 hours, after which the cells were rinsed twice with 200 µL serum free RPMI-1640. The C6 cells were then exposed to vehicle (200µL serum free RPMI-1640/well), with and without IL-1β and other agents for 24 hours. When inhibitors were used the cells were pretreated with the appropriate inhibitor for 1 hour in serum free RPMI-1640 and then exposed to the drugs with and without IL-1β. After 24 hours the conditioned media was transferred to a new 96-well plate, which was then stored at 4 °C until measurements for IL-6 by bioassay.

7TD1 Bioassay for IL-6

The 7TD1 cells were maintained in continuous culture in a humidified atmosphere of 5% CO₂-95% air at 37 °C in 10 mL RPMI-1640 supplemented with10% FBS, 5mL of antibiotic mix and 5 pg/mL rmIL-6. After 3 days in culture the 7TD1 cells were removed from the tissue culture flask using trituration and approximately 600 x 10^3 cells were distributed in a 25 cm^2 flask (Greiner) for continuous culture. We used the IL-6-dependent 7TD1 hybridoma bioassay to quantify the accumulation of IL-6 in the C6 conditioned media as 7TD1 cell growth depends on the presence of IL-6. Conditioned
medium (20 μL) is cultured in 96 well tissue culture plates in duplicate in 180 μL RPMI-1640 supplemented with 10% FBS and antibiotics in the presence of 4,000 7TD1 cells for 72-96 hours. Tetrazolium salt (MTT) was used to determine the growth of the 7TD1 cells. MTT is cleaved in active mitochondria to form a dark blue formazan crystal which can be correlated with OD and IL-6 release. After 72-96 hours 20 μL of 5 mg/mL MTT is added to each well for 3 ½ hours. 150 μL of medium was removed from each well and the dark blue crystals were dissolved by the addition of 150 μL of 0.04 M HCL/isopropanol to form a dark colored liquid. The plates are stored overnight in the dark after which the optical densities were determined by a microelisa instrument using a test wavelength of 570 nm and a reference wavelength of 630 nm (Dynatech MR5000). For each assay a standard curve of rmIL-6 (0.5 to 64 pg/well) was generated. The data are formed from treatment groups each of which consists of 4 observations per group and are presented as the mean ± SEM. A foe chart is provided in figure 9.

Protein Extraction

C6 glioma cells 2x10^6 were plated in a 6-well plate and incubated for 24 h. After 24 h incubation cells were washed 2 times with sf-RPMI 1640. Cells were treated with or without IL-1β and incubated for 5-60 min. Cells are pretreated 1 hr with selective inhibitors and then treated with IL-1β ranging from 5-100 ng/mL. After the required incubation with drugs, cells were washed twice with cold PBS and 200 μL of sample loading buffer (9mL PBS, 1mL of phosphatase inhibitors and one tablet of protease inhibitors) was added to each well. Cells were scraped with cell scraper and transferred into microfuge tube along with the extraction buffer. Protein is extracted on ice using
sonicator in the pulse mode (10 times for 1.5 sec) according to the manufacturer instructions. Protein thus extracted is quantified using Pierce BCA kit (procedure is shown in figure 10). Standards in triplicates and samples in duplicate were incubated for 2hrs in the incubator and read at 562 nm. Average blank values were subtracted from individual sample and standards. A standard curve of concentration on X-axis and OD on Y-axis is plotted. Quantified protein samples are either stored at -70° C or diluted 1:1 with 2x sample loading buffer.

SDS-PAGE and Western blotting analyses

The apparatus for SDS-PAGE and Western blotting was purchased from Amersham Biosciences. SDS-PAGE (10% gels) were performed at 40mA at 4°C. Resolving gel occupied 75% of the chamber and water (100-200 uL) was placed on top if it. Following polymerization water was removed and the stacking gel was added and combs are placed on the top of the gel. Combs were removed following polymerization, samples were introduced into the wells with a standard biotinylated protein ladder (15 uL) and pre-stained protein ladder (10 uL). Before samples are introduced the whole cassette including the wells was filled with the 1X running buffer which got Tris Hcl, glycine and SDS. SDS-PAGE is ran at a 40mA current and is terminated when the sample reach 1cm from the bottom of gel. Gel is transferred into western transfer apparatus along Membrane sandwich. In the sandwich first the sponge is placed and top of it blotting paper, membrane, gel, blotting paper and sponge are placed in the order. Sandwich is introduced in the apparatus and the gray side of the sandwich should face the positive end
filled with western transfer buffer. Western transfer apparatus was ran at 400mA at 4°C for 2 hrs.

