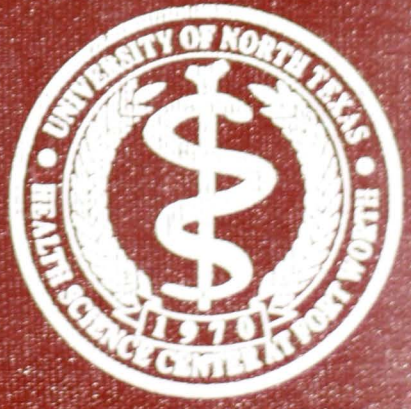
UNIVERSITY OF NORTH TEXAS
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ABSTRACT

The signaling pathways that mediate neurodegeneration are complex and involve a balance between phosphorylation and dephosphorylation of signaling and structural proteins. Estrogens have a variety of mode of action including transducing signaling events including the activation and/or suppression of survival pathways. The purpose of this study was to delineate the role of protein phosphatases (PP) in estrogen neuroprotection. We assessed the role of PP in neuroprotection mediated by estrogen and its analogues. We also determined the role of estrogen receptors (ER) and MAPK signaling. Okadaic acid (OA) and calyculin A (CA), non-specific serine/threonine PP inhibitors, were exposed to cells at various concentrations in the presence or absence of 17β-estradiol, 17α-estradiol, the enantiomer of 17β-estradiol (ENT E2), 2-(1-adamantyl)-3-hydroxyestra-1,3,5(10)-trien-17-one (ZYC3, non-ER binding estrogen analog) and/or glutamate. OA and CA caused a dose-dependent decrease in cell viability. None of the estrogen and its analogues showed protection against neurotoxic concentrations of either OA or CA, while all estrogens attenuated glutamate toxicity. However, in the presence of these PP inhibitors, estrogen mediated protection against glutamate toxicity was lost. Glutamate treatment caused a 50% decrease in levels of PP1, PP2A, and PP2B protein; while, co-administration of estrogen or its analogues with glutamate prevented the decrease in PP1, PP2A, and PP2B levels. In addition, PP2A and calcineurin activities were significantly suppressed with treatment of glutamate and/or OA; while, the presence

of these estrogens attenuated the decreases in PP activity. Moreover, an increase in reactive oxygen species, protein cabonylation, lipid peroxidation, caspase-3 activity, and mitochondrial dysfunction were evident in both glutamate and OA mediated cell death. Estrogens attenuate these increases in glutamate-mediated cell death, but were ineffective in OA-induced neuronal death. Furthermore, glutamate treatment caused a persistent increase in phosphorylation of ERK1/2 that corresponds with the decrease protein levels of PPs. Treatment of estrogens blocked the persistent increase in ERK phosphorylation and decreased PP protein expression associated with glutamate toxicity. These results suggest that estrogens protect cells against glutamate-induced oxidative stress and excitotoxicity through an ER-independent mediated mechanism that serves to preserve phosphatase activity in the face of oxidative/excitotoxic insults resulting in attenuation of the persistent phosphorylation of ERK1/2 associated with neuronal death.

ROLE OF SERINE/THREONINE PROTEIN

PHOSPHATASES IN ESTROGEN

MEDIATED NEUROPROTECTION

Kun Don Yi, B.S., M.S.

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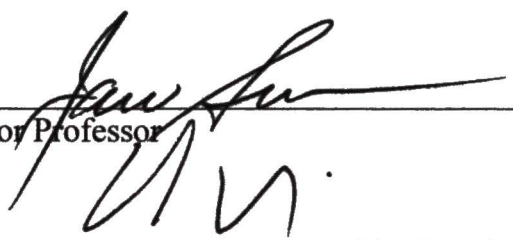

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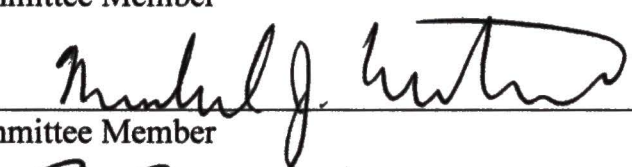
Committee Member



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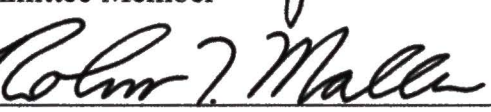

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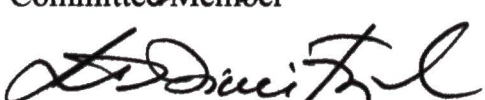

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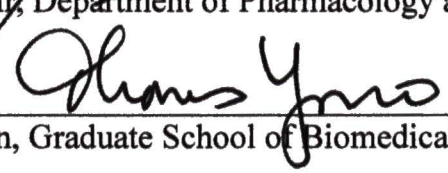

Dean, Graduate School of Biomedical Sciences

ROLE OF SERINE/THREONINE PROTEIN PHOSPHATASES IN ESTROGEN MEDIATED NEUROPROTECTION

DISSERTATION

Presented to the Graduate Council the

of the Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Kun Don Yi, B.S., M.S.

Fort Worth, TX

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LIST OF FIGURES

CHAPTER 1

Introduction To The World Of Estrogens And The Brain

Neurodegenerative Diseases

The mechanisms underlying the pathogenesis of neurodegenerative diseases are gradually being disclosed. The brain is an organ that consumes a large amount of oxygen, has a relatively low antioxidative capacity, and thus, the brain is highly susceptible to oxidative stress. It is also inadequately equipped with antioxidant defense systems to cope with continuous oxidative damage. Increasing evidence indicates that oxidative and nitrosative stress, excitotoxic insult, mitochondrial dysfunction (Reddy and Beal, 2005; Zeevalk et al., 2005), accumulation of oxidized aggregated proteins (Bence et al., 2001), inflammation (Griffin, 2006), ubiquitin-proteasome system dysfunction (McNaught et al., 2001; Ciechanover and Brundin, 2003; Stefanis and Keller, 2006), synaptic failure, and altered metal homeostasis represent the complex pathologies in many slowly progressive neurodegenerative disorders. These events probably constitute a vicious cycle, and any one of them could initiate neuronal cell death, rapidly recruiting or activating the others (Fig. 1.1 from (Halliwell, 2006)).

Many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and cerebrovascular stroke, show gender disparities. The incidence of a wide range of neurological disorders is higher in men than in women until women reach post-menopausal period, at which time, the incidence in women surpass that of men (Hurn and Macrae, 2000). Thus, many investigators have shown that female sex hormones are important regulators of neuronal function and may afford women protection from certain neurodegenerative diseases before the onset of menopause.

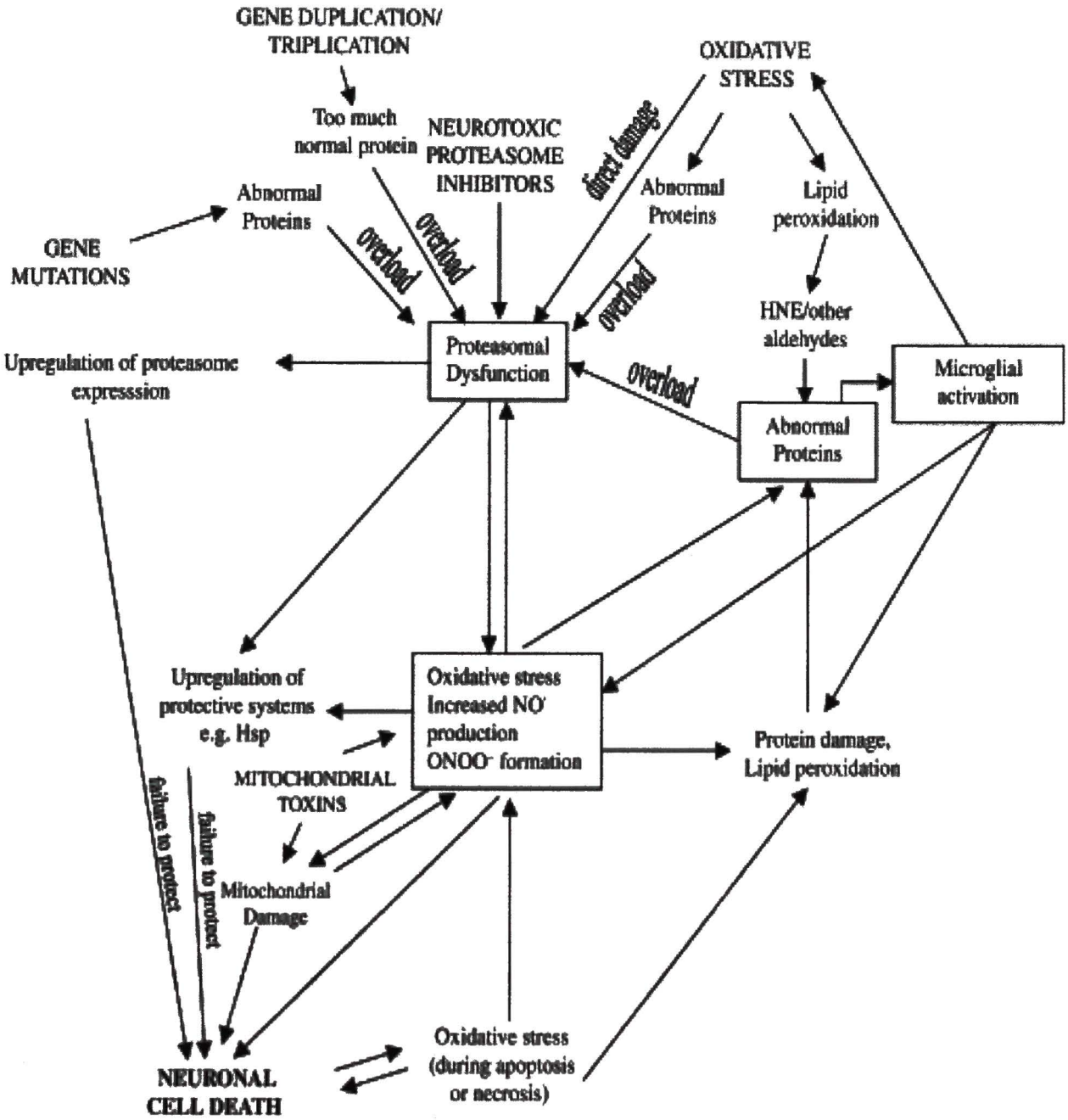

Figure 1-1. Schematice representation of oxidative damage, mitochondrial dysfunction, and proteosome dysfunction leading to neuronal dysfunction and/or cell death (Halliwell 2006).

Brain and Neurotoxic Events

All aerobic cells suffer oxidative damage, yet the mammalian brain is especially sensitive due to the fact that it is one of the highest O_2 consuming organs. In adult humans, the brain accounts for only a fraction of the total body weight, but about 20% of basal O_2 consumption. The discrepancy is even more striking in young children, who have much smaller bodies but not proportionately smaller brains. The major reason for the disproportionate O_2 uptake is the high amounts of ATP necessary for maintenance of neuronal intracellular ion homoeostasis in the face of the openings and closings of ion channels associated with propagation of action potentials and neurosecretions.

Therefore, interruption of mitochondrial function in neurons by various toxins or inadequate O_2 or substrate supply for energy production, results in rapid cellular damage. Interference with Ca^{2+} sequestration and/or disruption of the ATP supply produces especially rapid rises in intracellular free Ca^{2+}. Oxidative stress-dependent damage to plasma membrane Ca^{2+} exporters or to Ca^{2+} pumps in the endoplasmic reticulum has been shown to interfere with Ca^{2+} sequestration. In a cyclic manner, the rise in Ca^{2+} will further interfere with mitochondrial function as it will take up the excess intracellular Ca^{2+}, which in turn will increase mitochondrial $O_2^{\bullet-}$ formation. This excess $O_2^{\bullet-}$ can react with $NO^{\bullet}$ to form $ONOO^{-}$.

In addition, some neurons and glia contain transient receptor potential melastatin-related (TRPM)2 cation channels, which rapidly allow Ca^{2+} to enter when reactive oxygen species (ROS) such as H_2O_2 are present (Fonfria et al., 2005). Rises in Ca^{2+} can

activate neuronal nitric oxide synthase (nNOS), phospholipase A_2 and calpains, a family of Ca^{2+}-stimulated proteinases that can attack the cytoskeleton. In some animals calpains can convert xanthine dehydrogenase to the enzyme xanthine oxidase, which can generate $O_2^{\bullet-}$ and H_2O_2, but this is probably unimportant in human brain (Linder et al., 1999). The resulting $NO^{\bullet}$ is not only damaging to the neuron that produces it, but to near by neurons as $NO^{\bullet}$ can diffuse several cell lengths in the brain. The rise in arachidonic acid via activation of phospholipase A_2 coupled with increased lipid peroxidation (Smith, 2005) can promote eicosanoid formation (Farooqui et al., 2001; Phillis and O'Regan, 2004) and, if prostaglandins are not quickly removed, they can undergo conversion to the neurotoxic agents, cyclopentenone prostaglandins and levuglandins (Kondo et al., 2002; Musiek et al., 2005; Salomon, 2005).

Neuronal membrane lipids are rich in highly polyunsaturated fatty acid side-chains, especially docosahexaenoic acid ($C_{22:6}$) residues. Homogenization of isolated brain tissue causes rapid lipid peroxidation, which can be largely inhibited by iron-chelating agents such as desferrioxamine (Halliwell and Gutteridge, 1997). In addition, products of lipid peroxidation can injure the brain. 4-Hydroxynonenal is especially cytotoxic to neurons, increasing Ca^{2+} levels, inactivating glutamate transporters and damaging neurofilament proteins (Mark et al., 1997; Ong et al., 2000). It can also inactivate α-ketoglutarate dehydrogenase (αKGDH), a key enzyme of the tricarboxylic acid cycle (Sheu and Blass, 1999). Isoprostanes, which are also lipid peroxidation products, may act as vasoconstrictive agents in brain (Hou and Klann, 2004) and can damage developing oligodendrocytes in premature babies (Back et al., 2005). Other

products of the isoprostane pathway may also be neurotoxic by damaging the proteasome (Halliwell, 2006).

Hemoglobin is also neurotoxic. This protein is normally safely transported in erythrocytes, which are rich in antioxidant defense enzymes. However, isolated hemoglobin is degraded on exposure to excess H_2O_2, with release of pro-oxidant iron ions from the heme ring (Gutteridge and Hou, 1986; Puppo and Halliwell, 1988). Heme can also be released and is a powerful promoter of lipid peroxidation (Chiu et al., 1996) by decomposing peroxides to peroxyl and alkoxyl radicals (Phumala et al., 2003). In addition, hemoglobin reacts with H_2O_2 and other peroxides to form oxidizing species (heme ferryl and various amino acid radicals) capable of stimulating lipid peroxidation. Hemoglobin also binds $NO^{\bullet}$ avidly, producing vasoconstriction (Alayash, 2004) Both $NO^{\bullet}$ binding and oxidative damage are important in the vasoconstriction that can sometimes follow bleeding in the brain. For example, isoprostane levels were higher in the cerebral spinal fluid (CSF) of patients with subarachonoid hemorrhage who suffered vasospasm than in those who did not (Asaeda et al., 2005).

Concentrations of glutamate in brain extracellular fluids are normally low (< 1 μM). The death of cells or collapse of normal ion gradients due to severe energy depletion in neurons can cause massive glutamate release. This binds to receptors on adjacent neurons, leading to excessive and prolonged increases in intracellular free Ca^{2+} and Na^{+}. Neurons treated with excess glutamate or other excitotoxins rapidly die by apoptosis and/or necrosis. Oxidative stress can damage neurons and promote the release

of excitatory amino acids, generating a "vicious cycle" of events (Mailly et al., 1999). Other relevant events may be the ability of several reactive species (including $ONOO^-$) to decrease glutamate uptake by glial cells and to inactivate glutamine synthetase (Aksenov et al., 1997), preventing conversion of glutamate to glutamine. Hydroxynonenal can readily damage glutamate transporters, slowing glutamate clearance (Mattson and Chan, 2003).

Levels of 8-hydroxydeoxyguanosine (8OHdG), mutations and deletions increase with age in brain mitochondrial DNA. Several neurotransmitters are autoxidizable. Dopamine, its precursor L-DOPA, serotonin and norepinephrine can react with O_2 to generate not only $O_2^{\bullet-}$, but also quinones/semiquinones that can deplete reduced glutathione (GSH) and bind to protein SH groups (Spencer et al., 1998). Oxidation can be catalyzed by transition metal ions, as mentioned above, but if excess $O_2^{\bullet-}$ is present it can react with norepinephrine, dopamine and serotonin (Wrona and Dryhurst, 1998) to initiate their oxidation, which then continues with production of more ROS, quinones, and other products. Dopamine–GSH conjugates are degraded by peptidase enzymes to produce dopamine-cysteine conjugates (e.g. 5S-cysteinyldopamine), which can be detected in several brain regions, and levels are raised in PD (Spencer et al., 1998).

Brain metabolism generates a lot of H_2O_2, not only via superoxide dismutases (SODs) but also by other enzymes. Especially important are monoamine oxidases A and B, flavoprotein enzymes located in the outer mitochondrial membranes of neurons and glia. They catalyze the reaction

$$\underset{RCH_2NH_2}{\text{monoamine}} + O_2 + H_2O \rightarrow \underset{(RCHO)}{\text{aldehyde}} + H_2O_2 + NH_3$$

and generate substantial H_2O_2 in the brain (Gal et al., 2005). The ammonia is disposed of by several mechanisms, including its use by glutamine synthetase.

Antioxidant defenses are modest. In particular, catalase levels are low in most brain regions; levels are somewhat higher in hypothalamus and substantia nigra than in cortex or cerebellum (reviewed by (Halliwell, 2001)). Brain catalase is located in small peroxisomes (microperoxisomes) and its activity in rat or mouse brain is rapidly inhibited if aminotriazole is administered to the animals. This agent acts only on catalase complex I, confirming that brain generate H_2O_2 *in vivo* and that at least some of it reaches catalase (Sinet et al., 1980). The catalase probably cannot deal with H_2O_2 generated in other subcellular compartments.

Some glia are microglia, resident macrophage-type cells that arise from monocytes entering the brain during embryonic development. Normally, they help clear cellular debris (including apoptotic cells) and are alert for threats to neurons (Nimmerjahn and Ravetch, 2005). However, microglia can become activated to produce $O_2^{\bullet-}$, H_2O_2 and cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor α. In turn, such cytokines can cause microglia to generate more ROS upon activation and to produce inducible nitric oxide synthase (iNOS) and hence excess $NO^{\bullet}$. Cytokines can additionally be produced by activated astrocytes, which may again respond to them by

iNOS induction. Thus microglia and astrocytes are major players in inflammatory processes in the brain (Duncan and Heales, 2005).

Cytochromes P450 (CYPs) are present in some brain regions (Miksys and Tyndale, 2004). For example, CYP46 metabolizes cholesterol, and CYP2D6 is present in several human brain regions (Miksys et al., 2002). CYP2E1 is also found. Because CYP2E1 leaks electrons readily during its catalytic cycle, it produces more ROS than most other CYPs (Gonzalez, 2005). It is thus another potential source of oxidative stress. The magnitude of this may be small in normal brain because CYP2E1 levels are low. However, CYP2E1 metabolizes ethanol, acetone, halothane, related anesthetics and organic solvents such as CCl_4 and $CHCl_3$, and its levels may be increased in human brain by ethanol and smoking. Thus, it could contribute to solvent neurotoxicity (Howard et al., 2003). Brain CYP2D6 levels are also raised in human alcoholics (Miksys et al., 2002). RS, both directly (e.g. by decreasing the synthesis of proteins involved in tight junctions between cells; (Krizbai et al., 2005)) and/or by activation of matrix metalloproteinases, can contribute to 'opening up' the blood–brain barrier, allowing neurotoxins, endotoxin and inflammatory cells to enter the brain (Kim, 2003; Savaraj et al., 2005).

Like many other cells, neurons contain polyADP-ribose polymerase 1 (PARP-1), an enzyme that responds to DNA damage by cleaving NAD^+ and attaching ADP-ribose residues to nuclear proteins to facilitate DNA repair. Overactivation of PARP-1 can kill cells by depleting NAD^+, preventing energy production (Koh et al., 2005) and is involved in opening TRPM2 Ca^{2+} channels. Indeed, NAD^+ is neuroprotective; NAD^+ added to

neurons can slow axonal degeneration, an effect that seems to require the sirtuin, silent information regulator-like protein 1 (SIRT1), (Araki et al., 2004). Sirtuins are NAD^+-dependent protein deacetylase enzymes, intimately involved in the regulation of gene expression and of lifespan (Guarente, 2005).

Loss of trophic support can lead to oxidative stress and apoptosis in neurons, in part by activation of neuronal NADPH oxidase enzymes. NADPH oxidase (NOX) enzymes were first detected in phagocytes, but are now known to be widespread in animal tissues, seemingly producing $O_2^{\bullet-}$ for defense and/or signaling purposes (Krause, 2004). Neuronal NOX enzymes may promote necessary apoptosis during development of the nervous system but, if trophic support is lost in the developed brain, they may be activated inappropriately, leading to neuronal death (Sanchez-Carbente et al., 2005).

Oxidative Stress and Neurodegeneration

Oxidative damage is manifested as increases in lipid peroxidation end-products, DNA (and often RNA) base oxidation products and oxidative protein damage (Halliwell, 2001, 2002; Moreira et al., 2005; Sultana et al., 2006). The protein aggregates frequently contain proteins that are nitrated (Ischiropoulos and Beckman, 2003), bear carbonyl residues, have attached aldehydes such as HNE or acrolein and, sometimes, carry advanced glycation end-products (AGE products).

Increased oxidative damage occurs in all the human neurodegenerative diseases and seems especially important in AD and, perhaps to a lesser extent, in PD (Halliwell, 2001, 2006). Indeed, lipid peroxidation, measured as F2-isoprostanes in brain tissue or

CSF, is already increased in patients with mild cognitive impairment (MCI). Neuroprostane levels are also raised in AD brain, and levels tend to rise further as dementia progresses (Markesbery et al., 2005). Brain protein carbonyls (a marker of oxidative protein damage as well as oxidative DNA damage are also increased in MCI (Halliwell and Whiteman, 2004; Keller and Lauring, 2005). High doses of α-tocopherol (2000 units per day) were reported to produce a significant delay in the deterioration of patients with AD, although sadly the same dose did not affect the progression of patients with MCI to AD (Blacker, 2005). Given that α-tocopherol enters the brain only slowly, is very poor at decreasing lipid peroxidation in humans and is not depleted in AD brain, its apparent effect in AD gives hope that better antioxidants designed to target the brain could have a significant therapeutic impact (Halliwell and Gutteridge, 2006). Indeed, in mice overexpressing mutant human amyloid precursor protein, brain isoprostane formation preceded amyloid plaque deposition (Pratico et al., 2001) and inhibition of F_2-isoprostane formation by α-tocopherol administration delayed plaque formation and cognitive impairment (Sung et al., 2004). Unfortunately, α-tocopherol works better against lipid peroxidation, neurodegeneration and atherosclerosis in transgenic mice than it does in humans (Halliwell and Gutteridge, 2006). Thus, the development of novel antioxidants for AD, PD and other neurodegenerative diseases is a major research area (Halliwell, 2001; Moosmann and Behl, 2002; Mandel et al., 2005).

The effects of mutations in the ubiquitin–proteasome system, together with the finding that ubiquitin carboxyl-terminal esterase L1 (UCHL1) levels are decreased even in sporadic PD (Choi et al., 2004), suggest that all the events shown in Fig. 1.1 are

important. Indeed, this fall in UCHL1 activity involves oxidative damage, because the protein shows elevated levels of carbonyls and methionine sulfoxide (Choi et al., 2004). Mice lacking UCHL1 show widespread neurodegeneration, formation of protein aggregates and increased oxidative damage (Castegna et al., 2004). Damage to mitochondria [e.g. by neurotoxins such as 1-methyl-4-phenylpyridinium ion (MPP^+) or rotenone that target them generates more ROS from the electron transport chain and causes oxidative damage that modifies proteins and other biomolecules. Thus, in some studies, treatment of rats or monkeys with low-dose rotenone over long periods produces PD-like symptoms and neurodegeneration accompanied by oxidative damage, nitrotyrosine formation, and generation of protein aggregates containing α-synuclein (Moore et al., 2005). Unlike MPP^+, rotenone does not concentrate in dopaminergic neurons in this region, yet it can still induce fairly selective neurodegeneration in the substantia nigra. It follows that the neurons in the substantia nigra may be especially sensitive to inhibition of complex I. Another clue pointing to a key role for mitochondria is provided by the observation that early-onset PD can be caused by mutations in the nuclear gene encoding a mitochondrial protein, PINK1, a protein kinase that is somehow able to protect cells against apoptosis induced by proteasome inhibition (Moore et al., 2005).

Defects in mitochondria occur in the other common neurodegenerative disorders as well. Indeed, in AD the toxicity of amyloid peptides (Aβs) can involve direct mitochondrial damage (Yan and Stern, 2005). In addition, aggregating Aβs raise intracellular Ca^{2+}, increase NOX activity in astrocytes and directly produce ROS

(Barnham et al., 2004; Abramov and Duchen, 2005; Caspersen et al., 2005; Sultana et al., 2006). If mitochondrial damage significantly depletes the ATP supply, this will interfere with removal of proteins by the ubiquitin-proteasome system (ATP dependent), and it may even cause cells to increase their rates of Aβ production (Velliquette et al., 2005).

Oxidized and nitrated proteins are usually removed by the proteasome; its inhibition allows abnormal proteins to accumulate and produces oxidative stress. Exactly how this oxidative stress arises is unclear. Potential mechanisms include increased mitochondrial ROS production (Sullivan et al., 2004) and increases in nNOS activity, producing more $NO^{\bullet}$ (Lee et al., 2001). Formation of abnormal proteins resulting from gene mutations or of excessive amounts of normal proteins (e.g. α-synuclein, CuZnSOD) due to gene duplications or triplications (Halliwell, 2006) could overload the proteasome; its activity tends to decrease with age in the brain in any case. Indeed, injecting the proteasome inhibitor lactacystin into mouse or rat substantia nigra produced neurodegeneration, movement disorders and protein aggregates (Zhang et al., 2005). Protein aggregates may stimulate reactive oxygen and nitrogen species (RS) formation from neurons, and activate microglia. Both proteasome and complex I inhibitors are widespread in nature (for example rotenone and lactacystin are natural products), and consumption of too much of either or both could lead to sporadic PD or other neurodegenerative diseases (Halliwell, 2006). Finally, RS-producing agents could initiate neurodegeneration, because RS damage mitochondria, and may inhibit proteasome function directly by oxidative or nitrative inactivation of proteasome subunits) or indirectly by interfering with ubiquitination, or overloading the proteasome by creating

more oxidatively modified proteins such as those with HNE (Halliwell, 2006). Dopamine oxidation products, which accumulate in PD (Spencer et al., 1998), can both damage mitochondria and inactivate the proteasome (Keller et al., 2000).

Neuroprotective Effects of Estrogens

The neuroprotective effects of estrogens have been widely reported in various neuronal cells against different toxicities including serum deprivation, oxidative stress, and amyloid β peptide (Aβ) induced toxicity and excitotoxicity (Green and Simpkins, 2000). Clinical and experimental evidence have shown that both endogenous and exogenous estrogens exert neuroprotective effects against cerebral ischemia (Yang et al., 2000; Garcia-Segura et al., 2001; Wise et al., 2001; Yang et al., 2001). The pathological mechanisms that are activated during stroke include oxidative stress, free radical activity, excitotoxicity, inflammatory response, mitochondrial dysfunction, and apoptosis, which are antagonized by estrogens. While the classical estrogen effects have been indicated to be mediated by estrogen receptors (ERs)-dependent genomic mechanism, it is yet unclear whether the neuroprotective actions of estrogens are mediated by genomic or nongenomic mechanism. However, strong evidence from our laboratory and others indicate that neuroprotective effects of 17β-estradiol are receptor-independent since 17α-estradiol, the enantiomer of 17β-estradiol, and other non-ER binding estrogen analogues show equipotent protection (Behl et al., 1997; Green et al., 1997; Sawada et al., 1998; Green et al., 2001; Liu et al., 2005; Perez et al., 2005).

Clinical and Epidemiological Studies

Post-menopausal estrogen deficiency plays an important role in the pathogenesis of several age-related neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and cerebrovascular stroke.

Alzheimer's Disease

Alzheimer's disease afflicts more women than men and many epidemiological studies have suggested the protective role of estrogen in Alzheimer's disease. The earliest clinical trial assessing the potential effects of estrogen on Alzheimer's disease was a small pilot study of seven women who had been diagnosed with senile dementia of the Alzheimer's type (Fillit et al., 1986). Low dosages of estradiol therapy significantly improved attention, orientation, mood and social interaction in some women. A large prospective study (Henderson et al., 1994) also showed that postmenopausal estrogen replacement therapy may be associated with a decreased onset of AD and may improve cognitive performance of women with this disease. The cognitive function of women with Alzheimer's disease who had received estrogen therapy was well below non-demented control subjects, but better than the cognitive function of those Alzheimer's disease patients not taking estrogen. A placebo-controlled, double-blind, parallel-group design pilot clinical study evaluated the cognitive and neuroendocrine response to estrogen administration for postmenopausal women with AD. The findings of this study support that estrogen replacement may enhance cognition for postmenopausal women with AD (Asthana et al., 2001). Other studies also support a cognitive benefit of estrogen for women with AD (Kawas et al., 1997; Asthana et al., 2001).

