รายงานผลงานวิจัยฉบับสมบูรณ์

เรื่อง

"การศึกษาผลของเอสโตรเจนและลิเทียมต่อการอักเสบในสมองที่มีสาเหตุมาจากไมโครเกลียเซลล์ที่ถูกกระตุ้นในโรคอัลไซเมอร์"

(Effects of Estrogen and Lithium on Microglia-Mediated Neuroinflammation in Alzheimer's Disease)

ทุนวิจัยงบประมาณแผ่นดิน

ประจำปี 2549

โดย

รองศาสตราจารย์ ดร. พูลลาภ ชีพสุนทร

นางสาว นุชนาถ ไหมหรือ

ศาสตราจารย์ แพทย์หญิง นันทิกา ทวิชาชาติ

เมษายน 2551

Acknowledgements

The authors would like to thank Prof. James R Connor for kindly providing mouse microglial and neuroblastoma cells to do these experiments. We are so grateful to Chulalongkorn Medical Research Center (Chula MRC) for providing us with space and the cell culture facility. Finally, we are much indebted to the Government Research Budget fiscal year 2007 for sponsoring our research.

Project Title "Effects of Estrogen and Lithium on Microglia-Mediated Neuroinflammation in Alzheimer's Disease"

Investigators P. Cheepsunthorn, N. Mairuae, N. Thavichachart

Year April 2008

Abstracts

Regarding the pathogenesis of Alzheimer's disease (AD), increasing evidence indicates that activated microglia release neurotoxic inflammatory molecules that impair neuronal function, promote neuroinflammation and subsequently bring about neurodegenerative changes. As now, drugs that potentially dampen microglial activation may be beneficial for the treatment of AD. The present study aims to examine the potential application of mood stabilizing drug lithium chloride (LiCl) and valproic acid (VPA) in suppressing microglial activation endpoints using cell culture system supplemented with iron at the concentration reported in AD brain tissue. Here, we report for the first time that iron-enriched environment increases activity of GSK3 beta, a major kinase contributing to neurofibrillary tangle formation in AD. The presence of iron enhances LPS-induced microglial neurotoxicity, which is reversed by LiCl and to smaller scales by estrogen and VPA, respectively. Neuroprotective effects of these drugs correlate with their ability to suppress microglial proinflammatory potential including a significant lower in expression and secretion of TNF alpha and MMP-9. The presence of iron enhances the ability of these drugs to reduce secretion of MMP-9, IL-1 beta and nitric oxide from activated microglia without affecting MIP-1 alpha levels. We also show that the mechanisms of these drugs in suppressing microglial activation additional to increase inhibitory phosphorylation of GSK3 beta involve inhibition of NF-kB nuclear translocation. Taken together, our results show that LiCl and VPA, similar to estrogen, can suppress microglial proinflammatory potential. However, it should be note that LiCl shows a significant overall neuroprotective effects based on our neurotoxicity assay even in the presence of iron. Our results further suggest that pharmacological approaches using LiCl and VPA or a screening for its derivatives that simultaneously manipulate multiple targets involved in AD should be of great benefits to AD patients, alternative to the use of a single drug oriented towards a single molecular target possess limited ability to modify the course of the disease.

Contents

List of Figures

1. Introduction

There are two basic forms of Alzheimer's disease (AD). Early-onset AD is a rare form of the disease that strikes people younger than age 65 and is linked with genetic defects, whereas late-onset AD is more common, generally beginning after age 65 and the exact cause is unknown. The neuropathology of AD, considered the most reliable definitive diagnosis, has provided the starting point for defining its causes and mechanisms. Both the neurofibrillary tangles (NFT) and neuritic plaques have been subjected to intensive analyses by structural, biochemical and molecular biological approaches. It is established that paired helical filaments (PHF) comprising NFT distributed throughout the cortices of patients with AD are highly phosphorylated forms of the microtubule-associated protein tau (Mandelkow et al., 2007). The presence of extracellular amyloid deposits at the centers of neuritic plaques has led to the identification of the amyloid beta protein (AB) and eventually missense mutations in and around the AB region of the B-amyloid precursor protein (APP), in addition to mutations in presenillin genes, the most common known cause of early-onset AD (Walsh and Salkoe, 2004).

Within neuritic plaques, increased protein oxidation/nitration and lipid peroxidation have been reported, consistent with results from biochemical analysis revealing that plaques are rich in iron (Connor and Menzies, 1995; Smith et al., 1997; Lovell et al., 1998). Elevated levels of iron in AD brain are directly neurotoxic and contribute to the production and aggregation of AB. The presence of iron responsive element within the 5'UTR of the APP transcripts indicates that APP translation will be up-regulated in response to cellular iron influx by a regulatory mechanism similar to iron control of ferritin translation (Roger et al., 1999). Iron also facilitates AB aggregation by directly binding to AB (Atwood et al., 2004). Thus, iron appears to play diverse and complex roles in AD pathogenesis that awaited to be explored.

Recent studies have implicated the role of glycogen synthase kinase-3 (GSK3), a serine/threonine protein kinase ubiquitously expressed in the CNS, in AD pathogenesis. GSK3 exists in two isoforms, alpha and beta. The beta is pivotal for the induction of memory formation, since its over-expression in mice prevents the induction of long-term potentiation (LTP) (Lucas et al., 2001; Hooper et al., 2007). Increased

activity of GSK3 beta is involved in amyloid production (Ryder et al., 2003) and mechanism underlying tau hyperphosphorylation (Jope and Roh, 2006). Active GSK3 beta is detected in neurons with pre-tangle changes (Pei et al. 1999), in the hippocampus (Blalock et al. 2004) and in the frontal cortex of patients with AD (Leroy et al. 2007). As being necessary for the production of several proinflammatory cytokines including interleukin (IL)-1 beta and tumor necrosis factor (TNF) alpha in monocytes (Martin, 2005), perhaps GSK3 beta could function in cerebral inflammatory response, which is predominately mediated by microglia.

There is extensive evidence suggesting the key role of inflammatory cytokines as a driving force for pathology progression in AD. Preclinical studies in animal models relevant to AD demonstrated a correlation between early increases in the hippocampal levels of proinflammatory cytokines, such as IL-1 beta and TNF alpha, and the loss of synaptic function in the hippocampus (Mrak and Griffin, 2005; Tuppo and Arias; 2005). It is believed that AB itself is an important initiating factor in AD pathogenesis by stimulating microglial activation in its immediate vicinity to release of neurotoxic inflammatory molecules that impair neuronal function, promote neuroinflammation and subsequently bring about neurodegenerative changes (Giulian et al., 1995; McGeer et al., 2001; Rogers et al., 2002, Walsh and Selkoe, 2004). Furthermore, evidence provided by studied using inhibitor of glial activation which showed to attenuate AB-induced increase in proinflammatory cytokines production improving both the levels of synaptic markers in hippocampus and hippocampus-dependent behavioral deficits (Craft et al., 2004). These findings address a potential link between microglial activation and inflammatory processes that play important roles in the pathogenesis of AD and others neurodegenerative disorders. As now, agents that potentially dampen microglial activation may proof to be useful for the treatment of AD (Hunter et al., 2004; Seabrook et al., 2006).

Lithium chloride (LiCl) and valproic acid (VPA), commonly used mood-stabilizing drugs for the treatment of bipolar disorder, are GSK3 specific inhibitors. Inhibition of GSK3 beta by LiCl ameliorate AB-induced death of primary cortical and hippocampal neuronal cultures (Alvarez et al., 1999) and blocks the accumulation of AB and tau phosphorylation in several mouse models (Phiel et al., 2003; Parez et al., 2003; Noble et

al., 2005). Similarly, VPA has been shown to protect neurons from AB and glutamate-induced neurotoxic injury (Mark, 1995; Gobbi and Janin, 2006). It was shown that VPA possessed an anti-inflammatory effect by attenuating the production of TNF and IL-6 in a human monocytic leukemia cells through the inhibition of NF-kB (Ichiyama, 2000). The activation of NF-kB leads to inflammatory reaction through its regulatory role in the production of cytokines, chemokines, prostaglandin (Deshpande et al., 1997; Xing et al., 2007) and matrix metalloproteinases (Han et al., 1998).

Thus, the present study aimed to determine the potential application of GSK3 inhibitors; LiCl and VPA compared to estrogen, in the presence of iron in suppressing microglial activation endpoints that linked between neuroinflammation and progression of AD-relevant pathophysiology. Quantitative analyses to be included in this study were as follows: transcript expression and protein secretion of inflammatory cytokines (IL1 beta, TNF alpha), chemokine (MIp-1 alpha), inflammatory mediators (nitric oxide, MMP-9), as well as the activation of GSK3 beta and its down stream transcription factor NF-kB.

2. Materials and Methods

2.1 Chemicals and reagents

LiCl, VPA, estrogen, ferric ammonium citrate, lipopolysaccharide (LPS) and other reagents related to cell culture were purchased from Sigma, St. Louis, MO, unless otherwise stated.

2.2 Microglial and neuronal cell cultures

Mouse microglial BV2 and neuroblastoma cells were a kind gift from Dr. James R Connor, The Pennsylvania State University, USA. Cells were grown at 37°C in DMEM supplemented with penicillin/streptomycin and fetal bovine serum (FBS). Culture media were renewed approximately twice per week.

2.3 Cell viability assessment

Cells were plated at a density of $1x10^5$ cells/well in 24-well plates in DMEM containing 5% FBS. The viability of microglial cultures exposed to various concentrations

of LiCl or VPA or that of neuroblastoma cells challenged with microglia-conditioned media was determined by MTT assay. Briefly, A 10 ul of 0.5 mg/ml MTT reagent was added into each well and incubated at 37°C for 2 h. The media were removed and a 100 ul of DMSO was added to each well. Plates were covered in the dark for 2 h. Then, the absorbance at 570 nm was determined using a microplate reader.

