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17 β-ESTRADIOL AND PHYTOESTROGENS ATTENUATE APOPTOTIC CELL DEATH IN HIV-1 TAT EXPOSED PRIMARY CORTICAL CULTURES

by

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Submitted in Partial Fulfillment of the Requirements

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DEDICATION

To my Grandmama and Granddaddy, I know you are looking down upon me from heaven. Thank you for always believing in me and pushing me forward. I hope I have made you proud. I miss you both immensely.

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I would like to acknowledge first and foremost, my Lord and Savior, Jesus Christ, for HIS blessings.

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ABSTRACT

Despite the advent of highly active antiretroviral therapy (HAART), neurological disorders develop in most people infected with human immunodeficiency virus type 1 (HIV-1). The poor CNS delivery of anti-retrovirals allows continued virus production and renders neurons susceptible to the toxic effects of released viral proteins. The neurotoxic potential of HIV-1 viral protein Tat has been shown to include excitotoxicity, oxidative stress, and mitochondrial dysfunction leading to subsequent cell death. Thus, Tat is thought to have a key role in the pathogenesis of HIV-associated neurodegeneration. Estrogens are universally considered to be neuroprotective, as such; estrogens have positive implications in the treatment of neurodegenerative disease, as well as acute neuronal death. Estrogen neuroprotective mechanisms may include a direct neuronal anti-apoptotic effect as estrogen modulates actions of key regulators of the mitochondrial/intrinsic apoptotic cascade. However, estrogen therapy is associated with increased risk of breast and uterine cancers. Estrogen receptors (ERs) are present in the brain and are thought to mediate estrogen protective actions. Compounds that selectively target estrogens protective effects without eliciting its negative side effects may serve as viable therapeutic options.

In the present study, we tested the ability of estrogen and phytoestrogens

(genistein and daidzein) to protect against apoptotic signaling in cortical cell cultures exposed to Tat 1-86 (50 nM), and additionally, whether the beneficial actions of estrogen and phytoestrogens involved an estrogen receptor sensitive mechanism. We demonstrated that estrogen pretreatment significantly delayed Tat-induced cell death in primary cortical cultures. Pretreatment with 17β-estradiol or phytoestrogens attenuated the increased expression of anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax and activation of caspases linked to mitochondrial apoptotic pathway following Tat exposure. In addition, select components of apoptotic pathway signaling appear more sensitive to estrogen receptor (ER) activation, as the addition of ER antagonist ICI182,780 reversed downregulation of Bax and caspase 3, while effects on Tat-induced Bcl-2 and caspase 9 expression were maintained. Moreover, the addition of preferential ERα and ERβ antagonists (MPP dihydrochloride and PHTPP) indicated that 17β-estradiol and phytoestrogen effects on caspase 3 may be mediated by both receptor subtypes, while ERβ was more involved in effects on Bax. Our data suggest that 17β-estradiol and phytoestrogen are able to intervene against HIV Tat-induced cortical neuronal dysfunction via intersecting mitochondrial apoptotic pathway signaling in an ER-sensitive manner.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

1. Introduction

1.1. HIV-1 infection and consequences for the central nervous system

Over 33 million people worldwide are infected by HIV (UNAIDS/WHO), and while HIV is most well known for its devastating effects on the immune system and the resulting AIDS, it can also cause several neurological disorders, collectively known as HIV-associated neurocognitive disorders (HAND) (Dewhurst et al., 1996; Gray et al., 2001). HAND syndromes are characterized by cognitive, motor and behavioral abnormalities (Kaul et al., 2005) and are classified according to patient performance in areas of neurological and behavioral functioning and neuropsychological testing (Sacktor et al., 2001; Sactor, 2002; McArthur, 2004). Prior to the advent of highly active antiretroviral therapy (HAART), 20-30% of individuals with advanced HIV infection displayed symptoms of the severe HAND, HIV-associated dementia (HAD) (Kaul et al., 2005; Gonzalez-Scarano and Martin-Garcia, 2005). With the widespread use of HAART, the incidence of HAD has dramatically decreased; however, as many of 40% of HIV-positive patients continue to suffer from HAND (Dewhurst et al., 1996; Gray et al., 2001; Nath et al., 2008; Dewhurst et al., 1996; Gray et al., 2000; King et al., 2006; Li et al., 2005). Moreover, as the incidence of HAD has decreased, its prevalence is actually

increasing, due in part to the longer life expectancy of HIV-infected persons and to patient resistance to HAART drugs (Gonzalez-Scarano and Martin-Garcia, 2005; Gray et al., 2003; MacArthur et al., 2003; Sacktor et al., 2002; Maschke et al., 2000). The continued prevalence of milder forms of HAD suggests a shift in the distribution of HAND subtypes in the post-HAART era. In patients presenting even the milder forms of HAND, quality of life is greatly affected, with these individuals suffering from disruptions in ability to perform activities of daily living, perhaps most importantly, that of adherence to the HAART regiment (MacArthur, 2004). Manifestation of mild cognitive/motor disorder (MCMD) appears to indicate a worse prognosis for AIDS and has been highly correlated with the subsequent development of HAD and the detection of HIVE at autopsy (Sactor et al., 1996; Cherner et al., 2002). This continued prevalence of neurological dysfunction, suggests HAART fails to provide complete protection from the development of HAD (Antorini et al., 2007; Ellis et al., 1997; Liner et al., 2008; Boisse et al., 2008). This failure to prevent the deterioration or enable restoration of cognitive function has been linked to limited penetration of many antiretroviral drugs in the CNS (Enting et al., 1998). It is also of note that HAART, in the long term, poses a potential toxicological problem that may affect neurocognitve performance. Although HAART has impacted on the incidence of HAD, it still remains a serious concern during AIDS. A recent study reported that 90% of HIV-1 infected patients that died between 1996 and 2001 were newly diagnosed with HAD within 12 months of death (Welch and Morse, 2002). It is suggested that HAD is the most common form of dementia worldwide in people aged 40 years or less, and is a significant independent risk factor for death due to

AIDS (Ellis et al., 1997).

1.2. Pathology of HIV in CNS

HAD manifests as subcortical dementia characterized by psychomotor slowing, changes in mood and anxiety levels, deficits in memory, information processing, verbal fluency, decision making and attention (Navia et al., 1986; Grant et al., 1987; Price et al., 1988; Portegies et al., 1993). With such a range of symptoms, numerous brain regions commonly damaged in HAD are basal ganglia, deep white matter, hippocampus, and cerebral cortex (Berger et al., 2000; Dewhurst et al., 1996; Gray et al., 2001; Gray et al., 2003; Maschke et al., 2004; Sporer et al., 2005). Pathological features of HAD include multinucleated giant cells, activated microglia, dendritic simplification, and cell death (Kaul et al., 2005(Archibald et al., 2004). Clinical signs of HAD are more closely associated with increased numbers of microglia, evidence of excitotoxins, and selective neuronal damage and loss, rather than correlating with viral load in the CNS.

Cerebral atrophy and white matter abnormalities are common neuroimaging findings among HIV-infected individuals, and are evident in both cortical and subcortical regions of the brain (Paul et al., 2002). Several reports have shown large neuronal loss in cortical regions in AIDS (Everall et al., 1991; Everall et al., 1993; Fischer et al., 1999; Moore et al., 2006). In some studies, the severity of atrophy was correlated with advanced stages of HIV-1 infection or with clinical signs of cognitive impairment. Volumetric MRI analysis has shown that cortical atrophy associated with HIV infection might be caused by neuron loss and demyelination (Arendt and Nolting, 2008; Arendt,

1995; Bornstein et al., 1992; Boska et al., 2004; Chang et al., 2002; Chiang et al., 1998; Costello et al., 2007; Dal Pan et al., 1992; Di, V et al., 1997; Elovaara et al., 1990; Ernst et al., 2002). Synaptodendritic loss will reduce the overall volume of brain gray and white matter. In HIV-infected individuals, the degree of neurocognitive impairment was strongly related to loss of immunostaining for synaptophysin and MAP2, pre and post synaptic proteins, respectively (Arendt and Nolting, 2008; Arendt, 1995; Bornstein et al., 1992; Boska et al., 2004). The degree of atrophy is correlated to the degree of cognitive motor dysfunction. Quantitative MRI shows a correlation between cerebral atrophy and neuropsychological performance (Ho and Jay, 2010; Dewey et al., 2010; Johnson and Nath, 2010; Ances et al., 2010; Gongvatana et al., 2009; Descamps et al., 2008). Eventually, the correlation persists between an increase in atrophy and worsening in certain cognitive functions (Hall et al., 1996; Everall et al., 1999). Neuronal loss in the frontal cortex and in other regions of the neocortex has been estimated between 18 and 38% (Everall et al., 1991; Ketzler et al., 1990). Select neuronal subpopulations, including larger pyramidal cells within the cortex, are at greater risk of cell death and similarly neuronal populations that secrete certain neurotransmitters are more likely to be diminished in the HIV-1 infected brain (Masliah et al., 1992).

1.3. Oxidative stress and HIV dementia

There is overwhelming evidence that oxidative stress mediates neuronal injury and contributes to the pathogenesis of HIV dementia. Oxidative stress in HIV-dementia patients has been demonstrated in the brain and CSF (Chauhan et al., 2003; Turchan et

al., 2003; Pocernich et al., 2005; Steiner et al., 2006). Oxidative stress can be defined by the modification and accumulation of biological molecules altered by various reactive oxygen species (ROS) (Pocernich et al., 2005). ROS are highly reactive, toxic oxygen moieties, such as hydrogen peroxide, peroxynitrite and superoxide anion. ROS can lead to oxidation of proteins and DNA, peroxidation of lipids and ultimately cell death. Protein carbonyl groups are used as a marker of protein oxidation, and they are generated from direct oxidation of amino acids or from binding of the lipid peroxidation product 4-hydroxynonenal (HNE) to proteins (Pocernich et al., 2005). Staining of lipid peroxidation product HNE was found to be prominent in neurons, glial cells, perivascular cells in brain slices of patients with HIV encephalitis (Turchan et al., 2003). Protein oxidation was increased in the CSF of HIV-patients with mild and severe dementia compared to non-demented patients. Nitrated tyrosine residues, evidence of peroxytnitrite reaction with proteins, is increased in HIVD brains (boven , 1999). It was recently reported that levels of ceramide, sphingomyelin, and HNE are significantly increased in brain and CSF of HAD patient, and the HIV protein Tat can induce increases of all three in cultured neurons (Haughey et al., 2004; boven , 1999). Further, oxidative stress may be correlated with HIV-induced neuropsychological impairment (Turchan et al., 2003). This is partly due not only to the fact that HIV proteins and immune activation mediates the accumulation of ROS, but also to the fact that some antiretroviral therapy based on nucleoside reverse transcriptase inhibitors (NRTI) has been shown to increase oxidative stress (Hulgan et al., 2003) and may be toxic to mitochondria (Lewis et al.,

2003).

To counteract the damaging free radicals, antioxidant systems have evolved that include enzymes glutathione peroxidase, superoxide dismutase and catalase. Protection against oxidative stress also comes from small, nonprotein antioxidants, such as glutathione, vitamin C, flavonoids, and vitamin E (Butterfield and Stadtman, 1997). Antioxidant levels in HIV-infected patients are altered, and may lead to increase oxidative stress (Pocernich et al., 2005; Steiner et al., 2006). Glutathione, which protects neurons from ROS and binds lipid peroxidation products such as HNE, has decreased levels in HIV patients (Drake et al., 2002; Pocernich et al., 2000). Serum glutathione levels and glutathione peroxidase activity are significantly reduced in HIV patients compared to controls while an increase in lipid peroxidation products and DNA fragmentation were increased (Gil et al., 2003). Additionally, levels of glutathione synthase are decreased in the liver, blood, and CNS of HIV-infected persons and low concentration of glutathione synthase has been correlated with poor survival in HIV infected patients (Herzenberg et al., 1997;Castagna et al., 1995). These data indicate the detrimental effects of oxidative stress in HIV infection, increases production of reactive oxygen species along with suppression of endogenous antioxidant systems.

1.4. Tat

Given that the HIV-1 virus does not directly infect neurons, it has been determined that detrimental effects in the brain are mediated by released toxic viral

products (King et al., 2006; van de Bovenkamp et al., 2002). Both direct and indirect neurotoxic mechanisms are believed to operate in HIV-related neurotoxicity. In the direct pathway, infected microglia and macrophages release viral particles and proteins, some of which have been shown to be neurotoxic in vitro. The viral molecules which are particularly implicated include gp120 and Tat.

HIV-1 Tat is a viral nonstructural protein of 86-101 amino acids in length and it is the product of two exons. Tat, a transactivating nuclear regulatory protein, is critical for viral replication and is secreted by HIV-1 infected cells (King et al., 2006; Noonan and Albini, 2000; Wallace, 2006). Secreted Tat may be taken up by neighboring cells, and in this manner, Tat is able to elicit affects on both infected and uninfected cells. The HIV-1 protein Tat released from macrophages produces trimming of neurites, mitochondrial dysfunction, and cell death in neurons (Chauhan et al., 2003; (Aksenov et al., 2003; Aksenov et al., 2001a; Kruman et al., 1998). HIV Tat has been detected in sera of HIV-infected patients at the low nanomolar level, in the range of 2ng/mL to 40 ng/mL (Wiley et al., 1996; Xiao et al., 2001).

1.4.1 Tat-induced neurotoxicity

Neuronal injury is a primary basis/component of many neurodegenerative diseases, including HAD. Given that the HIV-1 virus does not directly infect neurons, it has been determined that detrimental effects in the brain are mediated by released toxic viral products (King et al., 2006; van de Bovenkamp et al., 2002). Several HIV-1 encoded proteins, including the transcription transactivator Tat, have been demonstrated

to directly induce neuronal cell death. HIV-1 viral protein Tat can be released by HIV-infected cells to the extracellular space, cerebrospinal fluid (CSF) and sera and is taken up by neurons (Chang et al., 1997; Ensoli et al., 1993; Sabatier et al., 1991). Tat has been detected in the brains of people with HIVE by mRNA and Western blotting analyses (Wiley et al., 1996; Hudson et al, 2000). The HIV Tat protein is thought to play a significant role in HIV related neuropathology. Tat is taken up by CNS cells, and often results in toxic consequences in both the cytoplasm and nucleus, including altered gene transcription, cytokine secretion, NMDAR activation in neurons, and the initiation of apoptotic cascades (Aksenov 2009; 2006; 2003; Silvers et al., 2007; Rappaport et al., 1999; Kumar et al., 1999). A combination of these cellular events are likely involved in neuronal apoptosis in response to HIV-1 infection and the secretion of Tat, however the exact mechanism is not well understood.

Tat induced neurotoxicity is thought to be mediated by various mechanisms including excitotoxic mechanisms involving calcium and oxidative stress. Tat toxicity has been associated with glutamate receptor activation, since antagonists of NMDA and non-NMDA receptors partially protect neurons from the toxic effects of Tat (Haughey et al., 2001; Magnuson et al., 1995; Nath et al., 1996). The neurotoxic effects of Tat are in part mediated by direct interactions with a polyamine-sensitive site on the NMDA receptor (Prendergast et al., 2001; Self et al., 2004). Studies have also suggested that Tat neurotoxicity is dependent on binding to low-density receptor (LPR) with subsequent activation of NMDAR (King et al., 2006). Several studies have shown that NMDAR

function may be modulated by dopamine D1-like receptors and a recent study has implicated D1-mediated pathways in the mechanism of Tat-induced neurotoxicity (Silvers et al, 2007). HIV-1 Tat may influence the activity of D1 receptors in postsynaptic neurons, thereby trigger NMDAR-regulated apoptotic cascades via D1/NMDAR interaction or, alternatively, NMDAR activation in D1-expressing neurons exposed to Tat may upregulate pro-apoptotic D1-mediated signaling (Silvers et al., 2007). Tat has been shown to activate phosphatidylinositol 3-kinase, increase levels of $IP_{3,}$ release calcium from endoplasmic reticulum internal stores, and increase activity of PKC isoforms, all precursors of oxidative stress (Aversa et al., 2004; Haughey et al., 1999; Borgatti et al., 1998; Haughey and Mattson, 2002).

Moreover, Tat exposure has been shown to increase markers of oxidative stress. Studies in our lab have demonstrated that striatal injections of Tat caused an increase in protein carbonyl formation that preceded Tat-mediated astrogliosis (Aksenov et al., 2001) and increased striatal tissue loss in rats (Bansal et al., 2000). Tat induced protein and lipid peroxidation has also been detected in synaptosomal membranes and neuronal cell cultures (Pocernich et al., 2004). Oxidative stress is an early step in Tat neurotoxicity as increases protein oxidation soon after Tat exposure, followed by pronounced neuronal degeneration and activation of macrophages/microglia (Aksenov et al., 2003). Tat may also compromise the functioning of antioxidant systems. Tat plays a major role in the glutathione system as demonstrated by a downregulation of glutathione synthetase, stopping glutathione synthesis, in the liver of tat-transgenic mice (Aksenov et al., 2001b;

King et al., 2006; Noonan and Albini, 2000; Pocernich et al., 2005b; Pocernich et al., 2005a; Steiner et al., 2006; Wallace, 2006; Choi et al., 2000). HeLa cells expressing Tat were found to have decreased glutathione peroxidase activity and mRNA levels and low levels Mn superoxide dismutase (Bouvard et al., 2002; Richard et al., 2001). Protein oxidation was increased and antioxidant systems were decreased in these HeLa –Tat cells, reflecting ongoing oxidative stress.

1.4.2. Effect of HIV-1 viral protein Tat on mitochondria

Mitochondria are considered the powerhouses of the cell. They are responsible for generating energy as ATP and are key regulators of cell survival and death. Thus, mitochondria may have a central role in numerous neurodegenerative diseases. Damage to mitochondria is caused primarily by reactive oxygen species (ROS) generated by mitochondria. Mitochondria consume 85% of the oxygen utilized by the cell during production of ATP (Shigenaga et al., 1994). Normally, 4% of all oxygen consumed is converted in mitochondria to the superoxide radical (Shigenaga et al., 1994; Evans et al., 2002; Carreras et al., 2004). Superoxide is transformed to hydrogen peroxide by the detoxification enzymes Mn superoxide dismutase (Wallace, 2005) and then to water by glutathione peroxidase (Green et al., 2004). However, when these enzymes cannot convert ROS to water fast enough, oxidative damage occurs and accumulates in the mitochondria (James and Murphy, 2002; Sies, 1993; Pieczenik and Neustadt, 2007). As mentioned previously, these endogenous antioxidant enzymes are compromised in HIV-

infected patients.

A general mitochondrial effect of HIV-1 has been obtained by treating HIV-1 transgenic mice with AZT, an anti-retroviral drug. AZT is discovered to negatively affect mitochondrial DNA replication and provoke persistent mitochondrial myopathy in AIDS patients. In mice, transgenic HIV sensitizes to AZT-induced myocardial mitochondriopathy (Kohler et al., 2009; Pupure et al., 2008; Chan et al., 2007; Collins et al., 2004). In Tat-expressing mice, AZT-treatment caused a greatly enhanced suppression of Mn superoxide dismutase (Prakash et al., 1997; Shi et al., 1998). In addition, Tat was found to exacerbate signs of AZT-induced oxidative stress(Prakash et al., 1997). Thus, HIV-1 and particularly Tat, can affect mitochondrial metabolism in vivo.

Cells stably transfected with Tat are more susceptible than control cells to undergo apoptosis upon serum withdrawal. According to one study, Tat causes a decrease in Bcl-2 expression and an increase in Bax expression (Sastry et al., 1996). Another study reports that Tat translocates to mitochondria concomitantly with the loss of mitochondrial membrane potential (Macho et al., 1999). Addition of extracellular Tat causes apoptosis (Li et al., 1995; Aksenov et al., 2009; Adams et al., 2010). Tat downregulates the mitochondrial isoform of superoxide dismutase (SOD2), either at the post-transcriptional or transcriptional levels (Briehl and Baker, 1996; Flores et al., 1993; Gil et al., 2003; Marecki et al., 2004). This downregulation of SOD is thought to sensitize cells to the lethal effects of reactive oxygen species produced in mitochondria. Exogenous Tat can also induce the expression of pro-apoptotic proteins (Adams et al., 2010). These proteins

include Bax and Par-4, which induces apoptosis via a mitochondrion-dependent mechanism (Kruman et al., 1998;1999). These results suggest multiple connections of between Tat-induced cell death and mitochondria.