A case-control (Resnick et al. 1998) and retrospective studies (Costa et al. 1999) have confirmed that estrogen protects postmenopausal women from cognitive deterioration. A case-control study nested within a prospective cohort study assessing the protective role of estrogen in Alzheimer's disease was based on postmortem follow-up reports of 3760 women who had answered a health survey that was administered 5 years before death (Paganini-Hill and Henderson, 1994, 1996). They found an inverse relationship between estrogen use and Alzheimer's disease; the risk of Alzheimer's disease decreased with increasing dose and duration of estrogen therapy. Results of many other epidemiological studies also demonstrated an inverse relationship between estrogen use and Alzheimer's disease (Tang et al., 1996; Costa et al., 1997; Yaffe et al., 1997; Resnick et al., 1998; Slooter et al., 1999; Waring et al., 1999).

Although a majority of studies on estrogen and cognition has found a positive effect of estrogen on memory processes, other studies have failed to demonstrate this effect (Rauramo et al., 1975; Vanhulle and Demol, 1976; Ditkoff et al., 1991). A randomized, double-blind, placebo-controlled 12-week trial has shown that a 1.25 mg/day dose of conjugated estrogen (Premarin) administered for 12 consecutive weeks does not produce a meaningful effect on cognitive performance, dementia severity, behavior, mood, and cerebral perfusion in female AD patients (Wang et al., 2000). Possible explanations for these discrepancies in the finding may include the differences in the selection of subjects and methodology. In particular, the differences in the type and route of administration of hormonal preparations that are employed may account for some of the inconsistencies in the results.

Moreover, recent reports from the Women's Health Initiative studies have indicated detrimental effects of HRT (Shumaker et al., 2003; Wassertheil-Smoller et al., 2003)) on cognition and increased risks of stroke and heart disease. These results are at odds with results of large epidemiological studies that showed protection. Although the latter data are, in part, confounded by a "healthy user bias," much of the inconsistency may be explained by the fact that women in the latter studies initiated HRT at the menopausal transition, whereas the WHI trial was conducted in older women who were approximately 12 yr postmenopausal. In addition, older trials included women on either unopposed estrogen therapy or cyclic HRT regimens. Further, a variety of other factors confound the issue of the beneficial effects of estrogen therapy, such as doses of hormones, type of hormones, the age at which HRT is administered, and a host of other factors. Therefore, a clearer understanding of the mechanism of estrogen-mediated neuroprotection is important.

Parkinson's Disease

Parkinson's disease (PD) is a chronic progressive degenerative disorder characterized by the selective degeneration of mesencephalic dopaminergic (DA) neurons in the substancia nigra pars compacta (SN). The loss of DA afferents to the striatum and putamen results in extrapyramidal motor dysfunction, including tremor, rigidity, and bradykinesia (Olanow et al., 2003), for extensive reviews). The mechanism(s) responsible for DA neuron degeneration in PD is still unknown, but oxidative stress, excitotoxicity, depletion of endogenous antioxidants, reduced expression of trophic factors and dysfunction of protein degradation system are believed to participate in the cascade of

events leading to DA neuron death (Di Monte et al., 1995; Jenner et al., 2000; Olanow et al., 2003). Recent evidence clearly indicates that neuroinflammatory mechanisms may play an important role in the pathogenesis of PD and inhibition of inflammation proposed as a promising therapeutic intervention (McGeer and McGeer, 2001; Chen et al., 2003; Gao et al., 2003; Hunot and Hirsch, 2003; Schiess, 2003; Marchetti et al., 2005).

Although several genes that cause certain forms of inherited PD have been identified, most cases of PD appear to be sporadic and likely represent an interplay between both genetic and environmental influences (Warner and Schapira, 2003). In particular, genetic factors may interact with early life events such as exposure to hormones, endotoxins or neurotoxins, thereby influencing disease predisposition and/or severity. In addition, developmental exposure to environmental toxins (such as pesticides/herbicides), either alone and/or in concert with other environmental (i.e. endotoxins; hormonal dysfunctions) and/or genetic "predisposing" factors, may synergistically increase DA neuron vulnerability (Warner and Schapira, 2003; Marchetti et al., 2005).

Importantly enough, gender and the sex steroid background also appear to strongly modulate vulnerability to PD, with mechanisms not completely elucidated. A number of epidemiological studies have reported that the incidence and prevalence of PD is higher in men than in women (Dluzen et al., 2003) for extensive review). Post-menopausal estrogen deficiency has been reported to cause a worsening of Parkinson-related symptoms, whereas the severity of symptoms in women with early PD is

diminished by the use of E_2 (Sandyk, 1989). Association between estrogen gene polymorphism and PD has also been reported (Westberg et al., 2004).

These clinical results are supported by a body of experimental evidence indicating that the nigrostriatal DA system is subject to modulation by estrogen in rodents and nonhuman primates (Dluzen and Horstink, 2003). Then, the nigrostriatal DA system is exquisitely sensitive to gonadal hormone influence and sexual differences are present in several parameters of the nigrostriatal DA neurons, as well as in the progression of diseases associated with this system (Miller et al., 1998; Callier et al., 2000; Dluzen and Horstink, 2003). Indeed, estrogens have been defined as a neuroprotectant for the nigrostriatal DA system (Dluzen and Horstink, 2003). The neuroprotective effects of estrogens have been reported against DA neurotoxicity induced by 6-hydroxidopamine (6-OHDA), methamphetamine, and MPTP model of PD (Miller et al., 1998; Callier et al., 2000; Grandbois et al., 2000; Dluzen and Horstink, 2003; D'Astous et al., 2004; Dluzen and McDermott, 2004).

Stroke

Stroke is the third leading cause of mortality and morbidity in the United States. Estrogen has been proposed to protect against stroke in humans (Paganini-Hill, 1995). Premenopausal women have a lower incidence of strokes and different types of strokes than observed in age-matched men. The incidence of stroke increase in postmenopausal women compared to age-matched pre-menopausal women. The effects of endogenous estrogen on lowering of cholesterol levels, on vascular endothelium, and on the

inflammatory response to infarct (Li et al., 1994) may contribute to the differences. The observations from epidemiological studies that moderate exposure to exogenous estrogen may decrease the risk of stroke; while tamoxifen (an estrogen receptor antagonist) treatment confers increased risk for stroke in women (Gail et al., 1999) suggests that estrogen plays a protective role in cerebrovascular disease.

The reports of epidemiological studies on the impact of estrogen replacement therapy on the incidence of stroke are conflicting (Paganini-Hill, 1995). The majority of studies have shown a 50% reduction in incidence of stroke with estrogen use while others have not demonstrated any effect of estrogen therapy (ET) on stroke risk (Petitti et al., 1998).

Even though the effect of estrogens on stroke incidence is unclear, many clinical trials consistently indicate estrogens plays a protective role on stroke-related mortality. An MRI analysis in a transient focal ischemia model demonstrates that estrogen decreases reperfusion-associated cortical ischemic damage (Shi et al., 2001). Estrogen replacement therapy is associated with a 40 to 60% reduction in death from stroke and other cerebral vascular diseases (Bush et al., 1987; Paganini-Hill et al., 1988; Hunt et al., 1990; Finucane et al., 1993; Petitti et al., 1998). However, the result of a randomized, double-blind, placebo-controlled trial of estrogen therapy (1 mg of 17β-estradiol per day) which was conducted in 664 postmenopausal women who had recently had an ischemic stroke or transient ischemic attack showed that estradiol did not reduce the risk of death or the risk of nonfatal stroke (Viscoli et al., 2001). In the past 25 years, 29 studies have

produced no conclusive evidence of a beneficial effect (Paganini-Hill, 2001). This may be due to the lack of consistency in stroke endpoints, definition of estrogen replacement therapy user, estrogen preparation, and influence of combined regimen.

The biological mechanisms by which estrogens may influence AD and stroke are still unknown. Experimental observations suggest that the protective effects of estrogen may be multifactorial, as discussed below.

Experimental Studies

***In vitro* evidence**

It has been known that addition of 17β-estradiol to serum-free media increases the viability, survival and differentiation of primary neuronal cultures from different neuronal populations (Faivre-Bauman et al., 1981; Bishop and Simpkins, 1994; Duenas et al., 1996; Brinton et al., 1997; Sudo et al., 1997). Studies using primary cortical cultures have shown that estradiol prevents neuronal death induced by many stimuli such as iron, glutamate, AMPA toxicity, anoxia, cytochrome oxidase inhibitor sodium azide, the pro-oxidant hemoglobin, kainate or NMDA (Regan and Guo, 1997; Vedder et al., 1999; Zaulyanov et al., 1999). Estrogens prevent cell death of cultured dorsal root ganglion neurons deprived of nerve growth factor (Patrone et al., 1999), hippocampal cell cultures exposed to NMDA (Weaver et al., 1997) and mesencephalic cultures subjected to glutamate, superoxide anions or H_2O_2 (Sawada et al., 1998). Other studies using neuronal cell lines have also demonstrated the neuroprotective effects of estrogens. 17β-estradiol protects NT2 neurons, PC12 cells and mouse neuroblastoma (Neuro 2a) cells from H_2O_2

or glutamate induced cell death (Bonnefont et al., 1998; Singer et al., 1998), SK-N-SH human neuroblastoma cells from serum deprivation and beta amyloid (Green et al., 1996; Green et al., 1997), hippocampal HT 22 cells from lipid peroxidation (Vedder et al., 1999). These *in vitro* studies have provided important insights into the possible mechanisms involved in the neuroprotective effects of estrogens.

***In vivo* evidence**

One of the models used to test the neuroprotective effect of estrgoens *in vivo* is experimental forebrain ischemia. 17β-estradiol pretreatment reduces animal mortality and ischemic area in ovariectomized rats after middle cerebral artery occlusion (Simpkins et al., 1997a; Simpkins et al., 1997b), and decreases the expression of amyloid precursor protein mRNA (Shi et al., 1998). 17β-estradiol reduces ischemia-induced neuronal damage to the CA1 region in gerbil hippocampus (Sudo et al., 1997; Chen et al., 1998). Neuroprotection by estrogen has also been shown in a mouse model of focal cerebral ischemia (Culmsee et al., 1999). Another experimental model is injury to the nigrostriatal system. Estrogen has neuroprotective properties against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in the nigrostriatal dopaminergic system (Callier et al., 2000; Dluzen and Horstink, 2003). Additionally, estrogens have been shown to protect against the vulnerability of the lateral striatal artery (a perforate artery that is most often affected by stroke) to 3-nitropropionic acid intoxication (Nishino et al., 1998). Administration of 17β-estradiol to ovariectomized rats is able to prevent the loss of hilus neurons of the dentate gyrus induced by kainic acid, a widely used experimental model of epilepsy and neurodegeneration (Azcoitia et al., 1998). In

agreement with clinical findings, several animal studies have shown that estrogens improve memory performance (Singh et al., 1994; Bimonte and Denenberg, 1999; Fader et al., 1999; Markowska, 1999; Rissanen et al., 1999; Frick et al., 2000)

Mechanisms of Neuroprotection by Estrogen

The steroidal compound estrogen is now well accepted as a central neuroactive and neuromodulatory molecule. One important aspect of estrogens' effect on neurons is its neuroprotective activity. The mechanisms of neuroprotective functions of estrogen are characterized by classical genomic effects as well as by rapid activities depending on estrogens interaction with neuronal membranes and intracellular signal transduction pathways. Estrogens hav also been shown to have an intrinsic antioxidant structure that lies in the phenolic ring of the compounds. This antioxidant activity of estrogens and estrogen-derivatives may provide antioxidant activity for neurons and may complement other neuroprotective activities of estrogen and therefore also mediate some of the beneficial effects of estrogen replacement with respect to Alzheimer's disease.

Mechanisms mediated by the activation of estrogen receptors and gene transcription

The estrogen receptors (ERs) are members of the steroid /thyroid/retinoid receptor superfamily. ERs are ligand-regulated transcription factors that are expressed in specific regions within target areas of the nervous system and other estrogen-sensitive organs (Katzenellenbogen et al., 2000). ERα and ERβ are the estrogen receptors which have been cloned. They share a structure that is similar to that of all members of the steroid receptor superfamily, and have highly conserved amino acid sequences in some domains,

particularly the DNA-binding domain. Both have an activation function domain in the amino terminus (AF-1), a central DNA binding domain and a carboxy-terminus ligand-binding domain, in which a second activation function (AF-2) resides (Garcia-Segura et al., 2001). Both ERα and ERβ are found throughout the rat brain. Levels of ER-α mRNA expression are higher in females than in males. The brain regions with the highest densities of cells expressing α or β estrogen receptors are found in the forebrain, the preoptic area, hypothalamus and amygdala (Li et al., 1997; Shughrue et al., 1997; Shughrue et al., 1998; Shughrue and Merchenthaler, 2001). Estrogen receptor β mRNA expression is higher than ER-α mRNA expression in the paraventricular nucleus and much higher in the medial tuberal nucleus, whereas ER-α mRNA expression is much greater than ER-β mRNA expression in the ventromedial and arcuate nuclei of the hypothalamus. The total number of estrogen-target cells in areas such as the cerebral cortex, hippocampal formation, and midbrain raphe may be much greater than found in the hypothalamus-preoptic continuum and amygdala (Garcia-Segura et al., 2001). Thus, the potential impact of estrogens, via estrogen receptor activation, in the vulnerable brain regions of AD should not be underestimated. Differences in expression of estrogen receptors in different brain regions also suggest that estrogens may not be universally neuroprotective.

A putative membrane receptor for estrogens has been proposed (Zheng and Ramirez, 1997; Becker, 1999) to function in estrogen signal transduction, in which estrogen rapidly activated G proteins, increased iniositol phosphate synthesis and

increased adenylate cyclase activity (Garcia-Segura et al., 2001). These rapid effects of estrogen may alter phosphorylation events or calcium release which then modifies the transcriptional regulatory effects of estrogen receptors. Some binding studies have shown that estradiol binds specifically to isolated synaptosomal mitochondria. Physiological concentrations of estradiol, acting on mitochondrial membrane properties, extragenomically modulate the mitochondrial, and consequently the synaptosomal content of Ca^{2+}, and in that way exert a significant change in nerve cell homeostasis (Horvat et al., 2001). Additionally, a substantial proportion of the total estrogen binding capacity and coexpression of the two isoforms was detected in mitochondria and microsomes in the uterus (Monje and Boland, 2001). This differential cellular partitioning of estrogen receptor α- and β-forms may contribute to the known diversity of 17β-estradiol effects. Both estrogen receptor α and β expression levels and cellular localization patterns implicated that estrogen may exert effects directly to mitochondria.

Putative estrogen response elements (EREs) have been identified in the promoter region of some genes that are both essential to normal brain function and implicated in brain disorders. Among these are the genes encoding choline acetyltransferase (Miller et al., 1999), the GABA transporter GAT-1 (Herbison et al., 1995), oxytocin (Young et al., 1996; Bale and Dorsa, 1997), somatostatin (Xu et al., 1998), glial fibrillary acidic protein (Stone et al., 1998), brain-derived neurotrophic factor (Sohrabji et al., 1995), transforming growth factor-alpha (El-Ashry et al., 1996), Fos (Weisz and Rosales, 1990), and Bcl-2 (Teixeira et al., 1995). Estrogens have been shown to increase the expression of these proteins. In addition to interacting with estrogen response elements, estrogen

receptors are able to affect transcriptional responses to other transcription factors, such as Jun and Fos at AP-1 sites (Paech et al., 1997), perhaps through protein-protein interactions that do not rely on binding to an estrogen response element (Webb et al., 1999).

Estrogen receptors may mediate the effects of estrogens via interaction with intracellular signaling cascades (Kelly and Wagner, 1999; Singer et al., 1999; Toran-Allerand et al., 1999; Singh et al., 2000). Estradiol can uncouple GABA-B and μ-opioid receptors from potassium channels via activation of protein kinase A and C (Lagrange et al., 1996; Lagrange et al., 1997; Kelly and Wagner, 1999). Estrogen activates mitogen-activated protein kinase (MAPK) and B-RAS (Singh et al., 2000), an effect that is involved in the ability of estrogen to protect against glutamate induced nerve cell death (Singh et al., 2000). G proteins are also activated by estrogen in the striatum (Mermelstein et al., 1996; Becker, 1999). All of these effects are rapid and depend on the presence of estrogen receptor (or a putative membrane receptor), but not on protein synthesis (Garcia-Segura et al., 2001). The trophic effects of estrogen and estrogen receptors involve activation of transcription via estrogen receptor binding to estrogen response elements, and estrogen receptor activation of signal transduction pathways via ligand-induced release of constituents of the multimeric complex of the estrogen receptor complex, or via a membrane receptor (Toran-Allerand et al., 1999).

Recent studies show that there is an interaction between the estrogen receptor and apolipoprotein E (ApoE). ApoE protein is important to membrane repair and synaptic

plasticity and is thought to reduce clearance of amyloid beta (Stone et al., 1998; Teter et al., 1999). In addition, estrogens upregulate choline acetyltransferase (Luine, 1985; Rabbani et al., 1997; Sohrabji et al., 2000), an important observation for AD, since the loss of choline acetyltranferase activity is correlated with the loss of cognitive function (Baskin et al., 1999). Furthermore, estradiol may improve cognitive function of AD patients by enhancing serotonin receptor signaling or increasing serotonin transporter expression (Halbreich et al., 1995; McQueen et al., 1997) since depression is one of the early symptoms of Alzheimer's Disease and abnormalities of serotonergic function have been proposed to be the causative factor. Finally, estrogen may upregulate the expression of antiapoptotic molecules, such as Bcl-2 and Bcl-xL, and downregulate the proapoptotic molecules Nip-2 and Par-4 in neurons and neuronal cell lines (Garcia-Segura et al., 2001).

It is well established that estrogen receptor and neurotrophin receptor co-regulate each other in many areas of the nervous system (Toran-Allerand et al., 1992; Miranda et al., 1994; Sohrabji et al., 1995; Toran-Allerand et al., 1999). The interaction of estrogen and neurotrophin may upregulate the specific genes responsible for neuronal survival through convergence of signaling pathways (Watters et al., 1997; Singh et al., 1999; Toran-Allerand et al., 1999). Estradiol may directly activate the signaling pathways of growth factors (Toran-Allerand et al., 1988; Duenas et al., 1996). Estradiol and NGF act synergistically to protect cells from apoptosis induced by serum deprivation (Gollapudi and Oblinger, 1999). Insulin-like growth factor I (IGF-I) has prominent neurotrophic effects, stimulating differentiation and survival of specific neuronal populations (Torres-

Aleman et al., 1994; de Pablo and de la Rosa, 1995). Estrogen and IGF-I have a synergistic action on neuritic growth (Fernandez-Galaz et al., 1997; Fernandez-Galaz et al., 1999). Collectively, estrogen can promote neuronal survival by interacting with growth factors and increasing the expression of anti-apoptotic proteins through classical nuclear receptor, as well as putative receptors located in plasma membrane and mitochondrial membrane.

Mechanisms independent of the transcriptional activation by estrogen receptors

The antioxidant activity of estrogen observed at high (non-physiological) concentrations (10^{-5} M) is dependent upon the presence of a phenolic A ring in the steroid structure and is independent of an activation of the estrogen receptors (Behl et al., 1995; Behl et al., 1997; Culmsee et al., 1999; Behl and Manthey, 2000; Moosmann and Behl, 2002). A large body of evidence has demonstrated that estradiol has antioxidant properties and suppresses the oxidative stress in neurons and neuronal cell lines induced by hydrogen peroxide, superoxide anions and other pro-oxidants (Behl et al., 1995; Behl et al., 1997; Bonnefont et al., 1998; Sawada et al., 1998; Behl, 1999; Behl and Holsboer, 1999; Calderon et al., 1999; Behl and Manthey, 2000). Both 17α- and 17β-estradiol have similar antioxidant effects (Behl et al., 1995; Behl et al., 1997; Green et al., 1997; Bonnefont et al., 1998), suggesting that the neuroprotective effects of estrogens are in part ER independent and developing estrogenic like compounds without the binding properties of 17β-estradiol to ER can serve as protective agents in both women and men against neurodegenerative processes.

Increasing evidence supports the mechanisms of the neuroprotective effects of estrogen independent of estrogen receptors. The finding that 17α- and 17β-estradiol protect SK-N-SH human neuroblastoma cells from serum deprivation and the effect was only partially reversed by the estrogen receptor antagonist tamoxifen suggests that estrogen may have neuroprotective effects not mediated by the estrogen receptors (Green et al., 1997). 17α- and 17β-estradiol also protect against beta-amyloid peptide toxicity in a HT-22 murine neuronal cell line lacking functional estrogen receptors (Green et al., 1998). Estrogen receptor antagonists do not block the neuroprotective effects of estradiol against NMDA-induced neuronal death in rat hippocampal cultures, glutamate neurotoxicity in mesencephalic cultures and pro-oxidants in rodent neuronal cultures (Behl et al., 1995; Weaver et al., 1997; Sawada et al., 1998; Moosmann and Behl, 2002). 17β-estradiol has also been shown to be a potent inhibitor of iron-induced lipid peroxidation of membrane and fatty acids in rat brain homogenates, rat cortical synaptosomes, hippocampal HT 22 cells and primary neocortical cultures (Keller et al., 1997; Vedder et al., 1999). Estrogens may decrease the production of cytokines in astroglial cells exposed to Aβ1-40, lipopolysaccharides (Dodel et al., 1999) or decrease the inflammatory response after brain injury (Garcia-Segura et al., 2001) by decreasing the activation of NF-κB, a potent immediate-early transcriptional regulator of numerous proinflammatory genes, through anti-oxidative mechanism.

In addition to anti-oxidant properties, the neuroprotective effects of estradiol may be independent of nuclear estrogen receptor activation. Estradiol may interact with

estrogen binding sites in the plasma membrane (Ramirez and Zheng, 1996) or mitochondrial membrane (Monje and Boland, 2001) and may have many different rapid effects on neuronal excitability and neuronal signal transduction.

Accumulating evidence has shown that neuroprotective effects involve hormonal activation of intracellular signaling pathways via G proteins (Moss and Gu, 1999; Raap et al., 2000), extracellularly regulated kinases (phosphoinositol 3-kinase and protein kinase B/AKT, and stimulates ERK and p38 MAP kinases) (Watters et al., 1997; Toran-Allerand et al., 1999; Singh et al., 2000), phosphorylation of the cAMP response element binding protein (Zhou et al., 1995; Zhou et al., 1996; Murphy and Segal, 1997; Watters and Dorsa, 1998; Walton and Dragunow, 2000), and alterations in intracellular calcium levels (Beyer and Raab, 1998; Improta-Brears et al., 1999; Pozzo-Miller et al., 1999). These actions suggest that there is a link between estrogen action at the cell membrane and discrete biological actions in the cell.

In conclusion, the etiology of Alzheimer's disease and other neurodegenerative diseases is multifactorial. Estrogens may provide protective effects for many of the factors by genomic or nongenomic mechanisms. Estrogen administration may increase total cerebral and cerebellar blood flow, augment cerebral glucose utilization, promote synaptic plasticity of damaged neurons, enhance cholinergic neurotransmission, normalize serotonergic function, promote the nonamyloidogenic proteolysis of the amyloid precursor protein and prevent neuronal death.

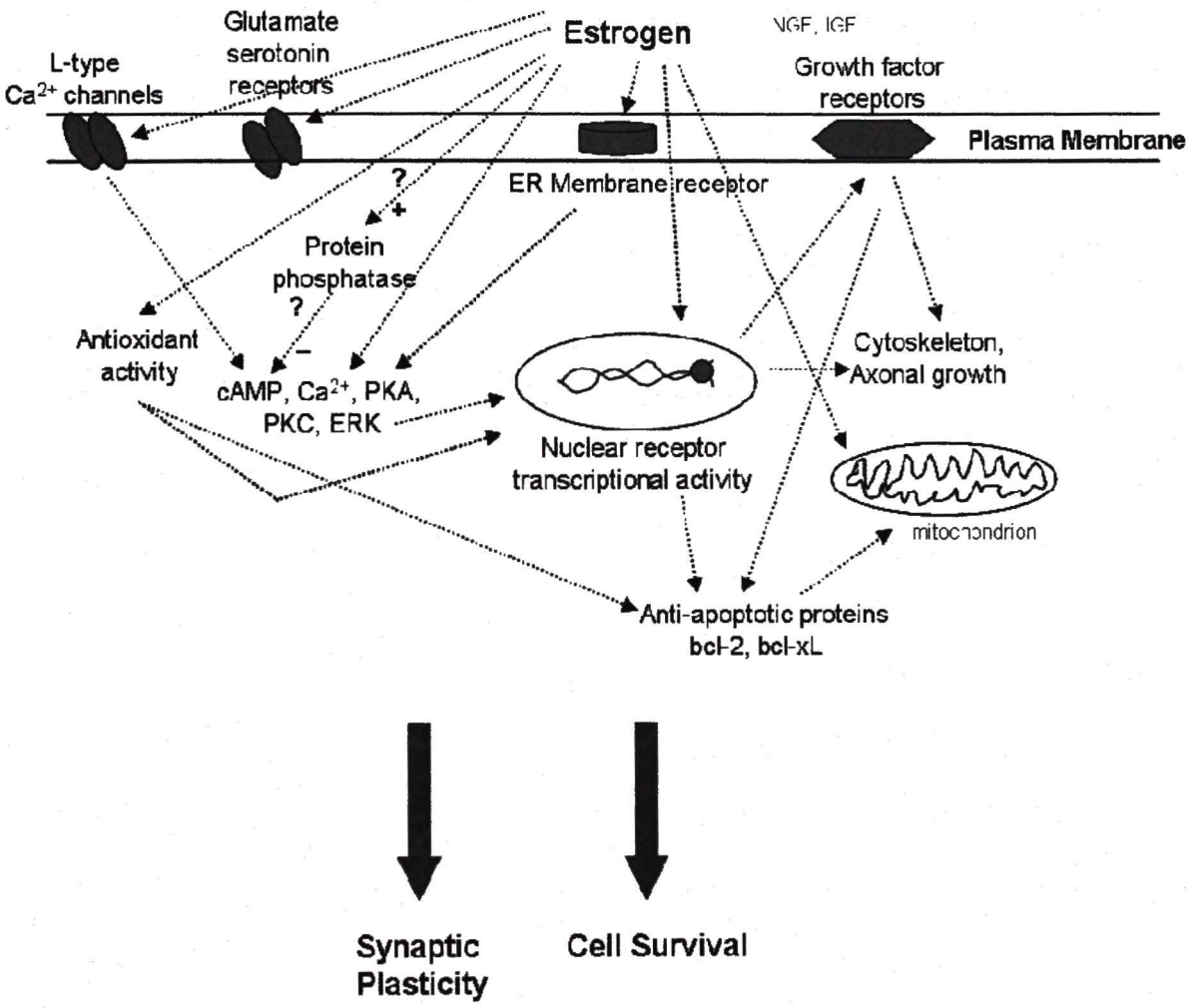

Figure 1-2. Potential mechanisms of neuroprotective effects of estrogens.

ERK and Neurodegeneration

The mitogen activated protein kinases (MAPK) comprise a ubiquitous group of signaling proteins that play a prominent role in regulating cell proliferation, differentiation and adaptation. Members of each major MAPK subfamily, the extracellular signal regulated protein kinases (ERK), c-Jun N-terminal kinases and p38 MAPK, have been implicated in neuronal injury and disease (Slevin et al., 2000; Zhu et

al., 2002a; Zhu et al., 2002b; Ferrer et al., 2003). The MAPK signaling module is defined by a three-tiered kinase cascade, resulting in phosphorylation of a conserved Thr-X-Tyr activation motif by an upstream dual specificity MAPK kinase. In particular, ERK1 and ERK2, which are activated by the MAPK/ERK kinase-1/2 (MEK1/2), are emerging as important regulators of neuronal responses to both functional (learning and memory) and pathologic (regulated cell death) stimuli. While ERK signaling plays a beneficial, neuroprotective role in many systems, there is growing evidence implicating these kinases in the promotion of cell death in both neurons and other cell types.

Initial indications that ERK1/2 activation may contribute to central nervous system (CNS) disease pathogenesis were noted in studies of diseased human brain tissues using antibodies that recognize the active, phosphorylated form of both ERK1 and ERK2. For example, Smith and colleagues noted aberrant neuronal expression of phosphorylated ERK1/2 and other MAPKs in Alzheimer's disease patients' brains in association with markers of oxidative stress (Zhu et al., 2002b). MAPK phosphorylation was also noted in a variety of sporadic and familial neurodegenerative diseases characterized by tau deposits (Ferrer et al., 2003). Phospho-ERK1/2 is increased in substantia nigra neurons of patients with Parkinson's disease and other Lewy body diseases, and the midbrains of these patients show elevated ERK activity (Zhu et al., 2002a). In addition to chronic neurodegenerative diseases, increased ERK1/2 phosphorylation has been noted in the vulnerable penumbra following acute ischemic stroke in humans (Slevin et al., 2000).

Protein Phosphatases

Phosphatases can be classified as serine–threonine phosphatases, 'classical' tyrosine phosphatases and dual-specificity phosphatases, which can dephosphorylate all three phospho-amino acids. The classical and dual-specficity phosphatase families are commonly referred to as protein tyrosine phosphatases (PTPs). Many serine–threonine phosphorylation cascades are stress-induced pathways involving, among others, mitogen-activated protein kinase (MAPK), Jun-kinase (JNK) and inhibitor of NF-κB (IKKB) kinases that activate transcription factors such as AP-1 (activator protein 1; fos–jun) and NF-κB, whereas tyrosine phosphorylation is associated with cytokine- and growth-factor-induced pathways (Cohen, 1989).