2.4 RNA isolation and RT-PCR

Total RNA at 6 h post-treatment was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. A volume of 2 ug RNA from each sample was subjected to reverse transcription (RT) to synthesize 1st strand cDNA. The RT reaction was performed at 42°C for 1 h. Then, AMV enzyme was inactivated at 99°C for 5 min. The cDNA samples were subjected to PCR reaction in the presence of Taq DNA polymerase (Fermentus) and primers specific for IL-1 beta forward (5'- gctatggcaactgtcccctgaac-3'), reverse (5'-tgagtgacactgccttcctcct-gaa-3') resulting in 200 bp products or TNF alpha forward (5'- atccgagatgtggaactggca-3'), reverse (5'- ccggactccgtgatgtctaagt -3') resulting in 659 bp products or MIP-1 alpha Forward (5'- taccagtcccttctctgctctg-3'), reverse (5'- ggaggtttgggggttccttg-3') resulting in 348 bp products or inducible nitric oxide synthase (iNOS); forward (5'-atcccgaaacgcta-cacttcc-3'), reverse (5'-ggcgaagaacaatccacaactc-3') resulting in 300 bp products or MMP-9 forward (5'- atccagtttggtgtcgcggag-3'), reverse (5'- aataggagcgtccctcgaagg-3') resulting in 225 bp products or GAPDH; forward (5'-aagctcactggcatggccttcc-3'), reverse (5'-ttggaggccatgtaggccatgag-3') resulting in 311 bp products. The PCR reactions were performed on Perkin-Elmer Thermal Cycle as followed: 95°C for 5 min followed by 35 cycles at 94°C for 1 min, 58-61°C for 1 min, and 72°C for 2 min. The amplification products were analyzed on 1.5% agarose gel. Band intensity will be quantified by densitometry.

2.5 Enzyme-linked immunosorbent assay

The presence of IL-1 beta, TNF alpha and Mip-1 alpha in culture supernatants at 24 h post-treatment were measured according to the manufacturer's protocol (R&D). Briefly, a 50 ul of assay diluent was added to each well followed by 50 ul of each sample or standard to the antibody coated plates. Plates was covered, incubated at room tem-

perature for 2 h, washed 4 times with wash buffer before adding biotinylated antibody and incubated at room temperature for additional 2 h. Then, substrate solution was added to each well and incubated for 30 min at room temperature. The enzyme reaction was stopped by adding stop solution into each well. The absorbance was read on a microplate reader using 450 nm as the primary wave length. The concentrations of IL-1 beta, TNF alpha and Mip-1 alpha in unknown samples was calculated using appropriate standard curves.

2.6 Nitric oxide quantification

The generation of NO at 24 h post-treatment was determined by measuring nitrite accumulation in the cell culture supernatants using Griess reagent (Invitrogen). Briefly, 150 ul/well of the nitrite-containing sample were place in 96-well plate, mixed with 20 ul of Griess reagent and 130 ul of deionized water and incubated for 30 min at room temperature. Optical density was read at 540 nm and compared with a standard curve generated in each experiment from known concentration of nitrites.

2.7 Gelatin zymography

The presence of MMP-9 in culture supernatant at 24 h post-treatment was visualized using gelatin zymography. Briefly, culture supernatant from each condition was centrifuged at 1500xg for 15 min to remove cellular debris. Proteins in the supernatant were concentrated in centriprep-10 concentrators (Millipore) to approximately 25 fold by centrifugation at 12000xg. Total protein concentration in each concentrated sample was determined using the BCA protein assay kit (Pierce). Samples (50 ug/lane) were mixed with 4x non-reducing sample buffer and incubated at room temperature for 10 min. Protein samples were separated on 10% SDS-PAGE containing 1 mg/ml gelatin. Then, the gelatin gels were washed in buffer containing Triton X-100 for 45 min to remove SDS and incubated overnight at 37^{o}C in developing buffer containing Tris-base, Tris-HCl, NaCl, CaCl2 and Brij-35. After staining gel with Coomassie blue, the gelatinolytic activity of MMP-9 appeared as clear bands against a dark blue background of the gels. The relative molecular weights of the clear bands were determined using pre-stained protein markers (Pierce).

2.8 Western blot analysis

Protein expressions of total and phosphorylated forms of GSK-3 beta at serine 9 indicating specific inhibition of this kinase were determined using the rabbit polyclonal antibodies (Cell Signaling). Total cells were collected at 1 h following each treatment and lysed in lysis buffer containing 1% Triton-X 100. Total protein content in each sample was quantified using BCA protein assay (Pierce). Equal amounts (80 ug) of total protein from each sample were mixed with sample buffer containing beta-mercaptoethanol, boiled for 10 min, and separated on 10% SDS-PAGE. Then, proteins were transferred to PVDF membranes, blocked in 5% dry milk for 1 h at room temperature, rinsed with TBS, and incubated overnight at 4°C in primary antibody specific to total form of GSK3 beta (1:1000). Subsequently, the membranes were washed with TBS containing 1% Tween 20 and incubated in the secondary antibody (1:2000) goat anti-rabbit IgG peroxidase conjugate (Sigma) for 2 h at room temperature. Bound antibodies were visualized using SuperSignal West Pico chemiluminescence (Pierce) and exposed on CL-X Posture film. The same membranes were washed intensively in TBS-Tween 20 to remove the first primary antibody before incubating in the second primary antibody specific to phosphorylation of GSK3 at serine 9 and subsequently in HRP conjugated secondary antibody. Then, band intensity of total and inhibitory phosphorylated forms of GSK3 beta was determined.

2.9 NF-kB assay

To prepare nuclear extracts, cells collected at 4 h post-treatment were washed, resuspended in iced cold PBS-PMSF and transferred into a microcentrifuge tube. Cells were pelleted at 1500 rpm at 4°C and resuspended in buffer containing 10mM HEPES (pH7.9), 1.5mM MgCl2, 10mM KCl, 0.5mM dithiothreitol, and 0.2 mM PMSF, followed by vigorous vortex for 15 sec before adding 10% detergent solution and vortexing for 10 sec. The samples were centrifuged at 14,000 rpm for 2 min. The pelleted nuclei were resuspended in buffer containing 20mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 Mm MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF and incubated for 30 min on ice and nuclear lysates were centrifuged at 14,000 rpm for 2 min. NF-kb activity in supernatants containing solubilized nuclear proteins was measured by a NF-

kb p65ActivELISA kit (Biocarta) according the manufacturer's instruction. The absorbance at 405 nm was determined using a microplate reader.

2.10 Statistical analysis

All values were expressed as the means ± standard error of means (SEM) of at least three independent experiments performed in triplicate. Comparisons were made with one-way ANOVA followed by LSD test. The statistical significance was established at a level of $p<0.05$.

3. Results

3.1 Cytotoxicity of LiCl and VPA in microglial cultures

To determine non-toxic concentrations of LiCl and VPA in microglia cultures, cells were exposed to either LiCl or VPA at various concentrations for 24 h and cell viability was determined by MTT assay. As shown in figure 1, at high concentrations, LiCl (20 mM) and VPA (3.2 mM) weakly induced cell death in microglial cultures. The maximum and non-toxic concentrations of LiCl and VPA for microglial cultures were observed at 10 mM and 1.6 mM, respectively. These concentrations are in a range of cell culture applications, as previously reported (Chen et al., 2006; Hashioka et al., 2007). Therefore, they were used in the following experiments and compared their anti-inflammatory properties with that of estrogen, which previously shown to exhibit its anti-inflammatory effects in microglial cells at a concentration of 10 nM (Cheepsunthorn et al., 2006).

3.2 Neuroprotective effects of LiCl, VPA and estrogen against microglial neurotoxicity in the presence of iron

In this experiment, microglial BV2 cells were activated by LPS at a concentration of 1 ug/ml to produce neurotoxic inflammatory molecules in the presence of LiCl, VPA or estrogen. Estrogen treatment served as positive control as previously reported for its neuroprotective effects (Pozzi et al., 2006; Tenenbaum et al., 2007). Cell-free supernatants were collected 24 h post-treatment and used to treat cultures of mouse neuroblastoma cells for 24 h. The viability of neurons was determined by MTT assay. As

shown in figure 2, media from LPS-activated microglia slightly decreased neuronal viability, whereas the presence of VPA in the media significantly enhanced this neurotoxicity. On the other hands, the presence of LiCl in the media from LPS-activated microglia compared to estrogen significantly increased neuronal viability.

Next, two non-toxic concentrations of iron, as determined by MTT assay in microglia and neuronal cultures at 24 h of exposure (data not shown),were included in the this experiment to determine neuroprotective effect of LiCl, VPA and estrogen against neurotoxic molecules produced by LPS-activated microglia in the presence of iron as seen in AD brain. The concentrations of 100 and 300 ug/ml of iron citrate represented the amount of iron reported in the brain tissues of normal elders and in the neuritic plaques of AD patients, respectively (Lovell et al., 1998). At 24 h, cell-free supernatants were collected and used to treat neurons for 24 h. As shown in figure 2, media from LPS-activated microglia containing 300 ug/ml of iron significantly decreased viability of neuronal cultures. VPA, only when 300 ug/ml of iron was presented, increased the viability of neuronal cells exposed to media from LPS-activated microglia. LiCl significantly increased viability of neurons exposed to media from LPS-activated microglia in an iron-dependent manner. Similar to LiCl, estrogen increased viability of neurons exposed to media from LPS-activated microglia, but not in an iron-dependent manner. These results demonstrate that LiCl, when compared to VPA and estrogen, is a potent neuroprotective agent against neurotoxic substances produced by LPS-activated microglia, even in the presence of high concentrations of iron.

3.3 LiCl, VPA and estrogen treatments decrease GSK3 beta activity in cultures of LPS-activated microglia in the presence of iron.