Moreover, numerous studies suggest that Tat-induced cell death is mediated through the intrinsic/mitochondrial apoptotic pathway. Tat has been shown to induce Ca2+ influx from the extracellular environment through various neuronal calcium channels (Bonavia et al., 2001; Haughey et al., 2001; Perez et al., 2001; Nath et al., 1996). The elevation in cytosolic Ca2+ is an early event in neuronal apoptotic cascade and appears to trigger mitochondrial calcium uptake and subsequent mitochondrial dysfunction. Studies in cultured embryonic rat hippocampal neurons demonstrated that HIV-1 Tat induced neuronal cell death involved caspase activation, disruption of Ca2+ homeostasis, and generation of mitochondrial reactive oxygen species (Kruman et al., 1998a).

1.5. Estrogen in neurodegeneration

Estrogens are primarily synthesized in the ovaries and in the suprarenal glands. The most potent and dominating estrogen is 17β-estradiol, but lower levels of estrone and estriol are also present (Gruber et al., 2002b). Following synthesis in the ovaries and suprarenal glands, 60% of estrogens are bound to plasma proteins and transported to the target tissue, including brain (McEwen, 2002; Gruber et al., 2002b). Estrogens are lipophilic and have a low molecular weight, which enables them to diffuse freely through

the blood-brain barrier (Samii et al., 1994).

In the developing brain, estrogens control the differentiation and plasticity of distinct neuronal populations (Beyer, 1999). Estrogens promote growth in the hypothalamus, hippocampus, midbrain and cortex (McEwen and Alves, 1999). In the adult CNS, in addition to its regulation of HPA and sexual behavior (Herbison, 1998), estrogens also powerfully influence cognition-related neuronal activity, and the modulation of mood, mental state and learning, and memory (Brinton, 2001; McEwen et al., 1995; McEwen et al., 2001).

Gender differences have been associated with decreased risk, delayed onset and progression, or enhanced recovery from numerous traumatic or chronic neurological and mental diseases. This involves many different types of diseases, classified as neurotransmitter system abnormalities, disorders caused by trauma (stroke) or by defects in the immune or cardiovascular systems, and neurodegenerative diseases (Rogers and Wagner, 2006; Farooque et al., 2006; Chen et al., 2005; Wooten et al., 2004; Bayir et al., 2004). Although the reason for the gender difference in these diseases is still unknown, the concentration of estrogen may be involved, or the expression of their receptors.

Collateral support for a neuroprotective role of endogenous E2 has also arisen from observations of greater brain damage in males than in female animals in ischemic stroke models (Dubal et al., 1998; Zhang et al., 1998; Rusa et al., 1999; Alkayed et al., 2000). Moreover, estrogen deficiency as seen during menopause has been correlated with increased incidence of stroke and cognitive defects, mood changes, and early onset

and severity of Alzheimer's (Correia et al., 2010; Aenlle et al., 2009; Rocca et al., 2007; Solenski, 2007; Genazzani et al., 2007).

1.5.1. Neuroprotective role of estrogen

There is recent yet still controversial evidence that estrogen treatment reduces the risk and delays the onset of some neurodegenerative diseases. In Alzheimer's disease (AD), the decline of estrogens is related to the risk of this disease in post-menopausal women (Tang et al., 1996). Early epidemiological evidence supported a role for estrogen replacement therapy (ERT) in reducing the incidence of AD in these women. Moreover, several studies indicated that ERT improves performance in some memory/cognition tests in AD patients (Henderson et al., 1995; Yaffe et al., 1998; Costa et al., 1999).

Both epidemiological and clinical reports indicate a positive role for estrogen use in Parkinson's disease (PD) (Saunders-Pullman et al., 1999). Reduction of estrogen supplementation in postmenopausal women causes a worsening of Parkinson-related symptoms, and the severity of symptoms in women with early PD is diminished by the application of estrogens (Morale et al., 2006). The neuroprotective acts of estrogen on the dopaminergic system may be indicated by epidemiological reports showing a sex difference in PD, as it is reported that PD has greater prevalence in males (Bourque et al., 2009; Dluzen, 2000; Liu et al., 2005; Miller et al., 1998; Sawada et al., 2002; Sawada et al., 1998).

Experimental investigations strongly support the idea that estrogens may play a neuroprotective role in neurodegenerative processes. Many in vitro studies indicate that

the addition of estrogen to culture media increases viability, survival, and differentiation of primary cultures from different populations, including those from the hypothalamus, amygdala, neocortex or hippocampus. The protective effects of estrogens have been widely reported in many different neuronal cell types against a variety of toxic insults, including oxidative stress (Behl et al., 1995; Borrβs et al., 2010; Gordon et al., 2005; Morale et al., 2006; Ozacmak and Sayan, 2009; Stirone et al., 2005; Strehlow et al., 2003), β-amyloid-induced toxicity (Bozzo et al., 2010; Carbonaro et al., 2009; Yang et al., 2008; Morinaga et al., 2007; Xu et al., 2006; guado-Llera et al., 2007; Ba et al., 2004), and excitotoxicity (Behl et al., 1995; Goodman et al., 1996). The effective concentrations for estrogen-mediated neuroprotection range from a low of 0.1 nM to a high of 100 μM. This points to the existence of differences in the sensitivity to estrogen-mediated protection in different neuronal types. Additionally, many studies have indicated that longer estrogen pretreatment times may increase neuroprotective potency of the hormone.

In addition to the extensive evidence for the neuroprotective effects of estrogens in vitro, there is now evidence for the neuroprotective effects of estrogens in animal models of the neurodegenerative process. Estrogen exposure yields a reduction in the degree of dopamine depletion resulting from treatment with the neurotoxic 6-hydroxydopamine, a model of PD (Murray et al., 2003; Quesada and Micevych, 2004). Moreover, estrogens were also shown to protect nigrostriatal dopaminergic neurons against MPTP-induced neurotoxicity in mice model of PD (Ramirez et al., 2003; Callier

et al., 2001). Estrogens also provided neuroprotection against glutamate-induced neurotoxicity (Sawada et al, 1998).

There are also recent reports that the most frequently prescribed estrogen replacement hormones in the United States, conjugated equine estrogens, are neuroprotective against neuronal cell death induced by β-amyloid$_{(25-35)}$, hydrogen peroxide, and glutamate, and induce neurite outgrowth in cortical, hippocampal, and basal forebrain neurons (Diaz-Brinton et al., 2000; Brinton et al., 2000). This finding raised the possibility that estrogen replacement therapy may also have beneficial neurotrophic and neuroprotective effects on the brain.

1.5.2. Potential mechanisms of estrogen neuroprotective actions

Although, numerous studies demonstrated that estrogens play an important trophic and protective role in brain, it is still unclear the precise mechanisms involved in the neuroprotective effects of estrogens. Many of the neuroprotective effects of estrogens appear to be mediated by the activation of classical estrogen receptor (ER). Several reports have also indicated that estrogen neuroprotective activity is exerted via intracellular receptor-mediated mechanisms. Another plausible mechanism to explain the protective effects of estrogens is related to the ability of the hormone to alter free radical production or act as an anti-oxidant, which is exerted by their chemical structure.

1.5.3. Estrogen receptor mediated mechanism of neuroprotection

The classical mechanism of estrogen action is through one of two estrogen

receptors (ERs), ERα and ERβ, which act as nuclear transcription factors. ERα and ERβ each are composed of several domains: a) a highly variable N-terminal domain containing a transactivation region that activates expression of target genes by interacting with core transcriptional machinery; b) a DNA binding domain that contains two zinc fingers and is involved in receptor dimerization and specific DNA binding; and c) a large and complex ligand-binding domain that is also involved in nuclear localization, receptor dimerization, and interactions with other transcriptional regulatory factors(Witkowska et al., 2008; Kuiper et al., 1997). ERα and ERβ share high sequence homology in their DNA and ligand-binding domains (95.5% and 59.7%, respectively, in the rat), but differ in other domains(Witkowska et al., 2008; Kuiper et al., 1997; Gruber et al., 2002a). When ERs bind estradiol, they can form homo-or heterodimers, which can bind to estrogen response elements (EREs) in DNA and recruit other components of transcription machinery to promote gene expression(Gruber et al., 2002a). Estradiol-bound ERα and ERβ also bind the Fos/Jun complex to regulate gene expression through activated protein-1 sites in a manner that is independent of EREs. Binding assays indicate that estradiol has similar binding affinity for ERα and ERβ. The two forms of ER are structurally and functionally distinct, each regulating unique sets of target genes in a tissue and cell type-specific manner (Kian Tee et al., 2004). This may be due to the net effect of homo- or heterodimerization of ERβ and ERα. Receptor-mediated transcription is also modulated by coregulators (activator and repressor proteins and protein complexes). The numerous coregulator proteins and various selective combinations

associate with ERs and critically determine the region and cell-type specificity of the effects of ER ligands. Furthermore, the recent discovery in rodent and human brains of ER splice variant proteins, which alter gene transcription in a promoter- and ligand-dependent fashion, adds further diversity to ER signaling mechanisms (Chung et al., 2007; Ishunina and Swaab, 2008). The expression, coexpression, and ratio of ERα/ERβ and their splice variants in addition to the presence of combination of coregulatory proteins in any cell, will have a profound influence on the estrogen response.

Steroid hormones have traditionally been thought to act exclusively through nuclear receptors (Levin, 2001). However, recently both nuclear and membrane/cytoplasmic pools of ERα and ERβ have been demonstrated and the receptors have similar affinity for the estrogen ligand. In addition, steroid actions occur at the cell surface, a function that is preserved from plants to humans (Levin, 2001; Kushner et al., 2000). It is widely accepted that membrane ER signaling through kinase cascades, calcium and other second messengers impacts transcription (Watters et al., 2000). Pharmacological and ultrastructural evidence demonstrates that classic ERs can also be localized at the cell membraneto effect rapid activation of intracellular signaling pathways and modulatory proteins within seconds to minutes of exposure to estrogen (McEwen and Alves, 1999; Toran-Allerand et al., 2002; Gorosito et al.,2008; Raz et al., 2008). These effects include increases in intracellular calcium concentrations and activation of protein kinases in the cAMP/camp-dependent protein kinase pathway, the mitogen-activated protein kinase (MAPK or ERK), and the PI3K/Akt pathway. These pathways may interact and converge, to effect gene transcription and protein synthesis

via rapid down-stream activation of transcription factors, such as the CREB or NFκB.

The mechanisms by which activated membrane ERs elicit cellular responses are not yet understood, but interaction with other cell-surface receptors and G-proteins, IGF-1 and glutamate receptors may be a means in which ERs trigger intracellular signaling systems and affect cellular responses (Garcia-Segura et al., 2001, Mermelstein, 2009). Estrogen-activated signaling pathways can also increase mitochondrial efficiency and lead to a reduction in free radical generation in the brain and mitochondrial dependent apoptosis (Nilsen et al., 2007; Brinton, 2008; Chen et al., 2009). Estradiol has been reported to act as an NMDA receptor antagonist (Weaver et al., 1997) and to block calcium entry through the calcium channel (Mermelstein et al., 1996). This inhibition of calcium entry was not mediated by the estrogen receptor but by a plasma membrane receptor for estradiol. It occurs rapidly in seconds after estradiol administration and diminished soon after the removal of estradiol. Furthermore, membrane-initiated and genomic actions of hormones may be coupled. Most of the cellular mechanisms described for estrogen actions, involving MAPK and PI3K/Akt signaling and mitochondrial function, have important roles in cell survival, apoptosis function, neurodevelopment, and may subserve the critical neurotrophic and neuroprotective effects of estrogens in brain physiology and pathological conditions.

Protective effects of estradiol could also be mediated by activation of mitochondrial ERs, which are present in many cell types, especially tissues such as the brain with demand for mitochondrial energy metabolism. Along with nuclear ERs and

their coactivators, mitochondrial ERs are involved in cytoprotection from oxidative stress and regulation of apoptosis (Razmara et al., 2008). It is suggested that a deficiency in this estrogen-dependent mechanism might be related to neurodegenerative disease pathogenesis.

1.5.4. HIV-1 infection in Women

In recent years, the rate of HIV infection in women has been climbing significantly. HIV infection is the third leading cause of death in women between the ages of 25-44y. Women account for half of all people living with HIV worldwide- at the end of 2007 and estimated 15.4 million women were infected with HIV (UNAIDS 2008). The proportion of women living with HIV varies significantly between different regions of the world, with a higher burden in sub-Saharan Africa and the Caribbean. In sub-Saharan Africa women account for nearly 61% of all adults living with HIV, while in the United States only 25% of people with HIV are women (CDC 2007; UNAIDS 2007). Over the last 10 years, the number of women living with HIV globally has remained stable, but in latin America, Asia and Eastern Europe this number is slowly increasing (UNAIDS 2007).

HIV-1 infected women have a lower plasma viral load compared to HIV infected men at similar stages of HIV-1 infection, suggesting that gender differences may affect HIV/AIDS pathogenesis (Sterling et al., 2001; Evans et al., 2001; Farzadegan et al., 1998). These differences suggest a higher risk to AIDS among women with lower viral

load measurements and significantly lesser eligibility of women for therapy after seroconversion according to current guidelines for initiating antiretroviral therapy in women (Zhang et al., 2008). Moreover, there is evidence that women are less likely to start HAART despite equal access to care (Berg et al, 2004; Gilad et al., 2003). With HIV detected early in the brain after infection, delayed initiation of antiretroviral treatment may diminish effectiveness against toxic challenges in the CNS. Alternate therapy, not dependent on viral load, may be considered to circumvent the detrimental effects of HIV infection on neuronal functioning and survival.

Hormone levels are thought to play an important role in the susceptibility and immune responses to HIV-1 infection in women. In women with HIV infection, menstrual abnormalities frequently develop with increased cycle variability, polymenorrhea or amenorrhea (Clark et al., 2001; Farzadegan et al., 1998; Harlow et al., 2000). Additionally, HIV-infected women have been shown to undergo menopause earlier than noninfected women. Perimenopausal and menopausal symptoms may be more prevalent among HIV-infected women (Kojic et al., 2007). Conversely, an estrogen deficient state such as menopause has been associated with a prolonged disease course, and with an increase incidence of AIDS-related dementia. Moreover, a European study showed that women are two times more likely to develop HIV dementia than men (Chiesi et al, 1996).

1.5.5. Estrogen in HIV dementia

Accumulating evidence suggests that estrogen is able to protect against various neurodegenerative insults, including the neurotoxicity of HIV viral proteins. For example, estrogen reduces the extent of cell death in response to a variety of noxious disease-related stimuli such as excitatory amino acids (Sribnick et al., 2004), oxidative stress (Nilsen, 2008; Wallace et al., 2006) and β-amyloid toxicity (Nilsen et al., 2006; Hosoda et al., 2001). These cellular actions may explain its ability to ameliorate cognitive decline and reduce the incidence and progression of neurodegenerative disease. Furthermore, estrogen was shown effective in preventing the neuronal damage associated with Tat-induced toxicity (Kendall et al., 2005; Wallace et al., 2006). Estradiol protected mitochondria in receptor-independent manner that may be related to its anti-oxidant properties. Estradiol was also shown to suppress the proinflammatory effects of HIV proteins (Bruce-Keller, 2001). 17 β-estradiol treatment was also shown to reduce the number of apoptotic neurons and modulate the expression of anti-apoptotic proteins including Bcl-2 in primary neuronal cultures (Nilsen et. al., 2006). The mechanism(s) of estrogen neuroprotection are not fully understood, but may be linked to regulation of proteins that either promote cell survival or induce cell death. Identifying estrogen regulated apoptotic factors may be vital to elucidating the underlying mechanism(s) of estrogen protection.

These observations warrant further studies in HIV infected women with cognitive impairment or at risk for developing dementia. However, because of the potential risk of uterine and breast cancer, estradiol cannot be used in this population. Furthermore, due to

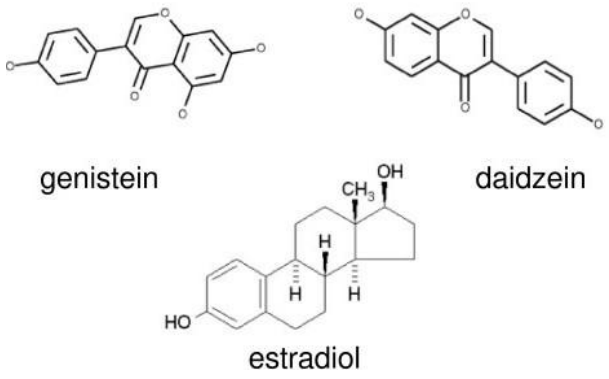
feminizing effects of estradiol, it cannot be used in men or children. Hence it is important to develop compounds that have the neuroprotective effects of estradiol but not the negative side effects.

1.6. Phytoestrogens

In recent years, phytoestrogens have been attracting increasing attention among the public and medical community because of evidence from a large body of literature suggesting that consumption of plant-based foods rich in these compounds may benefit human health (Than et al., 1998; Adlercreutz and Mazur, 1997; Setchel, 1998). Epidemiologic data and clinical experience indicate that estrogen therapy offers protection from cardiovascular disease, reduces the extent of osteoporosis, improves cognitive function and improves menopausal symptoms associated with ovarian estrogen loss. Substantial data from epidemiologic surveys and nutritional intervention studies in humans and animals suggest that dietary phytoestrogens have protective effects against menopausal symptoms and a variety of disorders, including neurodegenerative diseases.

Phytoestrogens are found in various plants consumed by humans, including legumes, seeds, and whole grains. Phytoestrogens in the diet may have a role in modulating hormone-related diseases based on their structural similarity to 17 β-estradiol. The majority of phytoestrogens found in typical human diets can be categorized into two primary classess, isoflavones and lignans. The most abundant food sources of

isoflavones are soybeans and its products. Isoflavones are the most common form of phytoestrogens. Two of the major isoflavones found in humans are genistein and daidzein. Genistein and daidzein are parent compounds, which are metabolized from their plant precursors, biochanin A and formononetin, respectively. In plants, isoflavones are inactive when present in the bound form as glycosides, but when the sugar residue is removed, these compounds become activated. These plant compounds undergo fermentation by intestinal microflora, with both metabolites and unfermented parent compounds now able to be absorbed. In the body, they do not undergo any further metabolism and are excreted in the urine (Sakai et al., 2008). In the colonic microflora, daidzein may be metabolized to equol. Daidzein, genistein, equol, and O-demethylangolesin are the major phytoestrogens detected in the blood and urine of humans and animals.



The molecular structure of biologically active isoflavones resembles that of 17β-estradiol.

1.6.1. Absorption and Bioavailability

It seems the bioavailability of isoflavones, particularly genistein and daidzein,

depends on the gut microflora activity. Despite considerable degradation of isoflavones in the gut, their plasma concentrations are significant. Isoflavones are mainly present as inactive glycosides and after enzymatic processes in the gut by bacteria, they become active compounds, taken up by enterocytes and entering the peripheral circulation (Branca and Lorenzetti, 2005). In humans, there is large variability in genistein and daidzein metabolism, due in part to variable individual intestinal flora and transit time. In an adult, consumption of 50 mg/day of isoflavones may give rise to plasma concentrations ranging from 200-3000 nM. Other studies evaluating plasma concentration of phytoestrogens have reported that plasma levels in humans range from 10 nM to 10 μM.

Numerous factors, such as light, environmental conditions, plant genetics, ripeness and species variety, affect the formation and the content of phytoestrogens in plants. It is also reported that the estimated daily intake of isoflavones differs among countries, ranging from 20-100 mg/day. In Asian countries, an average daily dietary intake of soy and isoflavones is estimated to 50 g and 20-150 mg, respectively. In western countries, intake of isoflavones is considerably lower, at 1g of soy daily and 2mg of isoflavones. Also of note, women consuming the commercially available phytoestrogen preparations are taking doses ranging from 50-150 mg aglycones/day, doses in range of what is consumed in Asian populations.

1.6.2. Effects of soy isoflavones on cognitive function

Various types of neural damage may induce neurodegenerative diseases associated with cognitive decline. Several studies have found that soy isoflavones can improve cognitive functions in both humans and rats, but underlying mechanisms remain unknown (File et al., 2001). Soy isoflavones have an estrogenic effect and it is reported that isoflavones may improve cognitive functions my mimicking the effects of estrogen in the brain (Pan et al., 2000).

Human studies

Although numerous experimental studies have shown that isoflavones are beneficial to cognitive functioning, in the clinical setting results vary on the cognitive effects of isoflavones. Some studies have shown that isoflavones improve cognitive function in postmenopausal women. The SOPHIA study examined cognitive functioning of postmenopausal women receiving soy isoflavones every day over 12 weeks. Women receiving soy supplementation showed significantly higher performance on memory and attention tasks compared to controls (Duffy et al., 2003;). However another study of isoflavone supplementation in postmenopausal women, found no improvement of cognitive functioning after 1 year. Additionally, young healthy adults receiving high dosage of soy showed significant improvement in short and long-term memory and mental flexibility; improvements were seen in both males and females. Although the clinical data examining the effects of isoflavones on cognitive function offer differing results, these clinical trials indicate that soy isoflavones might improve cognitive function

not only in postmenopausal women but also young adult women and males.