There is increasing evidence that relatively few Ser- or Thr specific protein phosphatases with pleiotropic action participate in cellular regulation. Four major classes of protein phosphatase, type 1 (PP1), type 2A (PP2A), type 2B (PP2B or calcineurin), and type 2C (PP2C), have been identified in mammalian cells (Cohen, 1989; Mumby and Walter, 1993). PP1 occurs in three isoforms (α, β/δ, and γ1) and contains a catalytic subunit (PP1C), regulatory subunit (inhibitor 2), a G subunit, and an M subunit. PP1C binds to two cytosolic thermostable proteins, termed inhibitor-1 (I-1) and inhibitor-2 (I-2), which completely abolish its activity at nanomolar concentrations (Nimmo and Cohen, 1978). The I-1 tightly binds to PP1 both *in vitro* and *in vivo* and requires phosphorylation by cAMP-dependent protein kinase (Huang and Glinsmann, 1976; Vulsteke et al., 1997), whereas phosphorylation of I-2 at Thr72 by glycogen synthase kinase leads to activation of the enzyme (Hemmings et al., 1982; DePaoli-Roach, 1984).

Several catalytic subunits of PP1, including α-,β-, γ1- and γ2-isoforms, are present in the brain (Strack et al., 1999), where they interact with various regulatory proteins. In cortical pyramidal cells, the β- and γ1-isoforms have divergent subcellular distributions, the β-isoform being restricted to the cell soma and the γ1-isoform being expressed in the soma, dendrites and presynaptic boutons. Although no pharmacological activators of PP1 are known, this phosphatase, along with PP2A, is inhibited by the cell-permeant toxins okadaic acid and calyculin A, and by the membrane-impermeant agent microcystin.

PP2A, which is pharmacologically similar to PP1, is a heterotrimeric enzyme consisting of a 36-kDa catalytic subunit (C), 65-kDa structural subunit (A), and a variable regulatory subunit (B). PP2A is effectively inhibited by low concentrations of okadaic acid (Takai, 1988; Cohen, 1989; Wera and Hemmings, 1995). Whereas subunits A and C are fairly broadly expressed, inhibitor of protein phosphatase types 1 and 2A. B subunits are present and/or enriched in the brain, immunohistochemistry showing Bα and Bβ expression in CA1 pyramidal cells (Strack et al., 1998). PP2A is active toward most substrates in the absence of divalent cation, whereas PP2B and PP2C have absolute requirements for Ca^{+2} and Mg^{+2}, respectively (Cohen, 1989).

Since the discovery of the first protein tyrosine phosphatase (PTP) (Tonks et al., 1988), many other family members have been identified, and the mammalian gene family is now known to include 90–100 members, as defined by their common ~250 amino acid catalytic domain and/or PTP signature motif ([H/V]C(X)$_5$R[S/T]) (Wang et al., 2003;

Tonks, 2006). Two distinct groups of PTP have been identified. The first group of PTPs has been described as receptor-like with a single transmembrane domain, one or two intracellular catalytic domains, and a unique extracellular domain of variable length. The second group consists of cytoplasmic enzymes, having a single catalytic domain and a variable amino or carboxyl-terminal regulatory domain (Wang et al., 2003; Tonks, 2006). Vanadate and pervanadate are two commonly used general PTP inhibitors.

Dual specificity phosphatases, emerging as crucial players in cell signaling roles, belong to two different groups. The first group has been shown to be induced by MAP kinase pathway and dephosphorylate MAP kinases, providing a negative feedback mechanism. Another group consists of cdc25, a dual specificity phosphatase which plays a critical role in the control of cell cycle by removing the inhibitory phosphates on amino-terminal threonine and thyrosine residues of cyclin-dependent kinases. At the present time, however, there are no specific pharmacological inhibitors of these phosphatases.

MKPs are a family of protein phosphatases that inactivate MAPKs through dephosphorylation of threonine and/or tyrosine residues within the signature sequence – pTXpY– located in the activation loop of MAP kinases, where pT and pY are phosphothreonine and phosphotyrosine, respectively. MKPs are divided into three major categories depending on their preference for dephosphorylating tyrosine, serine/threonine or both the tyrosine and threonine (dual specificity). The tyrosine-specific MKPs (TS-MKPs) include PTP-SL (Pulido et al., 1998), STEP (Pulido et al., 1998) and HePTP

(Saxena et al., 1999). The physiological significance of these dual-specificity phosphatases is now being elucidated, and appears to be very important in cellular function. However, whether these dual-specific phosphatases are involved in the pathology of neurodegeneration is not clear and has not been widely explored. Therefore, we chose to focus on the following phosphatases that have been implicated in the pathophysiology of various neurodegenerative diseases.

Protein Phosphatase 1

Protein Phosphatase 1 (PP1) is involved in regulation of various biological processes including synaptic plasticity, cell cycle, gene transcription, and carbohydrate and lipid metabolism. There are three genes that generate four mammalian isoforms of the PP1 catalytic subunit. Three of the isoforms, PP1α, β and γ1, are ubiquitously expressed in mammalian tissue while PP1γ2 is predominantly expressed in testes. The functional importance of PP1 isoforms is yet to be resolved. Hormones and growth factors regulate PP1 activity. Hormonal regulation of PP1 activity is thought to occur via endogenous inhibitors and regulatory subunits (Brautigan et al., 1990). PP1 inhibitors include Inhibitor-1 (I-1), Inhibitor-2 (I-2), dopmine- and cAMP-regulated phosphoprotein of Mr 32,000 (DARPP-32), nuclear inhibitor of PP1 (NIPP-1), C-kinase activated phosphatase inhibitor of Mr 17,000 (CPI17), and ribosomal inhibitor of PP1 (RIPP-1). There are also a number of PP1-binding proteins that inhibit the phosphorylase *a* phosphatase activity of PP1 *in vitro*. The role of these PP1-binding proteins in regulating PP1 activity in vivo remains to be elucidated, but it is possible that some of these proteins could be phosphatase inhibitors.

Protein phosphatase 2A

PP2A is also a major protein phosphatase in all eukaryotic cells and has a wide range of biological functions. These include the control of cell cycle, organization of cytoskeleton, transcription of immediate early genes, cholesterol and protein biosynthesis. PP2A is a heterotrimeric enzyme made up of a catalytic or C subunit, and two regulatory subunits, termed A and B. To date, cDNAs have been identified for two A subunits, two C subunits, and over twenty B subunits. This suggests the existence of numerous PP2A complexes in mammalian cells. It has been speculated that each PP2A complex serves distinct functions, although at this time, there is very little direct evidence to support this notion. PP2A regulation is further complicated by recent reports that A, B and C subunits all exist as phosphoproteins (Hemmings et al., 1982; Wera and Hemmings, 1995), but the physiological relevance of these findings remains to be established. The $PP2A_C$ subunit is also the major carboxymethylated protein in mammalian cells. This modification occurs at the C-terminal $leucine_{309}$ and has been credited with various functions, including enzyme activation, assembly and localization to membranes (Favre et al., 1994). The identification of additional regulators, specifically PP2A inhibitors, is very much in its infancy. However, two PP2A inhibitors have recently been identified. Termed I_1^{PP2A} and I_2^{PP2A}, these proteins have been implicated in both physiological and pathophysiological regulation of PP2A (Li et al., 1995).

Protein phosphatase 2B (Calcineurin)

PP2B is pharmacologically distinct from PP1 and PP2A; for example, PP2B is insensitive to most inhibitors of PP1/PP2A. However, the immunosuppressive

compounds FK506 and cyclosporine A, by an immunophilin intermediary, inhibit PP2B activity. Furthermore, the insecticide cypermethrin and its derivatives are potent inhibitors of PP2B, as is orthovanadate at high concentrations. PP2B consists of a regulatory subunit and a catalytic subunit that has several isoforms, two of which (Aα and Aβ) are present in the brain. Immunohistochemistry and *in situ* hybridization have shown the presence of catalytic PP2B at high levels throughout the hippocampus (Steiner et al., 1992; Morioka et al., 1997). Catalytic PP2B is enriched in dendritic spines (Halpain et al., 1998), where immunogold labelling reveals an association with small intracellular membranous structures (Sik et al., 1998). PP2B is largely absent from glia and interneurons in the hippocampus, and is not readily detected in presynaptic terminals (Sik et al., 1998).

PP2B, also termed calcineurin, is a calcium/calmodulin-activated protein serine/threonine phosphatase consisting of a catalytic A-subunit and a regulatory or calcium-binding B-subunit which makes this the only phosphatase directly regulated by second messengers (Cohen, 1989). PP2B has a much narrower *in vitro* substrate specificity than either PP1 or PP2A. This is consistent with its specialized functions in the nervous system, T lymphocytes and other cells. Interestingly, the best known *in vitro* and *in vivo* substrates of PP2B are the PP1 inhibitors, I-1 and DARPP-32. Thus, PP2B controls PP1 activity and the two together forms the first documented phosphatase cascade (Mulkey et al., 1994). PP2B has drawn much attention as the target of two clinically important immunosuppressive drugs, cyclosporin and FK506 (Liu et al., 1991). The complex of each drug with its cognate intracellular receptor, known as an

immunophilin, binds to and inhibits the PP2B heterodimer. It has been speculated that the immunophilins may be physiological regulators of PP2B and the immunosuppressive drugs simply stabilize the formation of immunophilin/PP2B complexes to potentiate phosphatase inhibition (Cardenas et al., 1994). A more convincing candidate for an endogenous PP2B inhibitor is the newly discovered cain. This 240 kDa protein associates directly with the A or catalytic subunit and suppresses PP2B activity at micromolar concentrations (Lai et al., 1998). Several other PP2B binding proteins have been identified. Some of these like the A-kinase anchoring protein, AKAP-79, may also inhibit PP2B activity (Coghlan et al., 1995). PP2B associates with its substrate, the transcription factor NFAT (nuclear factor of activated T-cells). Recent studies show that the PP2B-binding domain of NFAT inhibits PP2B activity *in vitro* and in intact T-cells (Aramburu et al., 1998). Whether native NFAT functions as a substrate and a regulator of PP2B in mammalian cells remains to be determined.

Other protein serine/threonine phosphatases

A number of protein phosphatases have been identified by their homology to PP1 and PP2A. Some of these enzymes, such as PPX (PP4) and PPV (PP6), show intriguing and highly restricted subcellular localizations, pointing to specialized functions in cells (Huang et al., 1977). However, the study of these enzymes has lagged behind that of PP1, PP2A, and PP2B in large part due to problems with their expression and subsequent biochemical characterization. Thus, we know very little about the physiological functions and regulation of these enzymes in eukaryotic cells. The nomenclature for these newly

discovered enzymes is also undergoing review and as such, they will not be discussed further.

Protein Phosphatase and Oxidative Stress

There are numerous reports that cellular redox status plays an important role in the mechanisms to regulate the function of growth factors, serine/threonine phosphorylation-dependent and tyrosine phosphorylation-dependent signal transduction pathways (Watson et al., 1996; Flohe et al., 1997; Leclerc et al., 1997; Muller et al., 1997; Suzuki et al., 1997; Ferri et al., 2000). Furthermore, reactive oxygen species such as H_2O_2 have been shown to be involved in growth factor signaling pathway (Sundaresan et al., 1995; Rao, 1996), perhaps as a second messenger. Therefore, protein phosphatases appear to be logical targets of oxidative stress, leading to transient and reversible inactivation of phosphatase activity by oxidizing catalytic cysteine residue to sulfenic acid (Denu and Tanner, 1998). It has been reported that low levels of H_2O_2 (200 μM) robustly activate Erk1/2 (Guyton et al., 1996; Kanterewicz et al., 1998; Wang et al., 1998; Todd et al., 1999) but only slightly activate p38 MAPK and c-Jun N-terminal kinase/stress-activated protein kinase in COS1 cells by inactivating endogenous MAPK phosphatases to dephosphorylate Erk (Guyton et al., 1996). It has also been shown that MAPK phosphatase activity in microsomal and soluble fractions of rat brain was attributable mainly to PP2A. Moreover, H_2O_2 and glutathione disulfide inhibited MAPK phosphatase activity by dithiothreitol-reversible mechanism (Foley et al., 2004).

Protein Phosphatases and Neurodegeneration

Experimental data show that activities of various protein phosphatases decrease in aging and neurodegenerative diseases, such as Alzheimer's disease (Foster et al., 2003). PP1 is thought to be a molecular constraint on learning and memory and a potential mediator of cognitive decline during aging (Genoux et al., 2002). It has been shown with *tet-O*-inhibitor-1 transgenic mice model that the transgene of PP1 was expressed in the hippocampus and cortex of the mice brain. Because these tissues have been implicated in the memory process, the above observation offers a great credence to the physiological relevance of the PP1-dependent mechanism of forgetting, particularly in aging-related memory decline (Gallagher and Rapp, 1997).

Hyperphosphorylation of tau is a hallmark of neurofibrillary tangles seen in AD brain. An imbalance between tau kinases and protein phosphatases is therefore proposed as a pathological mechanism of AD. Reports indicate a decreased PP2B activity in the frontal cortex of human AD brains compared to age-matched controls, without a change in PP2B protein levels or PP2B regulatory proteins (Lian et al., 2001). Moreover, the mRNA expression of DSCR1 (Adapt78), a PP2B inhibitory gene, in AD brains was found to be twice as high as control brains (Ermak et al., 2001). Immunoblotting analyses has also revealed that there is a significant reduction in the total amounts of PP2A in AD frontal and temporal cortices that matched the decrease in PP2A activity measured in the same brain homogenates (Sontag et al., 2004). Immunohistochemical studies also showed that neuronal PP2A expression levels were significantly and selectively decreased in AD-affected regions and in tangle-bearing neurons, but not in

AD cerebellum and in non-AD dementias. The role of PP1 in the neurodegenerative diseases involves not only in Aβ-deposition and hyperphosphorylated tau but also decrease in cognitive function and memory formation. It is well documented that PP1, PP2A, and PP2B are an essential modulators of LTP and LTD (Sun et al., 2003; Munton et al., 2004; Jouvenceau and Dutar, 2006; Jouvenceau et al., 2006). It has been shown that PP1 is a major tau phosphatase (Zambrano et al., 2004; Rahman et al., 2005), and that inhibition of PP1 stimulates the secretion of soluble amyloid precursor protein (da Cruz e Silva et al., 2004). PP1 activity is decreased in AD brains (Gong et al., 1995), indicating its importance in the pathophysiology of neurodegeneration.

Rationale For This Study

Clinical and experimental evidence have shown that both endogenous and exogenous estrogens exert neuroprotective effects against cerebral ischemia (Seeman, 1997; Lindamer et al., 1999; Wise et al., 2000; Yang et al., 2000; Asthana et al., 2001; Garcia-Segura et al., 2001; Shepherd, 2001). The pathological mechanisms that are activated during stroke include oxidative stress, free radical activity, excitotoxicity, inflammatory response, mitochondrial dysfunction, and apoptosis, which are antagonized by estrogens. While the classical estrogens' effects have been indicated to be mediated by estrogen receptors (ER)-dependent genomic mechanism, strong evidence from our laboratory and others indicate that neuroprotective effects of 17β-estradiol are also ER-independent since 17α-estradiol, the enantiomer of 17β-estradiol, and other non-ER binding estrogen analogues show equipotent protection (Green et al., 1997; Sawada et al.,

1998; Behl and Manthey, 2000; Green et al., 2001). The mechanism of estrogen mediated neuroprotection remains unclear.

Estrogens have been shown to activate a signal transduction pathway involving MAP kinase and/or PKC within minutes of estrogen treatment, which argues against genomic ER actions. Singh et al (1999) showed that estrogen treatment activates MAP kinases, ERK1 and ERK2, within 5-15 min and this activation persists for at least 2 hrs. Our laboratory has shown that inhibitors of extracellular signal-regulated protein kinase (ERK), U0126 and PD98059, are potent neuroprotectants (unpublished data). We have also shown that down-regulation and inhibition of PKC enhanced 17β-estradiol mediated neuroprotection. Likewise, estrogen mediated down-regulation of PKC is neuroprotective (unpublished data). It has been shown that AD brains have persistent activation of ERK and hyperphosphorylation of tau (Zhu et al., 2002b).

The brain is an extremely rich source of PPs. Historically, protein phosphatases were thought to be ubiquitous nonspecific scavengers of phosphoproteins, but recent data demonstrate that they are regulated by interactions with scaffolding and anchoring molecules (Price and Mumby, 1999). The association of catalytic subunits with interacting proteins results in a diversity of phosphatase holoenzymes that are targeted to specific substrates, signaling complexes, and intracellular locations (Price and Mumby, 1999). PPs are essential modulators of protein kinases as well as direct effectors. It is becoming increasingly clear that protein phosphatases are important modulators of cellular activity. PPs are important regulators of disease processes such as Alzheimer's

disease. Experimental data have shown decreased activities of PP1, PP2A, and PP2B in AD brains as compared to aged matched control brains. Protein phosphatases are important regulators of tau phosphorylation and ERK 1/2, as director and indirect effectors (Lian et al., 2001; Vogelsberg-Ragaglia et al., 2001; Gong et al., 2003; Gong et al., 2004). Therefore, these experiments were designed to the role of protein phosphatases in estrogen-mediated neuroprotection.

Specific Aims

The first specific aim of the investigation was to determine whether serine/threonine protein phosphatase inhibitors are neurotoxic. The effects of PP inhibitors, including OA (non-specific serine/threonine PPs), CA (PP1 and PP2A), endothall (PP2A), protein phosphatase inhibitor 2 (PP1), and cyclosporine A (PP2B) was assessed in primary cortical neurons via cell viability assay. We hypothesized that protein phosphatases are essential for cell survival; therefore, inhibition of protein phosphatases will result in cell death. We found that general inhibition of protein phosphatases indeed produced a dose-dependent cell death in all three cell types, while specific inhibition of individual phosphatases had very little effect on cell viability. Therefore, we concluded that serine/threonine protein phosphatases are essential for cell viability in primary cortical neurons.

The second aim of this investigation was to determine if serine/threonine protein phosphatase inhibition will attenuate the estrogen-mediated neuroprotection against oxidative or excitotoxic stress. We hypothesized that protein phosphatase inhibition will

attenuate the neuroprotective effects of estrogens. Cell viability was assessed in primary cortical neurons treated with various concentrations of phosphatase inhibitors, 17β-estradiol or estrogen analogues, and/or glutamate. We found that okadaic acid and calyculin A at sub-lethal concentrations were able to abolish neuroprotective effects of estrogen and estrogen analogues, while specific inhibitors partially attenuated the estrogen effects.

The third aim was to determine the expression and activity of protein phosphatases in the neuroprotective effects of estrogens. We hypothesize that the neuroprotective effects of estrogens are mediated through increased PP content and activity. Immunoblots and protein phosphatase activities were measured in primary cortical neurons treated with phosphatase inhibitors, 17β-estradiol or estrogen analogues, and/or glutamate. We found that the neuroprotective effects of estrogens are mediated through sustained phosphatase protein content and activity.

The fourth aim of this study was to determine the effects of serine/threonine phosphatase inhibition on estrogen-mediated down-regulation of ERK1/2. We hypothesized that estrogen-mediated activation of phosphatases will decrease the activity of ERK. Inhibition of ERK showed similar neuroprotective effects as seen with estrogens, and as with estrogens, ERK inhibition was unable to protect cells against okadaic acid induced cell death. Phosphorylation of ERK was greatly enhanced with treatment of glutamate or okadaic acid. The presence of estrogens prevented this glutamate- but not okadaic acid-induced ERK phosphorylation suggesting that

serine/threonine phosphatases activities are a necessary component of estrogen-mediated neuroprotection via inhibition of ERK activity during oxidative or excitotoxic stress.

Significance

Retrospective epidemiological studies in post-menopausal women indicate that HRT/estrogen replacement therapy (ERT) is associated with a reduction in the risk of Alzheimer's disease as well as a delay its the progression (Paganini-Hill et al., 1988; Paganini-Hill and Henderson, 1994; Simpkins et al., 1994; Tang et al., 1996). In addition, several clinical studies have reported improvement of cognitive functions in female AD patients receiving ERT (Henderson et al., 1994; Ohkura et al., 1994). Moreover, epidemiological and clinical reports have shown diminished severity of Parkinson related symptoms in estrogen treated post-menopausal women with early PD (Sandyk, 1989; Saunders-Pullman et al., 1999). Clinical evidence has also shown that estrogens exert neuroprotective effects against stroke (Wren, 1992; Finucane et al., 1993; Paganini-Hill, 1995). However, recent reports from the Women's Health Initiative studies have indicated detrimental effects of HRT (Shumaker et al., 2003; Wassertheil-Smoller et al., 2003) on cognition and increased risks of stroke and heart disease. These results are at odds with results of large epidemiological studies that showed protection. Although the latter data are, in part, confounded by a "healthy user bias," much of the inconsistency may be explained by the fact that women in the latter studies initiated HRT at the menopausal transition, whereas the WHI trial was conducted in older women who were approximately 12 yr postmenopausal. In addition, older trials included women on either unopposed estrogen therapy (ERT) or cyclic HRT regimens. Further, a variety of

other factors confound the issue of the beneficial effects of ERT, such as doses of hormones, type of hormones, the age at which HRT is administered, and a host of other factors.

Therefore, a clearer understanding of the mechanism of estrogen-mediated neuroprotection is important in determining the clinical application of ERT. This study provides an insight into one of the mechanisms involved in the estrogen-mediated neuroprotection. First, it has been shown in this study as well as others that the neuroprotective effects of estrogens are estrogen receptor independent. Estrogen analogues, which do not bind to the receptors, used in this study show equipotent, if not better, efficacy in cell survival in the presence of cell death inducing agents. Second, these estrogen analogues have the same mechanism of action as 17β-estradiol in that they attenuate or induce similar signaling pathways. Hence, these results show that estrogens are beneficial to neuronal survival and function during oxidative and /or excitotoxic stress.

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CHAPTER 2

Role of Protein Phosphatases in Estrogen-Mediated Neuroprotection

by

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ABSTRACT

The signaling pathways that mediate neurodegeneration are complex and involve a balance between phosphorylation and dephosphorylation of signaling and structural proteins. We have previously shown that 17β-estradiol and its analogs are potent neuroprotectants. The purpose of this study was to delineate the role of protein phosphatases in estrogen neuroprotection against oxidative stress and excitotoxicity. HT-22 cells, C6-glioma cells, and primary rat cortical neurons were exposed to the non-specific serine/threonine protein phosphatase inhibitors, okadaic acid and calyculin A, at various concentrations in the presence or absence of 17β-estradiol and/or glutamate. Okadaic acid and calyculin A caused a dose-dependent decrease in cell viability in HT-22, C6-glioma, and primary rat cortical neurons. 17β-estradiol did not show protection against neurotoxic concentrations of either okadaic acid or calyculin A in these cells. In the absence of these serine/threonine protein phosphatase inhibitors, 17β-estradiol attenuated glutamate toxicity. However, in the presence of effective concentrations of these protein phosphatase inhibitors, 17β-estradiol protection against glutamate toxicity was lost. Furthermore, glutamate treatment in HT-22 cells and primary rat cortical neurons caused a 50% decrease in levels of PP1, PP2A, and PP2B protein; while, co-administration of 17β-estradiol with glutamate prevented the decrease in PP1, PP2A, and PP2B levels. These results suggest that 17β-estradiol may protect cells against glutamate induced oxidative stress and excitotoxicity by activating a combination of protein phosphatases.

I. INTRODUCTION

Protein phosphatases are modulators and direct effectors of protein kinases. As such, they are essential in directing signaling toward neuroprotection or neurodegeneration. Protein phosphatases are important regulators of neurodegenerative disease processes such as Alzheimer's disease (AD) in which oxidative stress is an important contributing factor (Tuppo and Forman, 2001; Cecchi et al., 2002; Perry et al., 2002). Additionally, protein phosphatases are important regulators of tau phosphorylation, as director and indirect effectors. Protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), and protein phosphatase 2B (calcineurin) expression and/or activity have been found to be greatly reduced in AD brains as compared to aged-matched control brains (Gong et al., 1993; Gong et al., 1995; Lian et al., 2001; Vogelsberg-Ragaglia et al., 2001; Sontag et al., 2004).

Inhibition of PP1 and PP2A by the algal toxin okadaic acid (OA) leads to disruption of the cytoskeleton and cell death in several cell culture systems (Holen et al., 1992; Blankson et al., 1995; Benito et al., 1997; Rossini et al., 1997; Yan and Surmeier, 1997). In neurons, OA has been reported to cause hyperphosphorylation of tau, modification of synapse structure, destruction of stable microtubules, and apoptosis (Harris et al., 1993; Mawal-Dewan et al., 1994; Garver et al., 1995; Saito et al., 1995; Burack and Halpain, 1996; Fernandez-Sanchez et al., 1996; Garver et al., 1996; Malchiodi-Albedi et al., 1997; Merrick et al., 1997).

Excitotoxicity and oxidative stress are known to disrupt intracellular signaling by persistently activating several phosphorylation-dependent pathways. Insult-induced

persistent activation of ERK1/2 has been shown to play a role in oxidative stress induced cell death in HT-22 and primary neurons (Stanciu et al., 2000). Persistent activation of PKC associated with ethanol-induced neurotoxicity has also been shown (Jung et al., 2005). Also, in models of stroke, brain trauma, and neurodegenerative diseases, the detrimental effects of persistent activation of ERK1/2 during oxidative as well as excitotoxic neuronal injury has been documented (Slevin et al., 2000; Stanciu et al., 2000; Zhu et al., 2002a; Zhu et al., 2002b; Ferrer et al., 2003; Harper and Wilkie, 2003).

The pathological mechanisms that are activated during neurodegenerative diseases, including oxidative stress, excitotoxicity, inflammatory responses, mitochondrial dysfunction, and apoptosis, are antagonized by estrogens (Green et al., 1996; Green et al., 1997b; Green et al., 1997a; Gridley et al., 1997; Green et al., 1998). I*n vitro* studies indicate that estrogens increase the viability and differentiation of primary cultures from different neuronal populations from the hypothalamus, amygdala, neocortex or hippocampus. Numerous *in vivo* studies have also demonstrated the neuroprotective effects of estrogens in a variety of animal stroke models, including transient and permanent middle cerebral artery occlusion (Simpkins et al., 1997; Alkayed et al., 1998; Dubal et al., 1998), global forebrain ischemia (Sudo et al., 1997), photothrombotic focal ischemia (Fukuda et al., 2000), glutamate-induced focal cerebral ischemia (Mendelowitsch et al., 2001), and subarachnoid hemorrhage (Yang et al., 2001).

There is considerable evidence that estrogens act on neurons through a variety of signal transduction pathways to induce rapid, but acute phosphorylation of signaling proteins (Zhou et al., 1996; Watters et al., 1997; Singh et al., 1999). Estradiol-induced

phosphorylation of the adenylyl cyclase, Akt , PKA, PKC, and MAPK pathways have been reported (Migliaccio et al., 1996; Kelly and Wagner, 1999; Singh et al., 1999; Zhang et al., 2001). Changes in the activity of these enzymes can regulate the phosphorylation of numerous intermediary signaling proteins such as Rsk, p38 and JNK, and nuclear transcriptional factors, CREB and cfos/cjun, which may ultimately mediate cell survival changes (for review see (Lee and McEwen, 2001). Further, estrogens have been shown to block the persistent activation of both ERK and PKC (Watters et al., 1997; Singh et al., 1999; Bi et al., 2000; Kuroki et al., 2000; Singh et al., 2000; Jung et al., 2005).

We sought to determine the role of protein phosphatases in estrogen mediated neuroprotection against excitotoxicity and oxidative stress induced cell death. In this study we show that inhibition of protein phosphatases is neurotoxic and that 17β-estradiol is ineffective in protecting against this toxicity. Inhibition of protein phsophatases prevents the 17β-estradiol mediated neuroprotection against oxidative and excitotoxic stress. Oxidative stress and excitotoxicity cause a decrease in protein phosphatase content, which is antagonized by 17β-estradiol. In the presence of okadaic acid, the 17β-estradiol mediated stablization of protein phosphatase levels is abrogated. Collectively, these data implicate a role for protein phosphatases in estrogen-mediated neuroprotection.

II. METHODS AND MATERIALS

Chemicals

17β-estradiol was purchased from Steraloids, Inc. (Wilton, NH) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and diluted to appropriate concentration in culture media. Calcein AM was purchased from Molecular Probes, Inc. (Eugene, OR). Okadaic acid, L-glutamate, and DMSO were purchased from Sigma (Paris, KY). Calyculin A, endothall, and protein phosphatase inhibitor 2 were purchased from Calbiochem (San Diego, CA). Anti-PP1, Anti PP2A, and Anti PP2B were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture

HT-22 and C-6 glioma cells were cultured in DMEM supplemented with 10% charcoal-stripped FBS (HyClone, Logan, UT) and gentamicin (50 μg/ml), at 37° C in an atmosphere containing 5% CO_2 and 95% air. HT-22 cells were obtained from David Schubert (Salk Institute, San Diego, CA). C6-gilioma cells were obtained from ATTC. HT-22 and C6-glioma cultures were maintained at 50% and 100% confluency, respectively, in monolayers in plastic 75 cm^2 flasks.