As shown in previous experiment, the presence of iron enhanced neurotoxicity of activated microglia consistent with neuropathological findings in AD brain and such effect of iron could be reversed by treatment with LiCl, estrogen and somewhat VPA, suggesting a possible link between iron, GSK 3 beta activity and microglial neurotoxicity. We therefore hypothesized that iron may be one among other factors contributing to an increased activity of GSK3 beta, which may, in turn, play important role in AD pathogenesis involved microglial activation. To test this hypothesis, the

activation state of GSK3 beta in microglial cultures induced by LPS in the presence of iron, LiCl, VPA or estrogen as described was quantified by the measurement of inhibitory phosphorylation of GSK3 beta at serine 9. Accordingly, total cell lysates prepared at 1 h post-treatment were immunoblotted for phosphorylated form of GSK3 beta at serine 9 and compared to its total form. As shown in figure 3, in non-activated microglial cultures iron decreased, whereas LiCl, VPA and estrogen increased, inhibitory phosphorylation of GSK3 beta (lane 1-4 from the left). Treatment of microglial cultures with LPS did not affect basal levels of phosphorylated GSK3 beta at time point examined (lane 5 compared to lane 1). It was found that the presence of iron decreased inhibitory phosphorylation of GSK3 beta in activated microglia in a concentration-dependent manner (lane 5-7). LiCl, VPA and estrogen reversed such effect of iron (lane 9, 10). These findings indicate that iron can increase GSK 3 beta activity in microglia in the absence of microglial activation signal such as LPS. LiCl compared to VPA and estrogen is a potent GSK 3 beta activity in LPS-activated microglia, even in the presence of iron.

3.4 Effects of LiCl, VPA and estrogen on microglial inflammatory reaction induced by LPS in the presence of iron

Increasing evidence indicates that microglial activation and inflammatory processes play key roles in pathogenesis of AD and others neurodegenerative disorders. This set of experiments was aimed to determine whether neuroprotective effects of LiCl, VPA and estrogen against microglial activation in the presence or absence of iron observed in previous experiments could be associated with anti-inflammatory effects of these molecules. To do so, microglial cultures were activated by LPS for 6 h in the described conditions and total RNA was isolated to profile the expression of inflammatory genes. To be included in this list were IL-1 beta, TNF alpha, MIP-1 alpha, iNOS and MMP-9.

As shown in figure 4A, treatments with LiCl and estrogen significantly suppressed LPS-induced transcript expression of IL-1 beta in microglial cultures. When compared to LiCl, VPA exhibited minimal effect in reducing transcript levels of IL-1 beta despite the fact that both LiCl and VPA are known to share the same cellular target.

Furthermore, suppressive effects of LiCl and estrogen including that of VPA were enhanced by iron. At time point examined as shown in figure 4B, treatments with LiCl and VPA were more effective than estrogen in suppressing LPS-induced transcript expression of TNF alpha in microglial cultures. In the presence of iron, suppressive effects of LiCl and VPA were slightly reduced. As shown in figure 4C, in contrast to what observed for IL-1 beta and TNF alpha at time point examined treatment with LiCl, VPA or estrogen did not suppress LPS-induced expression of chemokine MIP -1 alpha. However, it was observed that VPA slightly suppressed transcript levels of MIP-1 alpha, whereas LiCl increased MIP-1 alpha transcript levels. Although it was not statistically significant, the presence of iron appeared to reduce suppressive effects of VPA on LPS-induced expression of MIP -1 alpha in microglial cultures. As shown in figure 4D, treatment with LiCl, VPA and estrogen suppressed LPS-induced transcript levels of iNOS. Suppressive effects of LiCl, VPA and estrogen were dramatically enhanced by iron. Treatment with LiCl, VPA and estrogen, as shown in figure 4E, effectively suppressed LPS-induced expression of MMP-9 by 50%. Suppressive effects of LiCl, VPA and estrogen remained at the same levels, despite the fact that iron synergistically increased LPS-induced expression of MMP-9. These findings demonstrate that LiCl and VPA suppress microglial inflammatory gene expression associated with their activation induced by LPS. Suppressive effects of LiCl are comparable to that of estrogen and are more potent than that of VPA. The presence of iron during the treatments appears to enhance suppressive effects of LiCl on the expression of IL-1 beta, iNOS and MMP-9.

The following experiments aimed to determine whether suppressive effects of LiCl, VPA and estrogen on the expression of microglial inflammatory genes described in previous experiments would lead to a reduction in secretion of their corresponding proteins. Cell-free supernatants were collected at 24 h post-treatment for determination of IL-1 beta, TNF alpha and MIP-1 alpha by ELISA, or NO by Greiss reaction; or MMP-9 by gelatin zymography, as described in the methods. As shown in figure 5A, treatment with LiCl, VPA and estrogen significantly suppressed LPS-induced secretion of IL-1 beta in microglial cultures. The presence of iron suppressed LPS-induced secretion of IL-1 beta and enhanced suppressive effects of LiCl, VPA and estrogen on the secretion of IL-1 beta from microglia. These findings were in line with what described for transcript

levels of IL-1 beta induced by LPS following treatments of LiCl, VPA and estrogen. At time point examined as shown in figure 5B, similar to what observed for transcript expression of TNF alpha, treatments with LiCl and VPA were more effective than estrogen in suppressing LPS-induced secretion of TNF alpha from microglia. The presence of iron did not significantly alter suppressive effects of LiCl, VPA or estrogen. As shown in figure 5C, unstimulated microglia secreted high levels of MIP-1 alpha into the medium. However, treatments with LiCl, VPA or estrogen with or without iron had no effects on the secretion of MIP-1 alpha induced by LPS. As shown in figure 5D, treatment with LiCl, VPA and estrogen suppressed LPS-induced production of NO. Suppressive effects of LiCl, VPA and estrogen were enhanced by 100 ug/ml of iron. As shown in figure 5E, LPS induced microglial secretion of MMP-9 and iron further enhanced such effect of LPS. Treatments with LiCl and estrogen reduced LPS-induced secretion of MMP-9, whereas VPA significantly enhanced the secretion. Furthermore, suppressive effects of LiCl were enhanced by a higher concentration iron. Thus, at time point examined these findings demonstrate that LiCl and VPA suppress microglial secretion of inflammatory cytokines and mediators associated with their activated state induced by LPS with an exception for the secretion of the chemokine MIP-1 alpha. LiCl compared to VPA and estrogen strongly suppresses the secretion of TNF alpha, nitric oxide and MMP-9. The presence of iron during the treatments enhances suppressive effect of LiCl on microglial secretion of IL-1 beta, nitric oxide and MMP-9 consistent with the reduction in transcript expression of their corresponding transcripts.

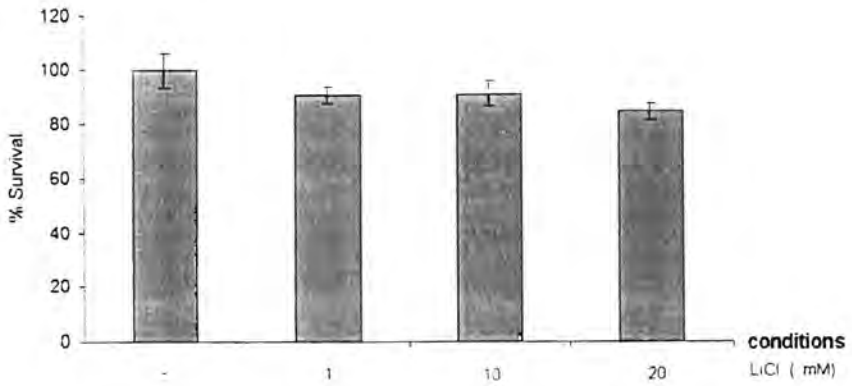
3.5 Effects of LiCl, VPA and estrogen on NF-kB-mediated microglial inflammatory reaction induced by LPS in the presence of iron

Because the activation of NF-kB is essential for microglial inflammatory reaction, the levels of NF-kB p65 subunit in nuclear extract of LPS-activated microglial cultures in the presence of LiCl, VPA or estrogen were determined by ELISA. It was found that treatment with LPS increased p65 nuclear translocation in microglia. The presence of LiCl and VPA reduced nuclear levels of p65 induced by LPS to approximately 50% and the greater effect was observed with estrogen. The presence of iron somewhat enhanced suppressive effects of VPA, whereas it greatly enhanced the effects of LiCl by

decreasing LPS-induced p65 levels to its baseline seen in the untreated control sample, as shown in figure 6. These findings suggest that suppressive effects of LiCl, VPA and estrogen on LPS-induced microglial inflammatory reaction partially involve the inhibition of NF-kB nuclear translocation.

Figure 1. Cytotoxicity of LiCl and VPA in microglial cultures. Cells plated in a 24-well culture plates at a density of $1x10^5$ cells per well were treated with indicated concentrations of LiCl (A) or VPA (B) dissolved directly into the culture medium. Twenty four hours later, the cell viability was determined by MTT assay. Values from three independent experiments performed in triplicate are expressed as mean ± SEM of percentage of untreated control cultures. *$p<0.05$ compared with untreated control cultures.