The membrane was washed with washing buffer for 5 min and blocked with blocking buffer for 1 hr. After blocking with blocking buffer, membrane was washed for 15 min by changing washing buffer every 5 min. After a 24 h incubation with phosphorylated p38 primary rabbit antibody (obtained from cell signaling #9211S) which recognizes the phosphorylated sites Thr180 and Tyr 182 amino acids of p38 (1:1000 dilution with primary antibody dilution buffer) membrane was washed for 15 min and incubated for an hour with a anti- mouse secondary antibody (obtained from KPL Inc. #074-1516, 1:5000 dilution with primary antibody dilution buffer) and anti-biotinylated antibody (1:1000 with primary dilution buffer). Anti-biotinylated antibody was used for detection of standard molecular weight ladder. After 2-3 h incubation with secondary antibody and anti-biotin antibody, membrane was washed for 15 min and treated with ECL Plus (Amersham) and scanned using Amersham typhoon scanner. ECL Plus purchased from Amersham is used to get Fluorescence signal.

**Enzyme Linked Immunosorbent Assay (ELISA)**

The ELISA kit was purchased from R&D systems. We want to confirm the release results between ELISA and Bio assay and want to use the best method for the further experiments. Kit consists of a 96 well rat IL-6 microplate, rat IL-6 conjugate, standard, control, assay diluent, control diluent, wash buffer, color reagent A and B, stop solution, and plate covers.
Reagents were prepared according to the manufacturer’s protocol and bought to room temperature before use. To the center of each well 50 μL of assay diluent 50 μL of standard, control, and sample were added. Plate was covered and gently tapped for 1 min to mix the wells and incubated for 2 h at room temperature.

Wells were washed with 400 μL of wash buffer for four times. Liquid was completely removed from the wells and plate was inverted against clean paper towels.

To the plate 100 μL of IL-6 conjugate was added to each well and covered with adhesive tape and incubated for 2 h at room temperature. Washing step was repeated for 5 times and a 100μL of substrate solution was added and incubated for 30 min protecting the plate from light.

Finally 100 μL of stop solution was added to each well and the plate read at 570 nm within 30 min of adding stop solution.
CHAPTER 3

RESULTS

7TD1 Bioassay Standard curve

The standard curve is shown in the (Figure 11, 12). A best-fit line equation was obtained from the graph plotted between rmIL-6 and optical density. Algorithm or linear regression was and used for the calculation of IL-6 (pg/well) in the samples. Samples were diluted so that results will fall within the standard values. Sometime we get a good linear curve and sometime a good logarithmic curve. Every time we construct both the curves and depending on the good standard curve we get, we utilize a logarithmic curve or a linear curve. Theoretically, we should get a good logarithmic curve.

C6 glioma Cell Density Effect on Rat Interleukin-1β release of IL-6

Cell density experiment was conducted to determine optimal cell number of C6 cells/well. We treated our cells with IL-1β (50 ng/mL) and incubated for 24 h and measured IL-6 using 7TD1 bioassay (Figure13). We observed that 100,000 cells/well gave a maximum stimulation of IL-6 and used this number for carrying out our future experiments.

Rat Interleukin-1β Time Course

IL-1β time course was measured using the 7TD1 bioassay. C6 cells are incubated
with IL-1β (50 ng/mL) for a time period of 3-48 h (Figure 14). 7TD1 bioassay was conducted on the samples. At 24 h we observed a maximum stimulation of IL-6 so we used a 24 h of incubation in our future experiment.

Rat Interleukin-1β dose response

7TD1 assay was conducted to see the IL-1β stimulated release of IL-6. We observed that as the concentration of IL-1β increases (5-200 ng/mL) there is an increase in the release of IL-6 (Figure 15). Sometimes we see biphasic stimulation for IL-6. By observing the curve we use an EC50 of 50 ng/mL of IL-1β for our further experiments when using inhibitors.