Culture of primary cortical neurons

Cerebral cortex rat embryo (18-day) were dissected and harvested in preparation medium (DMEM, glucose 4.5g/L, Penicillin 100u/ml, Streptomycin 100μg/ml). The

cortial tissue was treated with trypsin. The tissue was washed three times using washing medium (Hank's medium, glucose 4.5g/L, Penicillin 100u/ml, Streptomycin 100μg/ml) and individual cells will be isolated by mechanical trituration using three different sizes of fire polished Pasteur pipettes. The cells were harvested in seeding medium (DMEM, glucose 4.5g/L, penicillin 100u/ml, streptomycin 100μg/ml, glutamine 2mM, 19% horse serum) and filtered through 40μm filter. The cerebral cortical cells were seeded in various poly-L-lysine treated dishes and plates at various cell densities. The cells were incubated in neurobasal medium (DMEM, glucose 4.5g/L, Penicillin 100u/ml, Streptomycin 100μg/ml, glutamine 2mM) supplemented with B-27 with antioxidants in normal cell culture condition of 37°C in a humid atmosphere of 5% CO_2. Two hours before treatment with various inhibitors and/or 17 β-estradiol, the media was replaced with neurobasal medium supplemented with B-27 without antioxidants.

Calcein-AM viability assay

Cells were seeded 24 hr before initiation of the experiment at a density of 3,500 cells/well in 96-well plates. Cells were exposed to various treatments. After exposure to various treatment paradigms cells were rinsed with phosphate buffered saline, and cell viability was measured using the membrane-permeant calcein-AM dye (Molecular Probes, Eugene, OR). Calcein-AM is a fluorogenic esterase substrate that is hydrolyzed to a fluorescent product in cells having esterase activity and intact membranes. Cells were incubated in a solution of 2.5 μM calcein-AM in PBS. Twenty minutes later, fluorescence was determined using a Bio-Tek FL600 microplate reader (Winooski, VT)

with an excitation/emission filter set of 485/530 nm. Cell culture wells treated with methanol served as blanks. The results, obtained in relative fluorescent units (RFU), are expressed as the percentage of untreated or vehicle-treated control values.

Immunoblot analysis

Protein from whole cell lysates (25 μg) was separated by SDS-polyacrylamide gel electrophoresis and transferred to Immunobilon-P polyvinylidene difluoride (Millipore Corp., Bedford, MA) membrane. Membranes were rinsed in Tris-buffered saline (10 mM Tris-base, pH 8.0, 100 mM NaCl) containing 0.2% Tween 20 then blocked with 3% bovine serum albumin. Blots were then incubated with primary antibodies overnight at 4°C, rinsed and incubated in the appropriate secondary antibody before detection using enhanced chemiluminescence (ECL; Pierce Biotechnology, Inc., Rockford, IL). ECL results were digitized and quantified using UVP Bioimaging System.

Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. $p < 0.05$ was considered significant for all experiments. Each set of data represents three or more individual assays performed separately, with each containing 4-8 replicate wells. The values are reported as the mean ± SEM.

III. RESULTS

Effects of protein phosphatase inhibitors on estrogen-mediated neuroprotection against glutamate-induced cytotoxicity

To determine the effectiveness of 17β-estradiol to protect the cells against oxidative stress or excitotoxicity induced by glutamate in the presence of a PP inhibitor, we examined simultaneous treatment of 17β-estradiol with glutamate and the nonspecific PP inhibitors okadaic acid or calyculin A. Figure 1 shows the results for simultaneous treatment of okadaic acid with 17β-estradiol in HT-22, C6-glioma, and primary cortical neurons. In the absence of PP inhibitors, 17β-estradiol was neuroprotective in all three cell types (Fig. 1) against glutamate-induced cytotoxicity. 17β-Estradiol-mediated neuroprotection against glutamate induced toxicity was abolished in the presence of increasing concentrations of okadaic acid or calyculin A in HT-22, C6-glioma, and primary cortical neurons. Abolition of estrogen protection against glutamate toxicity was seen at 10 nM okadaic acid in HT-22 and C6 cells and at 5 nM okadaic acid in primary rat cortical neurons. Results from experiments in which cells were pretreated with 17β-estradiol for either 2 or 24 h before addition of glutamate and/or okadaic acid were similar to the simultaneous treatment data (data not shown). The presence of calyculin A (50 pM in HT-22 and primary neurons and 25 pM in C6-glioma) also abolished the neuroprotective effects of 17β-estradiol against oxidative- and excitotoxic stress-induced death caused by glutamate as seen with okadaic acid (data not shown). The concentrations at which either okadaic acid or calyculin A prevented the neuroprotective

effects of 17β-estradiol against glutamate-induced cell death were not neurotoxic in these cells (Fig. 2).

Effect of okadaic acid or calyculin A on cell viability

To determine the effects of PP inhibition on cell viability, cells were exposed to okadaic acid or calyculin A. Exposure to nonspecific PP inhibitor, okadaic acid, caused a dose-dependent cell death in both HT-22 and C6-glioma cells with an LD_{50} of 81 and 87 nM, respectively (Fig. 2*A*). Okadaic acid treatment in primary cortical neurons resulted in a dose-dependent decrease in cell viability similar to results seen in HT-22 and C6-glioma cells; however, okadaic acid was more potent in primary neurons, with an LD_{50} of 40 nM (Fig. 2*A*).

Calyculin A, a PP1 and PP2A inhibitor, also showed a dose-dependent decrease in viability in all three cell types (Fig. 2*B*). Cell viability decreased precipitously at a threshold concentration of 500 pM in C6-glioma cells, whereas HT-22 cells exhibited a more typical dose-dependent response with a 50% decrease in cell viability with >500 pM treatment. Calyculin A decreased cell viability in a dose-dependent manner in primary cortical neurons with a LD_{50} of 67 pM (Fig. 2*B*).

Estrogens did not prevent the protein phosphatase inhibitor-induced cell death

17β-Estradiol is a potent neuroprotectant; therefore, to assess the neuroprotective effects of 17β-estradiol, cells were exposed to 17β-estradiol simultaneously, 2 or 24 h before treatment with neurotoxic concentrations of okadaic acid or calyculin A. Neither

simultaneous nor 2 or 24 h pretreatment with 17β-estradiol protected HT-22, C6-glioma, or primary rat cortical neurons against okadaic acid- or calyculin A-induced cell death (Fig. 3). The 24 h pretreatment results are not shown but were same as those presented in Figure 3.

Time-dependent effects of 17β-estradiol and/or glutamate PP2A protein levels

We examined the time course of the response of PP2A protein expression to treatment with glutamate and/or 17β-estradiol in HT-22 cells. Glutamate treatment (10 mM) caused a reduction in PP2A levels that was significant at 30 min, and a 50% or greater reduction persisted through 24 h of treatment (Fig. 4). 17β-estradiol (10 μM) alone did not significantly affect PP2A levels at any given time compared with untreated controls. However, simultaneous treatment with 17β-estradiol and glutamate caused a rapid and profound increase in PP2A expression at 15 and 30 min, followed by a return to normal levels by 1 h after treatment that persisted through 24 h treatment.

Effects of 17β-estradiol on PP1, PP2A, and PP2B protein expression

Treatment of HT-22 cells with 10 μM 17β-estradiol alone for 24 h did not show any effect on the expressions of any PP compared with untreated control cells (Fig. 5). However, 24 h of 10 mM glutamate treatment caused a marked decrease in protein levels of PP1, PP2A, and PP2B in HT-22 cells (Fig. 5). Simultaneous treatment with 17β-estradiol in the presence of 10 mM glutamate restored PP1 and PP2A protein expression to levels comparable with untreated control cells (Fig. 5); however, although there was a

tendency for increased PP2B expression with simultaneous estrogen treatment, the difference was not statistically significant. The attenuation of glutamate induced declines in phosphatase expression by estrogen was abolished in the presence of 100 nM okadaic acid.

Primary rat cortical neurons showed a pattern similar to that seen in HT-22 cells (Fig. 6). As in HT-22 cells, PP1, PP2A, and PP2B protein expressions were decreased after 50 μM glutamate. The decrease in protein expression caused by glutamate was attenuated with simultaneous 100 nM 17β-estradiol treatment. Additionally, 17β-estradiol was unable to prevent the glutamate mediated decrease in protein expressions of PP1, PP2A, and PP2B in the presence of 50 nM okadaic acid.

DISCUSSION

The present study reports several significant findings. First, we confirmed that protein phosphatase inhibitors are potent neurotoxins in transformed neuronal and glial cell types as well as in primary cortical neurons. Second, we demonstrated for the first time that estrogen treatment regimens that are protective against other neurotoxic insults do not protect against the toxic effects of the phosphatase inhibitors. Third, the protective effects of estrogens against glutamate toxicity are antagonized by phosphatase inhibition. Finally, estrogens antagonize the reduction in PP1, PP2A and PP2B induced by glutamate, but not in the presence of okadaic acid. Collectively, these data support the

hypothesis that phosphatase induction is a major component of estrogen-mediated neuroprotection.

Protein phosphatase inhibitors are well known cytotoxins. Okadaic acid is a potent inhibitor of PP1 and PP2A at low concentrations (10 nM and 0.1 nM, respectively). It is also an inhibitor of PP2B at higher μM concentrations. In the present study, we observed a dose-dependent neurotoxic effect of okadaic acid in HT-22, C6-glioma, and primary cortical neurons. The neurotoxic effects of okadaic acid were clear with doses as low as 50 nM in primary neurons, which is in agreement with studies by other laboratories (Fernandez et al., 1991; Candeo et al., 1992; Nuydens et al., 1998; Kim et al., 2000). Numerous studies have also shown okadaic acid to be cytotoxic in a variety of other cell types (Morimoto et al., 1999; Huynh-Delerme et al., 2003; Morimoto et al., 2004; Parameswaran et al., 2004). Calyculin A is also a potent inhibitor of PP1 and PP2A. Calyculin A induces apoptosis in osteoblastic cells (Morimoto et al., 1997), oral squamous carcinoma cells (Fujita et al., 1999), submandibular gland cells (Morimoto et al., 1999), and neuronal cells (Ko et al., 2000; Li et al., 2004). In our hands, calyculin A was a more potent neurotoxin than okadaic acid.

Numerous studies from this laboratory, as well as others, have clearly established that estrogen compounds are potent neuroprotective agents, both *in vitro* and *in vivo* (for reviews see (Behl and Manthey, 2000; Green and Simpkins, 2000; Garcia-Segura et al., 2001; Lee and McEwen, 2001; Wise et al., 2001). These effects are mediated at least in part through the rapid, but acute phosphorylation of signaling proteins, such as adenylyl cyclase, Akt , PKA, PKC, and MAPK (Migliaccio et al., 1996; Kelly and Wagner, 1999;

Singh et al., 1999; Zhang et al., 2001). Changes in the activity of these enzymes can regulate the phosphorylation of numerous cellular substrates including intermediary signaling proteins such as Rsk, p38 and JNK, and the nuclear transcriptional factors, CREB and cfos/cjun, which may ultimately mediate cell survival changes (for review, see (Lee and McEwen, 2001).

The present study provides a potential mechanism for these effects of estrogens, through the prevention of insult-induced decrease in phosphatase activity and the resulting neurotoxic, persistent hyperphosphorylation of proteins in multiple signaling pathways. We have demonstrated that the neuroprotective effects of estrogens against glutamate toxicity are blocked by phosphatase inhibition and that estrogens prevent insult-induced reduction in protein phosphatase levels, suggesting that protein phosphatases play a role in estrogen-mediated neuroprotection against neurotoxic effects of oxidative stress and excitotoxicity.

A specific consideration of the effects of estrogens on signaling in the ERK1/2 pathway is informative. Estrogens cause a rapid, transient phosphorylation of ERK1/2 in neuroblastoma (Watters et al., 1997), non-neuronal cells (Migliaccio et al., 1996), and cortical explants (Singh et al., 1999), as well as in the cortex of estrogen receptor-α knock-out mice (Singh et al., 2000). This transient phosphorylation of ERK1/2 is believed to mediate a neuroprotective signal while persistent phosphorylation of ERK1/2 is associated with apoptosis, likely through nuclear translocation and retention of phosphorylated ERK1/2 (Kuperstein and Yavin, 2002; Kins et al., 2003). The transient nature of neuroprotective signaling indicates that dephosphorlyation though activation of

phosphatases is required. Indeed, pharmacological inhibition of ERK1/2 signaling is neuroprotective in a number of cell model systems (Murray et al., 1998; Barone et al., 2001), including the HT-22 cell oxidative stress model utilized in our studies (Satoh et al., 2000; Stanciu et al., 2000). Similarly, in models of stroke, brain trauma, and neurodegenerative diseases, there are detrimental effects of persistent activation of ERK1/2 during oxidative neuronal injury as well as excitotoxic injury (Alessandrini et al., 1999; Slevin et al., 2000; Stanciu et al., 2000; Zhu et al., 2002a; Zhu et al., 2002b; Ferrer et al., 2003; Harper and Wilkie, 2003; Wang et al., 2003; Wang et al., 2004).

A variety of insults have been shown to cause the hyperphosphorylation of tau (Guttmann et al., 1995; Schweers et al., 1995). The present data may provide a mechanism by which estrogens ameliorate the effect of insult on tau hyperphosphorylation *in vitro* (Alvarez-de-la-Rosa, 2005) and in ischemia/reperfusion-induced injury *in vivo* (Wen et al., 2004). By preventing insult-induced decreases in protein phosphatases, estrogens could allow for the de-phosphorylation of tau even in the face of an insult stimulation of tau phosphorylation. This could contribute to the beneficial effects of early post-menopausal estrogen therapy on the development of AD (Paganini-Hill and Henderson, 1994; Tang et al., 1996; Zandi et al., 2002; Bagger et al., 2005).

The mechanism by which estrogens increase protein phosphatase levels is not known. To date, we are not aware of an estrogen response element in the regulatory regions of the protein phosphatases under study. Therefore, we hypothesize that protein phosphatase signaling is through an alternative pathway. One intriguing possibility is that

the signaling pathways activated by neurotoxic insults suppress phosphatase expression or enhance the proteolytic breakdown of these important proteins. Estrogens then could induce an increase in phosphatase production and/or reduce proteolysis thereby normalizing protein phosphatase levels and regulating signaling along phosphorylation-dependent pathways.

We find it remarkable that all three protein phosphatases appear to respond similarly to estradiol, glutamate, okadaic acid, and their combination in HT-22, C6-glioma, and primary neuronal cultures. The robust decrease in these three protein phosphatases in response to glutamate in a model of oxidative stress (HT-22 and C6-glioma cells) and to excitotoxicity (primary cortical neurons) suggests that protein phosphatase inhibition may be a common neurotoxic event for these insults. Similarly, the ability of estrogens to antagonize these glutamate-induced reductions in protein phosphatases suggests that this action is common to estrogen protection in both oxidative stress and excitotoxicity in neurons. In addition, experimental data from various studies have shown not only the ability of estrogen to protect glial cells from various toxins (Bishop and Simpkins, 1994; Haghighat et al., 2004; Takao et al., 2004) but also the mediation of glial cells in estrogen neuroprotection (Sortino et al., 2004; Wynne and Saldanha, 2004). Therefore, it is important to understand the mechanism of estrogen actions in both neurons and glia. The results from the present study suggest that there is a common mechanism of neuroprotection by estrogens in neuronal and glial cells.

Although accumulating evidence from experimental studies using cell and animal models suggests that estrogens are potent neuroprotective agents against multiple types of

neurodegenerative diseases and injuries, recent clinical studies have reported either inconclusive or untoward effects of hormone therapy on the brain (Mulnard et al., 2000; Rapp et al., 2003; Shumaker et al., 2003, 2004; Espeland et al., 2004). The discrepancies in the results of basic science and clinical studies has been addressed by several recent reports (Brinton, 2003; Simpkins and Singh, 2004; Singh and Simpkins, 2005) and may be explained by a number of factors, including the preparation of the estrogen and progesterone used in the Women's Health Initiative Memory (WHIM) studies, the continuous exposure regimen, the advanced age of the women at the initiation of hormone or estrogen therapy, and the cardiovascular health of the women (Singh and Simpkins, 2005). It remains unknown to what extent these conditions of the WHIM studies contribute to the aforementioned differences in the reported effects of hormone therapy.

In summary, we have demonstrated that estrogen-mediated neuroprotection is antagonized by protein phosphatase inhibition in a manner associated with estrogen-induced increases in protein phosphatase levels. These data provide support for the hypothesis that estrogen-induced neuroprotection is mediated by protein phosphatases.

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FIGURE LEGENDS

Figure 1. Effects of okadaic acid on estrogen-mediated neuroprotection from glutamate neurotoxicity. HT-22 cells (passages 18-25), C6-glioma cells (passages 40-49), or primary rat cortical neurons were seeded into 96-well plates at a density of 3500 or 25,000 cells per well. ***(A)***, HT-22 cells treated simultaneously with 100 nM okadaic acid, 10 mM glutamate, and/or 10 μM 17β-estradiol. ***(B)***, C6-glioma cells treated simultaneously with 100 nM okadaic acid, 20 mM glutamate, and/or 10 μM 17β-estradiol. ***C***, Primary cortical neurons treated simultaneously with 50 nM okadaic acid, 50 μM glutamate, and/or 100 nM 17β-estradiol. Cell viability was determined by calcein AM assay after 24 h exposure to the various compounds. All data were normalized to percentage survival of vehicle control. Data are represented as mean ± SEM for $n = 6$. * P <0.05 vs. control; † P < 0.05 vs. glutamate treated group.

Figure 2. Dose-dependent neurotoxicity of okadaic acid and calyculin A. HT-22 cells (passages 18-25) and C6-glioma cells (passages 40-49) were seeded into 96-well plates at a density of 3500 cells per well, and primary rat cortical neurons were seeded into 96-well plates at a density of 25,000 cells per well. Okadaic acid **(*A*)** or calyculin A **(*B*)** were added at varying concentrations. Cell viability was determined by calcein AM assay after 24 h exposure to the various protein phosphatase inhibitors. All data were normalized to percentage survival of vehicle control. Data are represented as mean ± SEM for $n = 6$. $^{*}p$ < 0.05 versus vehicle control.

Figure 3. Effects of 17β-estradiol on okadaic acid- or calyculin A-induced neurotoxicity. HT-22 cells (passages 18-25) and C6-glioma cells (passages 40-49) were seeded into 96-well plates at a density of 3500 cells per well, and primary rat cortical neurons were seeded into 96-well plates at a density of 25,000 cells per well. HT-22 cells treated with 100 nM okadaic acid or 1 nM calyculin A and simultaneous ***(A)*** or 2 h pretreatment ***(B)*** of 17β-estradiol (E2) at varying concentrations are shown. C6-glioma cells treated with 100 nM okadaic acid or 500 pM calyculin A and simultaneous (***C***) or 2 h pretreatment (***D***) of 17β-estradiol at varying concentrations are shown. Primary cortical neurons treated with 50 nM okadaic acid or 10 nM calyculin A and simultaneous (***E***) or 2 h pretreatment (***F***) of 17β-estradiol at varying concentrations are shown. Cell viability was determined by calcein AM assay after 24 h exposure to the various protein phosphatase inhibitors and 17β-estradiol. All data were normalized to percentage survival of vehicle control. Data are represented as mean ± SEM for $n = 6$. $^{*}p < 0.05$ versus vehicle control.

Figure 4. Time course of the effects 17β-estradiol, glutamate, and their combination on PP2A protein levels. HT-22 cells were treated with either 10 mM glutamate (***A***) or 10 μM 17β-estradiol (***B***) and simultaneously with 10 mM glutamate and 10 μM 17β-estradiol (***C***). Cells were harvested at the times indicated for Western blot analysis of PP2A. The graphs represent relative OD as a percentage of time 0 control. Data are represented as mean ± SEM for $n = 3$ and normalized to β-actin (not shown). $^{*}p < 0.05$ versus time 0 control.

Figure 5. PP1, PP2A, and PP2B protein levels in response to 17β-estradiol in the presence and absence glutamate and/or okadaic acid in HT-22 cells. HT-22 cells were treated with 100 nM okadaic acid (OA), 10 mM glutamate (Glut), and/or 10 μM 17β-estradiol (E2). Cells were harvested after 24 h of treatment for Western blot analysis of PP1 (***A***), PP2A (***B***), and PP2B (***C***). Data are represented as mean ± SEM for $n = 3$ and normalized to β-actin (not shown). * $P < 0.05$ vs. control; † $P < 0.05$ vs. glutamate treated group.

Figure 6. PP1, PP2A, and PP2B protein levels in response to 17β-estradiol in the presence and absence of 50 μM glutamate and/or 50 nM okadaic acid in primary cortical neurons. Primary rat cortical neurons were treated with 50 nM okadaic acid (OA), 50 μM glutamate (Glut), and/or 100 nM 17β-estradiol (E2). Cells were harvested after 24 h of treatment for Western blot analysis of PP1 (*A*), PP2A (*B*), and PP2B (*C*). Data are represented as mean ± SEM for $n = 3$ and normalized to β-actin (not shown). * $P < 0.05$ vs. control; † $P < 0.05$ vs. glutamate treated group.

Figure 1

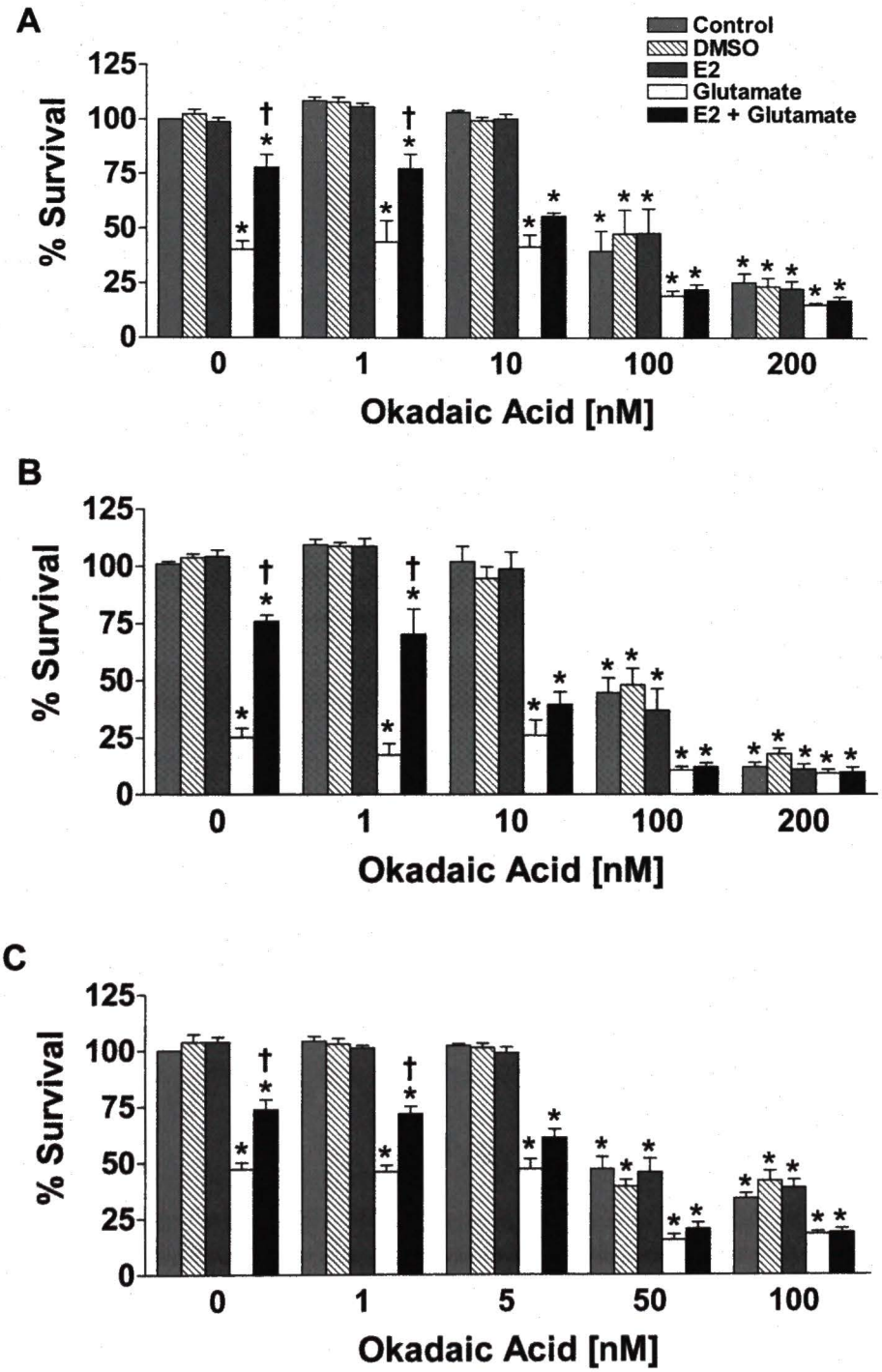

A
Control
DMSO
E2
Glutamate
E2 + Glutamate
% Survival
125
100
75
50
25
0
0
1
10
100
200
Okadaic Acid [nM]
B
% Survival
125
100
75
50
25
0
0
1
10
100
200
Okadaic Acid [nM]
C
% Survival
125
100
75
50
25
0
0
1
5
50
100
Okadaic Acid [nM]

Figure 2

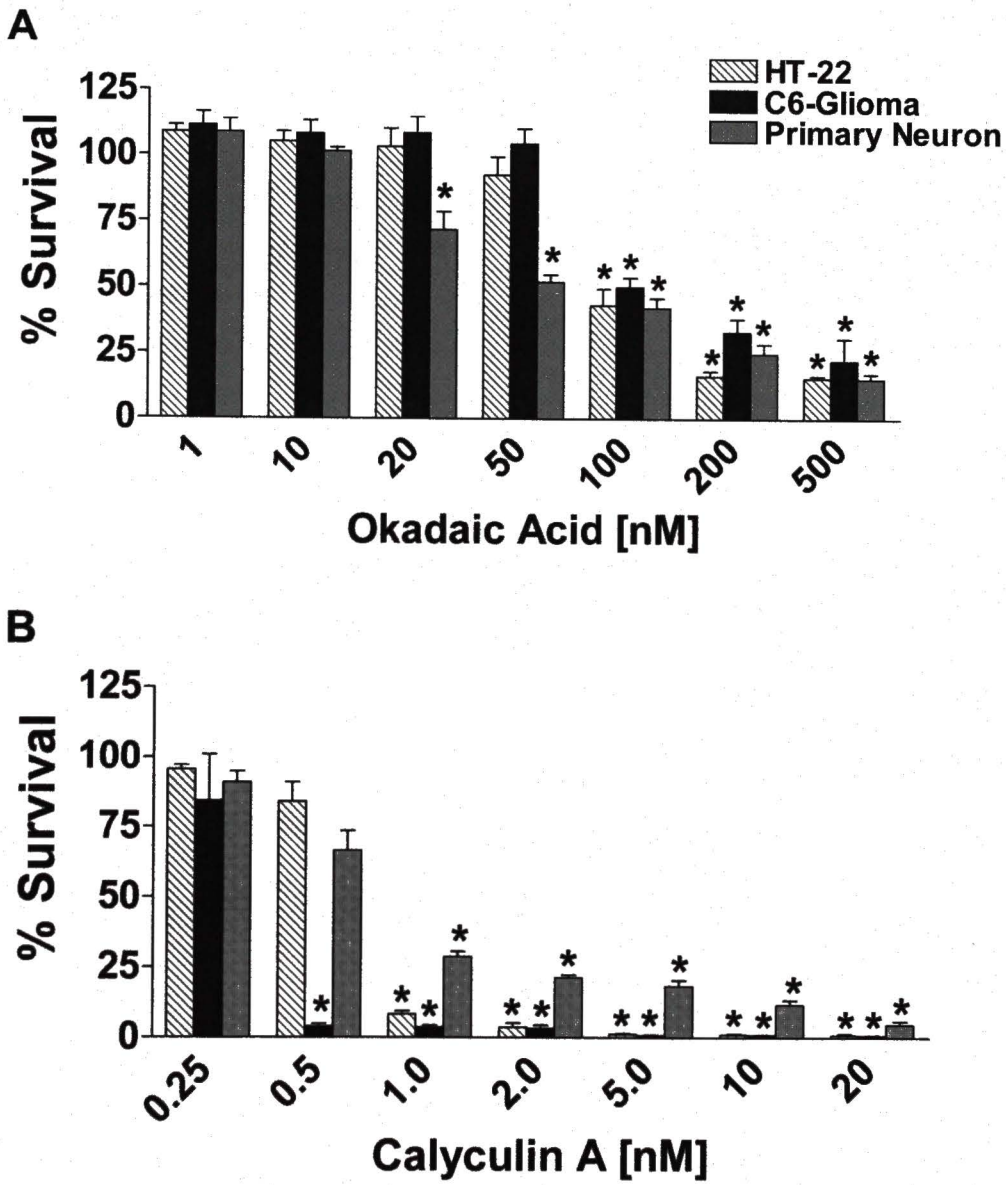

A
% Survival
125
100
75
50
25
0
HT-22
C6-Glioma
Primary Neuron
1
10
20
50
100
200
500
Okadaic Acid [nM]
B
% Survival
125
100
75
50
25
0
0.25
0.5
1.0
2.0
5.0
10
20
Calyculin A [nM]