A)



B)

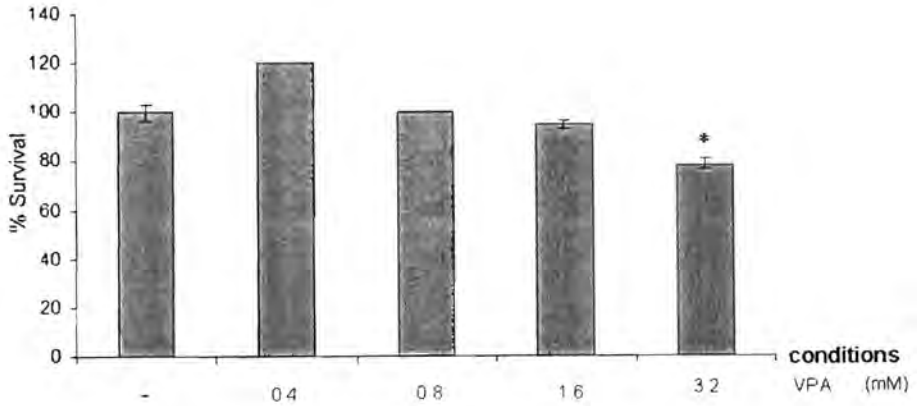


Figure 2. Neuroprotective effects of LiCl, VPA and estrogen against LPS induced microglial neurotoxicity in the absence or presence of iron. Concentrations of LiCl, VPA and estrogen examined were 10 mM, 1.6 mM, 10 nM, respectively. Neuroblastoma cells seeded in a 24-well culture plates at a density of $1x10^5$ cells per well were cultured with BV2 microglial conditioned-media as indicated. Twenty-four hours later, the cell viability was determined by MTT assay. Values from three independent experiments performed in triplicate are expressed as mean ± SEM of percentage of untreated control cultures. *; +; @ $p<0.05$ compared with untreated control cultures, LPS + 100 ug/ml iron, and LPS + 300 ug/ml iron, respectively.

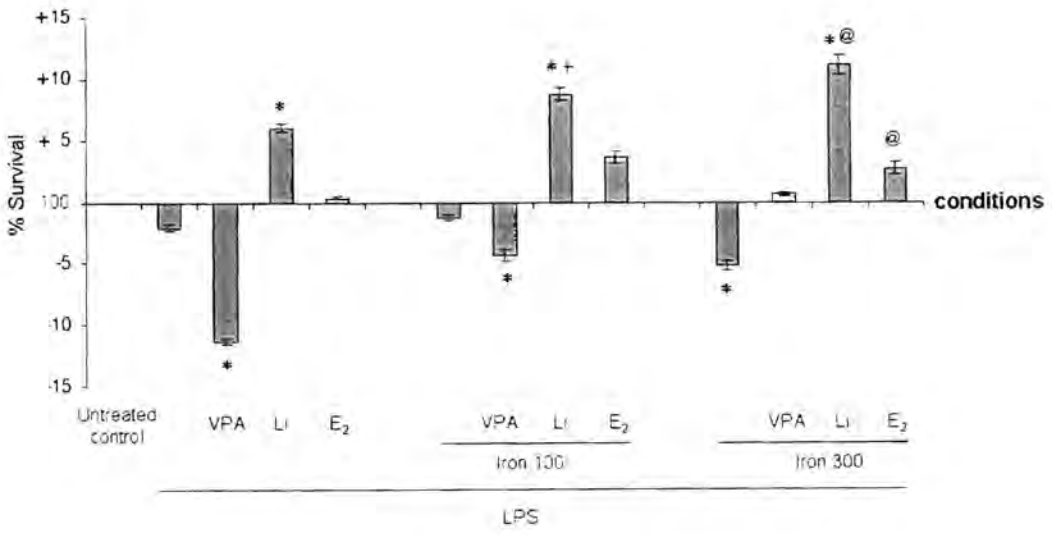


Figure 3. Iron increases GSK3 beta activity in LPS-activated microglial cultures, whereas LiCl, VPA and estrogen treatments decrease its activity. Cultures of BV2 microglial cells were activated by LPS and simultaneously treated with LiCl, VPA or estrogen as indicated. One hour later, level of inhibitory phosphorylation of GSK 3 beta at serine 9 was determined by western blot and compared with that of total form of GSK 3 beta as described in Materials and methods. Fold changes from three independent experiments are expressed as mean ± SEM of untreated control cultures. *; #; +; @ $p<0.05$ compared with untreated control cultures, LPS, LPS + 100 ug/ml iron, and LPS + 300 ug/ml iron, respectively.

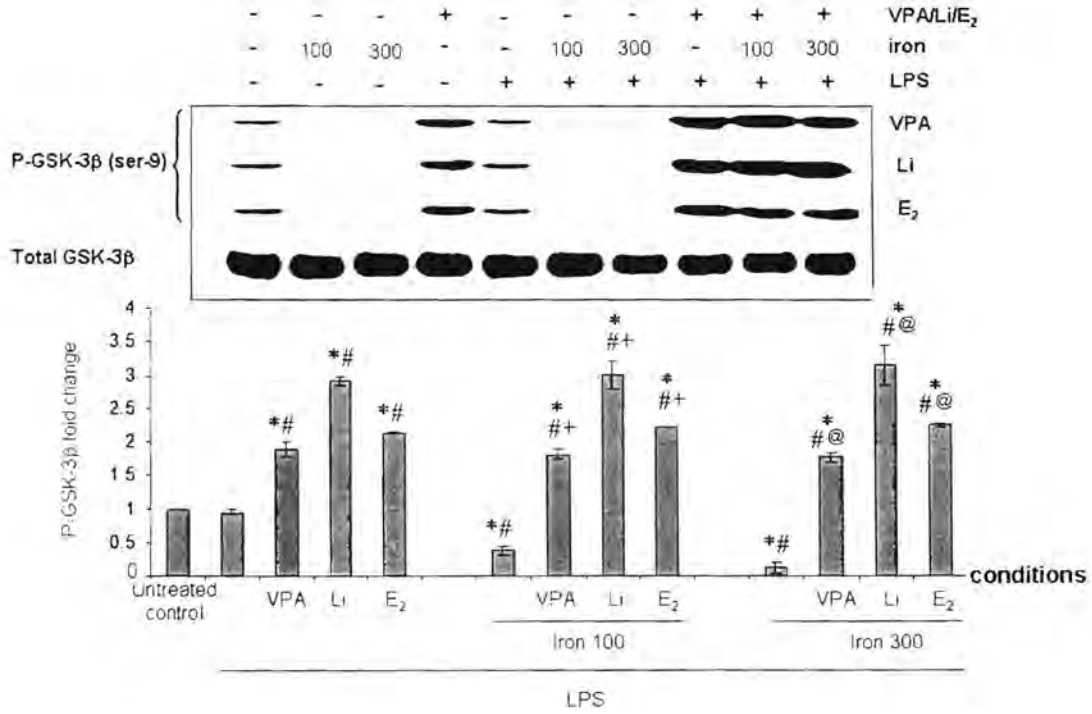
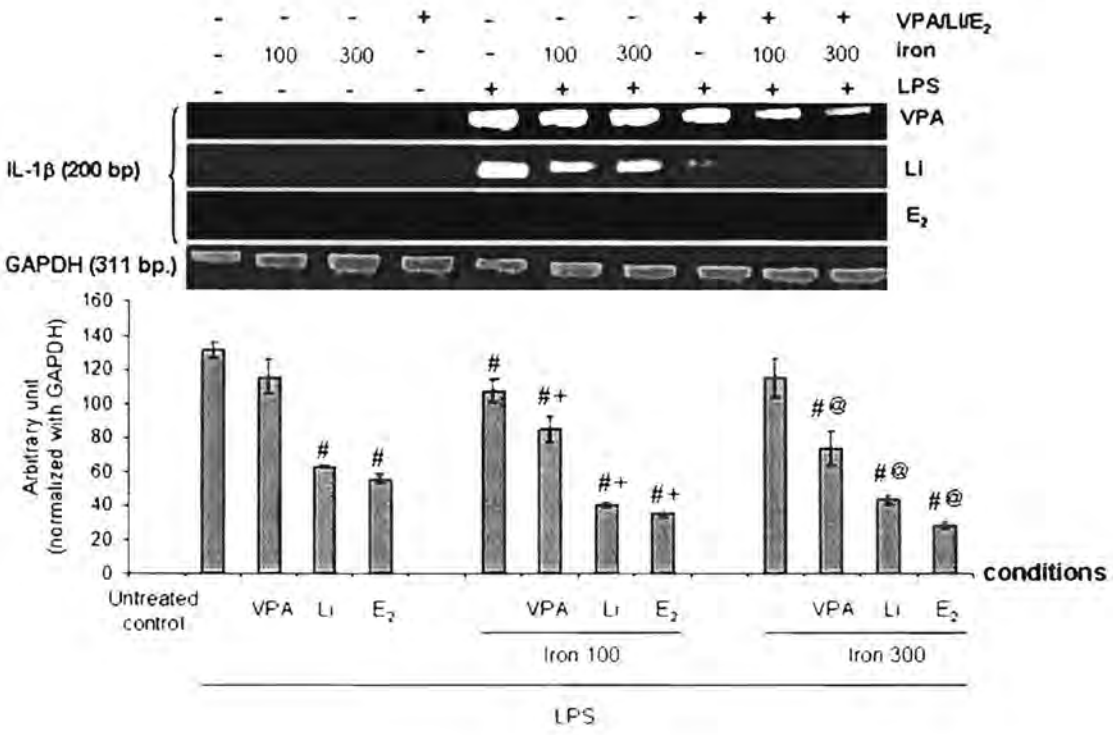
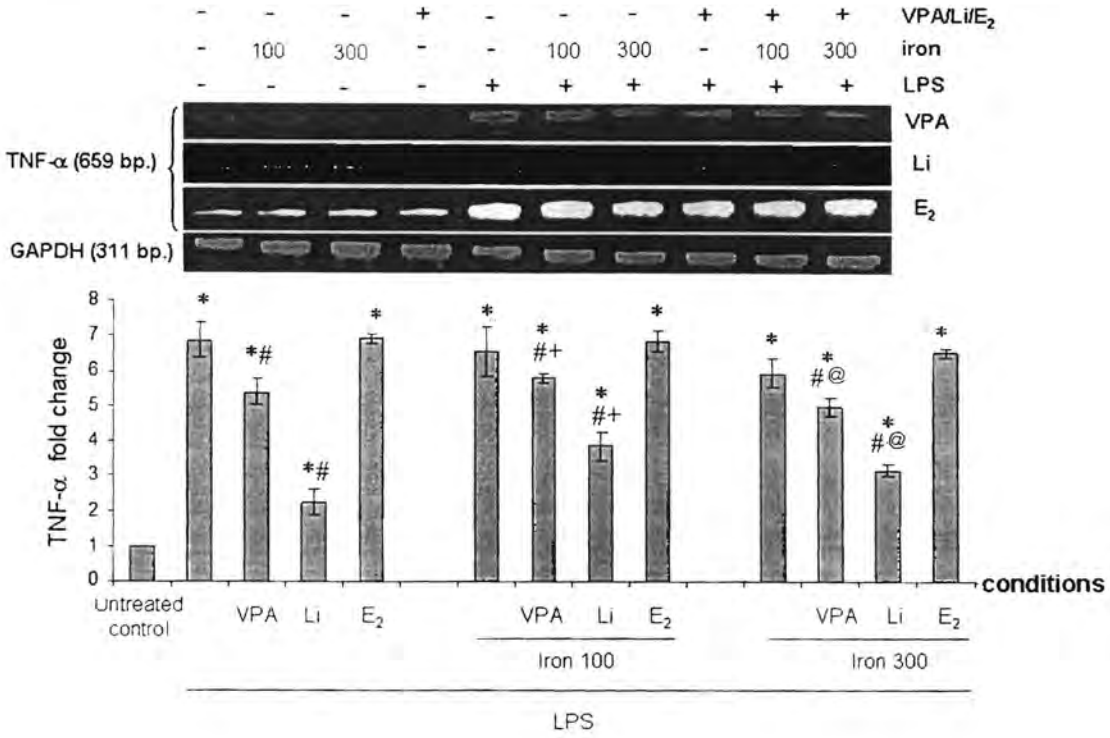