ELISA Standard curve on Rat Interleukin-1β stimulated release of IL-6

ELISA standard curve was obtained using standard concentrations of IL-6 provided by the manufacturer (Figure 16).

ELISA Dose Response on Rat Interleukin-1β stimulated release of IL-6

ELISA was conducted at different concentrations of IL-1β. From the graph (Figure 17) we saw that ELISA was not as sensitive as 7TD1 bioassay. So we performed 7TD1 bioassay for our future experiment.

Effect of SB203580 on IL-6 release

SB 203580 is a specific inhibitor of p38 MAPK. We wanted to investigate the involvement of p38 MAPK in the stimulated IL-6 release. We pre-incubated the C6 cells
for 1 h both with and without SB203580 (a specific inhibitor of p38) and incubated along with and without IL-1β for 24 h. SB203580 (10 µM) inhibited the IL-6 release (Figure 18). This suggested p38 MAPK was involved in the IL-1β stimulated mechanism.

Effect of PD98059 on IL-6 release

PD98059 is inhibitor of ERK MAPK. We want to investigate the involvement of ERK MAPK in the stimulated IL-6 release. We pre-incubated the C6 cells for 1 hr with and without PD98059 and incubated along with IL-1β for 24 hrs. PD98059 (10 µM) had no effect on IL-6 release (Figure 18). This suggested ERK MAPK was not be involved in the IL-1β mechanism.

Effect of GABA on IL-6 release

GABA is an inhibitory neurotransmitter. Decrease in the GABAergic neurons is seen in the patients with AD. We wanted to investigate the inhibitory effect of GABA on IL-6 release. A 500 µM concentration of GABA was used in the experiment. C6 cells were pre-treated with and without GABA for one hour followed by 24 h incubation with IL-1β. GABA inhibited IL-6 release (Figure 19).

Role of GABA subclass receptors in the inhibitory mechanism of GABA

As our results suggest that GABA inhibits the IL-6 we want to see which GABA subclass receptor is involved. GABA is composed of the two subclasses GABA_A and GABA_B. GABA_A acts through ion mediated channel while GABA_B acts through the metabotropic channels.
We used muscimol (10-50 μM) GABA_A receptor agonist which have a Kd of 16-20 nM (Eberta, 1999), its structure is provided in Figure 9 and baclofen (25-100 μM) a GABA_B receptor agonist which have a Kd of around 60nM (Ohmori, 1999), its structure is provided in Figure 10. We pre-treated our cells with and without muscimol and baclofen followed by incubation of 24 hrs with IL-1β. Our results suggested that baclofen play a role in the mechanism while the muscimol has no effect (Figure 20, 21).

This suggested that GABA_B receptor is involved in the GABA inhibition of p38 MAPK and IL-6.

Effect of IL-1β Time course on p38 MAPK

p38 MAPK is one of the three MAPK. As SB203580 inhibited the IL-6 release we wanted to investigate the activation of p38 by IL-1β. We treated our cells with IL-1β (25 ng/mL) and incubated for 5-60 min and extracted protein from the cells for western blotting analysis. Our results suggested that activation of p38 is maximum at 15 min (Figure 22) and we observed a biphasic stimulation of p38. Densitometry was performed on the stimulated bands for graphical representation.

Effect of IL-1β Dose Response on p38 MAPK

We investigated the dose of IL-1β which gives maximum stimulation of p38 MAPK. We treated our cells with IL-1β (5-100 ng/mL) and incubated for 15 min. Protein was extracted from incubated cells and analyzed using Western blotting. We observed 25 ng/mL of IL-1β gave the maximum stimulation of p38 (Figure 23). Densitometry was performed on the stimulated bands for graphical representation.
Effect of GABA on IL-1β stimulated p38 MAPK

As GABA inhibited the stimulated release of IL-6 release we wanted to investigate the mechanism of GABA inhibiting the stimulated release of IL-6. From our previous results we know that p38 MAPK is activated in the mechanism. We postulated that GABA might be acting through the inhibition of p38 MAPK. We pre-treated our cells with GABA (10-1000 µM) concentrations for 1 h, followed by 15 min incubation with IL-1β. Protein was extracted from incubated cells and analyzed with Western blotting technique. Our results suggested that GABA at 100 and 1000 µM completely blocked the p38 MAPK (Figure 24).