Figure 3

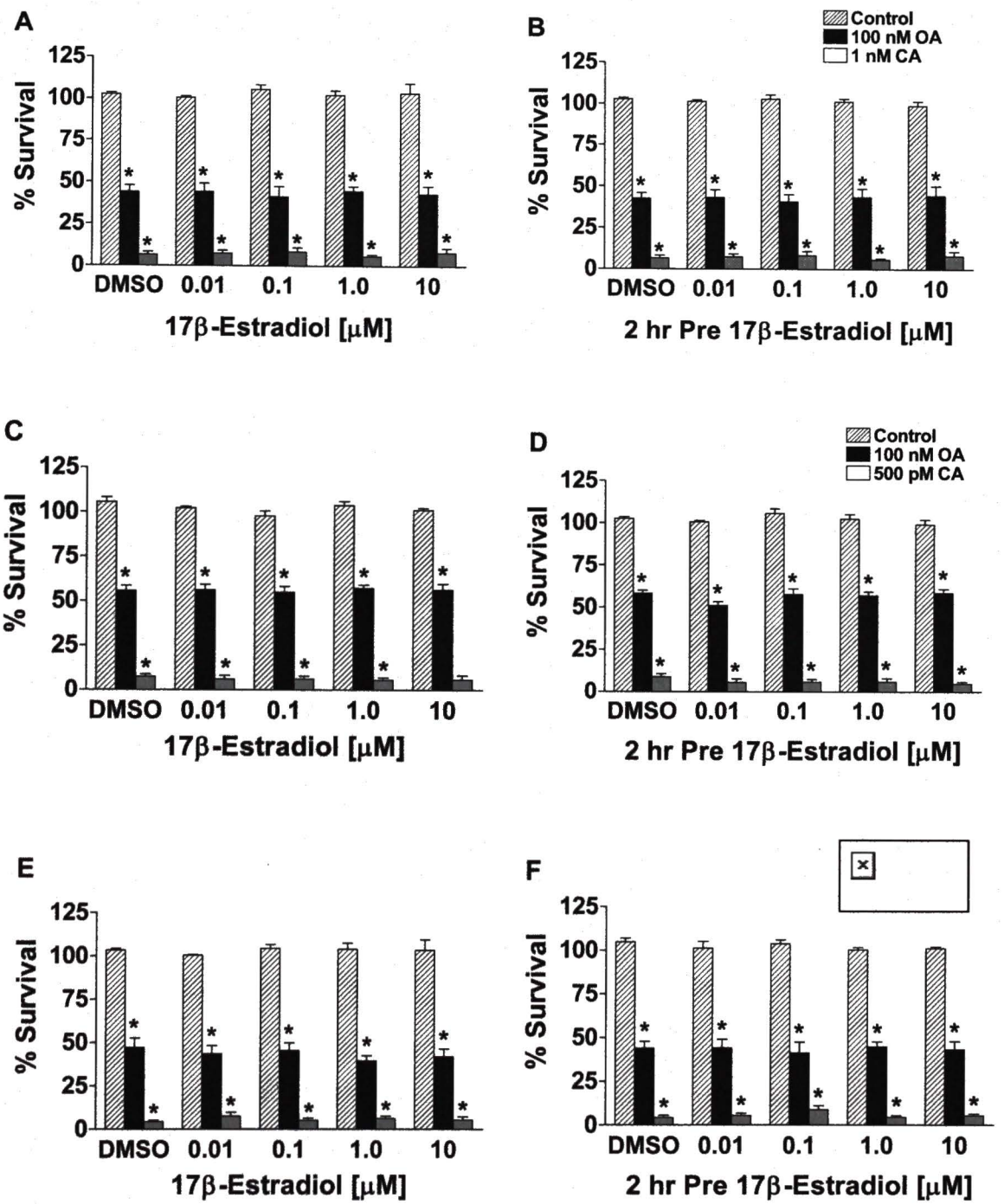

A
B
C
D
E
F
Control
100 nM OA
1 nM CA
500 pM CA
% Survival
125
100
75
50
25
0
DMSO
0.01
0.1
1.0
10
17β-Estradiol [μM]
2 hr Pre 17β-Estradiol [μM]

Figure 4

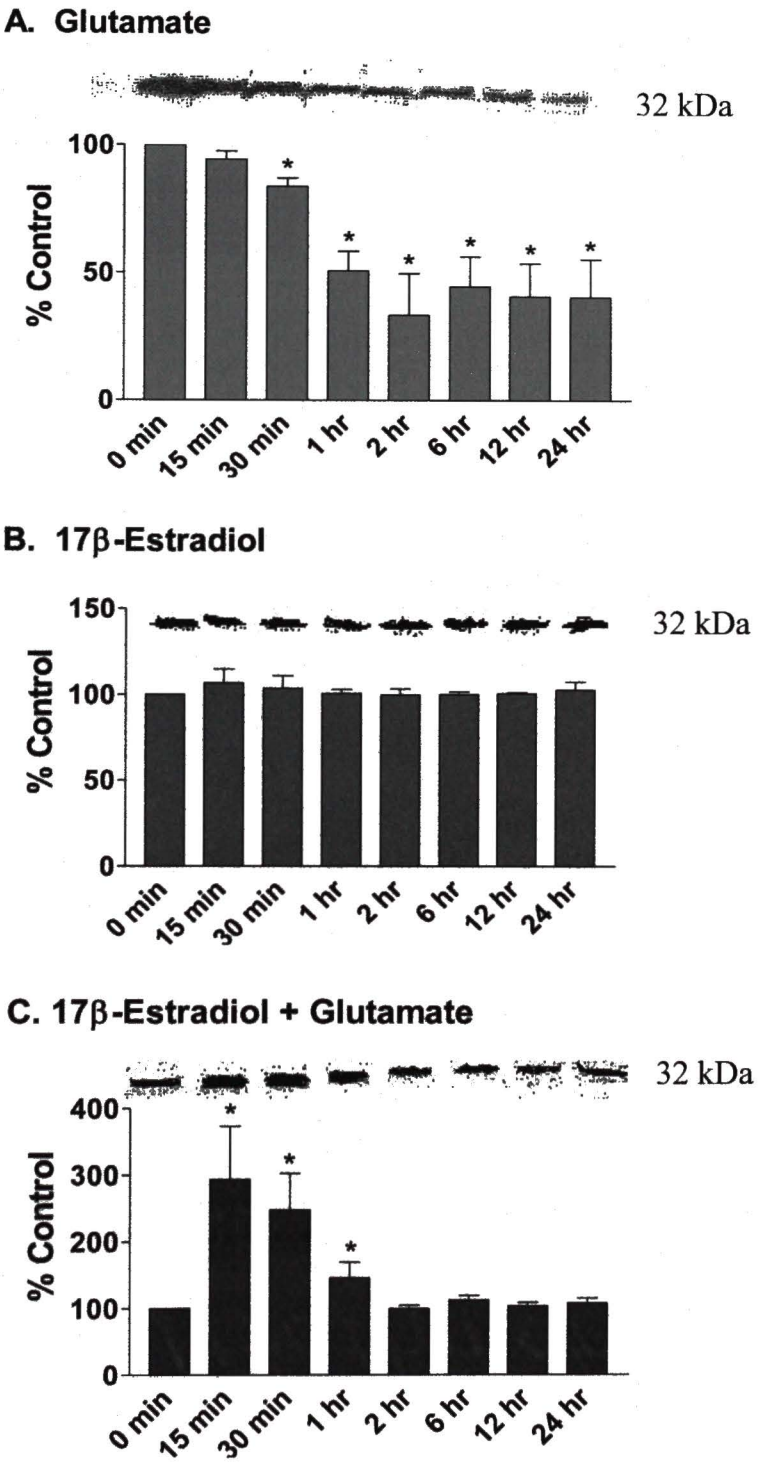

A. Glutamate
32 kDa
% Control
100
50
0
0 min
15 min
30 min
1 hr
2 hr
6 hr
12 hr
24 hr
B. 17β-Estradiol
32 kDa
% Control
150
100
50
0
0 min
15 min
30 min
1 hr
2 hr
6 hr
12 hr
24 hr
C. 17β-Estradiol + Glutamate
32 kDa
% Control
400
300
200
100
0
0 min
15 min
30 min
1 hr
2 hr
6 hr
12 hr
24 hr

Figure 5

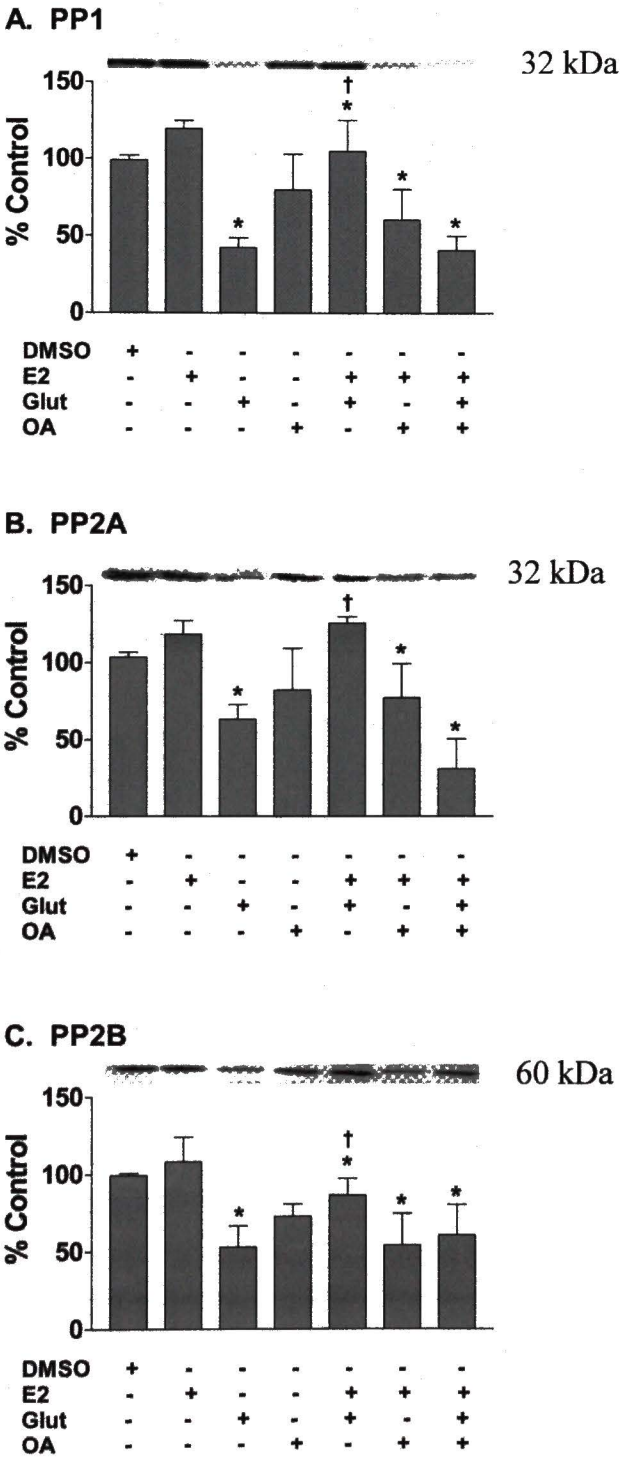
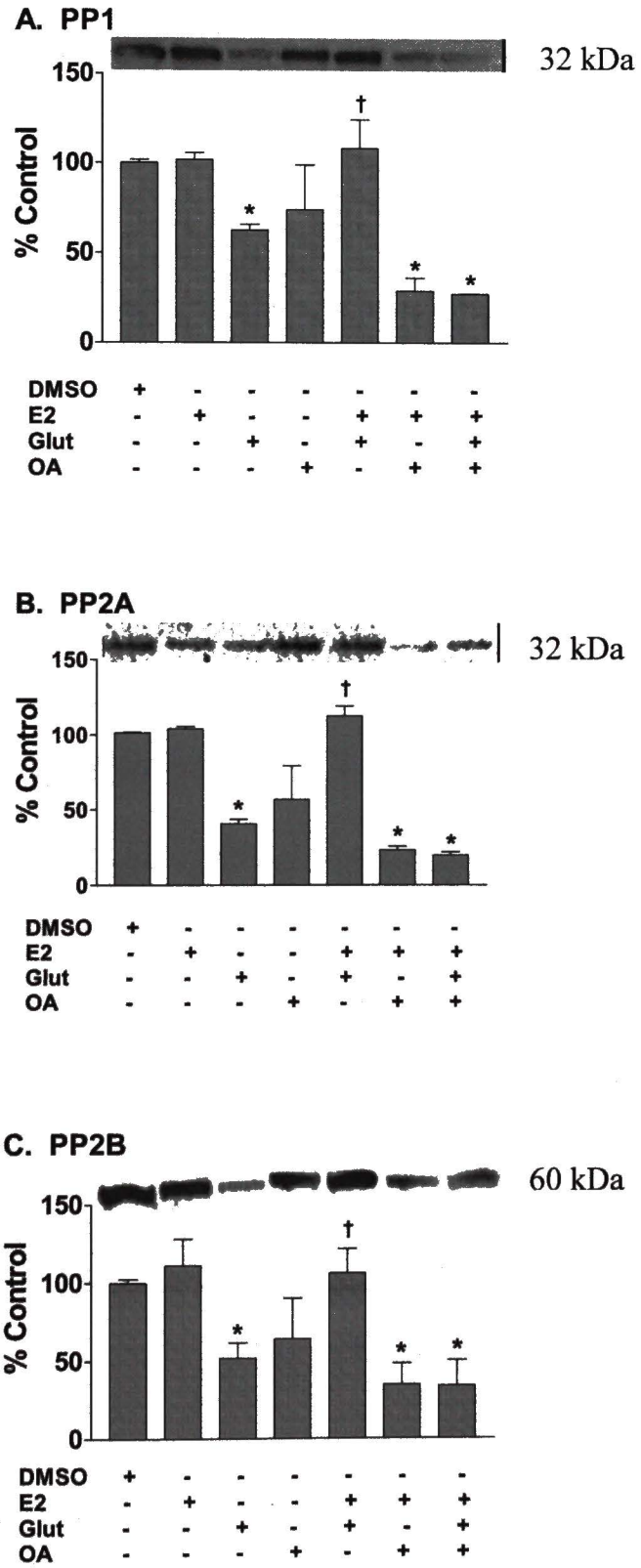

A. PP1
32 kDa
% Control
150
100
50
0
DMSO + - - - - - -
E2 - + - - + + +
Glut - - + - + - +
OA - - - + - + +
B. PP2A
32 kDa
% Control
150
100
50
0
DMSO + - - - - - -
E2 - + - - + + +
Glut - - + - + - +
OA - - - + - + +
C. PP2B
60 kDa
% Control
150
100
50
0
DMSO + - - - - - -
E2 - + - - + + +
Glut - - + - + - +
OA - - - + - + +

Figure 6


A. PP1
32 kDa
150
100
50
0
% Control
†
*
DMSO + - - - - - -
E2 - + - - + + +
Glut - - + - + - +
OA - - - + - + +
B. PP2A
32 kDa
150
100
50
0
% Control
†
*
DMSO + - - - - - -
E2 - + - - + + +
Glut - - + - + - +
OA - - - + - + +
C. PP2B
60 kDa
150
100
50
0
% Control
†
*
DMSO + - - - - - -
E2 - + - - + + +
Glut - - + - + - +
OA - - - + - + +

CHAPTER 3

Estrogen Receptor Independent Neuroprotection Via Protein Phosphatase Preservation and Attenuation of Persistent ERK1/2 Activation

by

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ABSTRACT

Estrogen-mediated neuroprotection has been well established; however, the mechanism of estrogen action is not yet clear. Estrogens have a variety of mode of action including transducing signaling events such as activation and/or suppression of the MAPK pathway. We have previously shown protein phosphatases (PPs) to be involved in 17β-estradiol mediated neuroprotection. In the present study, we assessed the role of estrogen receptors (ER) in PP protection from oxidative stress/excitotoxicity and the consequential effects on MAPK signaling. Okadaic acid (OA) and calyculin A (CA), non-specific serine/threonine PP inhibitors, were exposed to cells at various concentrations in the presence or absence of 17α-estradiol, the enantiomer of 17β-estradiol (ENT E2), 2-(1-adamantyl)-3-hydroxyestra-1,3,5(10)-trien-17-one (ZYC3, non-ER binding estrogen analog) and/or glutamate. All three compounds, which we have shown have little or no binding to ERα and ERβ, were protective against glutamate toxicity, but not against OA and CA toxicity. Additionally, in the presence of effective concentrations of these PP inhibitors, the protective effects of these estrogen analogues against glutamate toxicity were lost. Glutamate treatment caused a 50% decrease in levels of PP1, PP2A, and PP2B protein; while, co-administration of ZYC3 with glutamate prevented the decrease in PP1, PP2A, and PP2B levels. Furthermore, glutamate treatment caused a persistent increase in phosphorylation of ERK1/2 that corresponds with the decrease protein levels of serine/threonine PPs. Treatment of 17β-estradiol or ZYC3 blocked the persistent increase in ERK phosphorylation and decreased PP protein expression associated with glutamate toxicity. These results suggest that

estrogens protect cells against glutamate-induced oxidative stress and excitotoxicity through an ER-independent mediated mechanism that serves to preserve phosphatase activity in the face of oxidative/excitotoxic insults resulting in attenuation of the persistent phosphorylation of ERK1/2 associated with neuronal death.

I. INTRODUCTION

Estrogens have been shown to be potent neuroprotectants in a variety of systems and experimental models of neurodegeneration and cerebral ischemia (Green et al., 1996; Green et al., 1997b; Green et al., 1997a; Gridley et al., 1997; Simpkins et al., 1997; Sudo et al., 1997; Alkayed et al., 1998; Dubal et al., 1998; Green et al., 1998; Yang et al., 2001). However, the mechanism of action remains elusive. Potential mechanisms of estrogens neuroprotective effects include 1) classical ER-dependent genomic actions; 2) ER-dependent, non-genomic actions; 3) ER-independent genomic actions; 4) ER-independent non-genomic actions.

This laboratory and others have demonstrated that estrogens exert potent neuroprotective effects at physiologically relevant concentrations (for review, see (Green and Simpkins, 2000). Both the naturally occurring feminizing estrogen, 17β-estradiol and its inactive isomer, 17α-estradiol, can reduce toxicity caused by serum deprivation, β-amyloid treatment, and exposure to glutamate receptor agonists in cell model systems (Green et al., 1996; Green et al., 1997b; Green et al., 1997a; Gridley et al., 1997; Green et al., 1998; Gridley et al., 1998; Zaulyanov et al., 1999). Additionally, this laboratory has also demonstrated that treatment with either estradiol isomer markedly reduces ischemic brain damage produced by MCAO in ovariectomized rats (Simpkins et al., 1997; Yang et al., 2000; Yang et al., 2001), and that estradiol administration reduces secondary ischemic damage and mortality following subarachnoid hemorrhage in vivo (Yang et al., 2001). ZYC3, a non-receptor-binding estrogen analogue, possesses both neuroprotective and vasoactive effects, which offers the possibility of clinical application for stroke

without the side effects of estrogens (Liu et al., 2002). It also suggests that both the neuroprotective and vasoactive effects of estrogen are receptor independent as estrogen analogues with little or no binding to ERα and ERβ have been shown to be neuroprotective (Liu et al., 2002; Perez et al., 2005). In many studies, ER antagonists do not block or attenuate the protective action of 17β-estradiol (Weaver et al., 1997; Moosmann and Behl, 1999), and estrogens exert their neuroprotective action even in the presence of transcription and translation inhibitors (Regan and Guo, 1997; Sawada et al., 1998). Furthermore, estrogens are known to activate ERK1/2 within 5-15 min, and this activation persists for at least 2 hrs (Singh et al., 2000). It has been speculated that the time course of ERK activation, among other factors, determines whether a cell commits to survival or death, which can explain the contradictory effects of MEK1/2 inhibition on neuronal cell survival.

Persistent activation of ERK has been shown to be involved in neuronal cell death (Alessandrini et al., 1999; Wang et al., 2003; Wang et al., 2004). Aberrant neuronal expression of phosphorylation of ERK1/2 and other MAPKs are seen in AD brains (Zhu et al., 2002b) and other sporadic and familial neurodegenerative diseases characterized by tau deposits(Ferrer et al., 2003). In patients with Parkinson's disease and other Lewy body diseases, increased phosphorylation of ERK1/2 is seen in the substantia nigra and in the midbrain (Zhu et al., 2002a). It has also been shown that following acute ischemic stroke, there is an increased phosphorylation of ERK1/2 in the penumbra (Slevin et al., 2000).

In the present study, we hypothesized that the neuroprotective actions of estrogens do not require receptor binding but the activation of phosphatases to mediate signaling through the MAPK pathway. We have previously shown that PPs are involved in neuroprotective mechanism of 17β-estradiol (Yi et al., 2005). We show that inhibition of PPs attenuates the neuroprotective effects of estrogen analogues that have little or no ERα or ERβ binding properties and involves the MAPK signaling pathway.

II. METHODS

Chemicals

17α-estradiol was purchased from Steraloids, Inc. (Wilton, NH). ZYC3 and the enantiomer of 17β-estradiol (ENT E2) were prepared as described previously (Green and Simpkins, 2000; Liu et al., 2002). All steroids were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and diluted to appropriate concentration in culture media. Calcein AM was purchased from Molecular Probes, Inc. (Eugene, OR). Okadaic acid, L-glutamate, and DMSO were purchased from Sigma (Paris, KY). Calyculin A and PD98059 were purchased from Calbiochem (San Diego, CA). Anti-PP1, anti-PP2A, anti-PP2B, anti-ERK, and anti-pERK were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Culture of primary cortical neurons

Embryonic rat cerebral cortex (18-day) were dissected and harvested in preparation medium (DMEM, glucose 4.5g/L, Penicillin 100 U/ml, Streptomycin

100μg/ml). The cortical tissue was treated with trypsin. The tissue was washed three times using washing medium (Hank's medium, glucose 4.5g/L, Penicillin 100 U/ml, Streptomycin 100μg/ml) and individual cells isolated by mechanical trituration using three different sizes of fire polished Pasteur pipettes. The cells were harvested in seeding medium (DMEM, glucose 4.5g/L, Penicillin 100 U/ml, Streptomycin 100μg/ml, Glutamine 2mM, 19% horse serum) and filtered through a 40μm filter. The cerebral cortical cells were seeded in various poly-L-lysine treated dishes and plates at various cell densities. The cells were incubated in neurobasal medium (DMEM, glucose 4.5g/L, Penicillin 100 U/ml, Streptomycin 100μg/ml, glutamine 2mM) supplemented with B-27 with antioxidants in normal cell culture condition of 37°C in a humid atmosphere of 5% CO_2. Two hours before treatment with various inhibitors and/or 17α-estradiol, ENT E2, or ZYC3, the media was replaced with neurobasal medium supplemented with B-27 without antioxidants.

Calcein-AM viability assay

HT-22 and C6-glioma cells were seeded 24 hr before initiation of the experiment at a density of 3,500 cells/well in 96-well plates. Cells were exposed to various treatments. After exposure to various treatment paradigms cells were rinsed with phosphate buffered saline, and cell viability was measured using the membrane-permeant calcein-AM dye (Molecular Probes, Eugene, OR). Calcein-AM is a fluorogenic esterase substrate that is hydrolyzed to a fluorescent product in cells having esterase activity and intact membranes. Cells were incubated in a solution of 2.5 μM calcein-AM in PBS.

Twenty minutes later, fluorescence was determined using a Bio-Tek FL600 microplate reader (Winooski, VT) with an excitation/emission filter set of 485/530 nm. Cell culture wells treated with methanol served as blanks. The results, obtained in relative fluorescent units (RFU), are expressed as the percentage of untreated or vehicle-treated control values.

Immunoblot analysis

Protein from whole cell lysates (25 μg) was separated by SDS-polyacrylamide gel electrophoresis and transferred to Immunobilon-P polyvinylidene difluoride (Millipore Corp., Bedford, MA) membrane. Membranes were rinsed in Tris-buffered saline (10 mM Tris-base, pH 8.0, 100 mM NaCl) containing 0.2% Tween 20 then blocked with 3% bovine serum albumin. Blots were then incubated with primary antibodies overnight at 4°C, rinsed and incubated in the appropriate secondary antibody before detection using enhanced chemiluminescence (ECL; Pierce Biotechnology, Inc., Rockford, IL). ECL results were digitized and quantified using UVP Bioimaging System.

Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. $p < 0.05$ was considered significant for all experiments. Each set of data represents three or more individual assays performed separately, with each containing 4-8 replicate wells. The values are reported as the mean ± SEM.

III. RESULTS

Effects of PP inhibitors on estrogen analog mediated neuroprotection against glutamate induced cytotoxicity

17α-estradiol, ENT E2, and ZYC3 (Structures in Fig 1) have been shown to be as potent neuroprotectants as 17β-estradiol (Bishop and Simpkins, 1994; Green et al., 2001; Perez et al., 2005). In order to determine the effectiveness of 17α-estradiol, ENT E2, and ZYC3 to protect neurons against oxidative stress or excitotoxicity induced by glutamate in the presence of a PP inhibitor, we examined simultaneous treatment as well as 2 or 24 hr pretreatment of 17α-estradiol, ENT E2, or ZYC3 with glutamate and OA or CA. In the absence of PP inhibitors, the three estrogen analogues showed a dose-dependent neuroprotection against glutamate toxicity (Fig 2). ZYC3 showed a greater potency then either ENT E2 or 17α-estradiol, consistent with our previous structure-activity relationship assessment (Perez, et al., 2005). 17α-Estradiol, ENT E2, and ZYC3 were ineffective against OA or CA toxicity when the PP inhibitor was simultaneously administered with the steroids. In addition, non-lethal concentrations of OA and CA attenuated the ZYC3 mediated neuroprotection against glutamate-induced toxicity (Fig 3). Nearly complete abolishment of estrogen analogue protection against glutamate toxicity was seen at 5 nM OA and 0.5 nM CA (Fig 4). Twenty-four hr ZYC3 pretreatment did not prevent PP inhibitor-induced cell death or the PP inhibitor-induced abolishment of protection by ZYC3 of glutamate induced neurotoxicity (Fig 4). Data from 24 hr and 2 hr pretreatment with ENT E2 or 17α-estradiol are not shown, but are similar to those seen with the 24 hr pretreatment with ZYC3.

Time-dependent effects of ZYC3 and/or glutamate on PP1 protein level

We examined the time-course of the response of PP protein expressions to treatment with glutamate and/or ZYC3. Glutamate treatment caused a 50% reduction in PP1 protein levels that was significant at 15 min and persisted through 24 hours of treatment (Fig 5A). ZYC3 alone did not significantly alter the expression of PP1 at any given time point as compared to non-treated controls (Fig 5B). However, simultaneous treatment with ZYC3 and glutamate caused a rapid and profound increase in PP1 expression at 30 min and 1 hr followed by a return to control levels after 2 hr post-treatment that persists to 24 hr post treatment (Fig 5C). The time-course and magnitude of the response to ZYC3 was similar to that which we have reported for E2 (Yi et al., 2005). Similar trends were seen with PP2A and PP2B expression (data not shown). PP2A and PP2B protein expressions were decreased to ~ 30-50 % by one hour following glutamate treatment and this effect persisted for 24 hours. The presence of ZYC3 in the face of glutamate challenge caused a transient increase in PP2A and PP2B protein levels by 3-4 fold within 15 to 30 min. PP2A and PP2B protein levels return to control levels by 1 hr post-treatment and remained at control levels at 24 hr post-treatment.

Effects of estrogen analogues on PP1, PP2A, and PP2B protein expression

In order to determine the effects of ZYC3 treatment on PP expressions, we examined protein expressions of PP1, PP2A, and PP2B following 24 hr simultaneous treatment of glutamate, OA, and/or ZYC3 in cortical neurons. Twenty-four hour treatment of cortical neurons with ZYC3 alone did not alter levels of PP1 (Fig 6A), PP2A

(Fig 6B), and PP2B (Fig 6C) as compared to non-treated control cells. However, 24 hr glutamate treatment caused a marked decrease in protein levels of PP1, PP2A, and PP2B (Fig 6A,B,C). Simultaneous treatment with 10 nM ZYC3 in the presence of 50 μM glutamate restored PP1, PP2A, and PP2B protein expression to levels comparable to untreated control cells (Fig 6A,B,C). However, ZYC3 treatment was unable to block the glutamate induced decrease in protein expressions of PP1, PP2A, and PP2B in the presence of okadaic acid (Fig 6A,B,C).

MEK-dependent neuroprotection against toxicity

Experimental data have shown that inhibition of ERK phosphorylation via MEK inhibition is neuroprotective (Hetman et al 2004; Kummer et al 1997). Hence, the neuroprotective effects of MEK inhibitors PD98059 (Fig 7A) or U0126 (Fig 7B) were evaluated against glutamate toxicity. One hour pretreatment with PD98059 or U0126 had a neuroprotective effect against cytotoxicity caused by glutamate. This MEK-inhibitor-induced neuroprotection was blocked by increasing concentrations of OA (Fig 7).