Figure 4. Effects of LiCl, VPA and estrogen on microglial inflammatory gene expressions induced by LPS in the presence of iron. Cultures of BV2 microglial cells were activated by LPS and simultaneously treated with LiCl, VPA or estrogen in the absence or presence of iron as indicated. Six hours later, transcript levels of IL-1 beta (A), TNF alpha (B), MIP-1 alpha (C), iNOS (D) and MMP-9 (E) were determined by RT-PCR and normalized with that of GAPDH. Fold changes from three independent experiments are expressed as mean ± SEM of untreated control cultures. *; #; +; @ $p<0.05$ compared with untreated control cultures, LPS, LPS + 100 ug/ml iron, and LPS + 300 ug/ml iron, respectively.

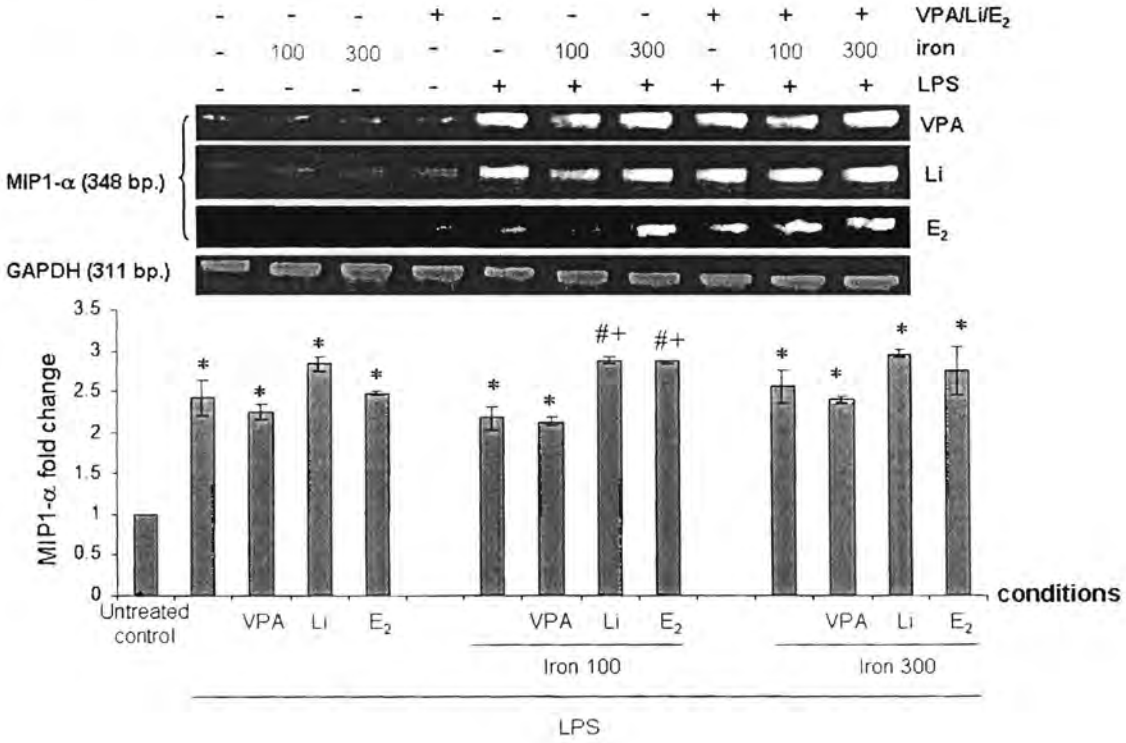
A) IL-1 beta



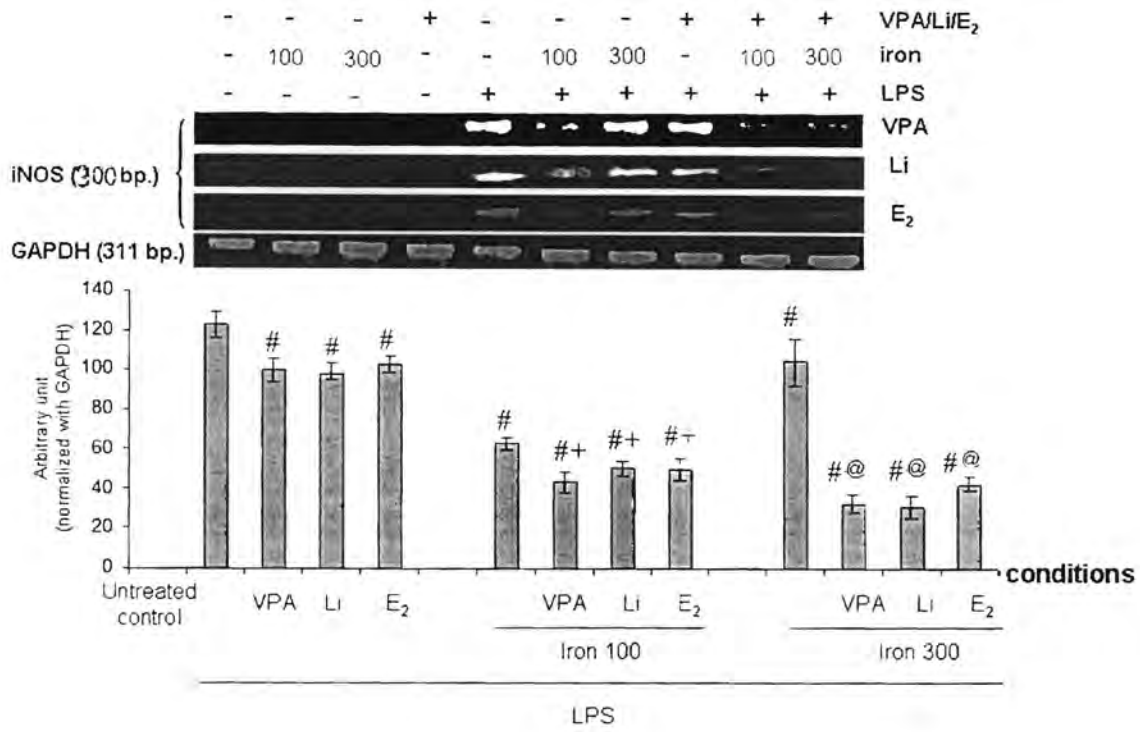
B) TNF alpha



C) MIP-1 alpha



D) iNOS


VPA/Li/E2
iron
LPS
VPA
Li
E2
iNOS (300 bp.)
GAPDH (311 bp.)
Arbitrary unit (normalized with GAPDH)
Untreated control
conditions
Iron 100
Iron 300
LPS