Effect of SRIF on IL-1β stimulated p38 MAPK

As SRIF inhibited the stimulated release of IL-6 release we wanted to investigate the mechanism of SRIF inhibiting the stimulated release of IL-6. From our previous results we know that p38 MAPK is activated in the mechanism. We postulated that SRIF might be acting through the inhibition of p38 MAPK. We pre-treated our cells with SRIF (25-100 nM) concentrations for 1 h, followed by 15 min incubation with IL-1β. Protein was extracted from incubated cells and analyzed with Western blotting technique. Our results suggested that SRIF enhanced the activation of p38 MAPK (Figure 25).
CHAPTER 4

DISCUSSION

Alzheimer's disease is a neurodegenerative disorder. Extensive research is on going but unfortunately, the cause of mechanism of AD is yet unclear. Approximately 2.5 million of cases of AD are registered alone in United States. Elevated levels of cytokines are seen in AD. Cytokines at normal levels are required for the cell to carry out normal function. But elevated and chronic levels of cytokines lead to neurodegeneration and disorders like AD. Increased levels of IL-1 and IL-6 in the senile plaques of AD are considered as important factors in the progression of AD.

Amyloid deposits associated with plaques are observed in AD. For the deposition of amyloid the precursor protein, amyloid precursor protein β (APP) is required as it the precursor for the formation of amyloid protein. IL-1 and IL-6 increases APP deposition and thus play a role in progression of AD. In patients with AD IL-1β and IL-6 enhance formation of APP which increases the formation of plaques which finally leads to AD (Nagele, 2004). We are interested in investigating the mechanism of IL-1β stimulation of IL-6 pathway. Cellular responses such as cell differentiation, apoptosis, and neurodegeneration must be defined and have important pharmacological significance for AD treatment.
Activated IL-1β forms a complex with the IL-1 receptor agonist and interleukin-1 accessory protein which is called complex 1. Complex 1 leads to the formation of complex 2 which is composed of transforming growth factor β activated kinase-1 (TAK1) and interleukin-1 receptor associate kinase 1 (IRAK1) and Toll like receptor accessory protein 6 (TRAF6). TRAF6 also plays an role in the activation of NFκB which further activates AP-1, a transcription factor. Complex II thus formed activates the MAPK signaling cascades which includes activation of MAPK kinase kinase which activates the MAPK kinase and finally activates MAPK. Activated MAPK thus activates its transcription factors and finally leads to the cell response (Figure 26).

We investigated the pathway of IL-1β stimulated IL-6 release in C6 glioma cells because the activation of these inflammatory cytokines leads to the progression of AD. As discussed, IL-1β stimulates IL-6 by activating the MAPK signaling cascade; which plays an important role in the stimulation of IL-6. We therefore want to investigate the role of p38, JNK, and ERK.

We used the SP600125 inhibitor of JNK, PD98059 inhibitor of ERK (Figure 7), and the SB203580 inhibitor of p38 (Figure 6). Previous studies indicated that SP600125 had no effect on the release of the IL-6. Therefore we suggest that JNK might not have an important role in the pathway. Our current results indicated that SB203580 inhibited IL-6 release while PD98059 has no effect on the release of IL-6. As JNK and ERK do not have a significant effect, we therefore examined p38 activation. We suggest that, p38 is activated while the other two MAPK are not involved. We proposed that IL-6 is stimulated by activating p38 and if we can inhibit the activation of p38 and thereby
inhibit the release of IL-6, this will be a great pharmacological significance in the progression of AD.

Our next focus is the role of inhibitory neurotransmitter GABA on p38 and IL-6. GABA is the major inhibitory neurotransmitter in the brain and is present in 40% of all neurons. It produces an hyperpolarizing inhibitory effects on neurons. By our results GABA inhibited the release of the IL-6. From the previous results we suggested the involvement of p38 MAPK in the stimulated mechanism of IL-6. We wanted to investigate the relationship between p38 and GABA. If GABA can inhibit the activation of p38 then it would be a great progression in the understanding of IL-1β signaling events. We performed western analysis to investigate the involvement of GABA in p38 activation. Our results suggested that GABA inhibited the activation of p38 dose dependently and at 1000µM it completely knocked out the p38. Here we concluded that GABA acts through inhibiting p38. However, a 100 µM of GABA completely inhibited the activation of p38 while it showed only some effect on IL-6 inhibition and this we suggested that for inhibition of IL-6 which takes place out side the cell may need higher concentration of GABA. As GABA inhibited both IL-6 and p38 we want to know more about the mechanism of action of GABA and investigated the GABA receptor subclass.