Animal studies

Several studies have shown that soy isoflavones improve cognitive function in female rats. In one study, OVX rats given isoflavone containing diets for 10 months showed a dose-dependent improvement in radial arm maze tests (Pan et al., 2000). Another study reported that female rats consuming a lifelong high-isoflavone diet showed acquisition of the radial arm maze faster than female rats consuming a isoflavone free diet (Lund et al., 2001).

The effects of soy isoflavones on cognitive function in male rats remains controversial. It is not yet clear whether isoflavones have beneficial or detrimental effects on cognitive function in male rats. It has been demonstrated that male rats consuming a high isoflavone diet performed worse on a visual spatial memory test than male rats consuming an isoflavone-free diet (Lund et al., 2001). However, it was recently reported that male rats consuming an isoflavone diet significantly outperformed male rats consuming a isoflavone free diet in a spatial delayed matching to place test (Lee et al., 2004).

1.6.3. Soy Isoflavones neuroprotective effects

Several studies have shown that isoflavones have neuroprotective effects against various insults. Genistein has been shown to have an antioxidant effect on neurons exposed to radical damage. In human cortical cell lines, pretreatment with 10 or 50μM of

genistein was found to prevent cell death due to tertiary butylhydroperoxide or oxidative stress (Sonee et al., 2004). Additionally, in hippocampal cells from rats, genistein was found to reverse effects of $A\beta_{25-35}$ on loss of cell viability, DNA fragmentation, intracellular increase in free calcium, the accumulation of reactive oxygen species and caspase 3 activation (Zeng et al., 2004). Another study reported that in thapsigargin-induced apoptosis, associated with mitochondrial dysfunction, DNA laddering, and caspase activation, the addition of genistein was able to decrease the number of apoptotic neurons and the number of neurons containing active caspase-3 (Linford and Dorsa, 2002). Genistein was also found to protect human neuroblastoma cells from Aβ-induced death and attenuated Aβ-induced apoptosis (Bang et al., 2004). Moreover genistein and daidzein were among six phytoestrogens that demonstrated neuroprotective efficacy against glutamate excitotoxicity and $A\beta_{25-35}$ in hippocampal neurons (Zhao et al., 2002).

1.6.4. Potential mechanisms of soy isoflavones actions in the brain

Estrogen receptor-mediated regulation

17β-estradiol, a mammalian estrogen, has been shown to protect neurons and affect cognitive function. It is thought that this effect is associated with estrogen receptors (ERs) in the brain, additionally is also suggested that isoflavones had neuroprotective and cognitive functions in the brain. Estrogen has been shown to affect neuron survival and growth, synaptic plasticity, brain function via a ER-mediated pathway and/or antioxidant properties. The effects of estrogen on nuclear and nonnuclear ERs can modulate brain

functions by regulating gene transcription and second messenger systems. Isoflavones may act in a similar way because they too exhibit estrogenic activity.

The estrogen effects of isoflavones appear to be dose dependent. Some studies suggest that the neuroprotective effects of isoflavones may be mediated by two mechanisms that operate at different concentrations. These studies demonstrate that at the nanomolar level, genistein protects neurons via an ER-mediated pathway, while at the micromolar level, genistein displays antioxidant abilities (Zeng et al., 2004).

As isoflavones can affect the viability of neurons and cognitive function by acting as estrogenic agonist, they can also utilize differential distribution and regulation of the ER subtypes, ERα and ERβ in the brain. The binding affinity for ERβ is higher than that for ERα by a factor of 20-fold in vitro. Additionally, genistein has been shown to affect ERβ- but not ERα-dependent gene expression in the hypothalamus. However, it is still unclear which ER subtype mediates the neuroprotective efficacy of estrogen/phytoestrogen. Treatment with 17β-estradiol prevented the apoptotic neuron death, and resulted in the strong expression of ERα and increased bcl-2 in primary cultures from rat brain (Harms et al., 2001). Several lines of evidence also suggest that ERβ is involved in neuroprotection. Estradiol exerted neuroprotective effects on dopaminergic neurons by suppressing proapoptotic gene transcription through the AP-1 site via activation of ERβ (Sawada et al., 1998). Genistein was also shown to attenuate neuronal apoptosis via an ERβ-mediated pathway. In this study, only ERβ was detected in a neuron culture, thus it was suggested that the neuroprotective effect of isoflavone is

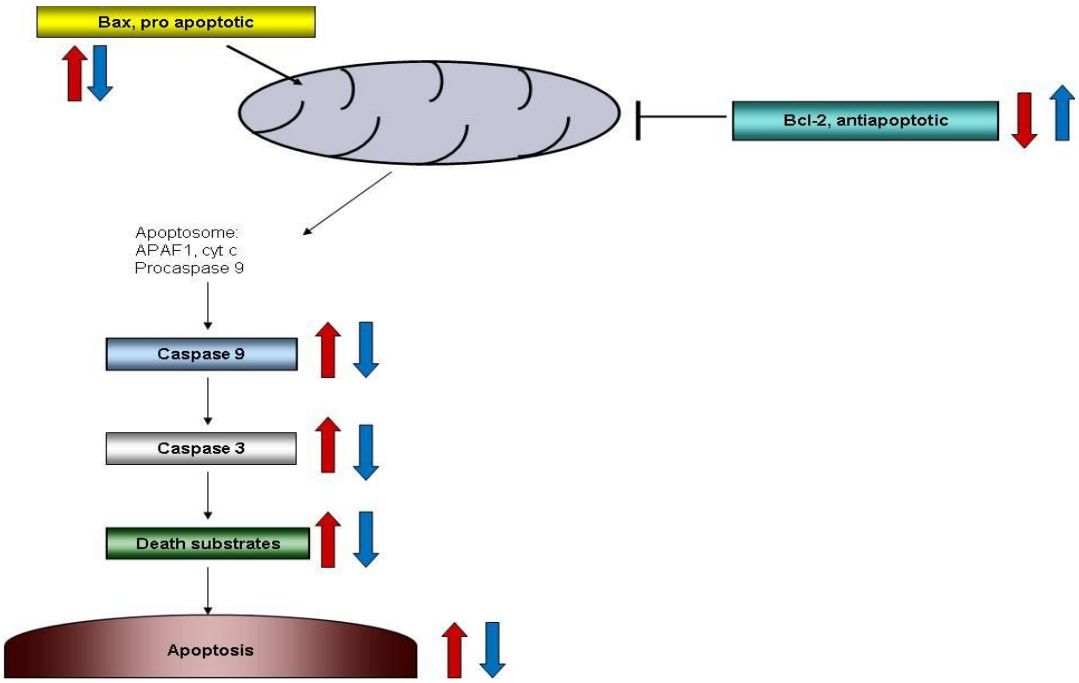
mediated through ERβ. However, there have been reports that demonstrated that both ER subtypes are involved in neuroprotective actions. Both ERα and ERβ agonists were found to protect hippocampal neurons from glutamate-induced neurotoxicity (Zhao et al., 2004).

Like estradiol, phytoestrogens are thought to have neuroprotective effects due to the ability to bind estrogen receptors and mediate estrogenic processes. Phytoestrogen preferential binding to ERβ is of significant consideration in neuroprotection as this ER subtype is highly expressed in the brain compared to ERα, which due to its high expression in the reproductive tissue, has been associated with the proliferative effects of estrogen. Elucidating whether phytoestrogen neuroprotection is mediated by ER selectivity is a central focus in research of neurodegenerative diseases.

Summary

Neuronal injury is a primary basis of many neurodegenerative diseases, including HIV-1 associated dementia (HAD). The HIV-1 viral protein Tat is thought to play a significant role in HIV related neuropathology and involving mitochondrial dysfunction and the potentiation of neuronal apoptosis. Hormone levels are thought to play an important role in the susceptibility and immune responses to HIV-1 infection in women. Estrogen deficiency has been associated with increased risk of neurodegenerative disease, including HAD. Accumulating evidence suggests that estrogen is able to protect against various neurodegenerative insults, which are mediated by two estrogen receptor (ER)

isoforms, ERα and ERβ. Despite its positive actions, the benefits of estrogen therapy on women's health are overshadowed by its association with an increased risk of reproductive cancers and cardiovascular disease. However, soy phytoestrogens represent a viable alternative to estrogen therapy as they have been shown to bind to ERs and affect estrogen mediated processes. In the current experiments, we seek to determine if treatment with 17 β-estradiol attenuates HIV-1 Tat-induced mitochondria associated apoptotsis in cortical cell cultures**;** whether estrogenic effects against Tat –induced mitochondria mediated apoptosis occurs through receptor mechanisms; and lastly, if phytoestrogens are equally effective as endogenous estrogen 17 β-estradiol against Tat-induced apoptotic signaling**.** We proposed that estrogen neuroprotection against HIV-1 viral protein Tat-induced neural toxicity involves ER-mediated attenuation of apoptotic signaling. Furthermore, soy phytoestrogens can mimic estrogen effects against Tat-induced apoptosis by targeting ER-subtype specific effects**.**



Mitochondria-mediated apoptotic cascade**.** HIV-1 Tat exposure (red arrows) has been shown to induce mediators of the apoptotic cascade leading to subsequent cell death**.** However, the antiapoptotic effects of estrogen (blue arrows) has been shown to involve regulation of Bcl-2- related proteins and downregulation of caspase activity.

CHAPTER 2

Experiment 1. To determine 17 β-estradiol effects on HIV-1 Tat –induced apoptotic signaling in primary cortical cell cultures.

2.1. Introduction

Estradiol is known to have protective properties for neural cells during injury and chronic diseases including neuro-AIDS (Nilson, S. et al., 2001; Wilson et al, 2006). It reduces the extent of cell death in response to variety of noxious stimuli such as excitatory amino acids, oxidative stress and β-amyloid toxicity by both in vitro and in vivo methods (Behl, 2002). Furthermore, estrogen was shown effective in preventing the neuronal damage associated with Tat-induced toxicity (Turchan et al., 2001; Kendall et al., 2005; Wallace et al., 2006). Estradiol treatment was also shown to reduce the number of apoptotic neurons and regulate the expression of anti-apoptotic proteins including Bcl-2 in primary neuronal cultures (Nilsen et. al., 2006). Studies have also shown that estrogen administration was able to attenuate caspase activation, which is responsible for many of the biological and morphological features of apoptosis, and mitochondrial signal transduction pathways induced by neurotoxic insults (Zhang et al., 2005). These cellular actions may explain its ability to ameliorate cognitive decline and reduce progression of neurodegenerative disease. As mitochondria play a central role in

apoptosis and neurodegeneration, we will further assess if Tat exposure induces mitochondria mediated apoptotic cell death. We will determine if the addition 17 β-estradiol prior to Tat exposure attenuates Tat-induced cell death signaling.

2.2. Experimental Design

Recombinant Tat 1-86 (50 nM) (Diatheva, Italy) was added to culture medium. To assess neurotoxicity, cortical cell cultures were exposed to Tat for 4, 24, 48, or 72 h for cell viability assays. In assessing estrogen effects against Tat toxicity, 17 β-estradiol (10 nM, Sigma) was added to neuronal culture 24 h prior to Tat exposure. Tat induction of apoptotic pathway was assessed by total caspase activity using FLICA in live Tat exposed cultures. A direct interaction of Tat and apoptotic neurons was determined via immunocytochemisty using antibodies against Tat and active caspase 3. Enzyme -linked immunosorbent assay (ELISA) was used to quantify expression of mitochondria apoptotic pathway regulatory proteins, pro- and anti-apoptotic Bcl-2 family proteins Bax and Bcl-2 respectively and active caspases in fetal rat cortical cultures exposed to Tat. We determined if Tat exposure increases expression of mitochondrial apoptotic markers Bax, Bcl-2 and caspase 9 and caspase 3, and whether estrogen effects on Tat-induced cell death involve attenuation of the mitochondria mediated apoptotic cascade. Data was analyzed with a one way ANOVA and planned comparison tests.

2.3. Materials and Methods

2.3.1. Cortical cell culture

Neuronal cultures were prepared from 18-day-old Sprague-Dawley rat fetuses. Rat cortex were dissected and incubated for 15 min in a solution of 2 mg/ml trypsin in Ca^{2+} and Mg2+ - free hanks balanced salt solution (HBSS) buffered with 10 mM HEPES (Invitrogen, Carlsbad, CA). The tissue was then exposed for 2 min to soybean trypsin inhibitor and rinsed three times with HBSS. Cells were dissociated by trituration and distributed to poly-L-lysine coated culture plates. At the time of plating each well contained DMEM/F12 medium supplemented with 100 ml/l fetal bovine serum. After a 24 h period, the DMEM/F12 medium was replaced with 2% v/v B-27 Neurobasal medium supplemented with 2 mM GlutaMax and 0.5% w/v D- (+) glucose. Cultures were used for experiments after 12 days in culture.

2.3.2. Cell Viability

Neuronal survival was determined using a Live/Dead viability/cytotoxicity kit (Molecular Probes, Eugene, OR) in rat fetal cortical cell cultures prepared in 96-well plates. In accordance with the manufacturer's protocol, neurons were exposed to cell-permeant calcein AM (2 μM), which is hydrolyzed by intracellular esterases, and to ethidium homodimer-1 (4 μM), which binds to nucleic acids. The cleavage product of calcein AM produces a green fluorescence ($F_{530\ nm}$) when exposed to 494-nm light and is used to identify live cells. Bound ethidium homodimer-1 produces a red fluorescence ($F_{645\ nm}$) when exposed to 528-nm light, allowing the identification of dead cells. Fluorescence

was measured using a Bio-Tek Synergy HT microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). Each individual $F_{530\ nm}$ and $F_{645\ nm}$ value on a plate was corrected for background fluorescence (readings obtained from cell cultures (wells) that were not exposed to calcein AM and ethidium bromide) by the microplate reader KC4 software package (Bio-Tek Instruments, Inc., Winooski, VT). For each individual cell culture (well) on a plate, ratios between corrected green and red fluorescence ($F_{530\ nm}/F_{645\ nm}$, Live/Dead ratios) were calculated. All individual relative numbers of live and dead cells were expressed in terms of percentages of average maximum Live/Dead ratio determined for the set of non-treated control cell cultures (8–16 wells) from the same plate: $[F_{530\ nm}/F_{645\ nm}]_{well\ n}/[F_{530\ nm}/F_{645\ nm}]_{average\ max} \times 100\%$.

2.3.3. Apoptosis detection

Following Tat treatment, neuronal apoptosis was analyzed by the Fluorochrome Inhibitor of Caspases (FLICA) Poly-Caspase (green fluorescence) Apoptosis Detection Kit (Immunochemistry Technologies LLC, Bloomington, MN). Poly-caspases Detection Kit contains a carboxyfluoroscein-labeled inhibitor of total caspase activity (CR-VAD-fluoromethyl ketone). VAD is an amino acid sequence targeted by all caspases. When covalently coupled to caspases, the dye is retained within the cell and the fluorescent signal is a direct measure of the number of active caspase enzymes that were present in the cell at the time the reagent was added. FLICA solution was added directly to the cell incubation medium and the plate was returned to the incubator for 1 additional hour. After 1h, Hoechst stain was added to the medium for neuronal nuclei

counterstaining and incubated for an additional 3-5 min at 37°C. Cells were washed twice with wash buffer supplemented with the kit. For the microplate readings of total caspase activity, rat fetal cell cultures were prepared in black wall 96-well culture plates (Costar). Green fluorescence produced as a result of interaction of CR-VAD fluoromethyl ketone with active caspases in Tat-treated and control cultures was determined using 488/530 nm filter set. Sensitivity was automatically adjusted to low signal wells that contained no fluorescent inhibitor. Blue fluorescent Hoechst neuronal nuclei counterstain was used to normalize differences in cell density between individual wells.

2.3.4. Immunocytochemistry

For immunocytochemistry experiments, Tat-treated cultures and non-treated controls were fixed in acetic alcohol (95% ethanol, 5% acetic acid) for 10 min and washed three times (5 min/wash) with Dulbecco phosphate-buffered saline (D-PBS): Na2HPO4 (1150mg/L), KH2PO4 (200 mg/L), NaCl (8000 mg/L), KCl (200mg/L) at pH 7.4. Following fixation, cultures were blocked with 10% normal horse serum (NHS) in PBS and used for the analysis.

2.3.5. Anti-Tat and active caspase immunofluorescence

Rabbit polyclonal Tat antibody (Diatheva, Italy) was used to determine Tat 1-86 immunoreactivity. Mouse monoclonal active caspase 3 antibody was used to determine caspase 3 activity. Primary antibodies were diluted 1:500 in 1% NHS/PBS. Cell cultures

were incubated with primary antibodies overnight at 4°C. Each set of experiments included cell culture dishes which were left without primary antibody. These cell cultures served as controls for non-specific binding of secondary antibodies. After incubation with primary antibodies was complete, plates were washed three times (5min/wash) with PBS. For the immunofluorescent detection of Tat immunoreactivity goat anti-rabbit IgG conjugated with Alexa 594 dye (Molecular Probes) diluted 1:500 in 1% NHS/PBS was used. Plates were incubated with secondary antibodies for 1h at room temperature and then washed 3 times with PBS.

Microscopic images of Tat and active caspase 3 immunoreactivity in primary cell cultures were captured using the computer-controlled inverted fluorescent microscope (Nikon Eclipse TE2000-E) under 20x magnification and analyzed using NIS-Elements BR 2.30 imaging software package (Nikon). Images of specific Tat immunofluorescence were merged with the subsequent differential interference contrast (DIC) images.

2.3.6. Detection of apoptotic proteins (ELISA)

Cortical cell cultures were prepared as described previously. 17 β-estradiol (10 nM, Sigma) was added to neuronal cell cultures 24 h prior to Tat exposure. After 24h estrogen pretreatment, recombinant Tat 1-86 (50 nM, Diatheva, Italy) was added to culture medium. Cell cultures were then exposed to Tat for 4, 16, and 24h before cell harvesting for ELISA experiments. Cell lysates were prepared from cultures grown in 24-well plates. At the time of harvesting, medium was removed and cells were washed three

times with Dulbecco phosphate-buffered saline, D-PBS, (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.137 M NaCl and 2.7 mM KCL at pH 7.4) and lysed with CellLytic ^{TM}- M mammalian cell lysis buffer (Sigma Chemicals) containing protease inhibitors (protease inhibitors cocktail, Sigma Chemicals). All samples in a group (6 sister culture wells) were pooled together and protein concentration was determined by BCA method (Pierce, Rockford, Ill.). Each well of Costar 96-well ELISA plates (Corning Inc, PA) was coated overnight at 4 °C using 100 µl of 20 mM carbonate coating buffer, pH 9.6. Cell culture lysates were diluted 1:10 with D-PBS and 2 ug of each sample were added to the plate wells in triplicates. After overnight incubation at 4 °C, plates were rinsed 5 times with PBST (0.05% Tween 20 in PBS, pH = 7.4) and blocked with 1% BSA in PBS for 2 h at room temperature. After blocking, plates were washed again as described above and primary anti-Bax, anti-Bcl-2, anti- active Caspase 9 and anti- active Caspase 3 antibodies (Abcam, Cambridge, MA) diluted 1:5000 in 0.1% BSA-PBST were added to each well except for blanks and no-primary antibody control wells. Plates were kept overnight at 4 °C. When the incubation with primary antibodies was completed, plates were again washed 5 times with PBST and secondary antibodies (goat anti-rabbit alkaline phosphatase conjugated, Sigma) diluted 1: 2000 in 0.1% BSA-PBST were added to each well except for blank and no-secondary antibody control wells. After 2 h of incubation, secondary antibody solution was removed, plates were washed 5 times with PBST and 100 µl of BluePhos phosphatase substrate mixture (KPL Research, Gaithersburg, MD) was added to the plate wells. After 30 min of incubation, the absorbance at 650 nm was determined using a Bio-Tek Synergy HT microplate reader. Multiple readings were taken

within a 1-h time period.

2.4. Statistical Analysis

Statistical comparisons were made using one way ANOVA and Tukey's multiple comparison tests were used to determine specific treatment effects. Significant differences were set at $p<0.05$. Data represent percent of control values.