E) MMP-9

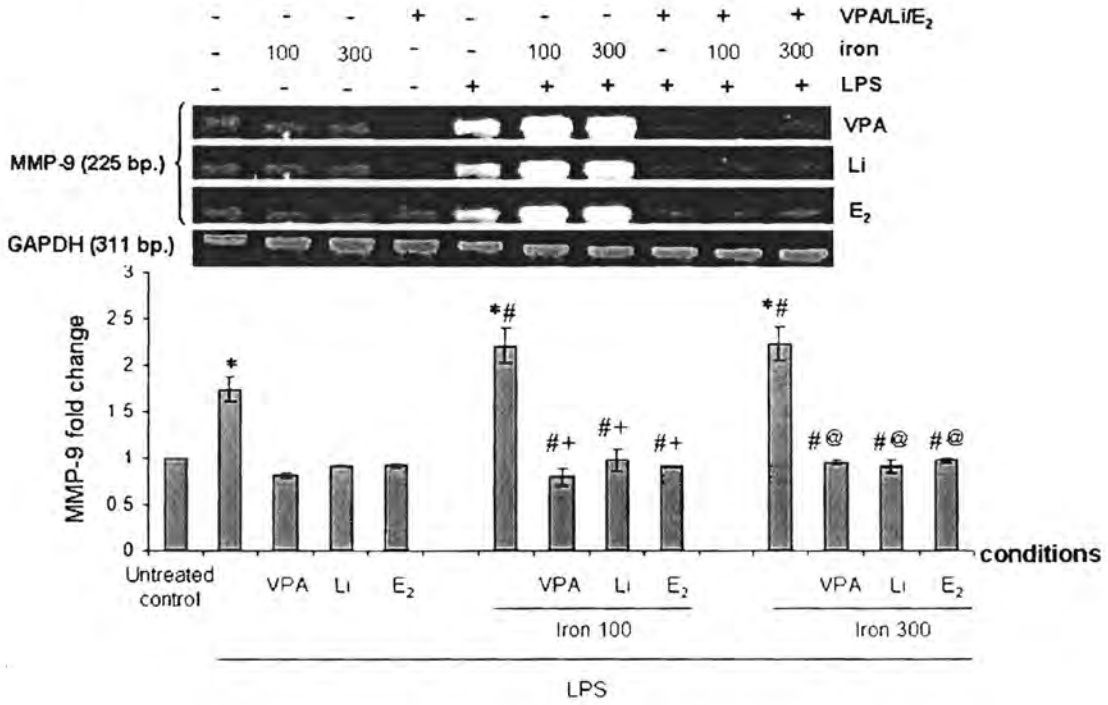
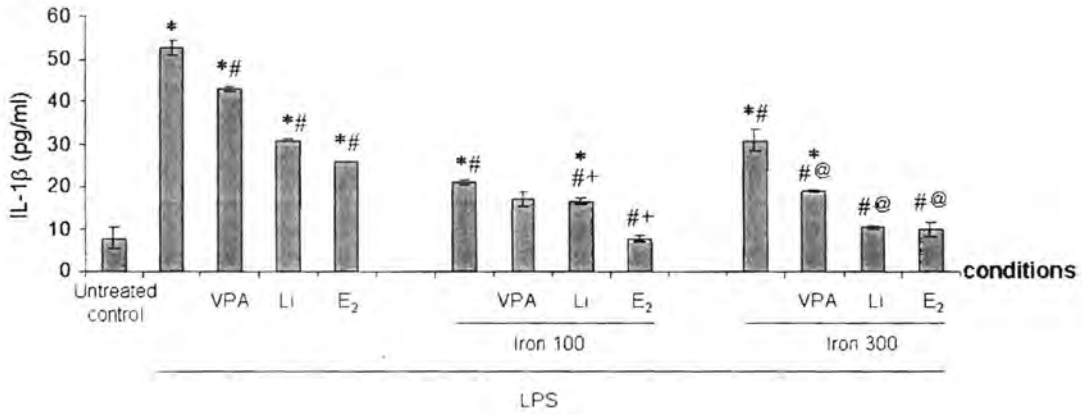
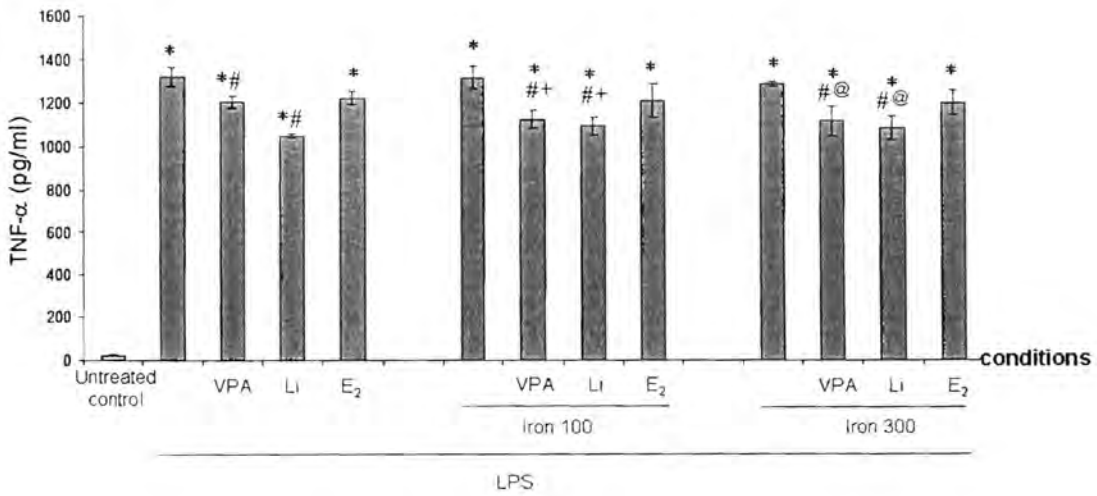

VPA/Li/E2
iron
LPS
VPA
Li
E2
MMP-9 (225 bp.)
GAPDH (311 bp.)
MMP-9 fold change
Untreated control
conditions
Iron 100
Iron 300
LPS

Figure 5. Effects of LiCl, VPA and estrogen on LPS-induced microglial secretion of inflammatory cytokines and mediators in the presence of iron. Cultures of BV2 microglial cells were activated by LPS and simultaneously treated with LiCl, VPA or estrogen in the absence or presence of iron as indicated. Twenty-four hours later, the presence of IL-1 beta (A), TNF alpha (B), MIP-1 alpha (C), iNOS (D) and MMP-9 (E) in the cell culture media were determined. Values from three independent experiments performed in triplicate are expressed as mean ± SEM. *; #; +; @ $p<0.05$ compared with untreated control cultures, LPS, LPS + 100 ug/ml iron, and LPS + 300 ug/ml iron, respectively.