Our next research goal involves the characterization of GABA subclass receptor. GABA is composed of two receptors GABA_A and GABA_B. GABA acts through binding one of its receptors or both of them. GABA_A acts through the ion channel receptor (Semyanov 2004; Lambert 2004). It is similar to the acetylcholine receptor. GABA_A increases the influx of Cl⁻ ion channels there by causes hyperpolarization and induces a hyperpolarization inhibition.
GABA\textsubscript{B} acts through G-protein coupled receptor. It creates an efflux of K\textsuperscript{+} channels from the cell and thereby creates hyperpolarization and inhibition (Bettler 2003). We treated our cells with muscimol which is a GABA\textsubscript{A} receptor agonist and baclofen which is a GABA\textsubscript{B} receptor agonist. Our results suggested that the GABA\textsubscript{B} receptor inhibited the release of IL-1\beta stimulated IL-6 from C6 glioma cells. GABA\textsubscript{A} has a moderate effect and thus we stated GABA\textsubscript{A} might not be involved in the mechanism. We hereby correlated that GABA inhibits p38 by binding through GABA\textsubscript{B} receptor and suggest that GABA acts through its metabotropic receptor.

From previous results, SRIF inhibits IL-1\beta stimulated release of IL-6 from C6 glioma cells. We want to know whether SRIF inhibits p38. But, SRIF did not inhibit p38 and we hereby suggest that SRIF may act through other MAPK (\textit{i.e.} ERK). We suggest that SRIF may act through ERK by crosstalk with other MAPK or through other transcription factors. One of the possible transcription factors may be nuclear factor – kappa B (NFkB). NFkB plays an important role in the inflammatory response of the cell (Azzolina, 2003). It is a heterodimer and helps in the activation of IL-6. Activation of IKK (IkB kinase) leads to the degradation of IkB which leads to the activation of NFkB. This mechanism is stimulated by the complex which includes the MAPKKK and TRAF6. Thus activated NFkB leads to the activation of the transcription factor AP-1. There may be a possible role of SRIF in the inhibition of IL-6 through NFkB. The role of NFkB will be one of our future studies. Their maybe a possible role for GABA acting through the NFkB and GABA may act through both mechanisms.
Our results are consistent with those observed in neuronal cell lines. LPS increases IL-1β stimulated IL-6 levels from neuronal cells (Li, 2003). Our future studies also include the role of the transcription factors like AP-1 and Elk-2 in the pathway. In the pathway we propose that MAPK leads to the activation of transcription factors which finally leads to the cellular responses like growth differentiation, apoptosis and inflammation. These inflammatory cytokines also cause apoptosis in the neuronal cell lines in a 24hr incubation time period.

From the Figure 27 we gave a possible mechanism of GABA inhibition of IL-6 and p38. When GABA binds with GABA_B besides creating hyperpolarization it inhibits cAMP there by inhibits release of IL-6 and activation of p38. Though hyperpolarization creates a difference in the electrochemical gradient and inhibits the transfer of message of message from one neuron to other, inhibition of cAMP may provide the reason for the effective GABA_B inhibition of IL-6 release and the difference in the mechanism of action between GABA_A and GABA_B.

Our results clarify the mechanism of IL-1β stimulated IL-6 release. We can also suggest that is pathway is common to Parkinson’s disease and we see elevated levels of TNF-α, IL-1β, and IL-6 in Parkinson’s disease. We suggests that p38MAPK plays an important role in the IL-1β stimulated release of IL-6 from C6 glioma cells. JNK and ERK might not be involved in the mechanism. We suggest that IL-1β stimulates the activation of p38 MAPK through the upstream MAPKKK and results in the stimulation of IL-6 release. SB203580 inhibits IL-6 release by inhibiting p38 kinase. GABA also inhibits the stimulated release of IL-6. This is achieved by inhibiting p38 acting through, the GABA_B subclass receptor. We proposed a signaling pathway from our results (Figure 25).
28). IL-1β binds to the IL-1 receptor 1 and activates ERK, and p38 kinase which activates IL-6 leading to responses like growth differentiation, apoptosis, and inflammation. GABA, SB203580, and SRIF inhibit the IL-1β stimulated IL-6 release. GABA and SB203580 achieves via inhibition of p38 while SRIF may act through ERK kinase. However, though PD98059 inhibits ERK kinase had no effect on IL-6. Our results are significant as the mechanism of IL-1β stimulated release of IL-6 in C6 glioma cells is now more clearly described. Treatment for the AD might involve manipulation of GABAergic neurons in the brain.
APPENDIX I