2.5. Results

2.5.1. Estrogen delays cell viability changes in primary neuronal cultures exposed to Tat 1-86

Previous studies have demonstrated that 17 β-estradiol was protective against neurotoxicity induced by HIV-1 viral proteins in cell culture (Kendall et al., 2005; Turchan et al., 2001), specifically using Tat 1-72. We evaluated the ability of 17β-estradiol to protect against recombinant HIV Tat1-86 -induced toxicity,the more stable tat variant, derived from the first and second exons of the tat gene. Preliminary dose response studies demonstrated no significant differences in protective efficacy of doses ranging from 10 nM-100 nM of 17β-estradiol. Additionally, prior studies have shown that 10nM dose of 17 β-estradiol was effective against HIV protein toxic insults in vitro (Kendall et al., 2005). Significant decreases in cell viability were detected in Tat-treated cultures after 48h and 72h, $p<0.05$. Pre-incubation with 10 nM 17 β-estradiol attenuated

the neurotoxic effects of HIV Tat 1-86 (**Figure 2.1**). Treatment with estradiol prevented the 20% reduction in cell viability observed in cultures treated with Tat alone. Repeated measures ANOVA revealed significant main effects of time [$F(3,27)=29.65$, $p<0.05$] and treatment [$F(1,9)=7.69$, $p<0.05$] and a significant time and treatment interaction [$F(3,27)=7.41$, $p<0.05$]. Specifically, 17 β-estradiol was effective in delaying the onset of Tat-induced cell death, as a significant difference in cell viability between Tat-treated vs. Tat + 17 β-estradiol cultures was detected at 48h and 72h, $p<0.05$.

2.5.2. Caspase activation in rat primary neuronal cell cultures exposed to Tat1-86

Execution of the apoptotic pathway leading to DNA degradation, subsequent cell death, involves activation of the caspase cascade. Results of microplate fluorescent detection of total caspase activity in cell cultures exposed to 50nM dose of Tat 1-86 for 2, 24, and 48 h are shown in **Figure 2.2A**. Significant increases in total caspase activity were detected in cell cultures exposed to Tat for 2h (127±11%, $p<0.05$) and 24 h (145±8.4, $p<0.05$). In cell cultures treated with 50 nM Tat for 48 h or longer, total caspase activity was not different from controls.

2.5.3. Tat immunoreactivity colocalize with active caspase expression in neuronal culture

We determined if Tat induction of cell death involves direct interaction of Tat with apoptotic neurons. **Figure 2.2B** demonstrates the immunodetection of cell-bound Tat immunoreactivity and active caspase 3 immunoreactivity in neuronal cultures exposed to 50 nM Tat for 2h. These results indicate the colocalization of bound Tat 1-86

with activated caspases in cultured neurons.

2.5.4. HIV/Tat exposure increases Bax, Bcl-2, and caspase activation in neuronal culture

Prior experiments have demonstrated that Tat treatment induced apoptosis in primary cell cultures (Aksenova et al., 2009; Bonavia et al., 2001). This Tat induced apoptosis may involve activation of the mitochondrial mediated apoptotic pathway. Therefore, we assessed markers specific to the mitochondrial apoptotic pathway including active caspase expression and pro- and anti-apoptotic proteins, Bax and Bcl-2 at different time points following exposure to 50nM Tat 1-86 (**Figure 2.3**). Results indicate that active initiator caspase 9 protein levels were significantly increased early (4h) after Tat exposure (122±9%, $p<0.05$); levels at later time points decreased and were not significantly different from untreated controls. Tat also induced a significant increase in active caspase 3 expression early (4h) (208 ± 9%, $p<0.05$) after treatment, and this increase was maintained throughout later time points, 16h (187± 7%, $p<0.05$) and 24h (211±7, $p<0.05$).

We also observed the expression of upstream apoptotic regulatory proteins Bcl-2 and Bax, which monitor mitochondrial membrane permeability and thus the release of apoptotic factors from mitochondria involved in caspase activation. Results indicated that Tat treatment caused a ~60% increase in pro-apoptotic Bax expression at 4h ($p<0.05$) that was maintained until 16h of exposure ($p< 0.05$) (**Figure 2.3**). Additionally, anti-apoptotic Bcl-2 protein levels reached significantly high levels only after 16h of Tat exposure (147±10%, $p<0.05$). No significant changes were seen at 4h or 24h of Tat

exposure, as levels were similar to that of controls.

2.5.5. 17 β-estradiol attenuates Tat-induced expression of Bcl-2, Bax and active caspases in neuronal cultures

We examined the effects of 17β-estradiol against Tat –induced Bcl-2 and Bax protein expression. Our findings demonstrate that pretreatment with estrogen caused a significant reduction in Tat-induced expression of Bax vs. cultures treated with Tat alone (83 ± 4% vs. 131±8%, $p<0.001$) (**Figure 2.4A**). Addition of 17 β-estradiol completely reversed Tat-induced changes in Bax, as exhibited by an approximately 45% reduction in Bax protein levels, relative to those generated by Tat alone. Results indicated that estrogen administration attenuated the Tat-induced increase in Bcl-2 observed at 16h of exposure (**Figure 2.4B**). Compared to cultures treated with Tat alone, cultures treated with estradiol (10nM) 24h before Tat exposure displayed a significant 20% decrease in Bcl-2 expression (103±7% vs. 123±7% tat-treated cultures, $P < 0.05$).

Caspase activation is known to be a critical phase in the cell death process. Activation of initiator caspases, such as caspase 9 leads to the further activation of effector caspase 3, which triggers the final proteolytic phase of apoptosis (Green, 1998; Haeberlein, 2004; Spierings et al., 2005). We examined if the addition of estradiol could eliminate Tat-induced increases in active caspase 9 and active caspase 3 expression. Results indicated that pretreatment with 10nM 17 β-estradiol significantly attenuated early (4h) Tat-induced caspase 9 expression ($p<0.05$) (**Figure 2.5A**). We then determined

if the inhibition of Tat-mediated caspase 9 activation by estrogen corresponded with reduced active caspase 3 immunoreactivity. Our results show that estrogen treated cultures displayed modest though significant attenuation of caspase 3 expression at 4h (141±4 vs. 166±8 tat-treated cultures, $p<0.05$), 16h (130±4 vs. 159 tat-treated cultures, $p<0.05$) and 24h (136±4 vs. 160±5 tat-treated cultures, $p<0.05$) (**Figure 2.5B**). However, estrogen down regulation of caspase 3 was not complete to the level of untreated controls.

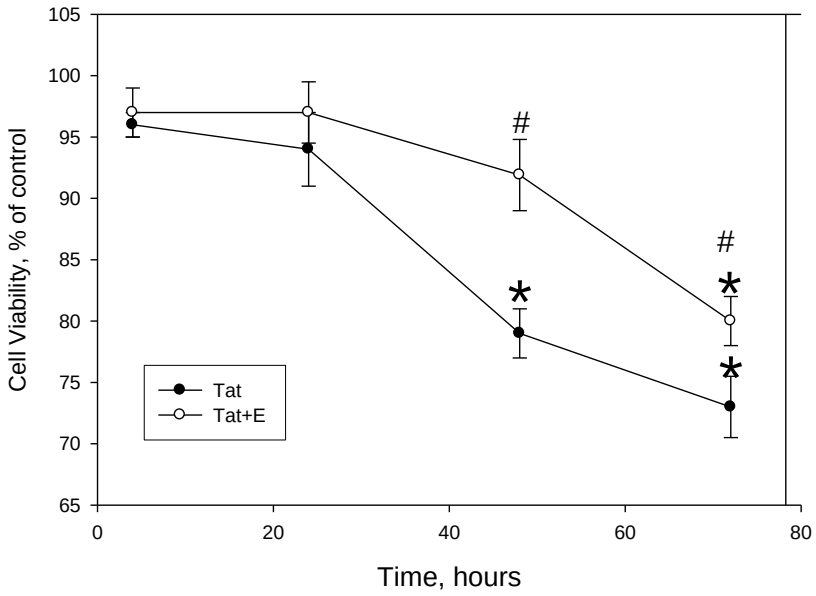
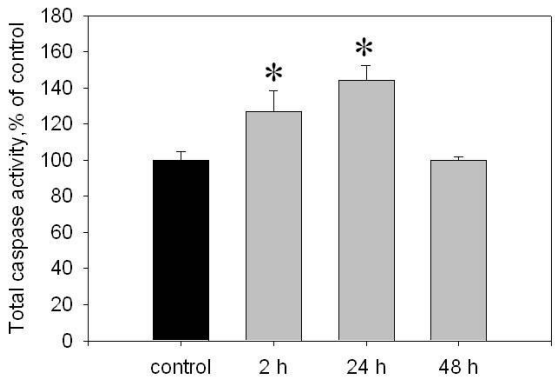


Figure 2.1 Estrogen treatment delays HIV-1 Tat 1-86 mediated decrease of cell viability in primary rat cortical cultures. The graph demonstrates the decrease in live cells in primary cultures following exposure to 50 nM Tat 1-86. Addition of 10nM 17β-estradiol 24h prior to Tat 1-86 exposure was able to significantly delay onset of cell death in primary neuronal cultures. Data presented as mean values, n of sister cultures analyzed 5-10 per each time point. *$p<0.05$ as compared to non-treated controls, #$p<0.05$ as compared to Tat-treated vs. Tat+E treated cultures.

A.



B.

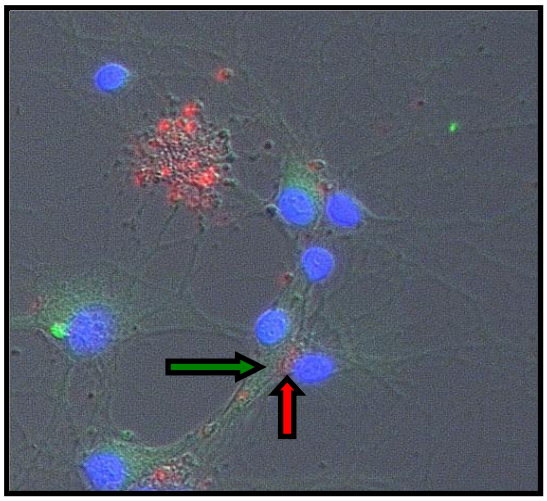


Figure 2.2. Caspase activity in rat fetal cortical cultures exposed to 50nM Tat1-86. (A). Time course of total caspase activity in cortical cell cultures continuously exposed to 50 nM Tat 1-86. Data presented as mean values±SEM, n of sister cultures analyzed 7-12 per each time point. *$p<0.05$ (B). Microscopic image of Tat immunoreactivity (red fluorescence) and active caspase 3 (green fluorescence) immunoreactivity in primary cortical cultures treated for 2h with Tat.

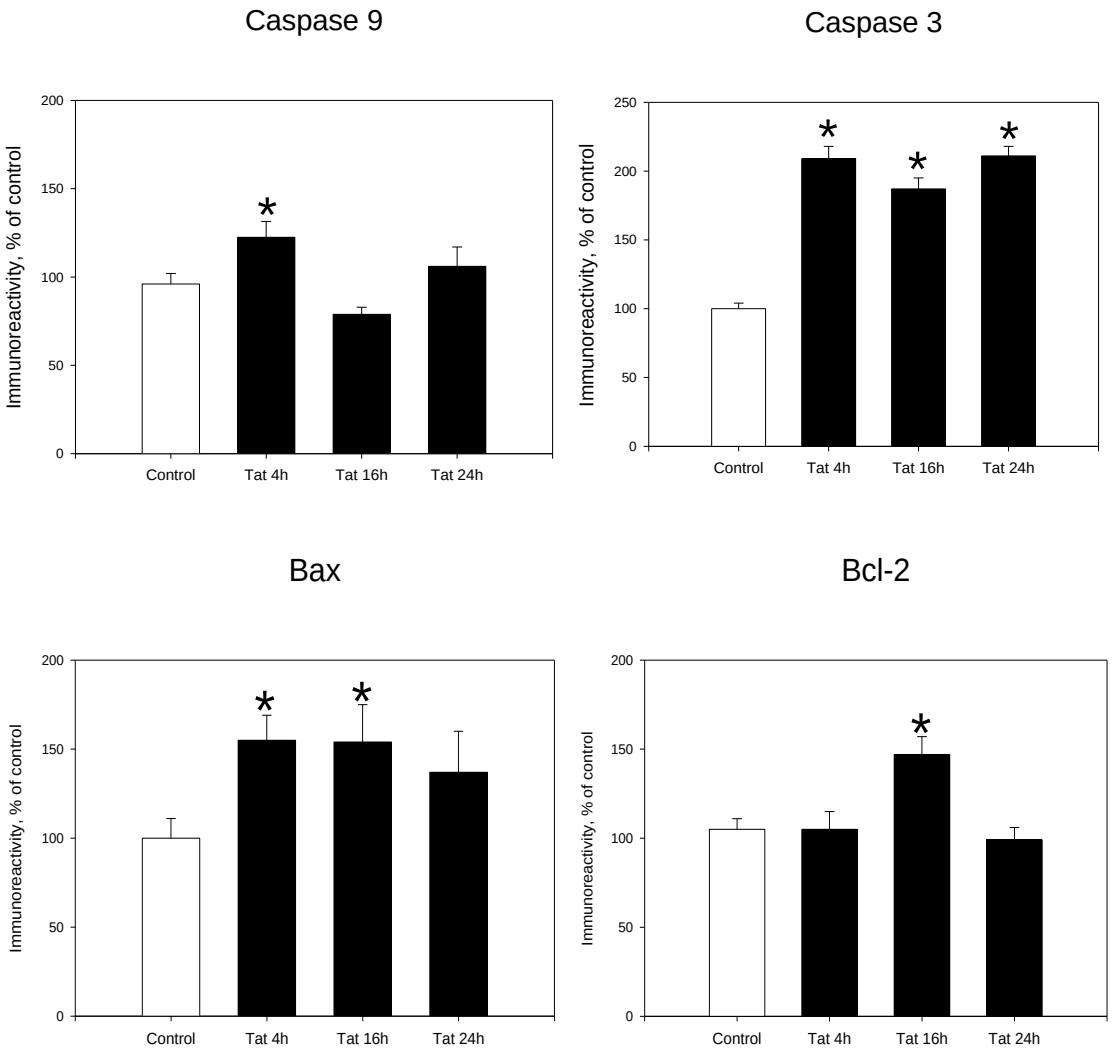
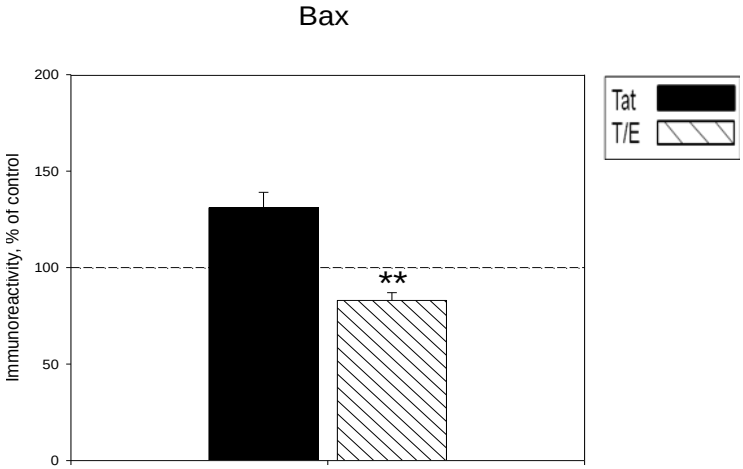


Figure 2.3. Time course of apoptotic protein expression following Tat exposure. Cortical lysates were collected at 4h, 16h and 24h following Tat 1-86 (50 nM) exposure. Expression of Bcl-2 (anti-apoptotic), Bax (pro-apoptotic), active Caspase 9 (initiator) and active Caspase 3 (effector) were assessed by ELISA experiments. Results are presented as % of control values, experiments performed in triplicate, *p< 0.05 as compared to controls.

A.



B.

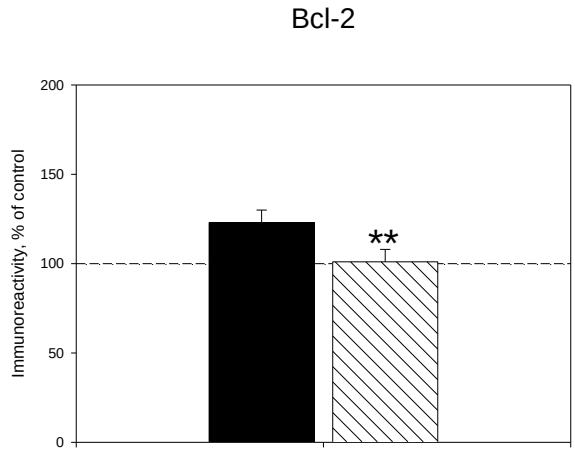
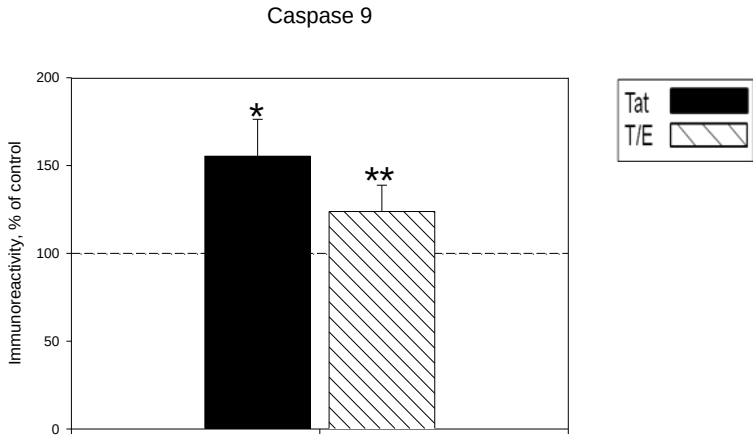


Figure 2.4. 17β-estradiol attenuates Tat-induced expression of Bcl-2 and Bax protein levels. Cortical cultures were treated with 10 nM 17β-estradiol 24h prior to Tat exposure. Expression of apoptotic proteins **A.** Bax (4h Tat exposure) and **B.** Bcl-2 (16h of Tat exposure) were assessed by ELISA experiments. Results are presented as % of control value, with experiments performed in triplicate, *p<0.05 as compared to controls, **p< 0.05 as compared to Tat-treated cultures. Legend box: Tat (T), Tat+Estrogen (T/E). Reference line in graph represents control group.

A.



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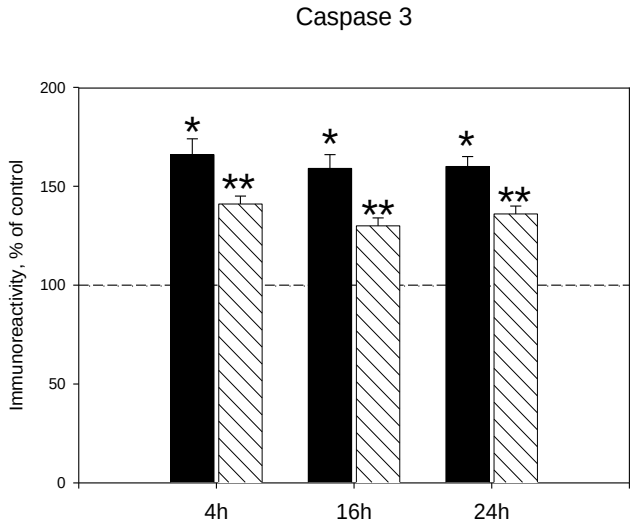


Figure 2.5. 17β-estradiol attenuates Tat-induced increases in active caspase expression Cortical cultures were treated with 10 nM 17β-estradiol 24h prior to Tat exposure. Expression of active **A.** Caspase 9 (4 h of Tat exposure) and **B.** Caspase 3 were assessed by ELISA experiments. Results are presented as % of control value, with experiments performed in triplicate, *p<0.05 as compared to controls, **p< 0.05 as compared to Tat-treated cultures. Legend box: Tat (T), Tat+Estrogen (T/E). Reference line in graph represents control group.

2.6. Discussion

The neuroprotective actions of estrogen have been demonstrated in many experimental models of neurodegenerative disease, including various dementias (Pike et al., 2009; Wilson et al., 2006). However, the mechanisms underlying the beneficial effect of estrogen are not well understood (Green & Simpkins, 2000). In this study, we sought to determine if estrogen attenuates Tat-induced cell death by inhibiting caspase cascade activation and modulating levels of anti-apoptotic and pro-apoptotic Bcl-2 related proteins, key regulators of mitochondrial/intrinsic apoptotic signaling.

Extensive cortical neuron damage and dropout is a significant consequence of NeuroAIDS, likely stemming from susceptibility of cortical cells to the various toxic mechanisms attributed to HIV-1 viral infection, contributed by the protein Tat. Tat may play a role in HIV-1 related neuropathology as prior studies have demonstrated the presence of Tat mRNA and protein in patients with HIVE and HAD. AIDS dementia is characterized by neuronal loss associated with synaptic damage (Weiss et al., 1999) and Tat exposure in vitro has been shown to induce similar pathology (Kim et al., 2008). As HIV-infected individuals are living longer, the rate of CNS disease is actually increasing, as well as the incidence of cognitive impairment, intensifying the need for therapeutic interventions capable of supporting neuronal functioning and viability.