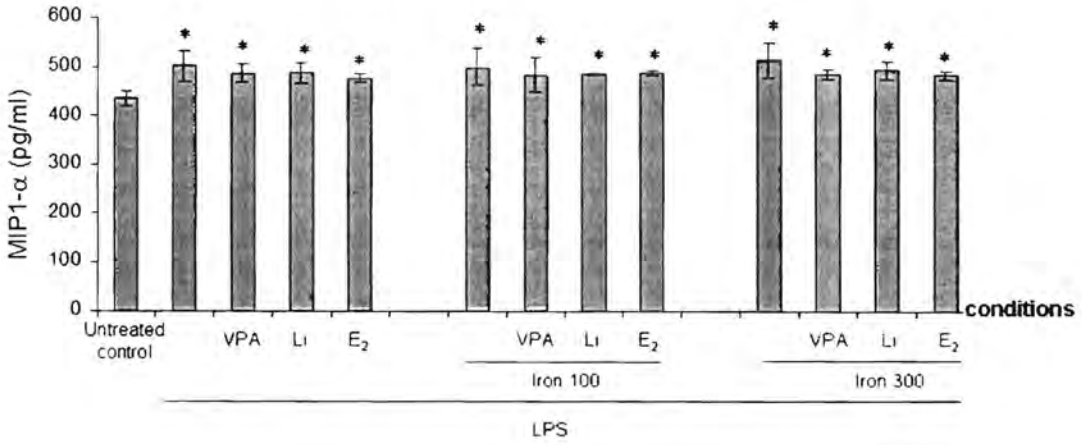
A) IL-1 beta



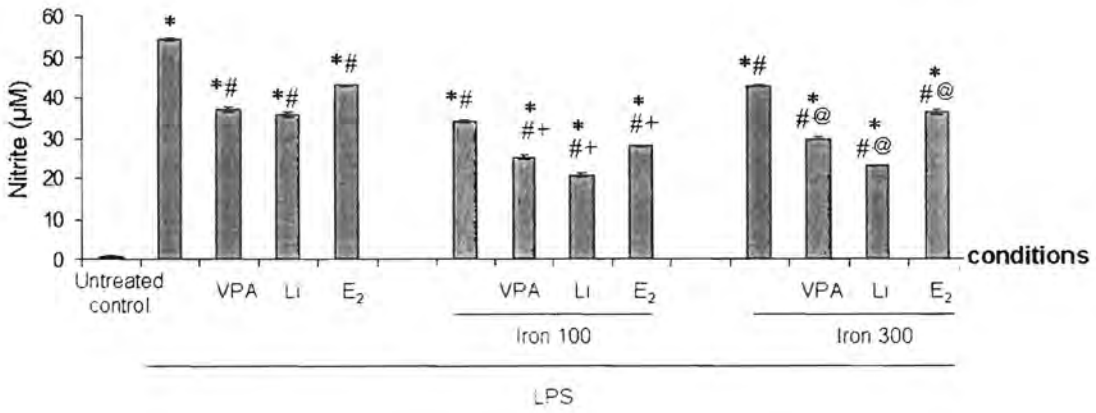
B) TNF alpha



C) MIP-1 alpha



D) Nitric oxide



E) MMP-9

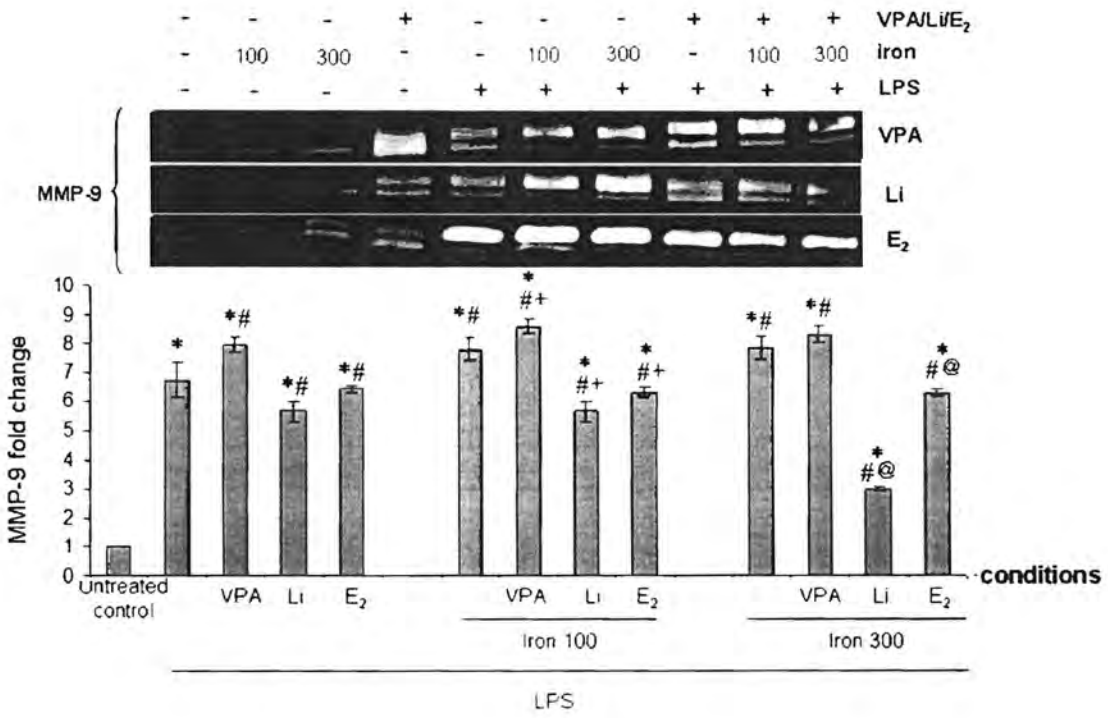
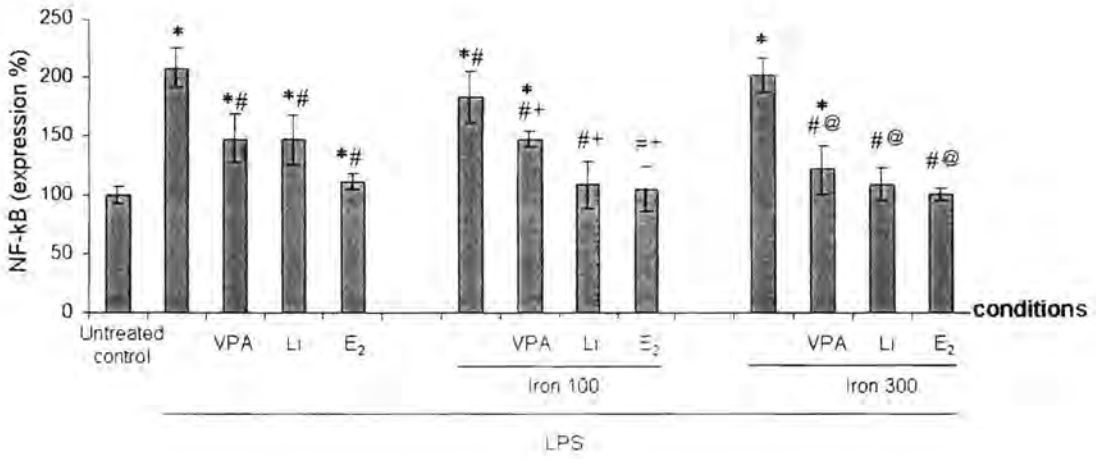

VPA/Li/E2
iron
LPS
MMP-9
VPA
Li
E2
MMP-9 fold change
conditions
Untreated control
VPA
Li
E2
Iron 100
Iron 300
LPS

Figure 6. LiCl, VPA and estrogen treatments decrease NF-kB nuclear translocation in cultures of LPS-activated microglia in the presence of iron. Cultures of BV2 microglial cells were activated by LPS and simultaneously treated with LiCl, VPA or estrogen in the absence or presence of iron as indicated. Four hours later, nuclei were isolated to determine the levels of NF-kB p65 by ELISA. Values from three independent experiments performed in triplicate are expressed as mean ± SEM. *; #; +; @ $p<0.05$ compared with untreated control cultures, LPS, LPS + 100 ug/ml iron, and LPS + 300 ug/ml iron, respectively.



4. Discussion

As reviewed earlier in this report, several key conditions have been implicated in AD neurodegeneration including elevated brain iron levels, aggregation of amyloid peptides that initiates amyloid/neuroinflammation cascades and increasing self-assembly of hyperphosphorylated tau leading to the formation of intraneuronal neurofibrillary tangles. The present study is, perhaps the first, to report that elevated extracellular iron levels increases kinase activity of GSK3 beta, a major kinase phosphorylating tau. However, the mechanism underlying this observation remains elusive. And this finding favors the tau hypothesis by suggesting that dysregulation of brain iron homeostasis as reported in aged individuals could contribute to neuronal loss in AD by promoting GSK3 beta-mediated tau hyperphosphorylation and eventually tangles formation. This finding also validates the relevance and significance of described tissue culture model used in this report to study neuropathogenesis in AD.

Among other trace metals in the brain, iron is, perhaps, a key catalyst in the process of amyloid aggregation/compaction (Atwood et al., 2004) and could possibly involve in microglia-mediated neuroinflammation. Studies show that amyloid beta peptides attract and activate microglia to increase cell surface expression of macrophage-associated markers and secretion of proinflammatory cytokines IL-1 beta, TNF alpha; chemokine MIP-1 alpha as well as inflammatory mediators NO and metalloproetinases (Rogers and Lue, 2001). Thus, the appearance of activated microglia surrounding iron-rich amyloid plaques and increased levels of inflammatory molecules within the plaque areas have led to the concept of self-propagating neurotoxic cycle of AD inflammation. We found that iron enhances neurotoxicity of activated microglia induced by LPS. This result is unlikely to be a direct effect of iron on cultured neurons (data not shown), but it is rather an indirect effect by which iron increases secretion of MMP-9 from activated microglia. Supporting evidence includes studies showing that MMP-9 induces neuronal apoptosis by regulating the expression of TNF-apha, Fas ligand and IL-1 beta (Kiaei et al., 2007; Russo et al., 2007). High levels of TNF-alpha previously shown to increase amyloid production persistently activate microglia feeding forward neurotoxic cycle (Blasko et al., 1999; Takeuchi et al., 2006). MMP-9 also contribute to chronic state of local inflammation by facilitating the

transmigration of leukocytes through microvasculature endothelium (Cross and Woodroofe, 1999), which could be enhanced by MIP-1 alpha highly secreted from activated microglia.

Regarding the concept of neuroinflammation in AD, supporting this view are reports showing that neuroprotection of nonsteroidal anti-inflammatory drugs (NSAIDs) and estrogen correlates with reduction in microglial activation and neuroinflammation in post-mortem and various cellular and animal studies relevant to AD (Mackenzie, 2001; Hoozemans et al., 2003; Yan et al., 2003; Xu et al., 2006; Pozzi et al., 2006, Yun et al., 2007). Unfortunately, prospective randomized studies have shown some controversies concerning their clinical efficacy and side effects (Hüll et al., 2006). In this report, using anti-inflammatory effects of estrogen as a reference we showed that LiCl and VPA, drugs clinically used for the treatment of patients with bipolar disorders, may have benefit as a therapeutic alternative to NSAIDs and estrogen when treating neuroinflammation in AD. Treatment with LiCl and VPA protected neurons from cytotoxicity of activated microglia. Their neuroprotective effects correlate with the suppression of microglial proinflammatory potential including the reduction in expression and secretion of MMP-9 and TNF alpha. The presence of iron appears to enhance neuroprotective effects of LiCl and VPA in reducing secretion of IL-1 beta, NO and MMP-9 suggesting the relevance of using these drugs for treating AD. It should be noted that LiCl is superior to VPA in protecting cultured neurons from microglial toxicity even under iron-enriched condition.

Mechanism by which LiCl/VPA suppress microglial production of cytokines and inflammatory mediators includes the prevention of NF-kB nuclear translocation. Although, LiCl and VPA reduce NF-kB levels closely to that of control, even in the presence of iron, they do not completely inhibit cytokine expressions suggesting that multiple NF-kB-independent pathways may be activated during microglial activation and actively involved in microglial inflammatory reaction. Recent studies indicate that LiCl independently from targeting GSK3 stimulates proliferation of neural progenitors in vitro and directly protects neuronal cultures from glutamate toxicity by inhibiting Src tyrosine kinase which phosphorylates and activates NMDA receptor and subsequently reducing

Ca2+ influx (Hashimoto et al., 2003a, b), supporting the regimen of using LiCl for the treatment of AD.

Taken together, our results show that LiCl and VPA, similar to estrogen, can suppress microglial proinflammatory potential. However, it should be note that LiCl shows a significant overall neuroprotective effects based on our neurotoxicity assay even in the presence of iron. Thus, considering the multi-etiological character of AD, our results suggest that pharmacological approaches using LiCl and VPA or a screening for its derivatives that simultaneously manipulate multiple targets involved in AD should be of great benefits to AD patients, alternative to the use of a single drug oriented towards a single molecular target possess limited ability to modify the course of the disease.

References

Alvarez G, Muñoz-Montaño JR, Satrústegui J, Avila J, Bogónez E, Díaz-Nido J. Lithium protects cultured neurons against beta-amyloid-induced neurodegeneration. FEBS Lett. 1999; 453(3):260-4.

Atwood CS, Perry G, Zeng H, Kato Y, et al. Copper mediates dityrosine cross-linking of Alzheimer's amyloid-beta. Biochemistry.2004; 43:560-8.

Blasko I, Marx F, Steiner E, Hartmann T, Grubeck-Loebenstein B. TNFalpha plus IFN gamma induce the production of Alzheimer beta-amyloid peptides and decrease the secretion of APPs. FASEB J. 1999; 13(1):63-8.

Cheepsunthorn P, Mairaue N, Nasee K. Estrogen enhances the inhibitory effect of iron on microglial nitric oxide production. J Med Assoc Thai. 2006; 89(6):840-5.

Chen PS, Peng GS, Li G, Yang S, et al., Valproate protects dopaminergic neurons in midbrain neuron/glia cultures by stimulating the release of neurotrophic factors from astrocytes. Mol Psychia-try. 2006; 11(12):1116-25.