FIGURES
Figure 1: Amino acid sequence of precursor protein for the cytokine IL-1β. It is a 30.75 kDa molecular weight protein. The region highlighted is the IL-1 domain and it corresponds from 117 to 269 amino acids. Precursor protein is cleaved by the proteases at the highlighted region and the active form of IL-1β is formed. IL-1β is a 17.3kDa protein. It is expressed in macrophage, lung and skin.
Figure 2: Amino acid sequence of precursor protein for the cytokine IL-6. It is 212 amino acid protein with molecular weight of 23.74 KDa. The region highlighted represents the IL-6 domain from 28-212 amino acids. Precursor protein is cleaved by the protease at the highlighted region shown by arrow and the active form of IL-6 is released. The sequence underlined represents the single peptide (SP) motif from 1-28 amino acids. IL-6 is expressed in the macrophages, brain, placenta, kidney, and skeletal muscles.
Figure 3: Schematic representation of MAP kinase pathway. Any of the three MAPK upstream targets are activated by stimulus such as, growth factors, stress, inflammatory cytokines, or mitogens. MAPKKK thus activated activates MAPKK. MAPKK in turn activates MAPK which finally lads to the biological response such as growth differentiation, inflammation, or apoptosis. ERK1/2 is activated by MEK1/2, three isoforms of p38 MAPK are activated by MKK3/6, and SAPK/JNK1/2/3 is activated by MKK4/7. (adapted from www.cellsignal.com)
Figure 4: Chemical structure of GABA. GABA inhibits the IL-1β stimulated release of IL-6 by acting through p38 MAPK. GABA acts by binding to one of its receptor either to GABA_A or to GABA_B receptor. Our results suggested that it acts through GABA_B receptor.
Figure 5: Chemical structure $\text{GABA}_A$ agonist muscimol. Its chemical name is 5-aminomethyl-3-hydroxy-isoxazole.
Figure 6: Chemical structure of GABA\textsubscript{B} agonist baclofen. Its chemical name is β-(aminomethyl)-4-chlorobenzene propanoic acid.
Figure 7: SB203580 is a p38 MAPK inhibitor. Its chemical name is Pyridine, 4-[5-(4-fluorophenyl)-2-[4-(methylsulfanyl)phenyl]-1H-imidazol-yl]. It inhibits the upstream activator of p38 MAPK (i.e. MKK3) and MAPKAPK 2 and thus inactivates the p38.
Figure 8: PD98059 is a ERK MAPK inhibitor. Its chemical name is 2'-Amino-3'-methoxyflavone. It inhibits the ERK 1/2 kinase and thus inhibits its downstream targets. Our results suggested that it has no effect on IL-1β stimulated release of IL-6.
Plate C6 cells in a 96 well plate; 100,000 cells/well and a volume of 200μL and incubate.

Wash the cells 2X with SF-RPMI; Add drugs and incubate.

Transfer the media into a new 96 well plate and label.

Take a 96 well plate and according to the number of samples set up for duplicates of experiment, concentrated and dilute.
Add 180μL of SF-RPMI to concentrated wells and 150μL in dilute wells.
Add 20μL of sample to concentrated wells and mix them 15X.
Discard 50μL media from concentrated wells.
Add 50μL of media from concentrated wells to diluted wells mix then 15X.
Discard 100μL media from diluted wells.
Add 100μL of 7TD1 cells 40,000 cells/wells to get a total volume of 200μL/well and incubate for 3-4 days.

Add 20μL of MTT to each well and incubate for 3-4 hrs.
Discard 150μL of media.
Add 150μL of acidified isopropanol.
Cover the plate with an aluminum foil and store the plates over night in dark.

Read the Plates and graph it.