Effects of ZYC3 on ERK phosphorylation during oxidative stress

Chronic phosphorylation of ERK1/2 is thought to send a neurotoxic or neurodegenerative signal (Stanciu et al., 2000; Kulich and Chu, 2001; Zhu et al., 2002a); thus, it has been speculated that estrogens attenuate the persistent ERK1/2 phosphorylation to mediate neuroprotection. Therefore, we examined the

phosphorylation of ERK1/2 in glutamate toxicity in the presence of ZYC3 and/or OA. Twenty-four hour glutamate treatment caused a ~2.5 fold increase in phosphorylation of ERK1/2 (Fig 8). ZYC3 alone did not alter the phosphorylation state of ERK1/2 as compared to vehicle control (Fig 8). However, the presence of ZYC3 attenuated the elevated phosphorylation of ERK1/2 induced by glutamate. The presence of ZYC3 had no effect on OA induced ERK1/2 phosphorylation, and the presence of OA abolished the ZYC3 mediated reduction in phosphorylation ERK1/2 caused by glutamate (Fig 8).

DISCUSSION

The present study demonstrates that one of the mechanisms of estrogen mediated neuroprotection is by preservation of PP activity via an ER-independent pathway that ultimately prevents persistent ERK1/2 hyperphosphorylation. These observations are important in view of the WHI-MS results that indicate chronic post-menopausal treatment of older women with estrogens (Rapp et al., 2003; Shumaker et al., 2003; Espeland et al., 2004; Shumaker et al., 2004) not only does not protect the brain from dementia, but appears to exacerbate the condition, through a mechanism that likely involves interaction of the steroid with ERs. Thus, alternatives to feminizing estrogens that protect the brain are needed.

We have previously reported that the non-feminizing analogues of estrogens used in this study are protective against a variety of cytotoxic insults (Green et al., 1996; Green et al., 1997b; Green et al., 1997a; Green et al., 1998) as well as in a rat model for middle cerebral artery occlusion (Simpkins et al., 1997; Yang et al., 2000; Liu et al.,

2002). The protective effects of these estrogen analogues against glutamate toxicity were antagonized by the protein phosphatase inhibitors, okadaic acid and calyculin A. In addition, we show that estrogen analogues rapidly and persistently antagonize the reduction in PP1, PP2A and PP2B protein expression induced by oxidative and excitotoxic stresses, but not in the presence of okadaic acid. Phosphorylation of ERK1/2 in response to glutamate toxicity corresponds to decreases in serine/theronine phosphatase expression, and inhibitors of ERK1/2 phosphorylation attenuate the glutamate induced cytotoxicity. Collectively, these data support the hypothesis that phosphatase regulation is a major component of estrogen-mediated neuroprotection that is not dependent upon the classical ER mediated genomic effects.

Of the three estrogen analogues assessed, ZYC3 was more potent in each cell type than either 17α-estradiol or ENT E2, consistent with our previous structure activity relationship studies (Green et al., 2001; Liu et al., 2002; Perez et al., 2005). Despite this, OA and CA were effective in blocking the neuroprotective effects of all three compounds. This antagonism by PP inhibition of estrogen neuroprotection occurred even at OA and CA concentration that did not induce cell death. This latter observation indicates that abolition of estrogen neuroprotection by PP inhibition is not due to an increase in the magnitude of the neurotoxic insult, which we have shown reduces the potency of estrogen neuroprotection (Perez et al., 2005).

The mechanism of this effect of estrogens on PP levels and activity is not clear. There are no previous experimental data suggesting a direct interaction between estrogens and PP. While PP2A has been shown to regulate ERα by mRNA stabilization

(Keen et al., 2005) as well as direct interaction with ERα in the absence of estrogen (Lu et al., 2003), our data indicate that effects of estrogens on PPs occur without ER interactions. An interesting, but yet unexplained observation is that while 17β-estradiol (Yi et al., 2005) or ZYC3 (present study) alone have little effect on PP levels, they cause a prompt increase in PP concentrations and a persistent resistance to glutamate-induced decline in PP levels. The absence of effects of estrogens alone and the rapidity of the response to estrogens in the face of insult suggest that estrogens either reduce or prevent the clearance of PPs that is activated by oxidative/excitotoxic insult, rather than causing expression of new protein. Therefore, it is likely that 17β-estradiol and its analogues are protecting cells by blocking the ubiquitination and/or degradation of protein phosphatases caused by oxidative or excitotoxic stresses.

In view of the relative lack of binding of these analogues to ERα or ERβ (Perez et al., 2005), intracellular signaling pathways may be involved in mediating their neuroprotective effects. Indeed, experimental studies have shown that the neuroprotective effects of estrogens are mediated at least in part through the rapid, but acute phosphorylation of signaling proteins, such as adenylyl cyclase, Akt, PKA, PKC, and MAPK (ERK) (Migliaccio et al., 1996; Watters et al., 1997; Kelly et al., 1999; Singer et al., 1999; Singh et al., 1999; Toran-Allerand et al., 1999; Zhang et al., 2001). Changes in the activity of these enzymes can regulate the phosphorylation of numerous cellular substrates such as intermediary signaling proteins Rsk, p38, JNK, and the nuclear transcriptional factors CREB and cfos/c-Jun, which may ultimately mediate cell survival changes (for review, see (Lee and McEwen, 2001)). The present study shows that

ERK1/2 is rapidly and persistently phosphorylated in response to oxidative and excitotoxic stresses caused by glutamate, and that the presence of estrogens prevents this persistent phosphorylation. These data suggests that prolonged phosphorylation of ERK1/2 is detrimental to cell survival, which is supported by the observations by Smith and colleagues who noted aberrant neuronal expression of phosphorylated ERK1/2 and other MAPKs in AD brains in association with markers of oxidative stress (Zhu et al., 2002b). Others have also shown that MAPK phosphorylation is associated with a variety of sporadic and familial neurodegenerative diseases characterized by tau deposits (Ferrer et al., 2003). Patients with PD and other Lewy body diseases have increased phosphorylated ERK1/2 in neurons of substantia nigra and midbrain (Zhu et al., 2002a). In addition, increased ERK1/2 phosphorylation has been noted in the vulnerable pneumbra following acute ischemic stroke in humans (Slevin et al., 2000).

The present study provides a potential ER-independent mechanism for the neuroprotective effects of estrogens, through the prevention of insult-induced decrease in phosphatase activity and the resulting neurotoxic, persistent hyperphosphorylation of proteins in multiple signaling pathways that are detrimental to cell survival. We have demonstrated that the neuroprotective effects of estrogen analogues against glutamate toxicity are opposed by phosphatase inhibition and that these analogues prevent insult-induced reduction in protein phosphatase levels as seen with 17β-estradiol (Yi et al., 2005). These data demonstrate that the protective effects of estrogens against neurotoxicity induced by oxidative stress and excitotoxicity are receptor-independent and

involve protein phosphatases activation through a signal transduction pathway mediated by MAPK (ERK 1/2).

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FIGURES LEGENDS

Figure 1. Structures of 17β-estradiol, and three non-feminizing estrogens, 17α-estradiol, ENT E2 and ZYC3.

Figure 2. Effects of okadaic acid and calyculin A on 17α-estradiol, ENT E2, or ZYC3 mediated neuroprotection in primary rat cortical neurons. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. **A**) Cells were treated simultaneously with 50 nM okadaic acid, 1 nM calyculin A, 50 μM glutamate, and/or varying concentrations of 17α-estradiol. **B**) Cells were treated simultaneously with 50 nM okadaic acid, 1 nM calyculin A, 50 μM glutamate, and/or varying concentrations of ENT E2. **C**) Cells were treated simultaneously with 50 nM okadaic acid, 1 nM calyculin A, 50 μM glutamate, and/or varying concentrations of ZYC3. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 3. Twenty-four hour pretreatment of primary rat cortical neurons with ZYC3 does not attenuate glutamate neurotoxicity in the presence of okadaic acid or calyculin A. Primary cortical neurons were seeded into 96-well plates at a density of

25,000 cells/well. **A**) Cells were treated simultaneously with 50 nM okadaic acid, 50 μM glutamate, and/or varying concentrations of ZYC 3. **B**) Cells were treated simultaneously with 10 nM calyculin A, 50 μM glutamate, and/or varying concentrations of ZYC3. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Gray bars, non-treated control; open bars glutamate treated group; black bars, okadaic acid (panel A) or calyculin A (panel B); hatched bars, glutamate and OA or CA treated group. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 4. Inhibition of protein phosphatases attenuates the neuroprotective effects of ZYC3 in primary rat cortical neurons. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. **A**) Cells were treated simultaneously with varying concentrations of okadaic acid, 50 μM glutamate, and 10 nM ZYC3. **B**) Cells were treated simultaneously with varying concentrations of calyculin A, 50 μM glutamate, and/or 10 nM ZYC3. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with three replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 5. Time-course of the effects of glutamate (A), ZYC3 (B), and their combination (C) on PP1 protein expression. Primary rat cortical neurons were treated with 50 μM glutamate and/or 10 nM ZYC3. Cells were harvested at the times indicated for western blot analysis of PP1. Depicted are mean ± SEM for n = 5 and normalized to β-actin (not shown). * $P < 0.05$ vs. time zero control.

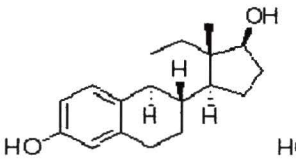
Figure 6. PP1 (A), PP2A (B), and PP2B (C) protein levels in response to ZYC3 in the presence and absence of 50 μM glutamate and/or 50 nM okadaic acid in primary rat cortical neurons. Primary cortical neurons were treated with 50 nM okadaic acid, (OA) 50 μM glutamate (Glut), and/or 10 nM ZYC3. Cells were harvested after 24 hr of treatment for western blot analysis of PP1, PP2A, and PP2B. Depicted are mean ± SEM for n = 5 normalized to β-actin (not shown). * $P < 0.05$ vs. control; † $P < 0.05$ vs. glutamate treated group.

Figure 7. Inhibition of MAPK pathway is the neuroprotective against glutamate-induced cytotoxicity. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. **A**) Cells were pretreated with 50 μM PD 98059 for one hour before treatment with varying concentrations of okadaic acid and 50 μM glutamate. **B**) Cells were pretreated with 10 nM U0126 for one hour before treatment with varying concentrations of okadaic acid and 50 μM glutamate. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are

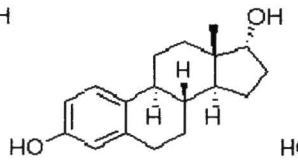
mean ± SEM for ten independent experiments with three replicates per experiment. * P <0.05 vs. vehicle control; † P < 0.05 vs. glutamate treated group.

Figure 8. Phosphorylation of ERK following glutamate treatment in the presence and absence of ZYC3 and/or OA. Primary cortical neurons were treated with 50 nM okadaic acid, (OA) 50 μM glutamate (Glut), and/or 10 nM ZYC3. Cells were harvested after 24 hr of treatment for western blot analysis of pERK. Depicted are mean ± SEM for n = 5. * P <0.05 vs. control.

Figure 1



17β-Estradiol



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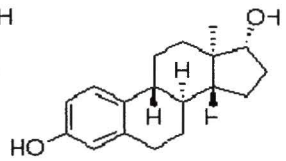
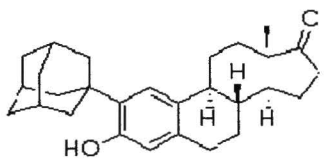


***ent*-Estradiol**
ENT E2



2-(1-Adamantyl)-3-hydroxy-estra-1,3,5(10)-trien-17-one
ZYC 3

Figure 2

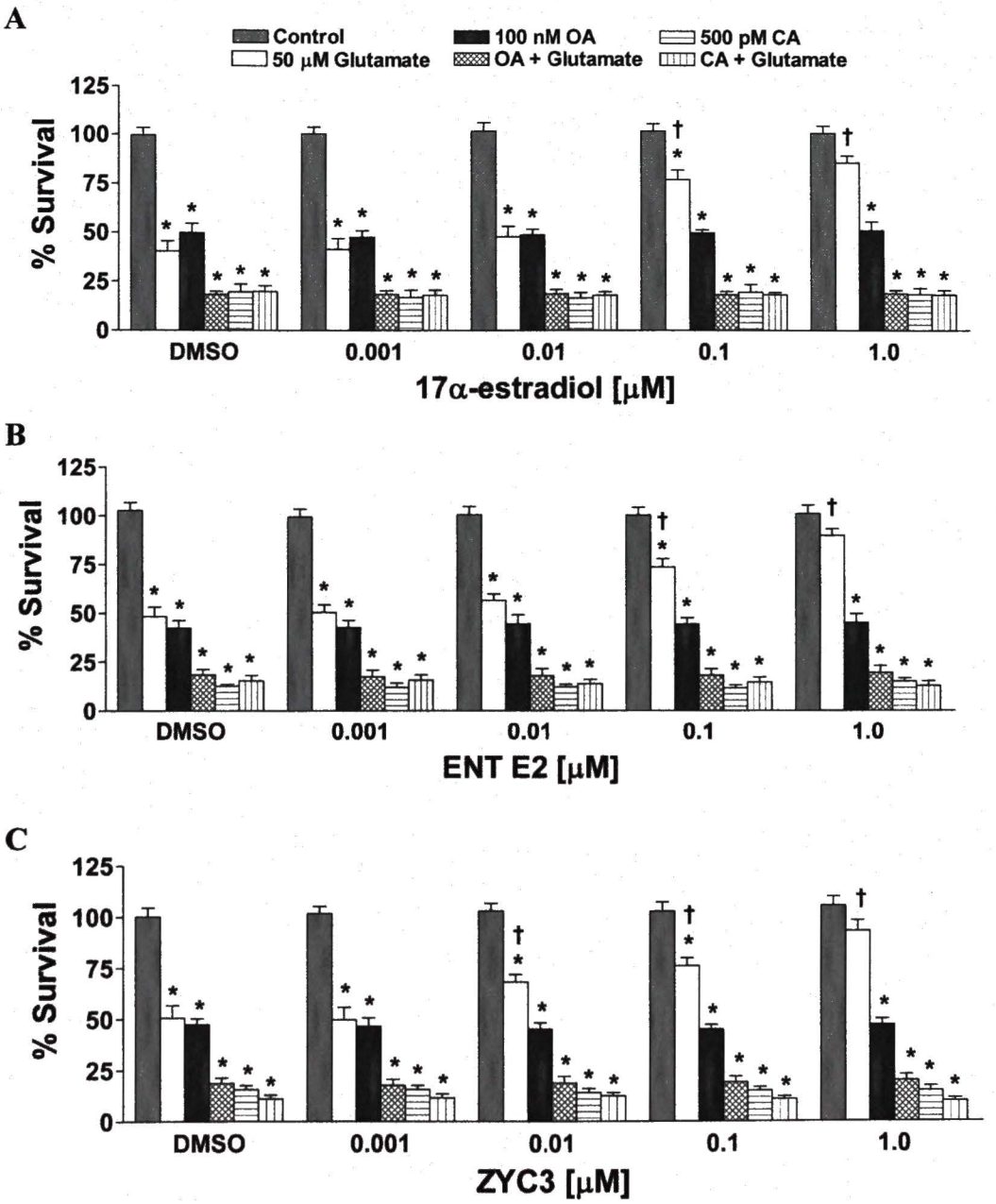

A
Control
50 μM Glutamate
100 nM OA
OA + Glutamate
500 pM CA
CA + Glutamate
% Survival
125
100
75
50
25
0
DMSO
0.001
0.01
0.1
1.0
17α-estradiol [μM]
B
% Survival
DMSO
0.001
0.01
0.1
1.0
ENT E2 [μM]
C
% Survival
DMSO
0.001
0.01
0.1
1.0
ZYC3 [μM]

Figure 3

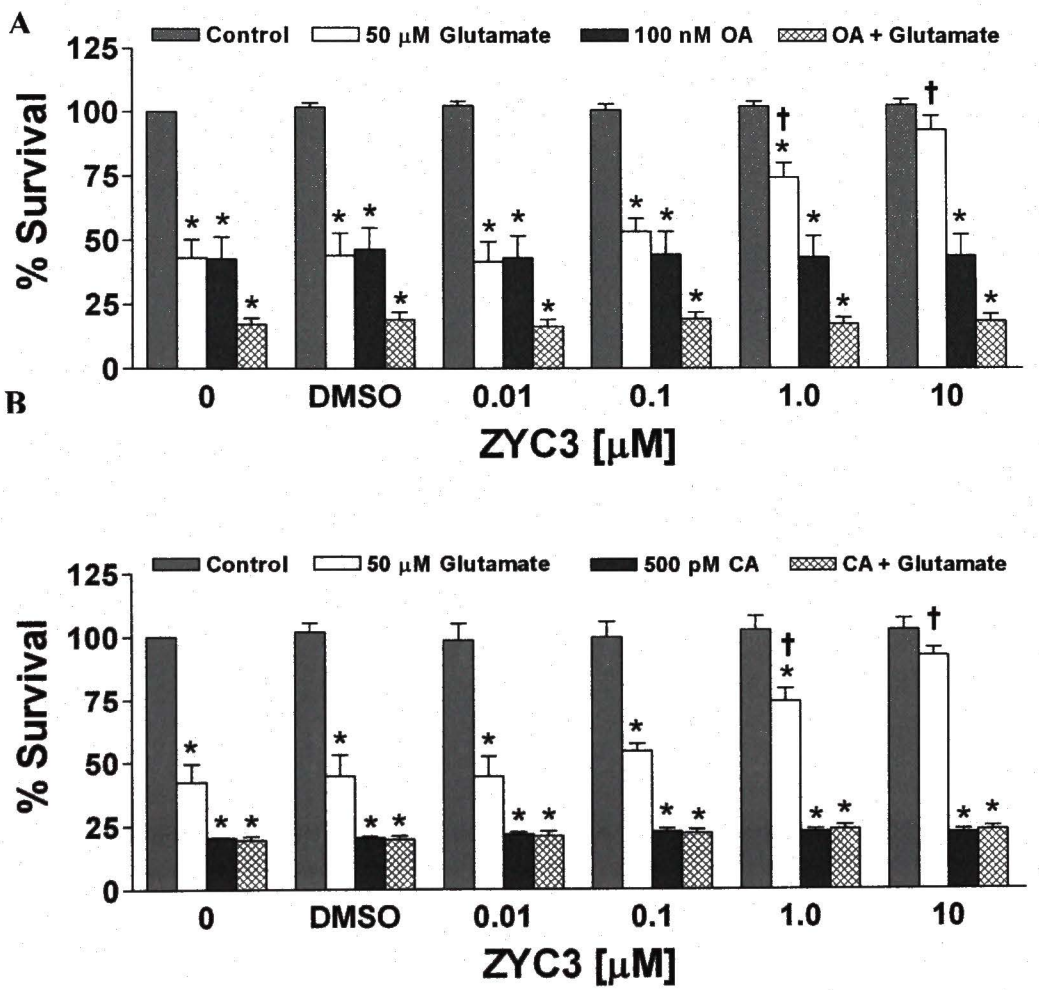

A
Control
50 μM Glutamate
100 nM OA
OA + Glutamate
% Survival
125
100
75
50
25
0
0
DMSO
0.01
0.1
1.0
10
ZYC3 [μM]
B
Control
50 μM Glutamate
500 pM CA
CA + Glutamate
% Survival
125
100
75
50
25
0
0
DMSO
0.01
0.1
1.0
10
ZYC3 [μM]

Figure 4

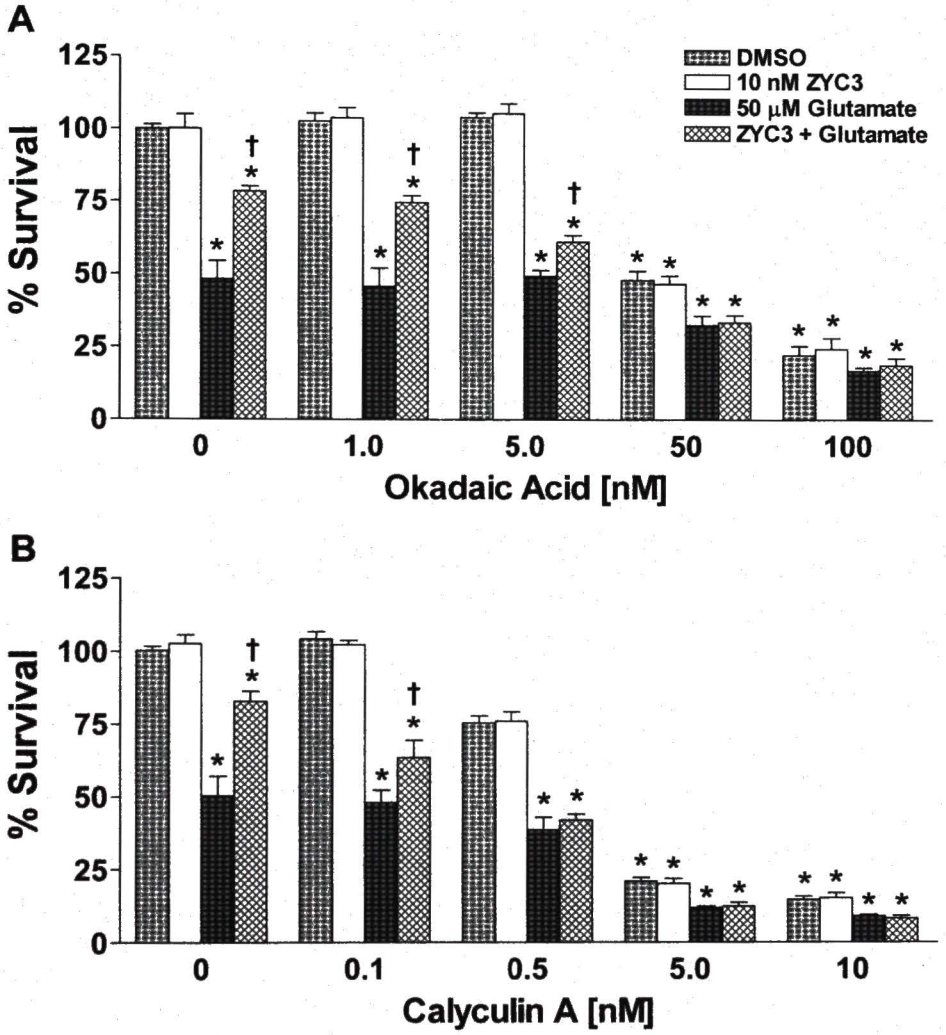

A
125
100
75
50
25
0
% Survival
DMSO
10 nM ZYC3
50 μM Glutamate
ZYC3 + Glutamate
0
1.0
5.0
50
100
Okadaic Acid [nM]
B
125
100
75
50
25
0
% Survival
0
0.1
0.5
5.0
10
Calyculin A [nM]

Figure 5

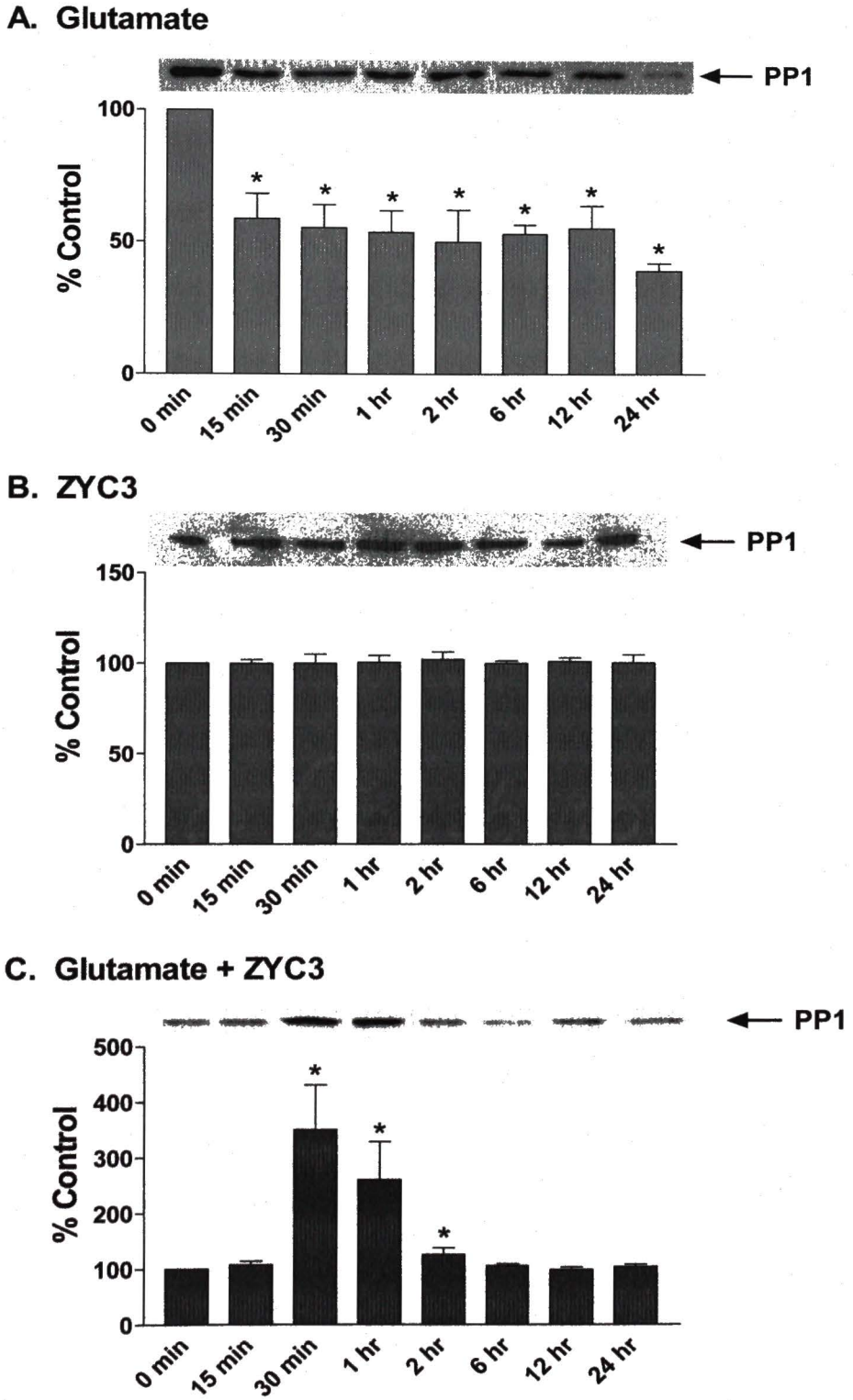

A. Glutamate
PP1
100
50
0
% Control
*
0 min
15 min
30 min
1 hr
2 hr
6 hr
12 hr
24 hr
B. ZYC3
PP1
150
100
50
0
% Control
0 min
15 min
30 min
1 hr
2 hr
6 hr
12 hr
24 hr
C. Glutamate + ZYC3
PP1
500
400
300
200
100
0
% Control
*
0 min
15 min
30 min
1 hr
2 hr
6 hr
12 hr
24 hr

Figure 6

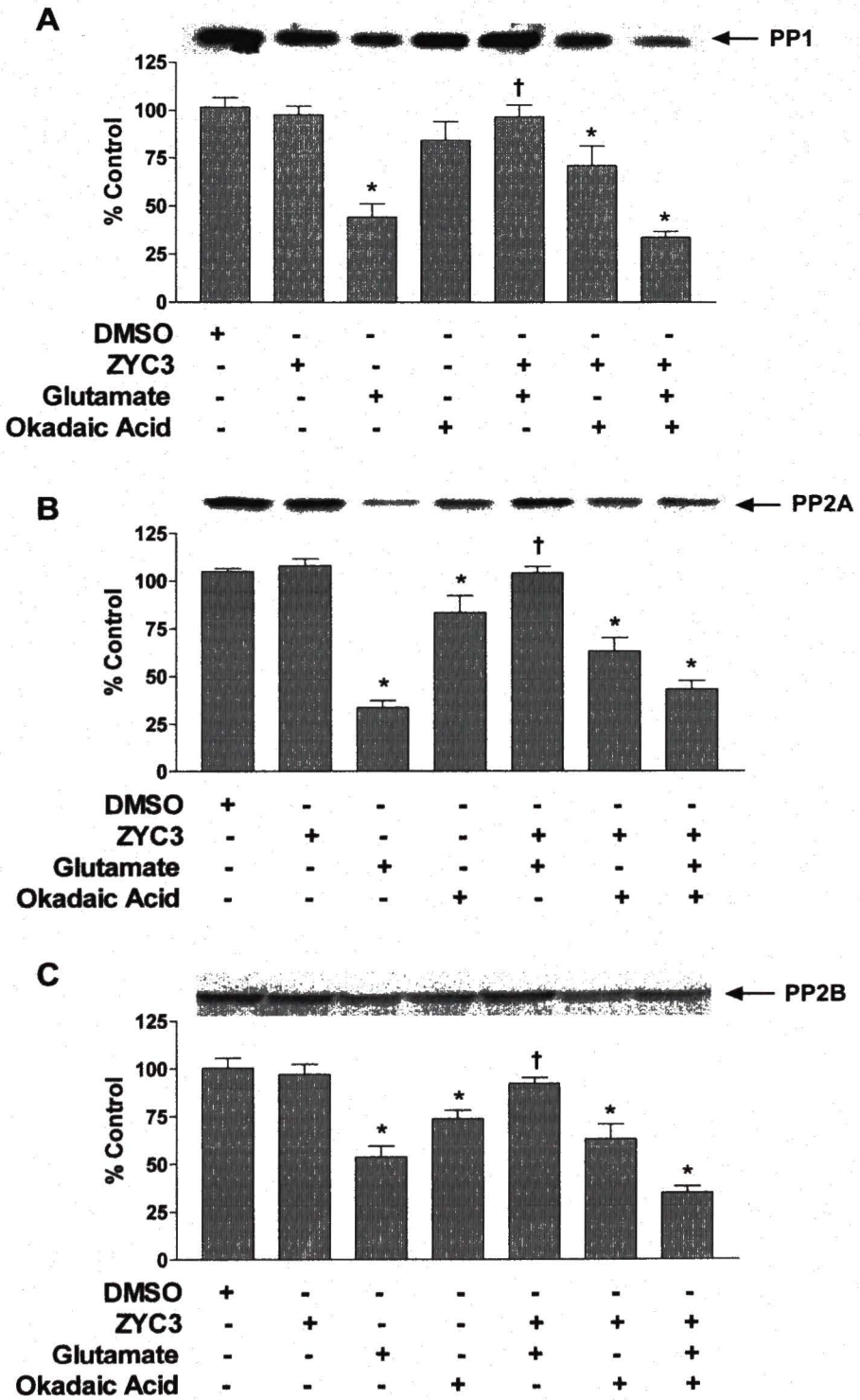

A
PP1
% Control
125
100
75
50
25
0
DMSO + - - - - - -
ZYC3 - + - - + + +
Glutamate - - + - + - +
Okadaic Acid - - - + - + +
B
PP2A
% Control
125
100
75
50
25
0
DMSO + - - - - - -
ZYC3 - + - - + + +
Glutamate - - + - + - +
Okadaic Acid - - - + - + +
C
PP2B
% Control
125
100
75
50
25
0
DMSO + - - - - - -
ZYC3 - + - - + + +
Glutamate - - + - + - +
Okadaic Acid - - - + - + +