Connor JR, Menzies SL. Cellular management of iron in the brain. J Neurol Sci. 1995; 134:33-44.

Craft JM, Watterson DM, Frautschy SA, Van Eldik LJ. Aminopyridazines inhibit beta-amyloid-induced glial activation and neuronal damage in vivo. Neurobiol Aging. 2004; 25(10):1283-92.

Cross AK, Woodroofe MN. Chemokine modulation of matrix metallopro-teinase and TIMP production in adult ratbrain microglia and a human microglial cell line in vitro. Glia. 1999; 28(3):183-9.

Deshpande R, Khalili H, Pergolizzi RG, Michael SD, Chang MD. Estradiol down-regulates LPS-induced cytokine production and NFkB activation in murine macrophages. Am J Reprod Immunol. 1997; 38(1):46-54.

Giulian D, Haverkamp LJ, Li J, Karshin WL, et al. Senile plaques stimulate microglia to release a neurotoxin found in Alzheimer brain. Neurochem Int. 1995; 27:119-37.

Gobbi G, Janiri L. Sodium- and magnesium-valproate in vivo modulate glutamatergic and GABAergic synapses in the medial prefrontal cortex. Psychopharmacology (Berl). 2006; 185(2):255-62.

Han Z, Boyle DL, Manning AM, Firestein GS. AP-1 and NF-kappaB regulation in rheumatoid arthritis and murine collagen-induced arthritis. Autoimmunity. 1998; 28(4): 197-208.

Hashimoto R, Fujimaki K, Jeong MR, Christ L, Chuang DM. Lithium-induced inhibition of Src tyrosine kinase in rat cerebral cortical neurons: a role in neuroprotection against N-methyl-D-aspartate receptor-mediated excitotoxicity. FEBS Lett. 2003a; 538(1-3): 145-8.

Hashimoto R, Senatorov V, Kanai H, Leeds P, Chuang DM. Lithium stimulates progenitor proliferation in cultured brain neurons. Neuroscience. 2003b; 117(1):55-61.

Hashioka S, Klegeris A, Monji A, Kato T, Sawada M, McGeer PL, Kanba S. Antidepressants inhibit interferon-gamma-induced microglial production of IL-6 and nitric oxide. Exp Neurol. 2007; 206(1):33-42.

Hooper C, Markevich V, Plattner F, Killick R, et al., Glycogen synthase kinase-3 inhibition is integral to long-term potentiation. Eur J Neurosci. 2007; 25(1):81-6.

Hoozemans JJ, Veerhuis R, Rozemuller AJ, Eikelenboom P. Non-steroidal anti-inflammatory drugs and cyclooxygenase in Alzheimer's disease. Curr Drug Targets. 2003 Aug;4(6):461-8.

Hüll M, Berger M, Heneka M. Disease-modifying therapies in Alzheimer's disease: how far have we come? Drugs. 2006; 66(16):2075-93.

Hunter CL, Quintero EM, Gilstrap L, Bhat NR, Granholm AC. Minocycline protects basal forebrain cholinergic neurons from mu p75-saporin immunotoxic lesioning. Eur J Neurosci. 2004; 19(12):3305-16.

Ichiyama T, Okada K, Lipton JM, Matsubara T, Hayashi T, Furukawa S. Sodium valproate inhibits production of TNF-alpha and IL-6 and activation of NF-kappaB. Brain Res. 2000; 857(1-2):246-51.

Jope RS, Roh MS. Glycogen synthase kinase-3 (GSK3) in psychiatric diseases and therapeutic interventions. Curr Drug Targets. 2006; 7(11):1421-34.

Kiaei M, Kipiani K, Calingasan NY, Wille E, et al. Matrix metalloproteinase-9 regulates TNF-alpha and FasL expression in neuronal, glial cells and its absence extends life in a transgenic mouse model of amyo-trophic lateral sclerosis. Exp Neurol. 2007; 205(1):74-81.

Leroy K, Yilmaz Z, Brion JP. Increased level of active GSK-3beta in Alzheimer's disease and accumulation in argyrophilic grains and in neurones at different stages of neurofibrillary degeneration. Neuropathol Appl Neurobiol. 2007; 33(1):43-55.

Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR. Copper, iron and zinc in Alzheimer's disease senile plaques.J Neurol Sci. 1998; 158(1):47-52.

Lucas JJ, Hernández F, Gómez-Ramos P, Morán MA, Hen R, Avila J. Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. EMBO J. 2001; 20(1-2):27-39.

Mackenzie IR. Postmortem studies of the effect of anti-inflammatory drugs on Alzheimer-type pathology and associated inflammation. Neurobiol Aging. 2001; 22(6): 819-22.

Mandelkow E, von Bergen M, Biernat J, Mandelkow EM. Structural principles of tau and the paired helical filaments of Alzheimer's disease. Brain Pathol. 2007; 17(1): 83-90.

Mark RJ, Ashford JW, Goodman Y, Mattson MP. Anticonvulsants attenuate amyloid beta-peptide neurotoxicity, Ca2+ deregulation, and cytoskeletal pathology. Neurobiol Aging. 1995; 16(2):187-98.

Martin, M., Rehani, K., Jope, R.S., Michalek, S.M. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. Nat Immunol. 2005; 6:777–784.

McGeer PL, McGeer EG. Inflammation, autotoxicity and Alzheimer disease.Neurobiol Aging. 2001; 22:799-809.

Mrak RE, Griffin WS. Glia and their cytokines in progression of neurodegeneration. Neurobiol Aging. 2005; 26(3):349-54.

Noble W, Planel E, Zehr C, Olm V, et al. Inhibition of glycogen synthase kinase-3 by lithium correlates with reduced tauopathy and degeneration in vivo. Proc Natl Acad Sci U S A. 2005; 102(19): 6990-5.

Pei JJ, Braak E, Braak H, Grundke-Iqbal I, Iqbal K, Winblad B, Cowburn RF. Distribution of active glycogen synthase kinase 3beta (GSK-3beta) in brains staged for Alzheimer disease neurofibrillary changes. J Neuropathol Exp Neurol. 1999; 58(9):1010-9.

Pérez M, Hernández F, Lim F, Díaz-Nido J, Avila J. Chronic lithium treatment decreases mutant tau protein aggregation in a transgenic mouse model. J Alzheimers Dis. 2003; 5(4):301-8.

Phiel CJ, Wilson CA, Lee VM, Klein PS. GSK-3alpha regulates production of Alzheimer's disease amyloid-beta peptides.Nature. 2003; 423(6938):435-9.

Pozzi S, Benedusi V, Maggi A, Vegeto E. Estrogen action in neuroprotection and brain inflammation. Ann N Y Acad Sci. 2006; 1089:302-23.

Rogers JT, Leiter LM, McPhee J, Cahill CM, et al. Translation of the alzheimer amyloid precursor protein mRNA is up-regulated by interleukin-1 through 5'-untranslated region sequences. J Biol Chem. 1999; 274:6421-31.

Rogers J, Lue LF. Microglial chemotaxis, activation, and phagocytosis of amyloid beta-peptide as linked phenomena in Alzheimer's disease. Neurochem Int. 2001; 39(5-6):333-40.

Rogers JT, Randall JD, Cahill CM, Eder PS, et al. An iron-responsive element type II in the 5'-untranslated region of the Alzheimer's amyloid precursor protein transcript. J Biol Chem. 2002; 277:45518-28.

Russo R, Siviglia E, Gliozzi M, Amantea D, et al. Evidence implicating MMPs in the mechanism underlying accumulation of IL-1beta and neuronal apoptosis in the neo-cortex of HIV/gp120-exposed rats. Int Rev Neurobiol. 2007; 82:407-21.

Ryder J, Su Y, Liu F, Li B, Zhou Y, Ni B. Divergent roles of GSK3 and CDK5 in APP processing. Biochem Biophys Res Commun. 2003; 312(4):922-9.

Seabrook TJ, Jiang L, Maier M, Lemere CA. Minocycline affects microglia activation, Abeta deposition, and behavior in APP-tg mice. Glia. 2006; 53(7):776-82.

Smith MA, Richey Harris PL, Sayre LM, Beckman JS, Perry G. Widespread peroxynitrite-mediated damage in Alzheimer's disease. J Neurosci. 1997; 17:2653-7.

Takeuchi H, Jin S, Wang J, Zhang G, et al. Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. J Biol Chem. 2006; 281(30):21362-8.

Tenenbaum M, Azab AN, Kaplanski J. Effects of estrogen against LPS-induced inflammation and toxicity in primary rat glial and neuronal cultures. J Endotoxin Res. 2007; 13(3):158-66.

Tuppo EE, Arias HR. The role of inflammation in Alzheimer's disease. Int J Biochem Cell Biol. 2005; 37(2):289-305.

Walsh DM, Selkoe DJ. A beta oligomers - a decade of discovery. J Neurochem. 2007; 101(5):1172-84.

Xing B, Liu M, Bing G. Neuroprotection with pioglitazone against LPS insult on dopaminergic neurons may be associated with its inhibition of NF-kappaB and JNK activation and suppression of COX-2 activity. J Neuroimmunol. 2007; 192(1-2):89-98.

Xu H, Wang R, Zhang YW, Zhang X. Estrogen, beta-amyloid metabolism/ trafficking, and Alzheimer's disease. Ann N Y Acad Sci. 2006; 1089:324-42.

Yan Q, Zhang J, Liu H, Babu-Khan S, et al. Anti-inflammatory drug therapy alters beta-amyloid processing and deposition in an animal model of Alzheimer's disease. J Neurosci. 2003; 23(20):7504-9.

Yun SH, Park KA, Kwon S, Woolley CS, Sullivan PM, Pasternak JF, Trommer BL. Estradiol enhances long term potentiation in hippocampal slices from aged apoE4-TR mice. Hippocampus. 2007; 17(12):1153-7.