In the present study , we used primary fetal rat cortical cell cultures to demonstrate that HIV- 1 Tat 1-86 can induce neuronal cell death by apoptotic mechanisms and that the process can be reversed or inhibited by 17β-estradiol. Our

results demonstrate that Tat exposure induced delayed cell death, as significant decrease in cell viability does not become apparent until 48h of Tat exposure. This suggests that in Tat-induced cell death, susceptible neurons are primed for death. Previous studies in our lab and others, demonstrate that Tat is rapidly taken up into cells with maximal absorption/binding detected at 2hr of Tat exposure. This coincides with our findings of increased ROS production and a decrease of mitochondrial membrane potential, and activation of caspases early (2h) after Tat exposure. These observations indicate that Tat initiate toxic events that primes susceptible neurons for cell death.

Estrogen pretreatment significantly attenuated Tat-induced cell death, thus it is plausible that estrogen effect on cell death involves attenuation of Tat neurotoxic mechanism(s). The delay in cell death represents a critical window of opportunity for therapeutic intervention to augment Tat-induced cell death. Understanding the toxic challenges associated with Tat exposure essential to preventing subsequent loss of cell viability and function. Although HIV-1 Tat has been shown to be neurotoxic, information about the time course of the development of Tat-mediated neurodegeneration is very limited. Direct interactions of Tat with cultured neurons, which includes binding of Tat to neuronal cell membrane, the uptake of the protein and translocation of Tat into the nuclei, require a very brief exposure time (Chandra et al., 2005; Aksenova et al., 2009). Studies in our lab demonstrate that the absorption of Tat by rat fetal neurons occurs within min after the addition of Tat to the culture medium. Previously, we reported increased ROS production and mitochondrial dysfunction following 2h exposure cell cultures to 50nM Tat 1-72 (Aksenov et al.,2006). In the current experiment we

demonstrate an increase in total caspase activity in 2hr of Tat exposure that was maintained over 24h. Levels of active caspases returned to that of controls by 48h. The loss of caspase activity coincides with significant decrease of cell viability, implicating that the dead neurons were expressing active caspases. As neurons are dying, caspase activity is loss.

Indeed the time period during which Tat 1-86 immunoreactivity is detectable in cell lysates overlaps with the increase in early and total caspase activities. Moreover, immunocytochemistry experiments detecting Tat and caspase 3 immunoreactivity demonstrate the colocalization of bound/internalized Tat with activated caspases in neurons. These observations confirm the theory that direct interactions of Tat induce oxidative stress-dependent apoptosis in neurons (Kruman et al., 1998; Aksenov et al., 2006; Aksenova et al., 2009).

Additionally, we determined that Tat –induced cell death was mediated by activation of caspases, a family of enzymes involved in signal transduction of apoptotic stimuli and cellular disassembly (Stennicke and Salvesen, 2000). Caspase 3, the downstream effector of the caspase cascade, is activated in several neurodegenerative disorders (Garden et al., 2002, Namura et al., 1998; Su et al, 2000). Elevated active caspase 3 immunoreactivity was reported in cerebrocortical neurons from patients with HAD and in cultured rodent neurons exposed to HIV viral proteins (Zheng et al., 1999). Our results demonstrate that active caspase 3 expression was significantly increased early following Tat exposure and these elevated levels were maintained for 24h after Tat

exposure. These findings support our findings that detected increases in total activated caspases in living cells and demonstrated increases in total caspase activity early after Tat exposure, peaking at 24h (Aksenov et al., 2009).

Previous studies have shown that the neurotoxic capabilities of Tat include inducing oxidative stress and disrupting mitochondrial membrane functioning (Aksenov et al., 2006; Aksenova et al., 2006; Kruman et al., 1998). Moreover, the mitochondria play a central role in mediating apoptotic signaling (Kroemer et al., 2007; Danial &Korsmeyer, 2004; Green& Kroemer, 2004:1998). As such, we determined if Tat-induced caspase 3 activation corresponds with the expression of key regulatory proteins linked to mitochondria mediated apoptotic pathway in cultured cortical neurons. Following the addition of Tat 1-86 to primary cultures, increased expression of active caspase 9, an initiator caspase associated with mitochondria-mediated apoptosis pathway, was apparent after 4h of Tat exposure. The activation of caspase 9 was detected as early as 2h following Tat treatment in living cells (Aksenova et al., 2009). However, active caspase 8 immunoreactivity, associated with extrinsic/death receptor mediated apoptotic cell death was not detected in Tat exposed cortical cultures (data not shown). Lack of caspase 8 activation in Tat treated cultures suggests that the death- receptor initiated apoptotic cascade may not play a major role in our model of Tat -induced caspase-dependent apoptosis.

Consistent with the suggestion that mitochondrial intrinsic pathway contributes to Tat-mediated apoptosis, our results demonstrated that pro-apoptotic Bax protein levels

were significantly increased early after the addition of Tat 1-86 to neuronal cultures. This increase in Bax expression was concurrent with expression of active caspases. Moreover, in our culture model, a ~20-30% reduction in cell viability with Tat exposure has been observed (Aksenova et al., 2009). Even with prolonged exposure and with Tat maintaining its toxicity, only a particular population of neurons appeared to be Tat-sensitive and die, whereas the remaining cells were resistant to Tat-toxicity. We observe that rat fetal neuronal cell cultures contain a subpopulation of neurons in which Tat interactions are able to induce a caspase-dependent apoptotic cascade. It is likely that the early induction of pro-apoptotic signals (Bax, caspase activation) may increase the susceptibility of Tat-sensitive cells to cell death. However, we found that an increase in anti-apoptotic protein Bcl-2 followed this induction of Bax expression and caspase activation. The significant increase in Bcl-2 expression may mediate a neuronal survival promoting response and thus reflect a compensatory mechanism to impede further apoptotic signaling and maintain the viability of remaining neurons.

Although Tat-induced apoptosis has been associated with caspase activation (Kruman et al., 1998), alternate mechanisms of cell death may also be activated by Tat toxicity. Caspase-independent apoptosis is well established and suggest the induction of alternate execution pathways. NMDA receptors are known to play the key role in the mechanism of Tat-mediated apoptosis (Haughey et al., 2001; Perez et al., 2001; Eugenin et al., 2007; Kim et al., 2008; Aksenova et al., 2009). Activation of NMDA receptors led to the development of apoptosis without involvement of caspases, due to the direct action of apoptosis-inducing factor (AIF) on neuron nuclei (Evstratova et al., 2009). This

mechanism involves the release of AIF, which is translocated from the mitochondrial membrane to the nucleus, inducing DNA degradation.

Estrogen deficient states may partly account for declines in cognition and neurodegeneration that are associated with dementia, such as HAD. The current experiments show estrogen treatment was able to delay the onset of cell death by attenuating Tat- induced apoptotic signaling. Upstream of caspase activation, we also show that Tat- induced increases in expression of Bcl-2 and Bax was reversed by estrogen. Since the Bcl-2 related proteins are made up of a group of apoptosis regulatory genes of which Bcl-2 is anti-apoptotic and Bax is pro-apoptotic, the ratio of Bcl-2 to Bax may determine whether the cells undergo apoptotic cell death or survive the toxic insult. Perhaps a key mechanism by which estrogens augment apoptotic signaling in Tat-induced cell death is by altering this ratio in favor of anti-apoptotic proteins (Zhang et al., 2003). The significant down regulation of proapoptotic protein Bax by estrogen could serve to balance this ratio and normalize mitochondrial function, preventing the release of apoptotic factors and initiation of the caspase cascade. This estrogen effect on Bax along with its effects on Bcl-2 and caspases serves to prolong the compensatory/survival promoting response of cells which corresponds with the significant delay of apoptotic cell death observed with estrogen treatment.

The current experiments show that upregulation of caspases and Bax, Bcl2 expression precede cell death, supporting the observation that Tat-induced cell death was the consequence of activation on the mitochondria-mediated apoptosis pathway. Furthermore, our observations indicate that estrogen protects against Tat-induced

apoptosis in primary cortical cultures in part by inhibiting caspase-activation and preventing mitochondrial membrane permeability.

CHAPTER 3

Experiment 2. To determine whether estrogen affects Tat-induced apoptosis through estrogen receptor- mediated mechanisms

3.1. Introduction

Estrogens regulate the expression of a number of target genes by binding the estrogen receptors ERα and ERβ, which function as ligand-activated transcription factors (Jover et al., 2002; Morrisette et al., 2008). However, there is still debate about which receptor subtype mediates the protective actions of estrogen (Dubal et al., 2001:2006). Studies have demonstrated that both receptor subtypes are capable of mediating estrogen neuroprotection (Zhao et al., 2004; Miller et al., 2005; Cordey and Pike, 2005; Liu et al., 2005; Zhao and Brinton, 2007). The differential expression and localization of ER isoforms underlies their distinct roles in various biological functions. Distinguishing the role of each receptor isoform in estrogen neuroprotection will assist the development of ER-subtype selective ligands to target the beneficial actions of estrogen and avoid unwanted side effects. In these experiments, we proposed that estrogen attenuation of Tat-induced apoptotic signaling was receptor mediated, furthermore, these estrogen effects are specific to ER-subtype.

3.2. Experimental Design

We investigated if estrogen effects on expression of apoptotic markers in Tat exposed

cortical cultures were estrogen receptor- mediated. We determined if estrogen effects on apoptotic markers were maintained in the presence of non-specific ER antagonist ICI182,780 (100nM), which blocks both ERα and ERβ. Additionally, ICI-sensitive apoptotic proteins were further evaluated to determine whether estrogen effects are specific to ER subtype. Specific antagonists of ERα (1000 nM, MPP dihydrochloride) and ERβ (1000 nM, PHTPP) were administered 1h before estrogen treatment.

3.3. Materials and Methods

3.3.1 .Immunocytochemistry

For immunocytochemistry experiments, Tat-treated cultures and Estradiol+Tat-treated controls were fixed in acetic alcohol (95% ethanol, 5% acetic acid) for 10 min and washed three times (5 min/wash) with Dulbecco phosphate-buffered saline (D-PBS): Na2HPO4 (1150 mg/L), KH2PO4 (200 mg/L), NaCl (8000 mg/L), KCl (200 mg/L) at pH 7.4. Following fixation, cultures were blocked with 10% normal horse serum (NHS) in PBS and used for the analysis.

3.3.2. Anti-ERα and anti-ERβ immunofluorescence

Rabbit polyclonal anti-ERα (Santa Cruz) and anti-ERβ (Affinity Biosciences) antibodies were used to detect expression of estrogen receptors in nontreated cortical cultures. Primary antibodies were diluted 1:500 in 1% NHS/PBS. Cell cultures were incubated with primary antibodies overnight at 4°C. Each set of experiments included cell

culture dishes which were left without primary antibody. These cell cultures served as controls for non-specific binding of secondary antibodies. After incubation with primary antibodies was complete, plates were washed three times (5 min/wash) with PBS. For the immunofluorescent detection of Tat immunoreactivity goat anti-rabbit IgG conjugated with Alexa 594 dye (Molecular Probes) diluted 1:500 in 1% NHS/PBS was used. Plates were incubated with secondary antibodies for 1h at room temperature and then washed 3 times with PBS. Cells were counterstained with DNA binding fluorescent Hoecsht dye**.**

3.3.3. Anti-Tat immunofluorescence

Rabbit polyclonal Tat antibody (Diatheva, Italy) was used to determine Tat 1-86 immunoreactivity. Primary antibodies were diluted 1:500 in 1% NHS/PBS. Cell cultures were incubated with primary antibodies overnight at 4°C. Each set of experiments included cell culture dishes which were left without primary antibody. These cell cultures served as controls for non-specific binding of secondary antibodies. After incubation with primary antibodies was complete, plates were washed three times (5min/wash) with PBS. For the immunofluorescent detection of Tat immunoreactivity goat anti-rabbit IgG conjugated with Alexa 594 dye (Molecular Probes) diluted 1:500 in 1% NHS/PBS was used. Plates were incubated with secondary antibodies for 1h at room temperature and then washed 3 times with PBS.

Microscopic images of Tat immunoreactivity in primary cell cultures were captured using the computer-controlled inverted fluorescent microscope (Nikon Eclipse TE2000-E)

under 20x magnification and analyzed using NIS-Elements BR 2.30 imaging software package (Nikon). Images of specific Tat immunofluorescence were merged with the subsequent differential interference contrast (DIC) images.

3.3.4. Detection of Apoptotic proteins (ELISA)

Expression of apoptotic signaling proteins in cell lysates was determined by ELISA. Cell lysates were prepared from cultures grown in 24-well plates. Cultures were treated with ICI182,780 (100 nM), MPP (1000 nM), or PHTPP (1000 nM) 1h prior to addition of 17β-estradiol (10 nM). After 24h estradiol pretreatment, cell cultures were exposed to 50nM Tat 1-86 for 4, 16, or 24 hours before harvesting. At the time of harvesting, medium was removed and cells were washed three times with Dulbecco phosphate-buffered saline, D-PBS, (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.137 M NaCl and 2.7 mM KCL at pH 7.4) and lysed with CellLytic ^{TM}- M mammalian cell lysis buffer (Sigma Chemicals) containing protease inhibitors (protease inhibitors cocktail, Sigma Chemicals). All samples in a group (6 sister culture wells) were pooled together and protein concentration was determined by BCA method (Pierce). Each well of Costar 96-well ELISA plates (Corning Inc, PA) was coated overnight at 4 °C using 100 µl of 20 mM carbonate coating buffer, pH 9.6. Cortical cell lysate samples were diluted 1:10 with D-PBS and 2 ug of each sample were added to the plate wells. After overnight incubation at 4 °C, plates were rinsed 5 times with PBST (0.05% Tween 20 in PBS, pH = 7.4) and blocked with 1% BSA in PBS for 2 h at room temperature. After blocking, plates were washed again as described above and primary anti-Bax, anti-Bcl-2, anti-

active Caspase 9 and anti- active Caspase 3 antibodies (all primary antibodies, Abcam, Cambridge, MA) diluted 1:5000 or 1:7500 (caspase 3) in 0.1% BSA-PBST were added to each well except for blanks and no-primary antibody control wells. Plates were kept overnight at 4 °C. When the incubation with primary antibodies was completed, plates were again washed 5 times with PBST and secondary antibodies (goat anti-rabbit alkaline phosphatase conjugated, Sigma) diluted 1: 2000 in 0.1% BSA-PBST were added to each well except for blank and no-secondary antibody control wells. After 2 h of incubation, secondary antibody solution was removed, plates were washed 5 times with PBST and 100 µl of BluePhos phosphatase substrate mixture (KPL Research, Gaithersburg, MD) was added to the plate wells. After 30 min of incubation, the absorbance at 650 nm was determined using a Bio-Tek Synergy HT microplate reader. Multiple readings were taken within a 1-h time period.

3.4. Statistical Analysis

Statistical comparisons were made using one way ANOVA and Tukey's multiple comparison tests to determine specific treatment effects. Significant differences were set at $p<0.05$. Data represent percent of control values.

3.5. Results

3.5.1. Estrogen receptor expression in cortical cell culture

Estrogen receptor expression was determined in non-treated primary cortical

cultures. Neurons positively stained for anti-ERβ and anti-ERα antibodies were present in cortical cultures (**Figure 3.1**). Anti-ERα immunoreactivity was mainly localized in nuclei, while ERβ immunofluorescence was also observed in nuclei, however, staining was also detected in cytoplasm with the nuclei unstained.

3.5.2. Estrogen effects on Tat interaction with neuronal membranes

Because neurons are not directly infected with HIV, Tat-induced neuronal apoptosis may be dependent upon Tat binding/interaction with neuronal membranes. The presence of estrogen membrane receptors are generally acknowledged and are thought to mediate estrogen rapid cellular effects. We determined if estrogen attenuation of Tat-induced apoptosis was mediated by preventing or interfering with mechanisms of Tat binding and uptake by neurons in culture. Immunostaining of cell cultures exposed for 2h to 50 nM Tat 1-86 alone or in the presence of 10 nM of 17β-estradiol with anti-Tat antibodies showed the presence of Tat in nuclei, but in some neurons anti-Tat immunofluorescence was localized in somata, leaving nuclei unstained (**Figure 3.2**). Tat immunoreactivity was assessed in estradiol pretreated and Tat-alone treated cultures. Results demonstrate no differences in anti-Tat immunoreactivity with estradiol pretreatment, as significant anti-Tat immunofluorescence was still detected and was comparable to that observed in Tat-alone treated cultures.

3.3.3. Estrogen receptors mediate 17β- estradiol effects against Tat-induced Bax not Bcl-2

To determine potential mechanisms of estrogenic action, we investigated the effects of ICI 182780, a specific ER antagonist, on 17β-estradiol- inhibition of apoptotic protein expression in Tat treated cell cultures. Cell cultures were incubated for 1hr with ICI 182,780 (100nM) before the addition of 17β-estradiol and then exposed to 50nM Tat 1-86 for 4, and 16 h. The addition of ER antagonist was able to block estrogen protective effects on Tat-induced Bax protein expression (4 h Tat exposure), as Bax levels were similar to that of cultures treated with Tat alone (134±6%, $p<0.05$, compared to Tat+E treated cultures) (**Figure 3.3A**). Results indicate that ICI 182,780 was not able to block estrogen actions on Bcl-2 expression with Tat exposure (16 h) (**Figure 3.3B**). There was no significant difference in Bcl-2 levels in 17β-estradiol treated and ICI 182,780 treated cortical lysates.

3.3.4. Estrogen receptors mediate 17β-estradiol effects on caspase 3 activity in Tat-exposed cortical cultures

We also evaluated if estrogen receptors play a role in regulation of active caspase expression. ICI treatment displayed no effect on active caspase 9 expression at 4h of Tat exposure, as estrogen effects on caspase 9 were maintained, suggesting the estrogen actions on caspase 9 are not receptor-mediated (**Figure 2.4A**). However, estrogen effects on caspase 3 expression were significantly attenuated by ICI treatment. At each time point evaluated, estrogen actions on Tat-induced caspase 3 expression were ICI-sensitive, as caspase levels in ICI treated cultures were not significantly different from those

present in cultures treated with Tat alone (**Figure 2.4B**).

3.3.5. ER subtypes differentially mediate estrogen effects on caspase 3 activity and Bax expression following Tat exposure

We then used ER subtype-selective antagonists to further define whether ER effects were mediated by either ERα and/or ERβ. ERα and ERβ specific antagonists, MPP dihydrochloride and PHTPP respectively, were added to cultures prior to17 β-estradiol treatment. Furthermore, results indicated that the addition of either MPP or PHTPP was unable to attenuate 17 β-estradiol effects on caspase expression, suggesting that 17 β-estradiol actions on Tat-induced caspase expression are mediated by both ER subtypes (**Figure 2.5A**).

In regard to Bax, the addition of MPP only partially attenuated estrogen effects on Bax expression, while the addition of PHTPP markedly abrogated estrogen downregulation of Bax (**Figure 2.5B**).