Figure 9: Flow chart for the 7TD1 Bioassay.
Step 1
Prepare working reagent in the ratio of 25:24:1 with solution A, Solution B, Solution C.
Working reagent can be stored at Room temperature.

Step 2
Prepare Series of standard solutions using serial dilution technique with the stock solution of 2ng/ML using PBS as a vehicle for dilution.
Prepare 7 standard solution and one blank which is just PBS.

Step 3
Add 150µL of working reagent to the well in a 96 well plate. Now add 150µL of samples and standards.

Step 4
Mix the plate on a plate shaker for 30 sec.

Step 5
Incubate the plate in the incubator for 2-3 hrs.

Step 6
Read the plate at 595 nm plot the graph and by interpolation from standard graph protein amount in the samples will be calculated.

Figure 10: Flow chart for the Micro BCA protein assay. Protocol is according to the manufactures protocol.
Figure 11: Standard curve of IL-6 is obtained from the known concentrations of the IL-6 and logarithmic curve was plotted between optical density (OD) and IL-6 concentration (pg/well). 7TD1 Bioassay was performed and equation was obtained from the graph. Equation is used for calculating the concentration of IL-6 in samples.
Figure 12: Standard curve of IL-6 is obtained from the known concentrations of the IL-6 and linear curve was plotted between optical density (OD) and IL-6 concentration (pg/well). 7TD1 Bioassay was performed and equation was obtained from the graph. Equation is used for calculating the concentration of IL-6 in samples.
Figure 13: Effect of cell number on IL-1β stimulated release of IL-6. Cultured C6 cells (nx10^3 cells/well) were plated in a 96 well plate. Cells were treated with and without IL-1β. Treated cells were thus incubated for 24hrs and samples are collected for IL-6 release studies. Release of IL-6 is measured by using 7TD1 bioassay. Samples are done in quadruplicates and the error value is calculated using SEM.
Figure 14: Time course of IL-1β stimulated release of IL-6. Cultured C6 cells (100,000 cells/well) were plated in a 96 well plate. Cells were treated with and without IL-1β and incubated for different time intervals. Samples are collected at different time intervals for IL-6 release studies. Basal release was not detectable at any time point. Release of IL-6 is measured by using 7TD1 bioassay. Samples are done in quadruplicates and the error value is calculated using SEM.
Figure 15: Dose response of IL-1β stimulated release of IL-6. Cultured C6 cells (100,000 cells/well) were plated in a 96 well plate. Cells were treated with and without IL-1β at different concentrations of IL-1β. Treated cells are thus incubated for 24hrs and samples are collected for IL-6 release studies. Release of IL-6 is measured by using 7TD1 bioassay. Samples are done in quadruplicates and the error value is calculated using SEM.
Figure 16: ELISA standard curve. Cultured C6 cells (100,000 cell/well) are plated in an ELISA 96-well plate. Different concentrations of IL-6 are placed in the cell and incubated for 24hrs. ELISA standard curve was plotted between OD and IL-6 concentration (pg/Well). Different standards are prepared using serial dilution technique. Stock solution of standard is provided by the manufacturer.
Figure 17: Dose response of IL-1β stimulated release of IL-6 using ELISA. Cultured C6 cells (100,000 cells/well) were plated in ELISA 96-well plate. Cells were treated with different concentrations of IL-1β and IL-6 release is measured after incubating for 24hrs. Samples are done in duplicates and the error value is calculated using SEM.
Figure 18: Effect of SB203580 and PD98059 on IL-1β stimulated release of IL-6. Cultured C6 cells (100,000 cells/well) were plated in a 96 well plate. Cells were treated with and with out IL-1β at different concentrations of IL-1β. Cells treated with inhibitors are pretreated with inhibitors for one hr and then treated with IL-1β. Treated cells are thus incubated for 24hrs and samples are collected for IL-6 release studies. Release of IL-6 is measured by using 7TD1 bioassay. Samples are done in quadruplicates and the error value is calculated using SEM.
Figure 19: Effect of GABA on IL-1β stimulated release of IL-6. Cultured C6 cells (100,000 cells/well) were plated in a 96 well plate. Cells were treated with and without IL-1β at different concentrations of IL-1β. Cells treated with inhibitors are pretreated with inhibitors for one hr and then treated with IL-1β. Treated cells are thus incubated for 24hrs and samples are collected for IL-6 release studies. Release of IL-6 is measured by using 7TD1 bioassay. Samples are done in quadruplicates and the error value is calculated using SEM.