Figure 7

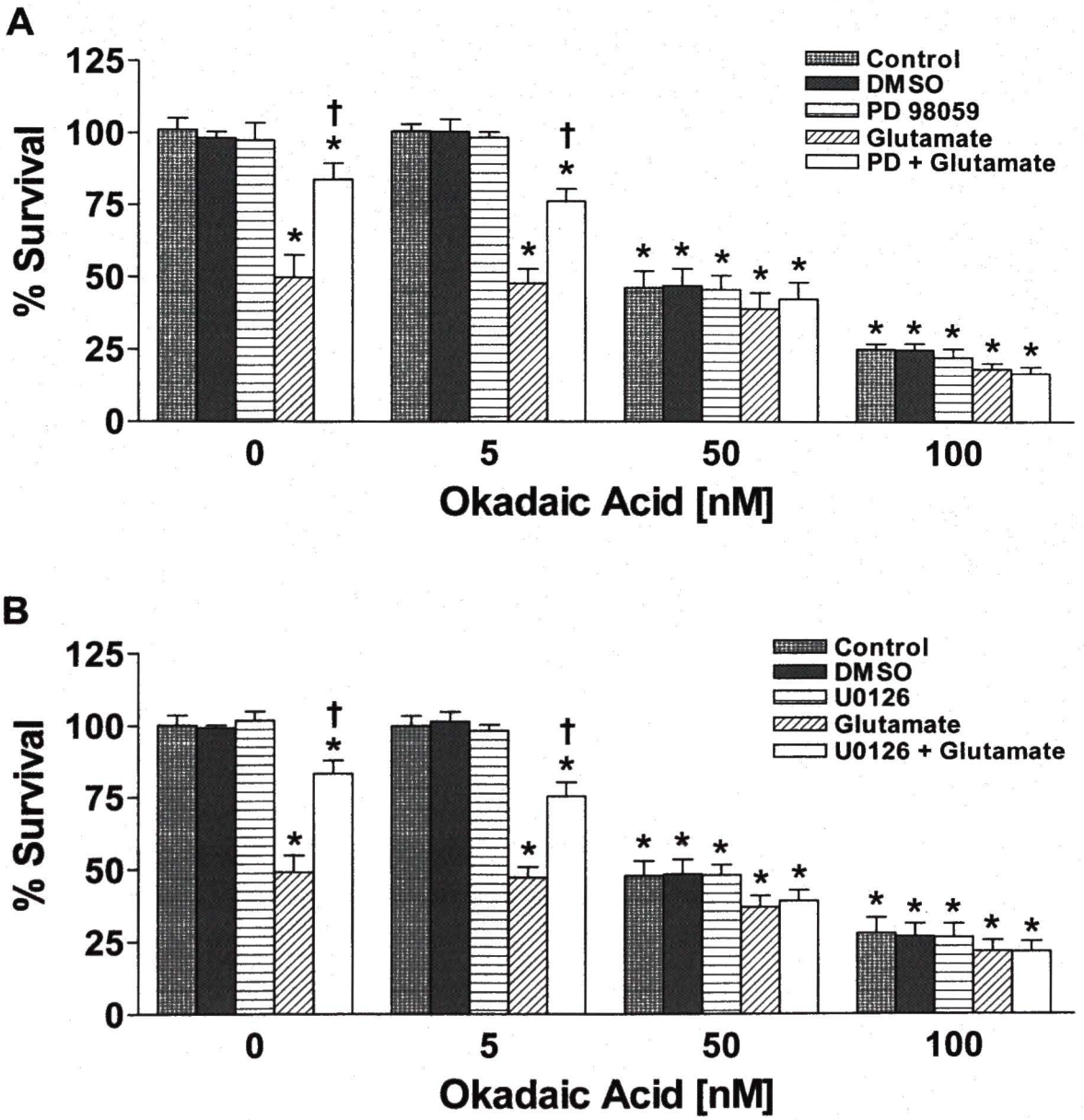
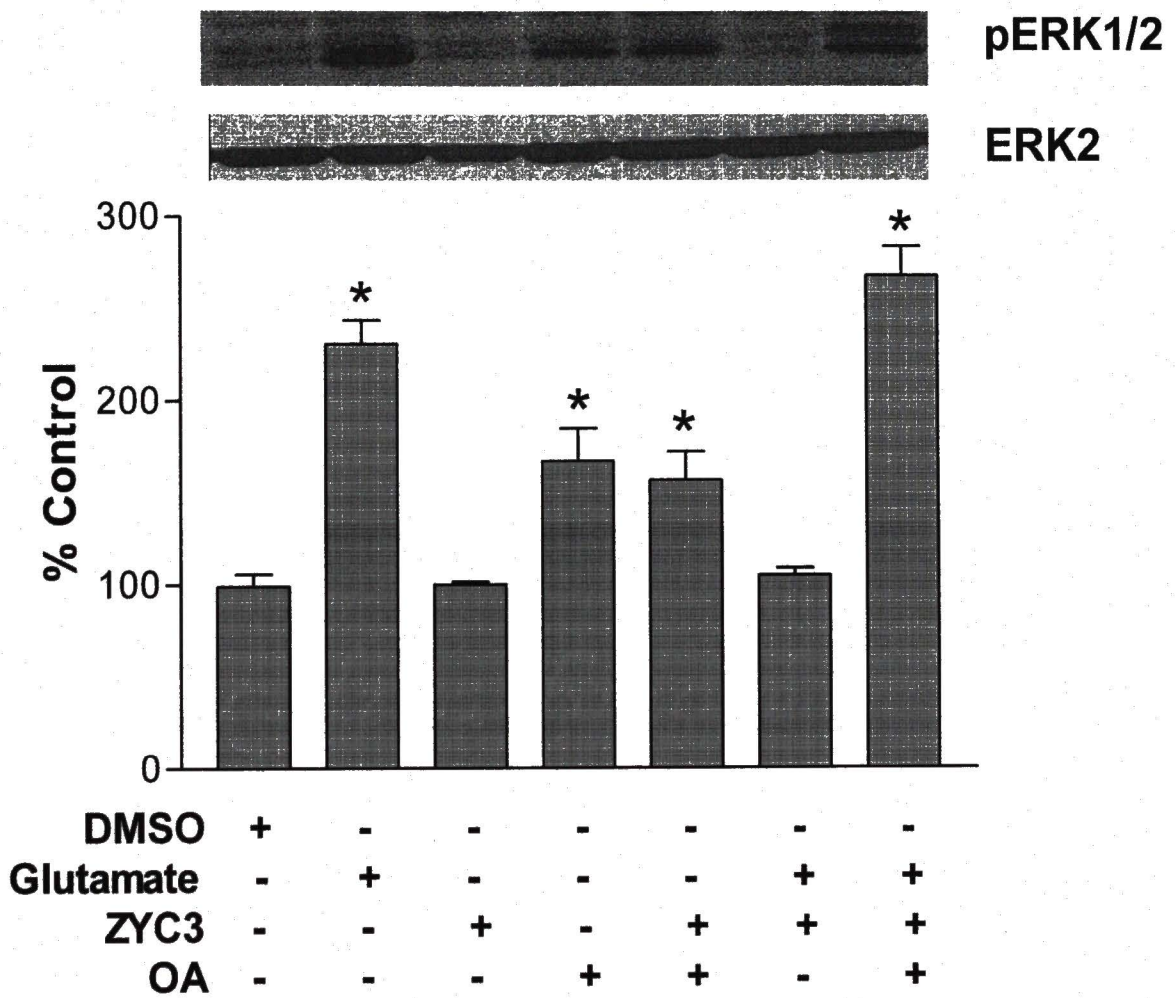

A
125
100
75
50
25
0
% Survival
Control
DMSO
PD 98059
Glutamate
PD + Glutamate
0
5
50
100
Okadaic Acid [nM]
B
125
100
75
50
25
0
% Survival
Control
DMSO
U0126
Glutamate
U0126 + Glutamate
0
5
50
100
Okadaic Acid [nM]

Figure 8



CHAPTER 4

PP1, PP2A, and Calcineurin Play a Role in Estrogen-mediated Neuroprotection

By

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Running Title: Serine/Threonine Protein Phosphatases and Estrogen

Key words: estrogen, PP1, PP2A, calcineurin, neurotoxicity, neuroprotection

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ABSTRACT

It is becoming increasingly clear that protein phosphatases are important modulators of cellular function and that disruption of these proteins are involved in neurodegenerative disease processes. Serine/threonine protein phosphatases such as PP1, PP2A, and PP2B (calcineurin) have been reported to be involved in hyperphosphorylation of tau as well as β-amyloid induced cell death. We have previously shown serine/threonine protein phosphatases to be involved in estrogen-mediated neuroprotection. The purpose of this study was to delineate the role of PP1, PP2A, and PP2B in the mechanism by which estrogens protect neurons against oxidative stress and excitotoxicity. Primary cortical neurons, HT-22, and C6-glioma cells were exposed to specific serine/threonine protein phosphatases inhibitors at various concentrations in the presence or absence of 17β-estradiol and/or glutamate. Treatment with protein phosphatase inhibitor II, endothall, or cyclosporin A which are specific inhibitors of PP1, PP2A, and PP2B, respectively, did not have an effect on cell viability. However, in combination, these inhibitors adversely affected cell survival in all three cell types, which suggests that protein phosphatases are important in maintenance of cellular function. Inhibitors of PP1, PP2A, and PP2B attenuated the protective effects of estrogen against glutamate induced-neurotoxicity, but did not completely abrogate the estrogen-mediated protection. The attenuation of estrogen-induced neuroprotection was achieved through decrease in the activity of theses serine/threonine phosphatases without the concomitant decrease in protein expression. Therefore, we conclude that in the face of cytotoxic challenge, estrogens maintain the function of PP1, PP2A, and PP2B.

Introduction

Neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), involve multifaceted processes that eventually lead to neuronal dysfunction and ultimately death. Much experimental data show that neurodegenerative diseases exhibit increased oxidative and excitotoxic stress (1-5), ubinquintin-proteosome system dysfunction (6-8), mitochondrial dysfunction (9, 10), synaptic failure, and abnormal phosphorylation/dephosphorylation (11-15) among other system failures. The question of cause and effect is yet unresolved, but these dysfunctional processes are unifying pathologies in many slowly progressive neurodegenerative diseases. It is clear, however, that phosphorylation and dephosphorylation processes are important not only in general maintenance of neuronal function, but also in diseased states.

Aberrant neuronal expression of phosphorylated ERK1/2 and other MAPKs in AD patients' brains is association with markers of oxidative stress (16). MAPK phosphorylation was also noted in a variety of sporadic and familial neurodegenerative diseases characterized by tau deposits (11, 13). Phospho-ERK1/2 is increased in substantia nigra neurons of patients with Parkinson's disease and other Lewy body diseases, and the midbrains of these patients show elevated ERK activity (13). In addition to chronic neurodegenerative diseases, increased ERK1/2 phosphorylation has been noted in the vulnerable penumbra following acute ischemic stroke in humans (17). Also, hyperphosphorylation of tau is one of the major hallmarks of AD. In addition, the activities of various serine/threonine phosphatases have been shown to be decreased in

AD brains compared to age-matched controls (18). Interestingly, tau and various MAPKs are substrates of serine/threonine phosphatases, such as PP1, PP2A, and calcineurin.

Experimental and epidemiological studies have demonstrated the beneficial effects of estrogens against neuronal dysfunction and memory loss (19-23). We and others have been shown that estrogens and estrogen analogues are potent neuroprotectants *in vitro* against a variety of toxicities, including serum deprivation, oxidative stress, β-amyloid-induced toxicity, and excitotoxicity (24-28). *In vivo* studies have also demonstrated the neuroprotective effects of estrogens in animal models of transient and permanent middle cerebral artery occlusion (26, 29, 30), global forebrain ischemia (31), photothrombotic focal ischemia (32), glutamate-induced focal cerebral ischemia (33), and subarachnoid hemorrhage (34).

The mechanisms of estrogen-induced neuroprotection are unclear. However, we have recently shown that inhibition of serine/threonine phosphatases completely abrogated the neuroprotective effects of 17β-estradiol (35). In the present study, we examined the specific protein phosphatases that are involved in the neuroprotective effects of estrogens.

Materials and Methods

Chemicals

17β-estradiol was purchased from Steraloids, Inc. (Wilton, NH) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and diluted to appropriate concentration in culture media. Calcein AM was purchased from Molecular Probes, Inc. (Eugene, OR). Okadaic acid, L-glutamate, and DMSO were purchased from Sigma (Paris, KY). Protein phosphatase inhibitor II, endothall, and cyclosporin A were purchased from Calbiochem (San Diego, CA). Anti-PP1, anti PP2A, and anti PP2B were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture

HT-22 and C-6 glioma cells were cultured in DMEM supplemented with 10% charcoal-stripped FBS (HyClone, Logan, UT) and gentamicin (50 μg/ml), at 37° C in an atmosphere containing 5% CO_2 and 95% air. HT-22 cells were obtained from David Schubert (Salk Institute, San Diego, CA). C6-gilioma cells were obtained from ATTC. HT-22 and C6-glioma cultures were maintained at 50% and 100% confluency, respectively, in monolayers in plastic 75 cm^2 flasks. For viability assays, HT-22 and C6-glioma cells were seeded in 96-well plates at a density of 3,500 cells/well, and for immunoblot analysis, the cells were seeded in 100 mm dishes at a density of 250,000 cells/ml.

Culture of primary cortical neurons

Cerebral cortex rat embryo (18-day) were dissected and harvested in preparation medium (DMEM, glucose 4.5g/L, Penicillin 100u/ml, Streptomycin 100 μg/ml). Individual cells were isolated by mechanical trituration using three different sizes of fire polished Pasteur pipettes. The cells were harvested in seeding medium (DMEM, glucose 4.5g/L, Penicillin 100 μg/ml, Streptomycin 100 μg/ml, Glutamine 2mM, 5% horse serum) and filtered through 40μm filter. Cerebral cortical cells were seeded in various poly-L-lysine treated dishes at a density of 500,000 cells /ml and 96-well plates at 25,000 cells/well. The cells were incubated in neurobasal medium (DMEM, glucose 4.5g/L, Penicillin 100u/ml, Streptomycin 100μg/ml, glutamine 2mM) supplemented with B-27 containing antioxidants in normal cell culture condition of 37°C in a humid atmosphere of 5% CO_2. Media was changed every third day, and experiments were performed following 14 days culture *in vitro*. Two hours before treatment with various inhibitors, glutamate, and/or 17β-estradiol, the media was replaced with neurobasal medium supplemented with B-27 minus antioxidants.

Calcein-AM viability assay

Primary cortical neurons, HT-22, and C6-glioma cells were exposed to various treatments, then were rinsed with phosphate buffered saline, and cell viability was measured using the membrane-permeant calcein-AM dye (Molecular Probes, Eugene, OR). Calcein-AM is a fluorogenic esterase substrate that is hydrolyzed to a fluorescent product in cells having esterase activity and intact membranes. Cells were incubated in a

solution of 2.5 □M calcein-AM in PBS. Twenty minutes later, fluorescence was determined using a Bio-Tek FL600 microplate reader (Winooski, VT) with an excitation/emission filter set of 485/530 nm. Cell culture wells treated with methanol served as blanks. The results, obtained in relative fluorescent units (RFU), are expressed as the percentage of untreated or vehicle-treated control values.

Immunoblot analysis

Protein from whole cell lysates (25 μg) was separated by SDS-polyacrylamide gel electrophoresis and transferred to Immunobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Membranes were rinsed in Tris-buffered saline (10 mM Tris-base, pH 8.0, 100 mM NaCl) containing 0.2% Tween 20 then blocked with 3% bovine serum albumin. Blots were then incubated with primary antibodies overnight at 4°C, rinsed and incubated in the appropriate secondary antibody before detection using enhanced chemiluminescence (ECL; Pierce Biotechnology, Inc., Rockford, IL). ECL results were digitized and quantified using UVP Bioimaging System. Protein concentration was determined by Bio-rad protein assay using BSA as the standard (Bio-Rad Laboratories, Hercules, CA).

Serine/threonine protein phosphatase activity assay

Calcineurin activity was quantitated using a non-radioactive assay system (Promega, Madison, WI), according to the supplier's instructions with a minor modification (36-38). Briefly, primary neurons were lysed on ice using 250 μl of storage

buffer containing 50 mM TRIS-HCl, pH 7.5, 1 mM EDTA, 0.1% β-mercaptoethanol, 0.1 mM leupeptin, and 75 μM pepstatin A. After centrifugation (100,000 *g* at 4°C for 1 h), the supernatant solution was applied to a Sephadex G-25 resin column, and centrifuged at 600 *g* at 4°C for 5 min. The sample lysate in storage buffer was obtained. The sample lysate (5 μg) was added to the reaction premix containing RRA(pT)VA in 5μl of phosphate free water, 10 μl PPTase-2B 5X buffer (250 mM imidazole, pH 7.2, 1 mM EGTA, 5 mM $NiCl_2$, 250 μg/ml calmodulin, 0.1% β-mercaptoethanol, 100 μg/ml bovine serum albumin, 200 μM sodium vanadate, and 500 nM okadaic acid) and 30 μl storage buffer in the well of a 96-well plate. After incubation for 30 min, 50 μl of molybdate dye/additive mixture was added to stop the reaction. The optical density of the samples was obtained using a microplate reader with a 630 nm filter. The calcineurin activity in each sample was calculated using a standard curve for free phosphate generated by a phosphate standard solution. After the calculation, phosphatase activity was divided by the protein content in each sample as measured by a protein assay system (Bio-Rad Laboratories, Hercules, Ca). All experiments were performed in triplicate.

PP2A activity was measured in the manner described above; however, instead if the PPTase-2B 5X buffer, 10μl PPTasc-2A 5X buffer (250mM imidazole, pH 7.2, 1mM EGTA, 0.1% β-mercaptoethanol, 500 μg/ml bovine serum albumin) was used.

Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. $p < 0.05$ was considered significant for all experiments. Each set of data represents three or more individual assays performed separately. For viability assays, three or more individual experiments, each containing 4-8 replicate wells, were performed. The values are reported as the mean ± SEM.

RESULTS

Effects of protein phosphatase inhibitor II, endothall, and/or cyclosporine A on cell viability in primary cortical neurons

Our previous studies showed that non-specific inhibition of serine/threonine protein phosphatases with okadaic acid or calyculin A was neurotoxic in primary cortical neurons, HT-22 cells, and C6-glioma cells (35). In order to determine the effects of individual protein phosphatase inhibition on cell viability, we examined the effects of protein phosphatase inhibitor II, endothall, and/or cyclosporin A on the viability of neurons and glia. Specific inhibition of PP1, PP2A, or calcineurin had no effect on cell viability in HT-22 cells (Fig 1A), C6-glia (Fig 1B), or primary neurons (Fig 1C). Interestingly, any combination of two inhibitors of PP1, PP2A or calcineurin caused a decrease in cell viability by approximately 25 - 35%, and inhibiting all three phosphatases caused 50% reduction in cell survival.

Effects of protein phosphatase inhibitor II, endothall, and/or cyclosporine A on estrogen-mediated neuroprotection against glutamate induced cytotoxicity in primary cortical neurons

Non-toxic concentrations of okadaic acid have been shown to abrogate the estrogen-mediated neuroprotection against oxidative stress and excitotoxicity (35). Therefore, we wanted to assess whether individually inhibiting PP1, PP2A, or calcineurin would have a similar effect. Although individual inhibition of PP1, PP2A, nor calcineurin had any effect on cell viability, inhibition of PP1, PP2A, or calcineurin attenuated but did not completely block the estrogen-mediated neuroprotection against glutamate induced neurotoxicity in primary cortical neurons (Fig 2), which suggests that inhibition of just one of these serine/threonine phosphatases are not sufficient to block the estrogen effect.

PP1, PP2A, and calcineurin protein expression in estrogen-mediated neuroprotection against glutamate in the presence of specific inhibitor of PP1, PP2A, or calcineurin in primary cortical neurons

Next, we examined the protein expressions of PP1, PP2A, and calcineurin in response to treatment with glutamate and/or 17β-estradiol in the presence of the specific inhibitors in primary cortical neurons. Twenty-four hour treatment with 17β-estradiol or PPI2 alone did not significantly alter the expression of PP1, PP2A, or calcineurin as compared to non-treated controls (Fig 3A, 3B, & 3C, respectively). Glutamate treatment caused approximately 50% reduction in PP1 protein levels. The presence of 17β-estradiol

prevented this glutamate-mediated decrease in PP1, PP2A, and calcineurin protein expressions; however, inhibition of PP1 with PPI2 did not block the decreases in PP1, PP2A, and calcineurin expressions caused by glutamate toxicity. In addition, the presence of PPI2 did not alter the 17β-estradiol attenuation of the glutamate-induced decreases in PP1, PP2A, and calcineurin protein expressions. PP2A inhibition with endothall (Fig 3D-F) or calcineurin inhibition with cyclosporine A (Fig 3G-I) also had no effect on the 17β-estradiol-mediated attenuation of decreased protein contents of PP1, PP2A or calcineurin caused by glutamate toxicity.

PP2A and calcineurin activity in estrogen-mediated neuroprotection against glutamate induced cell death in primary cortical neurons

We show that estrogens protect neurons and other types of cells via maintenance of serine/threonine phosphatase expression at control levels during oxidative and excitotoxic stress; however, protein expression does not always translates equally to activity. Therefore, the activities of PP2A and calcineurin were measured in primary cortical neurons to determine if estrogen-mediated neuroprotection required the activity of serine/threonine phosphatases. The effects of glutamate, 17β-estradiol, and various specific inhibitors of protein phosphatases on the activity of PP2A and calcineurin in primary cortical neurons were measured by simultaneously treating with glutamate, okadaic acid, 17β-estradiol, okadaic acid, PPI2, endothall, and/or cyclosporin A. Figure 4 and 5 shows the PP2A and calcineurin activities in cortical neurons, respectively. Glutamate, okadaic acid, and endothall treatment caused decreases in PP2A activity,

while PPI2 and cyclosporine A had no effect (Fig 4). The decreased PP2A activity mediated by glutamate was attenuated in the presence 17β-estradiol; however, 17β-estradiol was unable block the okadaic acid or endothall-mediated decrease in PP2A activity (Fig 4A & 4C). In fact, the presence of okadaic acid or endothall prevented the 17β-estradiol-mediated attenuation of the decreased PP2A activity caused by glutamate. Inhibition of PP1 with PPI2 or calcineurin with cyclosporin A had no effect on PP2A activity either in the presence or absence of glutamate and/or 17β-estradiol (Fig 4B & 4D). Calcineurin activity was also measured using the same treatment paradigm (Fig 5). Glutamate or okadaic acid treatment of neurons only reduced calcineurin activity by ~ 30-40% contrasted to the ~ 75-80% reduction seen in PP2A activity. Cyclosporine A inhibited calcineurin activity to ~ 50% of non-treated control (Fig 5D), while neither PP1 nor PP2A inhibitors had no effect on calcineurin activity (Fig 5B and 5C, respectively). Estrogen attenuated the glutamate-mediated decrease in calcineurin activity, but neither the okadaic acid- nor cyclosporine A-induced reduction in calcineurin activity was blocked by 17β-estradiol. Although glutamate treatment induced decreases in PP2A and calcineurin activities, the extent to which this reduction occurs was much greater for PP2A than calcineurin, suggesting that PP2A activity is more prominent than calcineurin activity in neurons in response to oxidative and excitotoxic stresses.

Discussion

These observations are important in view of the WHI-MS results that indicate chronic post-menopausal treatment of older women with estrogens (39-42) not only does

not protect the brain from dementia, but appears to exacerbate the condition. The WHI-MS results are inconsistent with numerous experimental, epidemiological, and clinical data that show the beneficial effect of estrogen replacement therapy on cognition and delayed onset of neurodegenerative diseases such as AD (23, 43-47). As the elderly population grows in magnitude, it is necessary to devise a treatment paradigm to cure, prevent, or delay the progression of neurodegenerative diseases, such as AD and PD. Yet, despite major efforts, the mechanisms responsible for neurodegeneration and estrogen-mediated neuroprotection remain unclear. The present study shows that maintenance of serine/threonine protein phosphatase activity is an important aspect of the estrogen-mediated neuroprotection against oxidative stress and excitotxicity.

We have previously reported that estrogens and non-feminizing analogues of estrogens are protective against a variety of cytotoxic insults (24-27) as well as ischemic conditions caused by middle cerebral artery occlusion in the rat (48-50). The protective effects of estrogens against glutamate toxicity were partially antagonized by specific protein phosphatase inhibitors, PPI2, endothall, and cyclosporine A. In addition, we show that estrogens persistently antagonize the reduction in PP1, PP2A and PP2B protein expression induced by oxidative and excitotoxic stresses, an effect that is not modified by specific inhibitors. The activities of PP2A and calcineurin are also maintained in cells treated with 17β-estradiol during oxidative and excitotoxic stress induced by glutamate. Collectively, these data support the hypothesis that serine/threonine phosphatase regulation is a major component of estrogen-mediated neuroprotection.

This is an important observation since AD affected brains have significantly decreased activities of PP1, PP2A, and calcineurin (18). With this down-regulation of phosphatase activities and up-regulation of protein kinase activities, it is likely that hyperphosphorylation of tau and activation of other pathological events involved in neurodegenerative diseases are triggered. In addition, these phosphatases are directly involved in synaptic plasticity and memory formation, which is affected in neurodegenerative diseases. The observation that estrogen-mediated neuroprotection involves maintaining the activities of these important phosphatases can lead to a more clinically relevant postmenopausal hormone replacement regime that would minimize the adverse effects of estrogens while maximizing the beneficial effects.

The inability of individual protein phosphatase inhibitors to prevent cell death following toxic treatment with glutamate in primary neurons cannot be explained by incomplete inhibition of the target protein phosphatase. The inhibitor concentrations used in this experiment were 10 to 100-fold higher than the reported IC_{50}s of each inhibitor. PPI2 has been shown to have an IC_{50} of 2 nM (51, 52). The IC_{50} of endothall has been reported to be approximately 90 nM (53-55). Various reports have shown that cyclosporin A inhibits 50 % of calcineurin in concentrations ranging from 2-8 nM (56, 57). Therefore, it is unlikely that there is incomplete inhibition of the various serine/threonine phosphatases used in this study.

The mechanism of estrogen-mediated attenuation of the decline in PP levels and activity during oxidative and excitotoxic stress is not clear. There are no previous experimental data suggesting a direct interaction between estrogens and PP. While PP2A has been shown to regulate ERα by mRNA stabilization (58) as well as direct interaction with ERα in the absence of estrogen (59), our data indicate that effects of estrogens on PPs occur without ER interactions since non-ER active estrogen analogues exert similar effects (60). The absence of effects of estrogens alone and the broad range of protein phosphatases affected by estrogens suggests that estrogens reduce the clearance of PPs that is activated by oxidative/excitotoxic insult, rather than causing expression of new protein. Our laboratory recently reported that estrogens attenuate ischemia induced increases in activities of MMP2 and MMP9, which belong to a class of proteases (61). We have also shown that transient cerebral ischemia causes neurofibrillary tangle like tauopathy involving cdk5 (62). This study showed that calpain II, a cytoplasmic cysteine protease, caused the cleavage of p35 to p25, which are co-activators of cdk5, and that the inhibition of calpain by MDL 28170 attenuated this cleavage, which prevented the activation of cdk5. It has also been shown that estrogen induced expression of secretory leukocyte protease inhibitor in the rat uterus (63). Because estrogens have been shown to modify the expression or functions of various proteases, it is likely that 17β-estradiol protect cells by blocking the ubinquitination and/or degradation of protein phosphatases caused by oxidative or excitotoxic stresses.