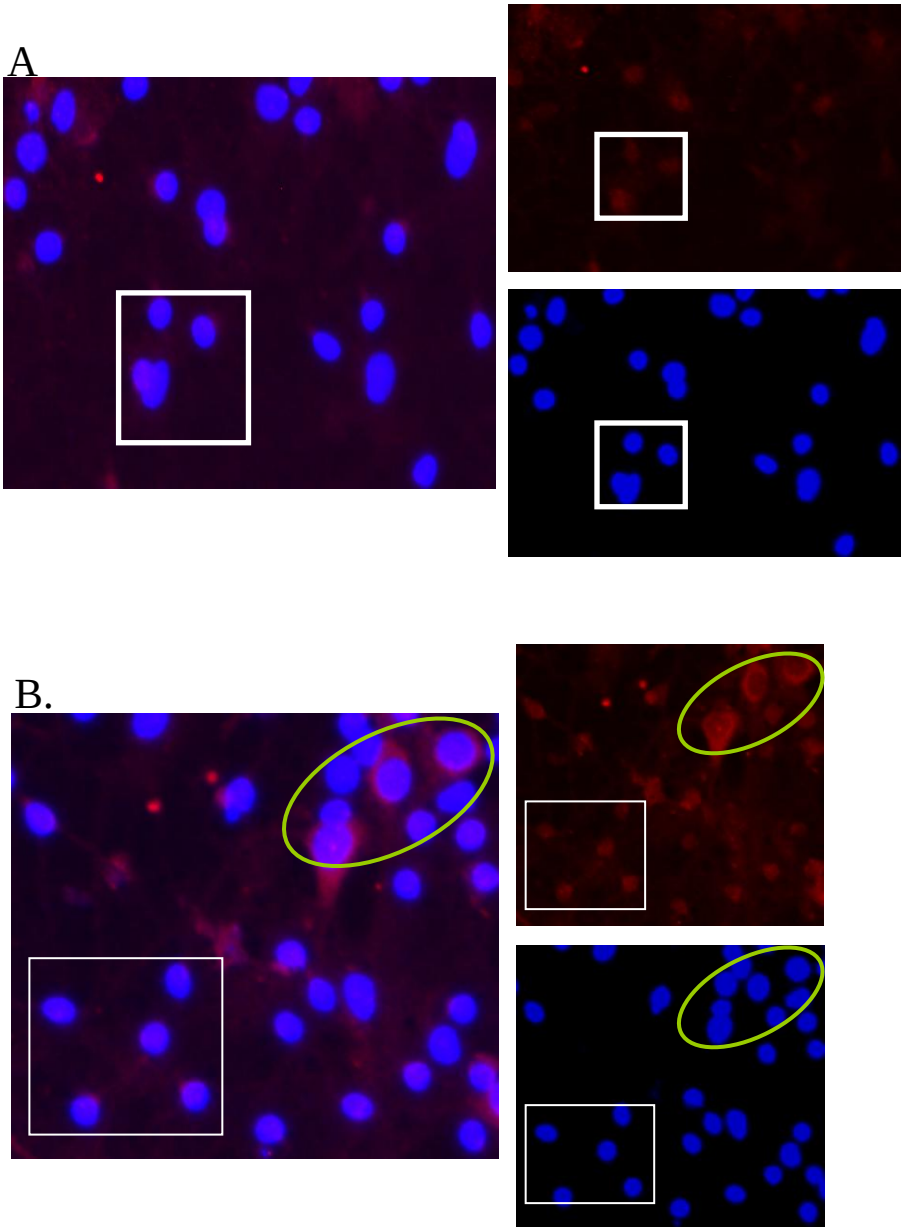


Figure 3.1. Estrogen receptor expression in primary cortical cultures. Microscopic images of ERα (A) and ERβ (B) expression (red fluorescence) in primary cortical cultures. Left panel displays overlap of anti-ER fluorescence and Hoechst nulear stain. Right panels show the individual images of ER expression and nuclear staining. White boxes demonstrate classic nuclear localization of ERα and ERβ. In green circles, ERβ immunoreactivity was also detected in cytoplasm of some neurons.

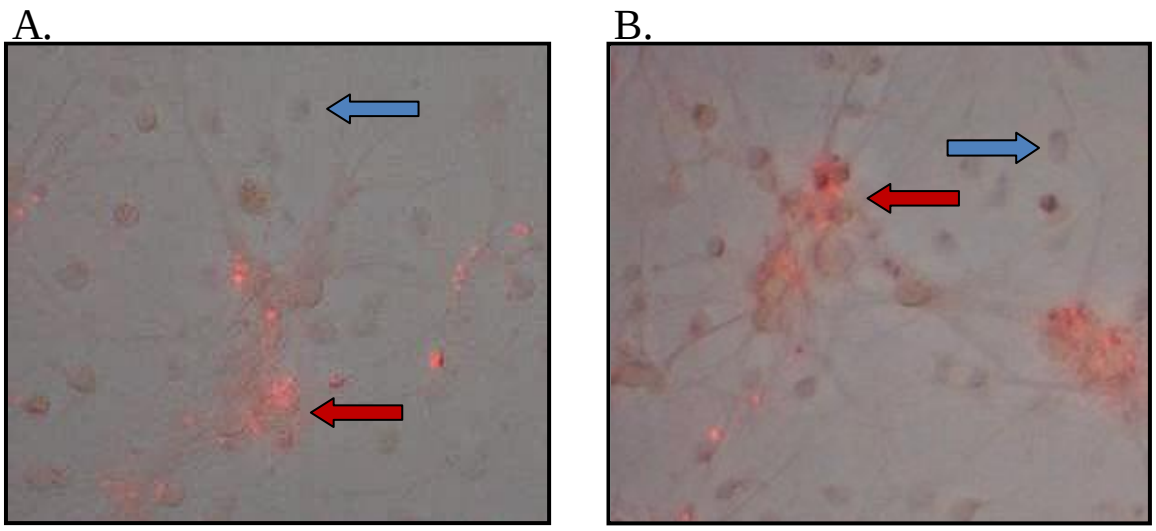
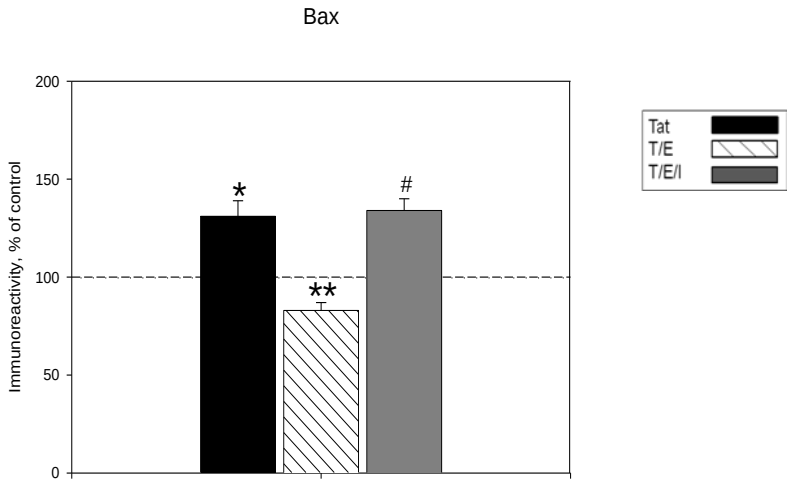


Figure 3.2. Image of Tat immunoreactivity in rat fetal cortical cell cultures. Tat-immunopositive cells were detected in cortical cultures pretreated with 10nM 17β-estradiol and/or exposed to 50nM Tat 1-86 for 2h. No differences in Tat immunoreactivity (Red arrows) was detected between estrogen pretreated (B) and Tat-alone treated cultures (A). Note not all cells express Tat immunoreactivity (Blue arrows).

A.



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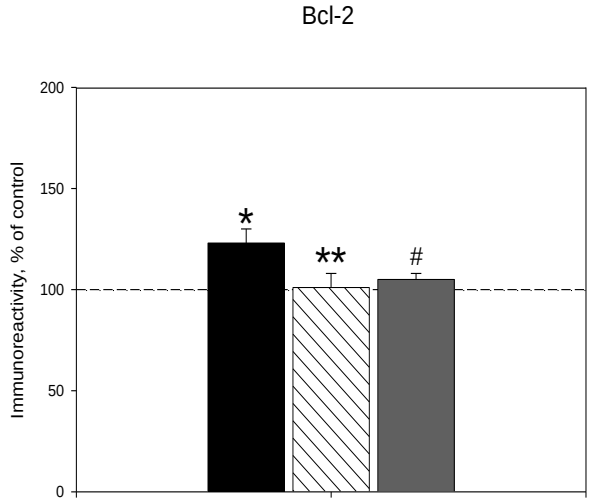
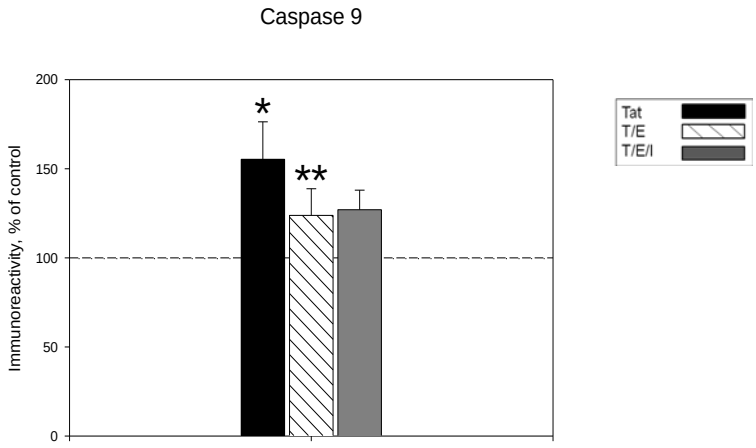


3.3. Tat-induced Bax expression sensitive to estrogen receptor -mediated estrogenic actions. A. Estrogen effects on Bax after 4h of Tat exposure were reversed by ICI 182,780, suggesting that estrogenic actions on Bax are mediated by ER signaling. **B.** Estrogen effects on Bcl-2 expression (16h Tat exposure) were not attenuated by the addition of ER antagonist. Experiments performed in triplicate, *p<0.05 as compared to Tat-treated cultures, **p<0.05 compared to Tat treated cultures, #p<0.05 as compared to Tat+E treated cultures.

A.



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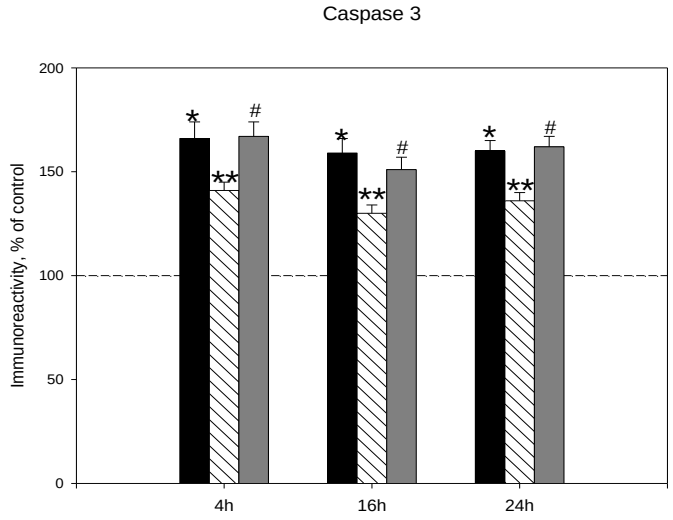
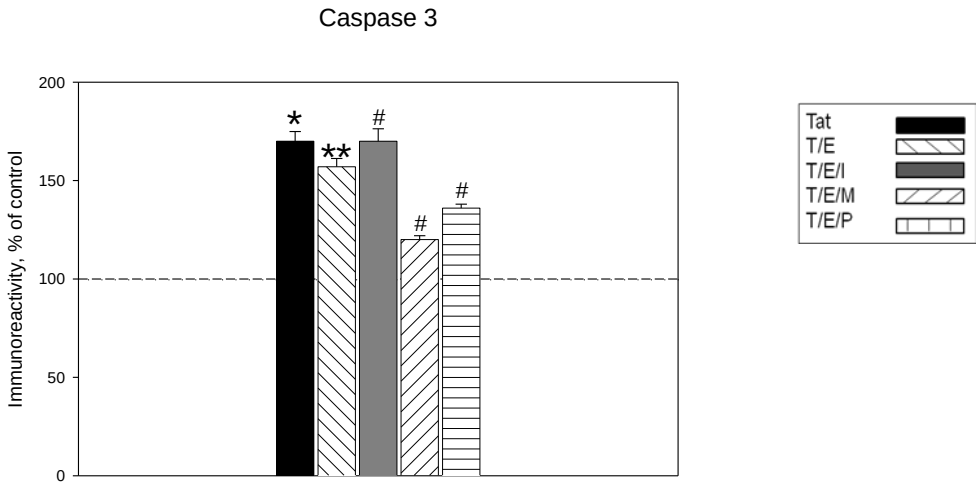


Figure3.4. Estrogen receptor mediates estrogen effects on Tat-induced Caspase 3 not Caspase 9 active expression. Estrogen receptor antagonist, ICI 182,780, was added to cultures 1h before treatment with 17β-estradiol. **A.** Estrogen effects on caspase 9 expression (4 h of Tat exposure) were maintained in presence of ER antagonist. **B.** Estrogen effects on Tat-induced expression of caspase 3 were reversed by ICI 182,780, suggesting that estrogenic actions on caspase 3 are ER mediated. Experiments performed in triplicate, *p<0.05 as compared to controls, **p<0.05 as compared to Tat-treated cultures, #p<0.05 as compared to Tat+E treated cultures.

A.



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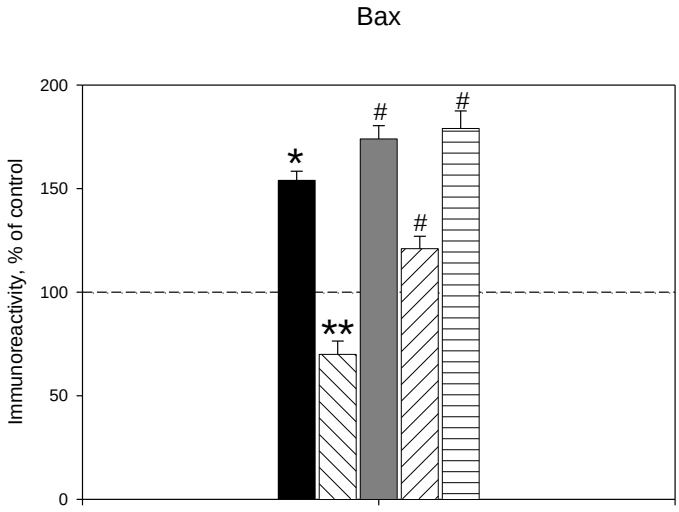


Figure 3.5. Estrogen receptor subtype specific effects against caspase activity and Bax expression. (A.) Estrogen effects against Tat-induced caspase 3 activity were maintained in presence of specific antagonists for ERα (MPP, 100nM) and ERβ (PHTPP, 100nM). (B.) ER subtype specific antagonists reveal that ERβ mediated signaling preferential for estrogen effects on Bax. Experiments performed in triplicate, *$p<0.05$ as compared to controls, **$p<0.05$ as compared to Tat treated cultures, #$p<0.05$ as compared to Tat+E treated cultures.

3.6. Discussion

Recently, much attention has been given to the neuroprotective properties of estrogens for chronic neurodegenerative diseases. In vivo and in vitro studies show that estrogens can protect against a number of neurotoxic compounds (Green and Simpkins, 2000; Turchan et al., 2001). The mechanisms by which estrogen protects cells can include receptor-mediated and non-receptor-mediated effects. Estrogen receptors are widely expressed in the brain with some regional differences. We demonstrated the presence of estrogen receptors in the primary cortical cultures by immunocytochemistry. We found that estrogen receptors are localized in neurons and display nuclear and cytoplasmic distribution.

Images of Tat immunoreactivity in cultured rat fetal cortical neurons treated with 50nM Tat 1-86 shows that when levels of cell bound Tat was maximal, the culture contained populations of Tat positive and Tat negative neurons. This indicates rat fetal neurons in cell culture may differ in their ability to interact with extracellular Tat. Moreover, this pattern of Tat immunoreactivity did not change in the presence of estrogen. Such observations seem to indicate that membrane actions of estrogen are not involved in its attenuation of Tat-induced cell death, as it does not augment the direct interaction of Tat with neurons.

The direct interaction of Tat with neuronal cell membranes involves binding and uptake into the cell and translocation of Tat into the nuclei. The nuclear localization and Tat transcriptional effects may contribute to neuronal apoptosis. The nucleus represents

an ideal site for estrogen protection. Although not assessed in these experiments, the nuclear colocalization of Tat and estrogen receptors supports the notion of direct estrogen effects against Tat induced transcriptional events. And suggests a more classical action of estrogen via its nuclear receptors

It is widely accepted that the biological actions of estrogen are mediated by two different receptor subtypes, ERα and ERβ. We next determined that 17β-estradiol protected against the neurotoxic insult of HIV-1 Tat via an ER-mediated mechanism. Results demonstrate that the addition of a nonspecific ER antagonist ICI 182,780, which blocks both ERα and ERβ, attenuated the estrogen down-regulation of Bax and caspase 3 with Tat exposure. Using subtype-specific antagonists we sought to further determine if these estrogen effects were selectively mediated by either ERα and/or ERβ. The ERα-specific antagonist MPP and ERβ-specific antagonist PHTPP did not attenuate the estrogen effects on caspase 3 expression. Our results indicated that estrogen receptor-mediated effects on Tat-induced caspase 3 expression are not selective to a specific ER subtype. However, estrogen effects on Bax are preferential for ERβ mediated signaling, although ERα did contribute to these effects as well.

Estrogen actions on caspase 9 expression and Bcl-2 levels were maintained in presence of antagonists, implying these estrogen actions are not receptor-dependent. These results suggest that ERs may be involved in select aspects of apoptotic signaling. Indeed, a previous report has shown that HIV-1 Tat toxic effects on mitochondria and neuronal cell survival may be independently regulated (Turchan et al., 2001). It seems

that estrogens are protective against both mitochondrial dysfunction and cell death by receptor and non-receptor mediated mechanisms. More evidence indicates that in addition to the genomic ER-mediated effects, many effects of estradiol may involve cross talk with other signal transduction pathways (Mhyre & Dorsa, 2006). Estrogen may act via an indirect, non-genomic response involving activation/phosphorylation of Akt which can mediate the anti-apoptotic signaling pathway and involves regulation of the antiapoptotic protein Bcl-2 (Honda et al.,2000; Singh, 2001).

Recent reports are suggesting the mitochondria as a promising target for estrogen-mediated protection (Arnold and Beyer, 2009; Klinge, 2008; Simpkins and Dykens, 2008). There is evidence that estrogen receptors and estrogen-binding proteins are located in the mitochondrial matrix or mitochondrial membranes. Activation of the nuclear ERs may directly regulate mitochondria by controlling the transcriptional activity of genes coding for mitochondrial associated proteins (Arnold and Beyer, 2009). The substantial effect of estrogen on Bax expression may relate to the mitochondrial localization of ERs, particularly as a recent study reported that ERβ colocalized with a mitochondrial marker in rat primary cortical and hippocampal neurons (Yang et al., 2004).

Exposure to HIV-1 viral proteins initiates a significant toxic cascade that likely involves activation of multiple cell death pathways. It is clear, that estrogen was able to attenuate Tat-induced apoptotic signaling and prolonged cell viability. With the induction of many apoptotic pathways and different factors/mediators of execution, it is difficult to delineate a point of convergence of the various pathways, which would be ideal as a target to interrupt the apoptotic process and support cell survival. However, the

ER-β appears to be a primary point for pharmacological targeting.

Although it is popular theory that these apoptotic processes occur in a sequential pattern, this may not be the case in all cell death mechanisms. Disruption of mitochondrial function can kill cells without caspase activation (Hirsch et al., 1997). In contrast, in several cases, caspase inhibition delays and sometimes prevents mitochondrial dysfunction (Hirsch et al., 1997; Susin et al., 1997; Bossy-Wetzel et al., 1998), caspases can cause mitochondrial dysfunction in vitro, and mitochondrial events can cause caspase activation (Zhou et al., 1997;Li et al., 1997; Kluck et al., 1997; Yang et al., 1997). It is plausible that caspase activation and disruption of mitochondrial function occur in a circular feedback loop. In such a scenario, mitochondrial dysfunction would release caspase activators at the same time as caspases might act on mitochondrial membranes. Caspase inhibition might retard but not prevent cell death because it slows down a caspase-dependent self-amplification loop resulting in acceelerated mitochondria dysfunction.

Both mitochondrial membrane function and caspases are crucially involved in cell death and seem to represent key checkpoints in the regulation of cell death. The circular model of apoptotic events reflects our observations of simultaneous upregulation of proapoptotic factors Bax, indicating mitochondria membrane permeability, disruption of mitochondrial membrane potential, and caspase activation early after Tat exposure. More interesting is the specific estrogen receptor mediated effects on Bax expression and caspase 3 activity, further indicating the important role of membrane permeability and

execution phase of apoptotic cell death. Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases. Additionally, these events might constitute a point of integration of multiple apoptotic pathways as several pro-apoptotic second messengers, including Ca2+ ions, ROS, ceramides, caspases and Bax facilitate their function. Multiple apoptotic pathways end at the point of the execution phase, considered the final pathway of apoptosis.

In summary, the present study suggests the neuroprotective actions of estrogen against HIV-1 Tat-mediated toxic insults involve regulating ER-responsive apoptotic factors. Our results indicated that HIV-1 Tat-mediated cell death induces key regulatory factors of the mitochondria-linked apoptotic pathway. Estrogen treatment was effective in delaying cell death that involved attenuating Tat- induced caspase activity and pro-apoptotic protein expression. The protective estrogenic effect was mediated by an ER-sensitive mechanism specific to Bax and caspase 3. Furthermore, the present study indicated that both ERα and ERβ may contribute to the beneficial actions of estrogen, with some selective effects of ERβ.

CHAPTER 4

Experiment 3. To determine if phytoestrogens are equally effective as endogenous estrogen against Tat-induced apoptotic signaling.