Figure 20: Effect of muscimol (GABA\textsubscript{A} receptor agonist) on IL-1\(\beta\) stimulated release of IL-6. Cultured C6 cells (100,000 cells/well) were plated in a 96 well plate. Cells were treated with and without IL-1\(\beta\) at different concentrations of IL-1\(\beta\). Cells treated with receptor agonist are pretreated with inhibitors for one hr and then treated with IL-1\(\beta\). Treated cells are thus incubated for 24 hrs and samples are collected for IL-6 release studies. Release of IL-6 is measured by using 7TD1 bioassay. Samples are done in quadruplicates and the error value is calculated using SEM.
Figure 21: Effect of baclofen (GABA\textsubscript{B} receptor agonist) on IL-1\textbeta stimulated release of IL-6. Cultured C6 cells (100,000 cells/well) were plated in a 96 well plate. Cells were treated with and without IL-1\textbeta at different concentrations of IL-1\textbeta. Cells treated with receptor agonist are pretreated with inhibitors for one hr and then treated with IL-1\textbeta. Treated cells are thus incubated for 24hrs and samples are collected for IL-6 release studies. Release of IL-6 is measured by using 7TD1 bioassay. Samples are done in quadruplicates and the error value is calculated using SEM.
Figure 22: Western analysis was conducted on C6 samples. Cells are treated with IL-1β (25 ng/mL). Samples are collected at different time intervals and protein is extracted from the samples. Extracted protein thus analyzed using Western blotting technique. Membrane is treated with phosphorylated p38 primary antibody (Cell signaling) and Anti-mouse secondary antibody (KPL Inc). Bar graph represents the densitometry values and fold increase compared to control.
Figure 23: Western analysis was conducted on C6 samples. Cells are treated with different concentrations of IL-1β. Samples are collected at 15 min after the cell treatment with the drug and protein is extracted from the samples. Extracted protein thus analyzed using Western blotting technique. Membrane is treated with phosphorylated p38 primary antibody (Cell signaling) and Anti–mouse secondary antibody (KPL Inc). Bar graph represents the densitometry values and fold increase compared to control.
Figure 24: Western analysis was conducted on C6 samples. Cells are pretreated with GABA for 1hr and treated with different concentrations of IL-1β (25ng/mL). Samples are collected at 15 min after the cell treatment with the drug and protein is extracted from the samples. Extracted protein thus analyzed using Western blotting technique. Membrane is treated with phosphorylated p38 primary antibody (Cell signaling) and Anti-mouse secondary antibody (KPL Inc). Bar graph represents the densitometry values and fold increase compared to control.
Figure 25: Western analysis was conducted on C6 samples. Cells are pretreated with SRIF for 1 hr and treated with different concentrations of IL-1β. Samples are collected at 15 min after the cell treatment with the drug and protein is extracted from the samples. Extracted protein thus analyzed using Western blotting technique. Membrane is treated with phosphorylated p38 primary antibody (Cell signaling) and Anti-mouse secondary antibody (KPL Inc). Bar graph represents the densitometry values and fold increase compared to control.

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Figure 26: Schematic diagram of GABA signaling pathway. When GABA binds to GABA_A it creates an influx of Cl^- ion which causes the hyperpolarization of the membrane. When it binds to GABA_B receptor it creates hyperpolarization by efflux of K^+ ion. GABA_B inhibits cAMP.
Figure 27: Schematic diagram of IL-1β stimulation of IL-6. We found GABA, SB203580 inhibit MAPK-p38 thereby inhibiting IL-6. PD98059 though inhibits ERK do not have a significant effect in inhibiting IL-6 stimulated release.
Early effect (death of GABAergic neurons) → ↓ GABA → ↑ IL-6 → AD

GABA
↓
IL-1β → IL-6

Figure 28: Pathway and relation between death of GABAergic neurons and Alzheimer's disease. Death of GABAergic neurons which is an early effect causes the reduction in levels of GABA which leads to increase in the IL-6 levels leading to AD. At normal conditions GABA inhibit the IL-1β stimulated release of IL-6.
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