4.1. Introduction

Since estrogen acts as a neurotrophic and neuroprotective factor, women in a hypoestrogenic state are more vulnerable to neurodegenerative conditions. However, in spite of the beneficial effects of estrogen, hormone therapy is met with resistance due to its association with reproductive and breast cancers. Therefore, there is great need to establish the mechanism(s) underlying estradiol mediated protection. Many studies suggest that estradiol-mediated protection may involve attenuation delayed cell death (Dubal et al., 2006). Estrogen can protect against neuronal apoptosis by regulating caspase activities and expression of anti-apoptotic proteins and maintaining mitochondrial functioning (Wang et al., 2001; Sawada et al., 2000). There is growing interest in the functions of phytoestrogens, such as soy isoflavones (genistein and daidzein), including whether they have beneficial effects on neurodegenerative disease. These soy isoflavones may mimic the actions and functions of estrogen in the brain, as they bind to the estrogen receptor and affect estrogen mediated processes. Several studies have shown that soy isoflavones have neuroprotective effects against various neurotoxic

insults that involved preventing oxidative stress, regulating anti-apoptotic proteins, and reducing caspase activation. In this experiment we propose that phytoestrogens mimic estrogenic actions against Tat-induced apoptotic signaling via a similar ER mediated mechanism.

4.2. Experimental Design

Enzyme-linked immunosorbent assay (ELISA) was used to quantify levels of apoptotic signaling proteins Bax, Bcl-2, active caspase 9 and active caspase 3 in cortical lysates incubated with phytoestrogens, genistein (GEN), and daidzein (DAI) prior to Tat exposure. Cells were incubated with 17β-estradiol (10 nM), GEN, or DAI (1000 nM) 24 hours prior to Tat exposure. Tat treated cell cultures were exposed to 50 nM Tat 1-86 for 4, 16, or 24 hours before harvesting. ELISA experiments were performed as described in Experiment 1. We determined if phytoestrogens are as effective as endogenous estrogen, 17β-estradiol, in attenuating Tat-induced cell death signaling.

4.3. Materials and Methods

4.3.1. Detection of Apoptotic proteins (ELISA)

Expression of apoptotic signaling proteins in cell lysates was determined by ELISA. Cell lysates were prepared from cultures grown in 24-well plates. Tat treated cell cultures were exposed to 50 nM Tat 1-86 for 4, 16, or 24 hours before harvesting. At the time of harvesting, medium was removed and cells were washed three times with Dulbecco phosphate-buffered saline, D-PBS, (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.137

M NaCl and 2.7 mM KCL at pH 7.4) and lysed with CellLytic ^{TM}- M mammalian cell lysis buffer (Sigma Chemicals) containing protease inhibitors (protease inhibitors cocktail, Sigma Chemicals). All samples in a group (6 sister culture wells) were pooled together and protein concentration was determined by BCA method (Pierce, Rockford, Ill.). Each well of Costar 96-well ELISA plates (Corning Inc, PA) was coated overnight at 4 °C using 100 µl of 20 mM carbonate coating buffer, pH 9.6. Cortical cell lysate samples were diluted 1:10 with D-PBS and 20ug of each sample were added to the plate wells. After overnight incubation at 4 °C, plates were rinsed 5 times with PBST (0.05% Tween 20 in PBS, pH = 7.4) and blocked with 1% BSA in PBS for 2 h at room temperature. After blocking, plates were washed again, as described above, and primary anti-Bax, anti-Bcl-2, anti- active Caspase 9 and anti- active Caspase 3 antibodies (all primary antibodies, Abcam, Cambridge, MA) diluted 1:5000 or 1:7500 (caspase 3) in 0.1% BSA-PBST were added to each well except for blanks and no-primary antibody control wells. Plates were kept overnight at 4 °C. When the incubation with primary antibodies was completed, plates were again washed 5 times with PBST and secondary antibodies (goat anti-rabbit alkaline phosphatase conjugated, Sigma) diluted 1: 2000 in 0.1% BSA-PBST were added to each well except for blank and no-secondary antibody control wells. After 2 h of incubation, secondary antibody solution was removed, plates were washed 5 times with PBST and 100 µl of BluePhos phosphatase substrate mixture (KPL Research, Gaithersburg, MD) was added to the plate wells. After 30 min of incubation, the absorbance at 650 nm was determined using a Bio-Tek Synergy HT

microplate reader. Multiple readings were taken within a 1-h time period.

4.4. Statistical Analysis

Statistical comparisons were made using one-way ANOVA and Tukey's multiple comparison tests were used to determine specific treatment effects. Significant differences were set at $p<0.05$. Data represent percent of control values.

4.5. Results

4.5.1. Effects of genistein and daidzein on Tat-induced caspase activation in primary fetal rat cortical cell cultures

We compared the protective effect of 17 β-estradiol and phytoestrogens genistein (GEN) and daidzein (DAI) in cortical neuronal cultures exposed to 50 nM Tat 1-86. Previous experiments demonstrated that Tat exposure caused a significant increase in caspase 9 activation. Moreover, the addition of 17 β-estradiol was able to significantly attenuate this induction of caspase 9 activity. In the present experiments, we evaluated the protective efficacy of GEN and DAI against Tat-induced upregulation of caspase 9 activity. **Figure 4.1A** shows significant caspase 9 activation following only 4 hr of Tat exposure ($p<0.05$). Preincubation with 10nM estradiol or 1000nM of phytoestrogens genistein (GEN) or daidzein (DAI) prevented the increase in Tat-induced caspase 3 activation. Similar to estradiol, in the presence of genistein or daidzein, the expression of active caspase 9 was diminished by ~20% vs. Tat-alone treated cultures ($p<0.05$).

Moreover, analysis revealed that caspase 9 activity was not statistically different between the 17β-estradiol, GEN and DAI pretreated cultures.

In regard to effector caspase 3 activity, a similar effect was observed with phytoestrogen pretreatment prior to Tat exposure. Results demonstrate a significant increase in caspase 3 activation in cortical cultures following 4h exposure to Tat ($p<0.05$). The addition of 17β-E2 was able to attenuate the increase in caspase 3 observed with Tat exposure. The current experiments show that the addition of GEN or DAI prior to incubation with 50nM Tat significantly attenuated the upregulation of active caspase 3 expression ($p<0.05$, **Figure 4.1B**). The level of activation of caspase 3 in GEN and DAI pretreatment groups was not significantly different from that of vehicle-treated controls, furthermore these levels were very similar to that of the endogenous estradiol. These results indicate that isoflavones GEN and DAI downregulate Tat-induced caspase activation to a level comparable to that of 17β-E2, suggesting that isoflavones and estradiol may share a common neuroprotective mechanism.

4.5.2. *Effects of genistein and daidzein on pro- and anti-apoptotic proteins Bax and Bcl-2 upregulation in Tat-exposed primary cortical cell cultures*

Bcl-2 is an anti-apoptotic protein, thought to regulate the permeability of the mitochondrial membrane, and prevent the release of apoptotic factors that initiate the caspase cascade leading to cell death. Our prior experiments have shown that incubation with Tat (16 h) caused a significant upregulation of Bcl-2 expression in cortical cell cultures. With the addition of 17β-E2, we observed an attenuation of Tat-induced Bcl-2

expression. Results of the current experiment indicate that similar to estradiol, GEN and DAI significantly attenuated the increase of Bcl-2 expression following Tat exposure ($p<0.05$). Bcl-2 levels in the estradiol and isoflavone treated cultures were not significantly different from that of vehicle-treated controls, nor was there any statistical difference between the treatment groups.

We also evaluated the efficacy of isoflavones against Tat-induced Bax expression. Bax is a pro-apoptotic protein, that opposes the actions of Bcl-2, that also effects mitochondrial membrane permeability. Over-expression of Bax increases the permeability of the mitochondrial membrane, allowing the release of apoptotic factors which activate the caspase cascade. Results demonstrate that 24h pretreatment with GEN or DAI was able to significantly block the induction of Bax expression following exposure to Tat ($p<0.05$). In addition, this effect on Bax expression was similar across the 17β-estradiol, GEN and DAI treatment groups.

4.5.3. Estrogen Receptor-Mediated effects of genistein and daidzein against Tat-induced caspase 3 activation and Bax expression in primary cortical neuronal cultures

As phytoestrogens have been shown to affect estrogenic processes, it is thought that they may act via a similar mechanism as estradiol. Many of estrogens effects are mediated by estrogen receptors, ERα and ERβ. The expression of both receptor subtypes has been determined in cortical regions of the human and rat brain, moreover the expression of estrogen receptors has been observed in vitro in cortical cell cultures. In

the present experiments, we sought to determine if the protective actions of phytoestrogens against Tat-induced apoptotic process were mediated by estrogen receptors. The ER antagonist, ICI182,780 (ICI,100 nM) was added to cultures 1h prior to incubation with GEN and DAI. Prior experiments have shown that endogenous 17β-estradiol effects against caspase 3 were receptor mediated, as the addition of ICI prior to estradiol returned caspase 3 activation levels to that of Tat-alone treated cultures. Results of the current experiments indicate that ICI prevented the downregulation of caspase 3 activation observed with GEN pretreatment (**Figure 4.3A**). Caspase 3 activity in ICI treated cultures was significantly higher than GEN+Tat treated cortical cultures (177±10 vs. 107±8, $p<0.05$) and caspase 3 activity was returned to levels similar to that of Tat-alone treated cultures. We also observed that DAI effects on caspase 3 were mediated via an ICI-sensitive mechanism. There was a 40% increase in active caspase 3 expression with the addition of ICI compared to DAI+Tat treated cultures ($p<0.05$). Moreover, caspase 3 activity did not differ statistically from that of Tat-alone treated cultures.

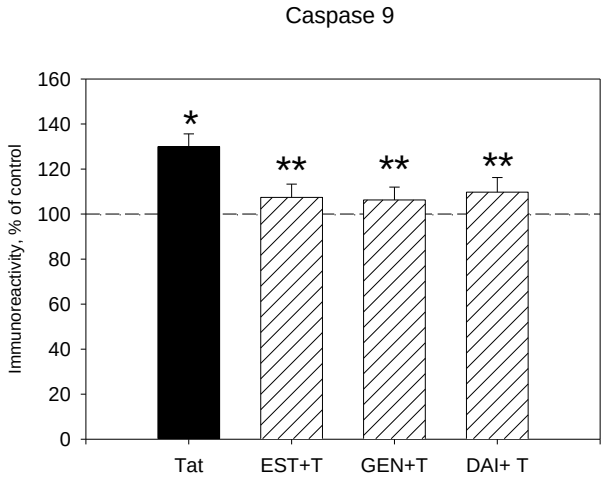
We then evaluated if GEN and DAI effects were specifically selective to ER subtype. The ER-subtype specific antagonists MPP dihydrochloride (ERα) and PHTPP (ERβ) (1000 nM) were added to cortical cultures prior to incubation with GEN or DAI and subsequent exposure to 50 nM Tat. The addition of ERα or ERβ antagonist did not attenuate the protective effects of GEN or DAI against Tat-induced caspase 3 activation (**Figure 4.4A**). The protective effect of GEN and DAI was maintained in presence of ERα and ERβ specific inhibitors. The ER subtype selective antagonists on partially

attenuated the down regulation of caspase 3 activation in cultures treated with isoflavones GEN and DAI. Even in the presence of the antagonist a similar effect on caspase activation is seen in GEN and DAI pretreated cultures. Interestingly, no difference in caspase activity is observed between the GEN and DAI treated cultures. Moreover, this profile of caspase activation is quite similar to that observed in 17β-estradiol treated cultures with the addition of ER subtype antagonists.

We have also previously shown that estrogen downregulation of Tat-induced Bax expression was mediated by estrogen receptors. To evaluate if GEN and DAI effects on Bax expression are ER-mediated, cultures were incubated with the general ER antagonist ICI 182,780 (ICI). ICI exposure blocked GEN and DAI effects against Tat-induced Bax expression. A significant 25% increase in Bax expression was seen with ICI treatment prior to incubation with GEN or DAI ($p<0.05$) (**Figure 4.3B**). We then sought to determine if the protective effects of GEN and DAI against Tat-induced expression of Bax was mediated by a specific ER-subtype. The ER-subtype specific antagonists MPP dihydrochloride (ERα) and PHTPP (ERβ) (1000 nM) were added to cortical cultures prior to incubation with GEN or DAI and subsequently exposed to 50 nM Tat. The addition of ERα antagonist MPP attenuated GEN effects on Bax expression, however, GEN downregulation of Bax was markedly abrogated in the presence of ERβ antagonist PHTPP (**Figure 4.4B**). A similar profile of Bax expression was observed in DAI+Tat treated cultures in the presence of the ER subtype antagonists. The addition of MPP and PHTPP significantly blocked DAI downregulation of Bax expression; however this

suppression of DAI effect was slightly higher in the presence of the ERα antagonist MPP. Although, results suggest that both ER subtypes mediate DAI effects on Bax expression.

A.



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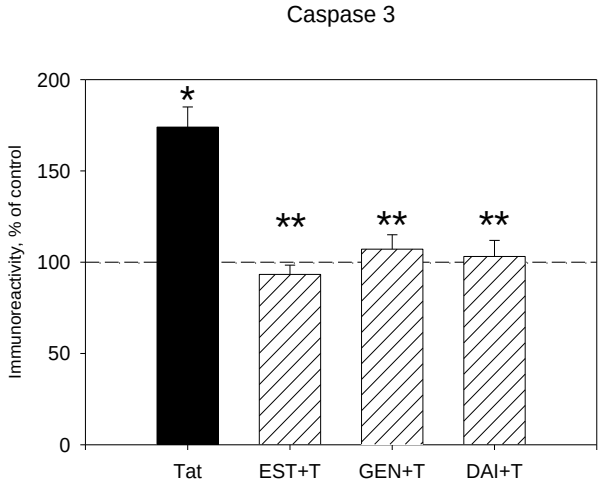
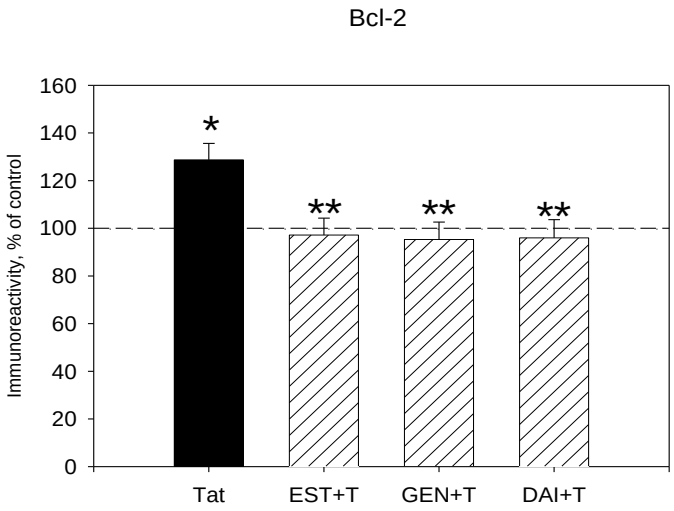


Figure 4.1. Phytoestrogens attenuate Tat-induced caspase activation in primary cortical cultures. Cortical cultures were treated with 1μM GEN or DAI 24h prior to Tat exposure. Expression of apoptotic proteins **A.** Caspase 9 (4h of Tat exposure) and **B.** caspase 3 activity (4h Tat exposure) was assessed by ELISA experiments. Results are presented as % of control value, with experiments performed in triplicate, * $p < 0.05$ as compared to control cultures, ** $p < 0.05$ as compared to Tat-treated cultures.

A.



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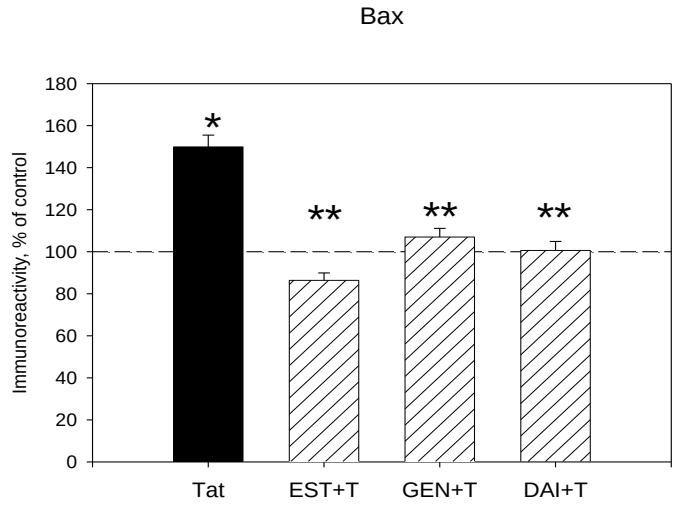
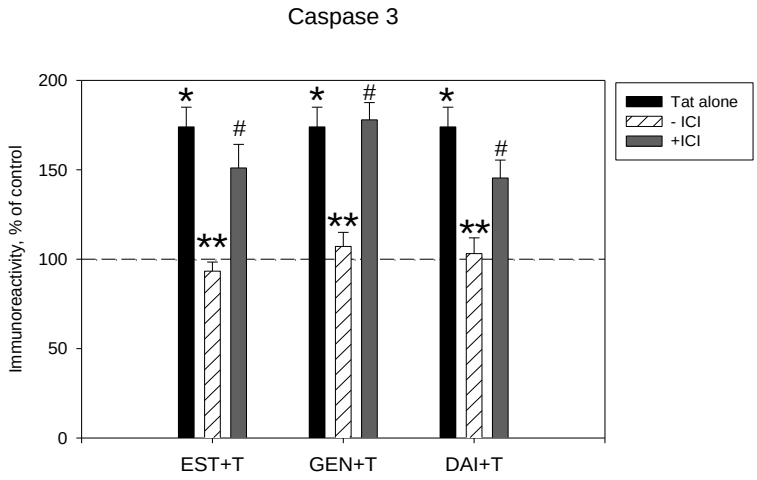


Figure 4.2. Phytoestrogens attenuate Tat-induced expression of Bcl-2 and Bax protein levels. Cortical cultures were treated with 1μM GEN or DAI 24h prior to Tat exposure. Expression of apoptotic proteins **A.** Bcl-2 (16h of Tat exposure) and **B.** Bax (4h Tat exposure) were assessed by ELISA experiments. Results are presented as % of control value, with experiments performed in triplicate, * $p < 0.05$ as compared to control cultures, ** $p < 0.05$ as compared to Tat-treated cultures.

A.



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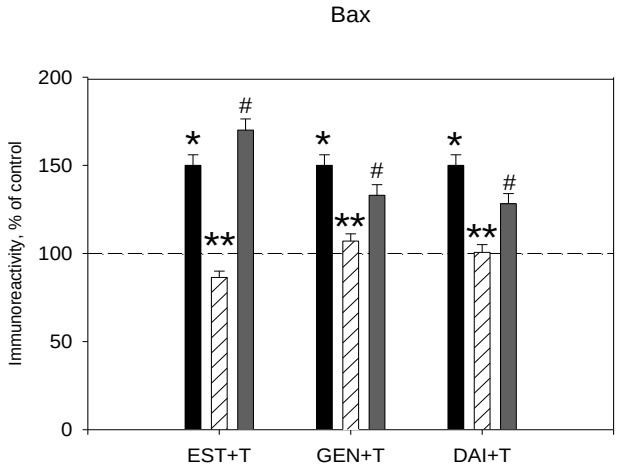
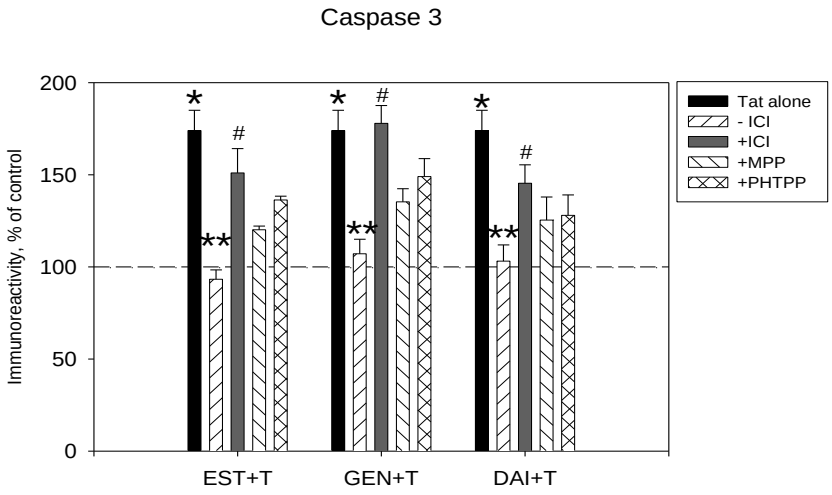


Figure 4.3. ERs mediate phytoestrogen effect on Caspase 3 and Bax expression in Tat exposed cortical cultures. A. GEN or DAI effects on caspase 3 expression were blocked in the presence of ER antagonist, ICI 182,780. **B.** GEN or DAI effects on Tat-induced expression of Bax were reversed by ICI , suggesting that estrogenic actions on caspase 3 and Bax are ER mediated. * $p < 0.05$ as compared to control cultures, ** $p < 0.05$ as compared to Tat-treated cultures.# $p < 0.05$ vs. GEN/DAI+T treated cultures.

A.



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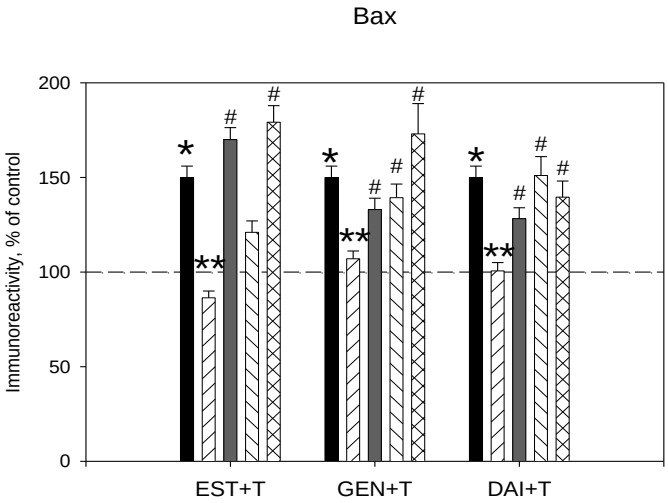

Figure 4.4. ER subtype specific effects against caspase activity and Bax expression. (A)GEN and DAI effects against Tat-induced caspase 3 activity were maintained in presence of specific antagonists for ERα (MPP) and ERβ (PHTPP). (B)ER subtype antagonists reveal that ERβ signaling preferential for GEN effects on Bax. DAI effects on Bax were blocked in presence of both ER subtype antagonists. Experiments performed in triplicate, * $p < 0.05$ as compared to control cultures, ** $p < 0.05$ as compared to Tat-treated cultures. # $p < 0.05$ as compared to GEN/DAI +Tat treated cultures.

4.6. Discussion

In the present study, we evaluated the neuroprotective efficacy of phytoestrogens genistein (GEN) and daidzein (DAI) against HIV-1 Tat-triggered mitochondrial-mediated apoptosis pathway. The goal of the present study was to evaluate the protective effect of phytoestrogens on Tat-induced activation of the mitochondrial- mediated apoptotic pathway. The neurotoxic potential of the HIV-1 Tat protein has been well documented in various cell culture models (Bonavia et al., 2001; Aksenov et al., 2006:2009; Adams et al., 2010). Recent studies have indicated that an apoptotic process is involved in Tat – induced cell death. Mitochondria are known to be involved in apoptosis by releasing apoptogenic substances into the cytoplasm to activate the caspase cascade, including key apoptotic initiatior caspase 9 and executor caspase 3. We found that exposure to 50nM Tat 1-86 caused significant increases in caspase 9 and caspase 3 activities in primary cortical culture. As observed previously with endogenous 17β-estradiol, phytoestrogens genistein and daidzein prevented the upregulation of caspase activity in Tat-exposed cultures. In addition, upstream of caspase activation, we show that Tat exposure significantly increased expression of pro- and anti-apoptotic proteins Bax and Bcl-2, which regulate mitochondrial membrane permeability and thus, the release of apoptogenic substances. Our results indicate that treatment with phytoestrogens genistein and daidzein also markedly reduced the expression of Bax and Bcl-2 in Tat-exposed cortical cultures. These results indicate that the beneficial/protective effect of genistein and daidzein is via inhibition of the mitochondria-mediated apoptotic pathway.

Both soy isoflavones genistein and daidzein exhibited protective effects similar to that achieved by 17β-estradiol. Since phytoestrogens are able to bind ERs, such actions may be produced through activation of the ER. In the present study, we found that the addition of the non-specific ER antagonist ICI 182,780, which blocks both ERα and ERβ, attenuated genistein and daidzein downregulation of caspase 3 and Bax with Tat exposure, suggesting that these effects were mediated by ERs. The addition of ICI had a more robust effect against genistein actions on caspase 3 activity, as levels were nearly the same as that of Tat-alone treated cultures. ICI only partly attenuated the daidzein effects on caspase activation. Furthermore, phytoestrogen effects on Bax expression were also shown to be ER-dependent, as the addition of ICI significantly attenuated genistein and daidzein downregulation of Tat-induced Bax expression. These results suggest that genistein and daidzein act as estrogen receptor agonist in primary cortical neurons and that they may have neuroprotective effects in vivo.

Caspase 3 is a member of the caspase family of proteins that is activated in response to apoptotic signals coming either from the mitochondria via caspase 9 or from death receptor signaling via caspase 8, independent of mitochondria, as well as through other pro-apoptotic pathways inside the cell (Cohen, 1997; Porter and Janicke, 1999). Caspase 3 cleaves a variety of crucial intracellular proteins including endonucleases, cytoskeletal components, and enzymes that lead to characteristic morphology of apoptosis. More upstream, Bax is a proapoptotic Bcl-2 protein. In healthy cells, the majority of Bax is found in the cytosol, but upon initiation of apoptotic signaling, bax

undergoes a conformation shift and inserts into the outer mitochondrial membrane. Thus upregulation of Bax is associated with mitochondrial membrane permeabilization and release of pro apoptotic factors from mitochondria, leading to activation of caspases. The reduction of caspase 3 activity and Bax expression by estradiol and phytoestrogens suggest that these compounds disrupt apoptotic signaling by downregulating key pro apoptotic factors in the cell death cascade. As multiple apoptotic pathways converge on mitochondria functioning and caspase 3 activation, Bax and caspase 3 represent potential upstream and downstream check points for the attenuation of apoptosis by estrogenic compounds in response to neurodegenerative insults.

We then sought to determine if these receptor-mediated effects were specific to a particular ER-subtype. The ERα-specific antagonist MPP dihydrochloride and ERβ-specific antagonist PHTPP did attenuate genistein and daidzein downregulation of caspase 3 activity. Similar to previous results observed with estradiol, both ER subtypes seem to play a role in genistein and daidzein actions on Tat activation of caspase 3. A comparable profile was observed with genistein effects on Bax in the presence of ER subtype antagonists. Both ER subtypes appear to mediate genistein effects on Bax expression as significant attenuation was achieved with both antagonists. However, a more robust attenuation of genistein effects on Bax expression, even above levels achieved in cultures treated with Tat alone, was observed in the presence of PHTPP. These results indicate that genistein effects on Bax expression may be preferential for ERβ-mediated signaling. Our results also demonstrate that significant attenuation of daidzein effects on Bax expression was achieved with the addition of both ER subtype

antagonists, suggesting involvement of both ERα and ERβ in daidzein downregulation of Bax. It is possible that daidzein's lower binding affinity for ERs compared to that of genistein and estradiol may explain the absence of the preferential mediation of a specific ER subtype.

Importance of ERβ

The recent discovery of a second isoform of the estrogen receptor (ERβ) has generated intense interest in its potential role in estrogen signaling. In the adult rat, the cerebral cortex appears to predominantly express ERβ (Shughrue et al., 1997). Studies using a rat model of stroke found estadiol to ameliorate the tissue loss associated with stroke in the cerebral cortex, initially implicating ERβ as the mediator of this protective effect (Dubal et al., 1998). However, studies using transfected cells as well as knockout mice have suggested that ERα. not ERβ, mediates protection against toxicity associated with middle cerebral arterial occlusion in addition to other toxic insults including β-amyloid toxicity. Some studies have found that ERα does not appear to mediate the protective effects of 17β-estradiol, suggesting that there may be multiple mechanisms mediating estrogenic effects depending on the trigger and specific cellular responsiveness. The present study demonstrates a potential role for ERβ in protection. Although both ER subtypes were detected in our cultures, this was not a quantitative measure and it is likely that ERα expression may be very low in our primary cortical cultures as seen in prior studies assessing ER expression on cortical cultures (Linford and Dorsa, 2002). Due to the high ERβ presence in the cerebral cortex, combined with the in

vitro selectivity of genistein and daidzein for ERβ, this subtype likely mediates the protective effects of phytoestrogens. Recent evidence using an ERβ mouse knockout model shows that the absence of ERβ in development leads to an increase in cortical neuron loss, suggesting that ERβ may play an anti-apoptotic role in normal cerebral cortical development (Wang et al., 2001).

Mitochondria have a vital role in neurodegeneration as well as neuroprotective mechanisms. The role of mitochondrial in apoptotic pathways is well established. In addition, recent studies report the ERβ is localized in the mitochondrial membrane. Our results suggest that the preferential involvement of ERβ- mediated signaling in 17β-estradiol and genistein effects on Bax may be related to localization of ERβ in mitochondria membrane. Estrogen and phytoestrogen attenuation of Tat-induced apoptotic signaling likely occurs via binding of estrogen receptors on the mitochondrial membrane and the direct regulation of proapoptotic proteins.

It is widely accepted that estrogen actions are mediated by its receptors ERα and ERβ. These receptors can exist and act as homodimers as well as heterodimers, suggesting functional interaction between these two ERs (Toran-Allerand et al., 1999). Classical genomic mechanisms of estrogen action may not completely explain the complex and extensive range of estrogen action in the brain. In addition to direct ER mediated effects, the estrogenic response has been shown to interact with many second messengers, which may mediate receptor independent effects. Indirect nongenomic pathways have been shown to mediate estrogenic responses. Estrogenic neuroprotection

has been associated with PI-3 kinase activation, CREB phosphorylation, MAPK activation, and elevation in Bcl-2 mRNA. Additionally it has been determined that estrogenic compounds can confer protection independent of estrogen receptor signaling (Linford and Dorsa, 2002). PI-3 kinase activation and MAPK activation have been directly linked to a reduction in intracellular caspase 3 activity, as well as being downstream of estrogen receptor signaling in neurons (Zhou et al, 2000; Zhang et al., 2001). For instance, the phosphorylation of the ER is now well established and it was shown to modulate ER ability to promote the transcription of estrogen-response-element controlled sequences as well as via the MAP-kinase pathway. In addition, several studies provide evidence for the existence of signal cross-talk between the ER and other second messengers. Although the question of whether phytoestrogens act via the same cellular pathways as estradiol or involve post-transcriptional events and/or membrane effects remains a topic of debate; however, our results have interesting implications in the understanding of the effect of phytoestrogens on brain function.

Studies have indicated that estrogen and phytoestrogens exhibit different effects depending on the concentration. In addition to the genomic and nongenomic mechanisms, these compounds are can act as potent anti-oxidants. Genistein is known to inhibit the activity of tyrosine kinases (Akiyama and Ogawara, 1991) and topoisomerase II (Markovits et al., 1989). However, these effects are thought to occur at high µM concentrations. Neurotoxic effects of genistein and daidzein were also demonstrated with high doses in range of 50-100µM, which are significantly higher than the doses used in the present experiments. Moreover, this antioxidant and tyrosine kinase inhibition

activities usually require higher concentrations than needed for receptor-mediated activity. Our data suggest that there is specific ER modulation of the apoptotic cascade, thus attenuating neuronal cell death, rather than antioxidant or tyrosine kinase inhibition effects.

Soy-derived extract preparations have been the most common form used in phytoestrogen intervention studies. However, variations in relative composition could be significant among soy extracts obtained from different sources of soy plants and manufactured using different protocols, bringing into question the safety and efficacy of random mixtures. The relative content of each isoflavone in soy-derived extract preparations may greatly influence the efficacy and safety phytoestrogen supplements. Although our studies demonstrate that individual isoflavones were capable of producing neuroprotective effects of the same magnitude as estradiol, Some studies suggest that select combination of phytoestrogens can elicit a stronger neuroprotective response and enhance ERβ-binding selectivity (Zhao et al., 2009). With the wide use of phytoestrogen supplements as alternative to hormone therapy, understanding the efficacy of individual isoflavone versus when they are used in combination would be of great value as possible antagonistic interactions could occur in random mixtures from soy extracts.

As estrogen receptors are thought to play a major role in mediating estrogen effects, delineating their role in the neuroprotective versus negative proliferative actions of estrogen remains a central focus of current research. Studies have reported that soy isoflavones preferentially bind ERβ over ERα. With the more abundant expression of

ERβ in the brain, it is suggested that this ER subtype is more involved in the neuroprotective actions of estradiol and phytoestrogens. The use of an ERβ selective agonist represents a viable therapeutic option to elicit the beneficial effects of estrogens without inducing the negative proliferative effects associated with estrogen therapy.

CHAPTER V

General Discussion

Estrogen deficient states may partly account for declines in cognition and neurodegeneration that are associated with dementia, such as HAD. The neuroprotective actions of estrogen have been demonstrated in many experimental models of neurodegenerative disease, including various dementias (Pike et al., 2009; Wilson et al., 2006). However, the mechanisms underlying the beneficial effect of estrogen are not well understood (Green & Simpkins, 2000). It is widely accepted that the biological actions of estrogen are mediated by two different receptor subtypes, ERα and ERβ. However, prolonged estrogen therapy is associated with increased risk of uterine and breast cancers. Thus, deciphering the roles of estrogen receptors in estrogenic processes is critical to developing therapeutic approaches that target the beneficial effects of estrogen and avoid unwanted side effects.

The observations that the viral regulatory protein Tat is actively secreted by infected cells, and that Tat mRNA is elevated in the patients with HIV dementia suggest a possible role of extracellular Tat in the progression of HAD. Accordingly, Tat has been shown to have direct toxic effects on neurons that included increased production of ROS indicating oxidative stress, mitochondrial dysfunction, and apoptotic cell death.

The current series of experiments determined the neuroprotective efficacy of endogenous estrogen, 17β-estradiol and phytoestrogens, genistein and daidzein, against HIV-1 Tat induced cell death. The results observed in the present series of experiments, demonstrate a common magnitude of neuroprotective effect of endogenous 17β-estradiol and phytoestrogens, mediated by similar estrogen receptor activation.

Estrogen deficiency is associated with increased risk for dementia such as Alzheimer's disease indicating that estrogens are a key factor in susceptibility to neurodegenerative disease and thus may be essential to neuroprotective processes. In Experiment 1, we demonstrate that estradiol was effective in attenuating HIV-1 Tat-induced cell death. Numerous studies have evaluated the neurotoxic potential of Tat, which includes the induction of apoptotic cell death. However, the mechanism(s) underlying this cell death and the time course of Tat-mediated neurodegeneration is not well-understood. In accordance with previously reported results, that Tat exposure induces mitochondrial dysfunction and production of ROS (Aksenov et al., 2001:2003), we show that apoptotic cell death, as determined by the activation of caspases, was initiated early after Tat exposure. This caspase activity was maintained over 24hrs and preceded the significant loss of cell viability in Tat exposed cultures. Moreover, we demonstrate a direct interaction of Tat with neurons expressing caspase activity. In addition Tat-induced cell death involved the activation of the mitochondria-mediated apoptotic cascade. Key regulators of this cascade such as the proapoptotic protein Bax and active caspase 9 and caspase 3 were increased early after Tat exposure. Estrogen

attenuation of cell death involved down regulation of these mitochondria specific apoptotic markers. Such observations support the findings that that estrogen is protective against oxidative stress-mediated apoptosis.

Many of the physiological actions of estrogens are mediated by the two estrogen receptor subtypes, ERα and ERβ that belong to the nuclear receptor superfamily. The differential expression and distribution of the ERs play a major role in the many physiological processes mediated by estrogens. However, estrogen therapy is associated with negative side effects, mainly increased risk of ovarian and breast cancer. Delineating the mechanisms underlying the actions of estrogen will be essential in targeting its beneficial effects with minimal side effects. This experiment illustrates the ability of 17β-estradiol to attenuate some aspects of Tat-induced apoptotic cell death. Since estradiol pretreatment was able to downregulate the expression/activity of mitochondria specific apoptotic markers, this supports a strong role for genomic effects in estradiol-mediated attenuation of the mitochondrial apoptotic cascade. Our experiments evaluating the mechanism of estradiol neuroprotection against Tat-induced neurodegeneration reveal that ERs mediate select aspects of apoptotic cascade. Particularly, estrogen receptors mediate the pro-apoptotic protein Bax expression, which along with Bcl-2, monitors mitochondria membrane permeability, and capsase 3, an effector caspase. This indicates that mitochondria membrane function and the execution phase of the apoptotic cascade represent key targets of apoptotic intervention. We also demonstrate the differential involvement of ER subtypes in mediating estrogen effects. Both ER subtypes seem to

play a role in estrogen effects on caspase 3 while estrogen effects on Bax expression are mediated more so by ERβ. The ERβ specific effects on Bax, may be related to the recent discovery of ERβ localization in mitochondria, suggesting a direct estrogen effect on mitochondria function via ERβ activation and signaling.

The functions of ERβ are of particular importance in understanding the neuroprotective strategies exhibited by estrogen. It has become clear that ERβ has functions that are distinct from those of ERα. Studies in ER knockout mice indicate that ERα is the main player in mediating female reproductive functions whereas ERβ is more important in non-classical target tissues, such as central nervous systems (Harris, 2007). Targeting ERβ function may be a potential therapeutic option in neurodegenerative disease, since it is highly expressed in the brain, specifically in cortical regions responsible for executive cognitive functioning, which is significantly affected in HIV-1 associated neurocognitive disorders (HAND).

Further experiments sought to evaluate if potential ERβ selective agonists are equally effective as endogenous estradiol against Tat-induced apoptotic cell death. Phytoestrogens are plant-derived nonsteroidal compounds that are structurally similar to estrogens and thus induce estrogenic responses. Since the discovery that phytoestrogens can bind and activate ER, particularly interesting was the finding that many phytoestrogens bind with a higher affinity to ERβ compared to ERα, suggesting that they may induce physiological effects through this ER subtype. We observe that phytoestrogens genistein and daidzein exhibit a similar magnitude of protection as

estradiol against Tat-induced apoptotic cell death. Genistein and daidzein significantly attenuated the expression of apoptotic markers specific to the mitochondria apoptotic pathway. Moreover these effects were also mediated by estrogen receptors. However, receptor mediated effects of genistein were more similar to estradiol than daidzein. These observations indicate that phytoestrogens attenuate Tat-induced cell death at a comparable magnitude and via estrogen receptor signaling. In addition, genistein closely modeled the receptor-mediated effects of estradiol, unlike daidzein.

Although phytoestrogens are thought to preferentially bind ERβ, the degree to which a compound binds the ER may not necessarily reflect its ability of activate ER signaling. In a study using radioligand binding assay, genistein was shown to have a higher binding affinity than daidzein for ERβ and have a higher/stronger transcriptional potency. Of note is that the concentrations of phytoestrogen required to activate transcription through both ERα and ERβ are generally 1μM or less, which are concentrations physiologically achievable in humans after consumption of phytoestrogen rich foods or supplements. In tissues, expressing both ER subtypes activation of ERα and ERβ may occur.

It should be considered that even though the individual purified phytoestrogens and their metabolites can bind and activate ERs, in regard to human exposure to phytoestrogens do not represent single compounds, but a mixture of different phytoestrogens and metabolites. The mixtures may induce different physiological effects compared to a single phytoestrogenic compound. A recent study found that a select

combination of phytoestrogens may enhance ERβ activation and binding. It was shown that when genistein, daidzein, and equol were combined, the selectivity for ERβ binding increased, compared to genistein alone (Zhao et al.,2009). Moreover, the effects of these combinations in cells coexpressing ERα and ERβ may also display differential transcriptional activity when exposed to mixtures of different phytoestrogens.

In addition to neuronal loss, synaptodendritic pruning has also been implicated to underlie the cognitive dysfunction observed with HIV infection in the CNS. Studies have shown that Tat exposure caused loss of synapses before cell death was observed. Moreover, in contrast to cell death, synaptic degradation can be reversed. Future investigations should consider if estradiol and phytoestrogen protective effects against Tat induced neurodegeneration involves preservation of neuronal networks and synaptic density. Furthermore, to better model human consumption of soy products and easily available supplements, a combination of phytoestrogens should be used to determine their effects against neurogenerative insults.

With the success of HAART on viral suppression, viral production still persists in the brain, allowing the continued release and exposure to toxic viral proteins. The inability of anti-retroviral therapy to prevent the development of cognitive dysfunction indicates the need for alternative therapies that may ameliorate the neurodegenerative and subsequent neurologocial disturbances associated with NeuroAIDS. Findings in the present study demonstrate that phytoestrogens offer a similar magnititude of protective effect as endogenous estradiol via a selective estrogen receptor -mediated mechanism

against HIV-1 Tat-induced cell death. Phytoestrogens, acting as selective ER agonists targeting the neuroprotective effects associated with estradiol, may be represent a viable adjuvant therapy with HAART combat NeuroAIDS.

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