The present study provides a potential ER-independent mechanism for the neuroprotective effects of estrogens, through the prevention of insult-induced decrease in phosphatase activity and the resulting neurotoxic, persistent hyperphosphorylation of proteins in multiple signaling pathways that are detrimental to cell survival. Inhibition of individual phosphatases does not abolish estrogen-mediated neuroprotection as seen with general inhibition with okadaic acid or calyculin A (35). In light of the data that shows AD brains have decreased activities of protein phosphatases and our results that demonstrate estrogens maintain phosphatase activities during oxidative stress, it seems likely that clinical studies that show improved cognition and delayed progression of AD with estrogen treatment maybe due to through this action of estrogens.

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Figure Legends

Figure 4-1: Effects of PPI2, endothall, and cyclosporine A on cell survival.

HT-22 cells and C6-glioma were seeded into 96-well plates at a density of 3,500 cells/well. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. **A**) HT-22 cells were treated with varying concentrations of PPI2, endothall, and/or cyclosporine A. **B**) C-6 cells were treated with varying concentrations of PPI2, endothall, and/or cyclosporine A. **C**) Primary cortical neurons were treated with varying concentrations of PPI2, endothall, and/or cyclosporine A. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. two combination treatment.

Figure 4-2: Effects of PPI2, endothall, or cyclosporine A on 17β-estradiol mediated neuroprotection in primary cortical neurons.

Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. **A**) Cells were treated simultaneously with with 200 nM PPI2, 50 μM glutamate, and/or 100 nM 17β-estradiol. **B**) Cells were treated simultaneously with 9 μM endothall, 50 μM glutamate, and/or 100 nM 17β-estradiol. **C**) Cells were treated simultaneously with 500 nM CsA, 50 μM glutamate, and/or 100 nM 17β-estradiol. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are

mean ± SEM for ten independent experiments with two replicates per experiment. * P <0.05 vs. vehicle control; † P < 0.05 vs. glutamate treated group.

Figure 4-3: PP1, PP2A, and calcineurin protein expression in response to glutamate and/or 17β-estradiol treatment in the presence of specific inhibitors of PP1, PP2A, or calcineurin in primary cortical neurons.

Primary cortical neurons were seeded in 100 mm dishes at a density of 500,000 cells/ml. Cells were treated simultaneously with 200 nM PPI2, 50 μM glutamate, and/or 100 nM 17β-estradiol. Cells were harvested after 24 hr of treatment for western blot analysis of PP1 (**A, D, and G**), PP2A (**B, E, and H**), and calcineurin (**C, F, and I**). Depicted are mean ± SEM for n=6 and normalized to β-actin (not shown). * P <0.05 vs. control; † P < 0.05 vs. glutamate treated group.

Figure 4-4: PP2A activity in primary cortical neurons following treatment with glutamate and/or 17β-estradiol in the presence of specific inhibitors of PP1, PP2A, or calcineurin.

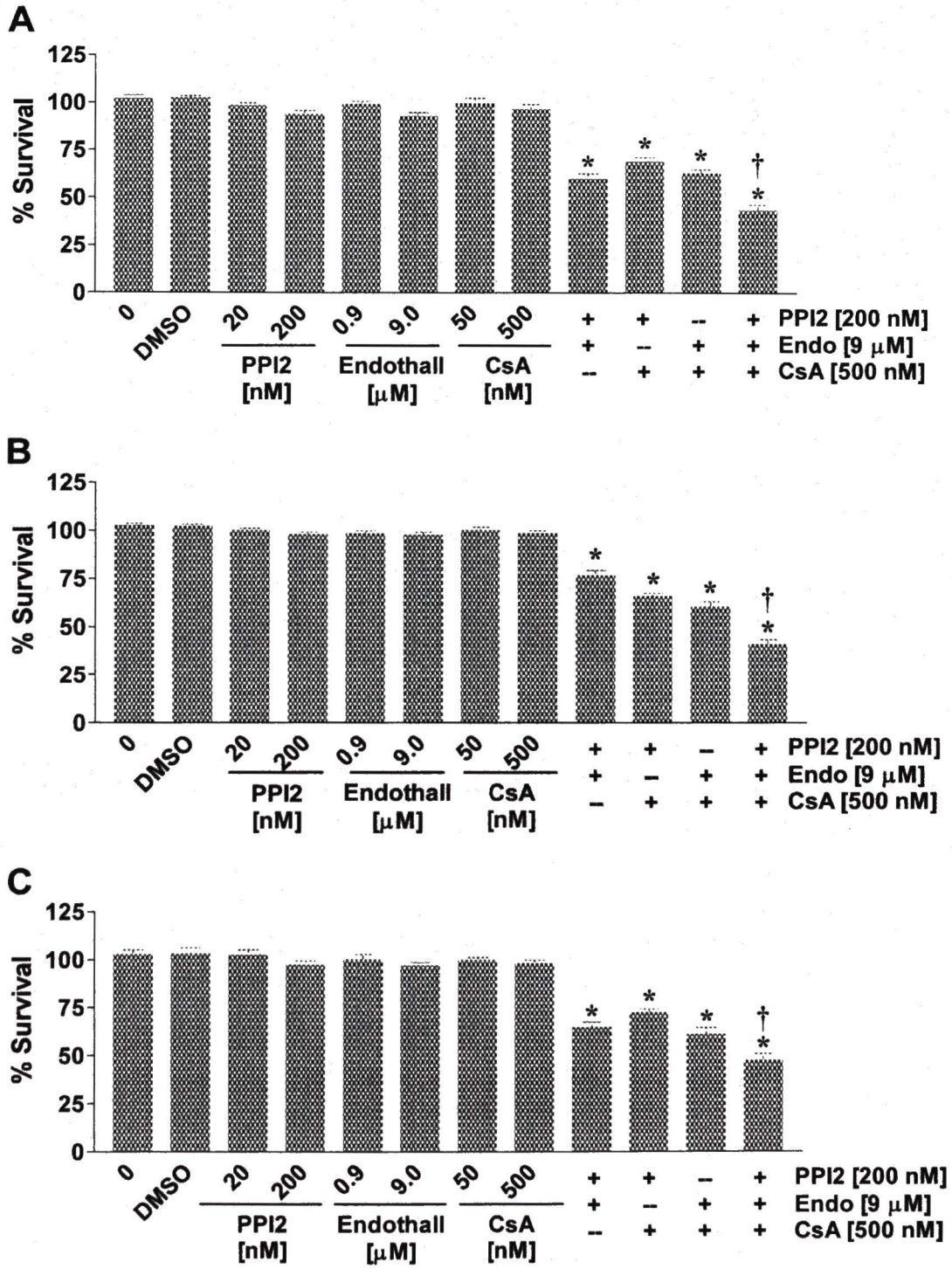
Primary cortical neurons were seeded in 100 mm dishes at a density of 500,000 cells/ml. **A**) Cells were treated simultaneously with 50 nM okadaic acid, 50 μM glutamate, and/or varying 100 nM 17β-estradiol. **B**) Cells were treated simultaneously with 200 nM PPI2, 50 μM glutamate, and/or 100 nM 17β-estradiol. **C**) Cells were treated simultaneously with 9 μM endothall, 50 μM glutamate, and/or 100 nM 17β-estradiol. **D**) Cells were treated simultaneously with 500 nM CsA, 50 μM glutamate, and/or 100 nM 17β-

estradiol. PP2A activity was determined using a serine/threonine phosphatase activity assay (Promega, Madison, WI) after 24 hr exposure to the various compounds. All data were normalized to % survival of vehicle treated control. Depicted are mean ± SEM for six independent experiments with triplicates per experiment. * P <0.05 vs. glutamate treated group.

Figure 4-5: Calcineurin activity in primary cortical neurons following treatment with glutamate and/or 17β-estradiol in the presence of specific inhibitors of PP1, PP2A, or calcineurin.

Primary cortical neurons were seeded in 100 mm dishes at a density of 500,000 cells/ml. **A**) Cells were treated simultaneously with 50 nM okadaic acid, 50 μM glutamate, and/or varying 100 nM 17β-estradiol. **B**) Cells were treated simultaneously with 200 nM PPI2, 50 μM glutamate, and/or 100 nM 17β-estradiol. **C**) Cells were treated simultaneously with 9 μM endothall, 50 μM glutamate, and/or 100 nM 17β-estradiol. **D**) Cells were treated simultaneously with 500 nM CsA, 50 μM glutamate, and/or 100 nM 17β-estradiol. Calcineurin activity was determined using a serine/threonine phosphatase activity assay (Promega, Madison, WI) after 24 hr exposure to the various compounds. All data were normalized to % survival of vehicle treated control. Depicted are mean ± SEM for six independent experiments with triplicates per experiment. * P <0.05 vs. glutamate treated group.

Figure 4-1


A
% Survival
125
100
75
50
25
0
0
DMSO
20
200
PPI2
[nM]
0.9
9.0
Endothall
[μM]
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500
CsA
[nM]
+ + – + PPI2 [200 nM]
+ – + + Endo [9 μM]
– + + + CsA [500 nM]
B
% Survival
125
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75
50
25
0
0
DMSO
20
200
PPI2
[nM]
0.9
9.0
Endothall
[μM]
50
500
CsA
[nM]
+ + – + PPI2 [200 nM]
+ – + + Endo [9 μM]
– + + + CsA [500 nM]
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% Survival
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DMSO
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PPI2
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Endothall
[μM]
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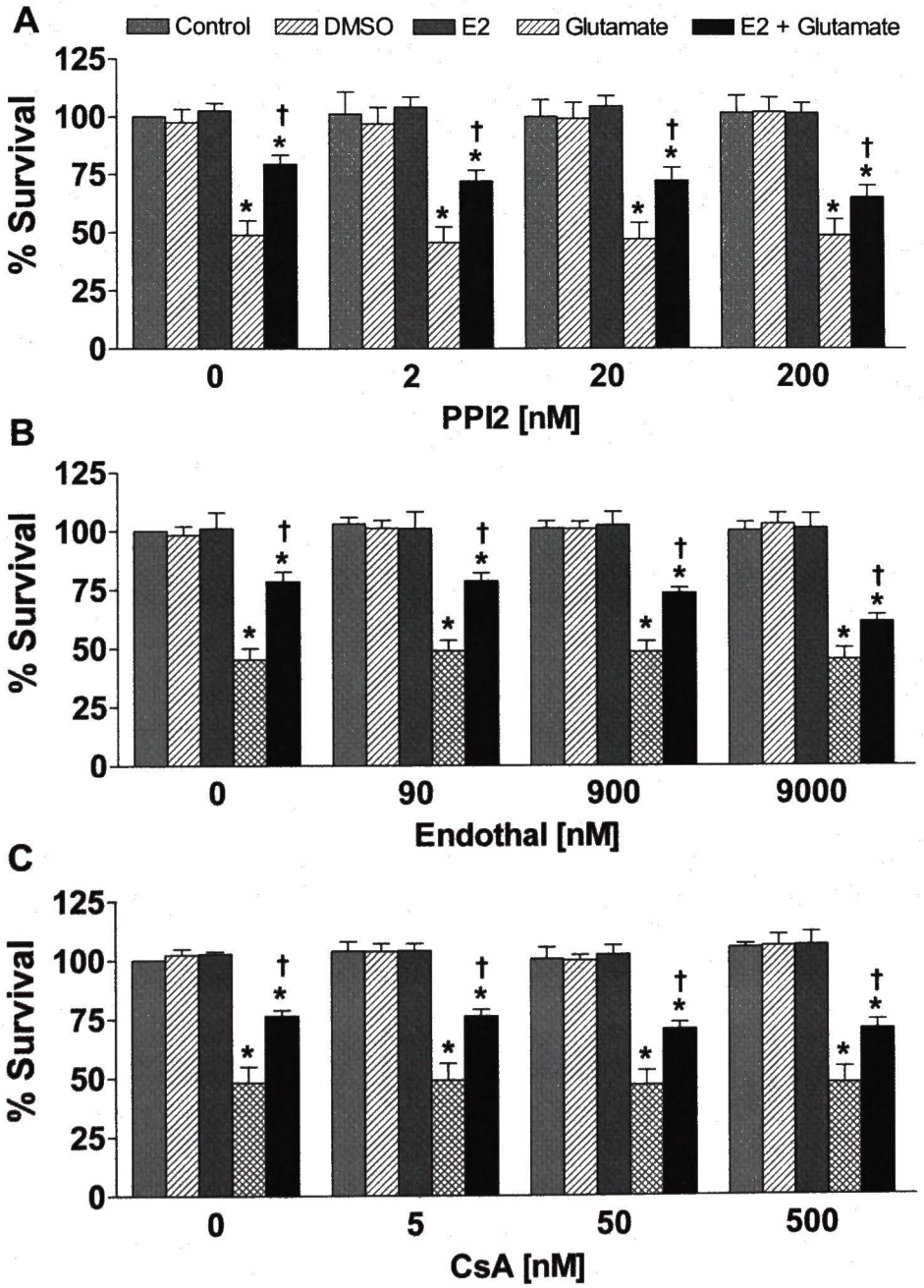
+ + – + PPI2 [200 nM]
+ – + + Endo [9 μM]
– + + + CsA [500 nM]

Figure 4-2


A
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Glutamate
E2 + Glutamate
% Survival
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PPI2 [nM]
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Figure 4-3

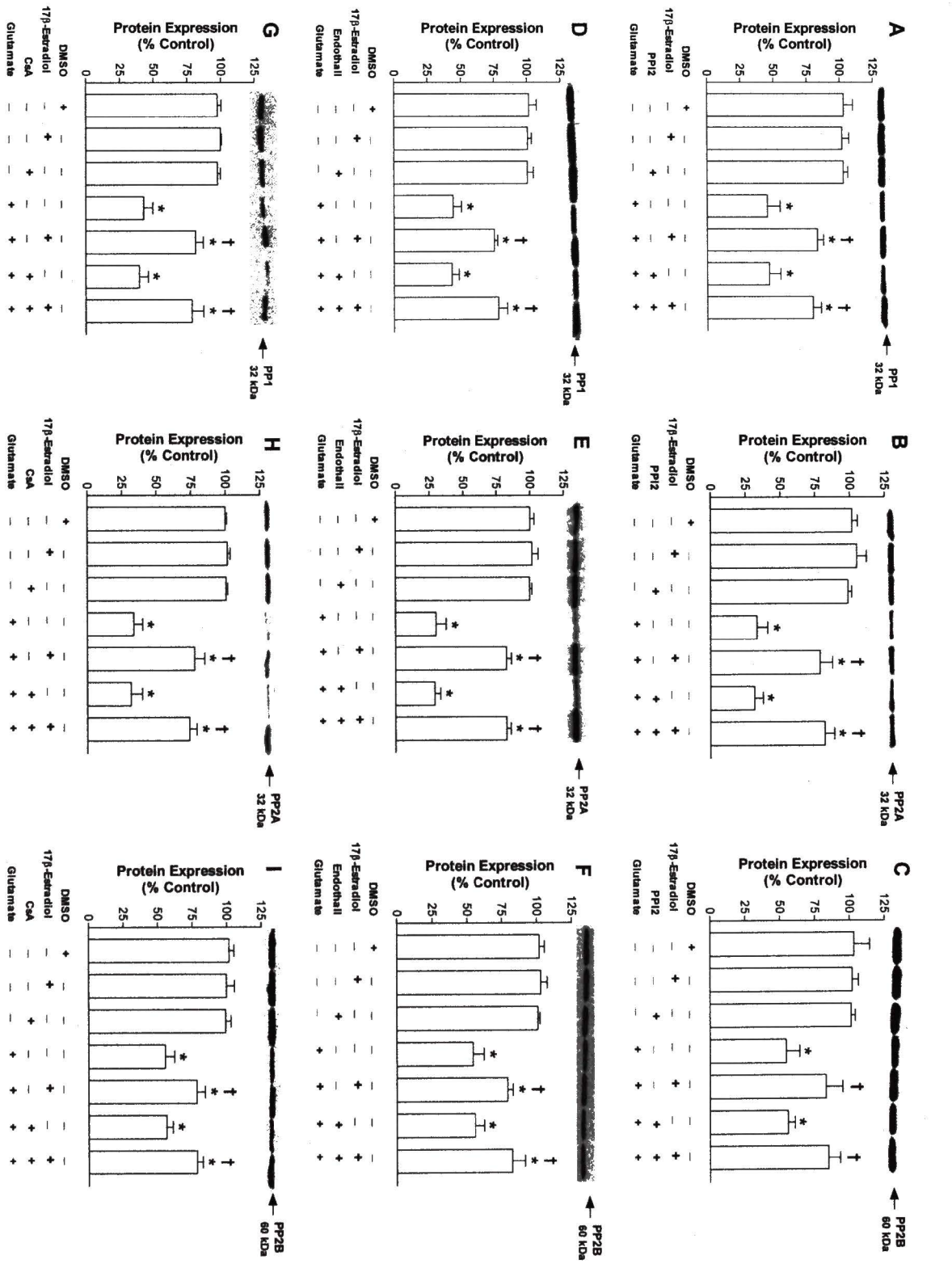
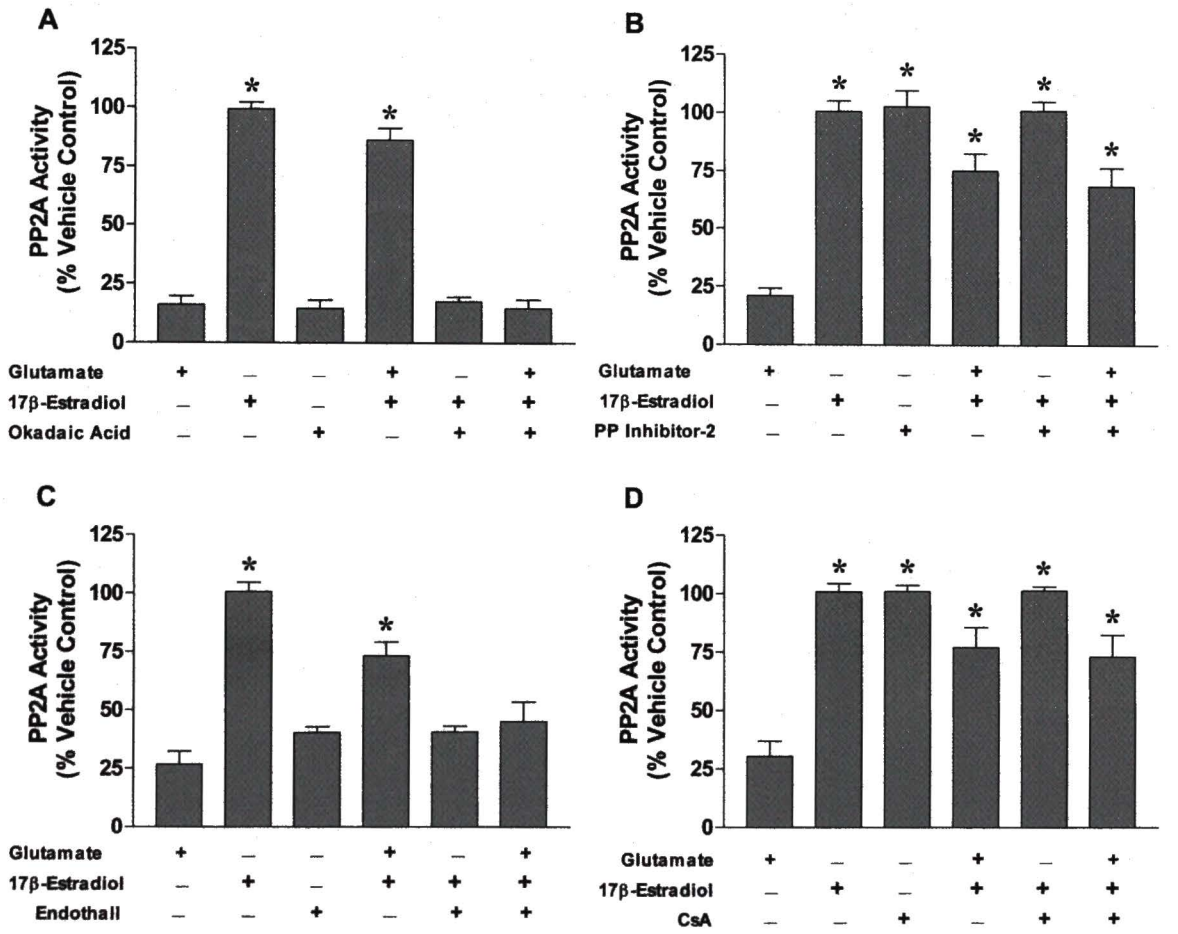

A
B
C
D
E
F
G
H
I
Protein Expression (% Control)
0
25
50
75
100
125
DMSO
17β-Estradiol
PPI2
Endothall
CsA
Glutamate
PP1 32 kDa
PP2A 32 kDa
PP2B 60 kDa

Figure 4-4


A
PP2A Activity (% Vehicle Control)
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17β-Estradiol – + – + + +
Okadaic Acid – – + – + +
B
PP2A Activity (% Vehicle Control)
Glutamate + – – + – +
17β-Estradiol – + – + + +
PP Inhibitor-2 – – + – + +
C
PP2A Activity (% Vehicle Control)
Glutamate + – – + – +
17β-Estradiol – + – + + +
Endothall – – + – + +
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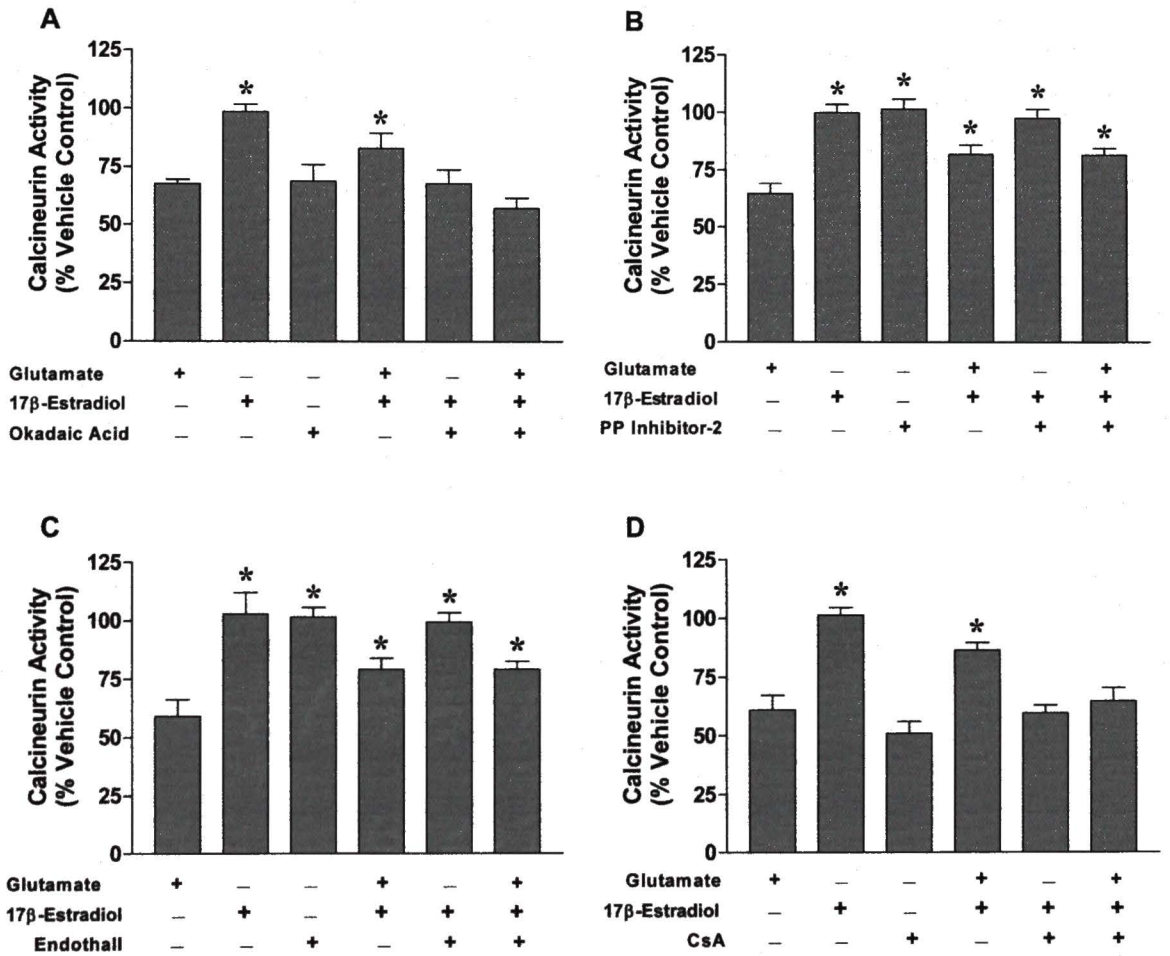
Glutamate + – – + – +
17β-Estradiol – + – + + +
CsA – – + – + +

Figure 4-5


A
Calcineurin Activity (% Vehicle Control)
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Glutamate + – – + – +
17β-Estradiol – + – + + +
Okadaic Acid – – + – + +
B
Calcineurin Activity (% Vehicle Control)
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17β-Estradiol – + – + + +
PP Inhibitor-2 – – + – + +
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Calcineurin Activity (% Vehicle Control)
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Glutamate + – – + – +
17β-Estradiol – + – + + +
Endothall – – + – + +
D
Calcineurin Activity (% Vehicle Control)
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50
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0
Glutamate + – – + – +
17β-Estradiol – + – + + +
CsA – – + – + +

CHAPTER 5

Mechanism of Okadaic Acid Induced Neuronal Death and the Effect of Estrogens

by

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ABSTRACT

Accumulating evidence has shown that serine/theronine protein phosphatases (PPs) are important mediators of general cellular function as well as neurodegenerative processes. We have previously shown inhibition of protein phosphatases (PPs) to be as neurotoxic as glutamate-induced neuronal death, but resistant to the neuroprotective effects of estrogens. We wanted to determine the mechanism by which phosphatase inhibition is neurotoxic as well as the signaling pathways involved. Primary cortical neurons were exposed to okadaic acid (OA) in the presence or absence of various protein kinases inhibitors, 17β-estradiol or 2-(1-adamantyl)-3-hydroxy-estra-1, 3, 5(10)-triene-17-one (ZYC3), and/or glutamate. In OA induced cell death, an increase in reactive oxygen species (ROS), protein cabonylation, lipid peroxidation, caspase-3 activity, and mitochondrial dysfunction were evident, which are similar to the events that occur in glutamate- induced toxicity. Estrogens attenuate these increases in glutamate-mediated cell death, but are ineffective in okadaic acid induced neuronal cell death. In addition, inhibition of PKA and PKB was not protective against neither glutamate nor OA toxicity; however, inhibition of PKC and MAPK pathway was neuroprotective against glutamate toxicity but not OA toxicity. Interestingly, inhibition of MAPK pathway with PD98096 or U0126 caused a decrease in ROS production suggesting that activation of ERK1/2 could further exacerbate the oxidative stress caused by glutamate-induced toxicity; however, these inhibitors had no effect on OA-induced toxicity.

I. INTRODUCTION

Oxidative stress has been shown to cause protein oxidation, lipid peroxidation, DNA, oxidation, and accumulation of reactive oxygen species (ROS), which are increased in a diverse group of neuropathological conditions such as Alzheimer's disease, stroke, and Parkinson's disease (Butterfield, 2006), suggesting a role for oxidative stress in the pathogenesis of these neurodegenerative diseases (Coyle and Puttfarcken, 1993; Mattson et al., 2001; Sultana et al., 2006). It has been demonstrated by various laboratories that dysfunction of protein phosphatases are evident during oxidative stress. For example, calcineurin activity was recently shown to be reduced in lymphocytes of amyotrophic lateral sclerosis patients due to an effect thought to be mediated by oxidation (Ferri et al., 2004). Indeed, it has been known for some time that PP2A activity is reduced in the cortices of Alzheimer's patients as compared with control patients (Gong et al., 1995), and ERK has been found to be activated in these tissues (Pei et al., 2002). Tau is a well-described ERK1/2 target (Pei et al., 2003), and the hyperphosphorylation of tau protein and the development of neurofibrillary tangles in Alzheimer's disease pathology could reflect aberrant ERK1/2 activity (Pei et al., 2003). Interestingly, mice expressing a dominant negative form of PP2A in neurons displaying features of Alzheimer's pathology (Kins et al., 2003). Inactivation of PP1 via oxidative stress has been shown *in vitro* and *in vivo* to be involved in hyperphosphorylation of tau, prolonged phosphorylation of ERK 1/2 (Rahman et al., 2005; Poppek et al., 2006). Thus, it is intriguing to postulate that oxidative stress mediated PP1 and PP2A inhibition in Alzheimer's disease may account

for enhanced ERK1/2 activity and subsequent tau hyperphosphorylation and neurofibrillary tangle formation.

Okadaic acid, a potent inhibitor of serine/threonine phosphatases, has been shown to be cytotoxic in a variety of cell lines. Okadaic acid increases phosphorylation of microtubule –associated protein and tau, which are concomitant with early changes in neuronal cytoskeleton that ultimately leads to cell death in primary cortical neurons and in neuroblastoma cell lines (Arias et al., 1993). In cerebellar granule cells, okadaic acid induces disintegration of neuritis and swelling of cell bodies (Fernandez-Sanchez et al., 1996). Okadaic acid has also been shown to produce condensation of chromatin, reorganization of cytoskeleton, DNA fragmentation characteristic of apoptosis (Boe et al., 1991; Fernandez-Sanchez et al., 1996). We have previously shown okadaic acid induces neuronal death, and estrogens, which are known potent neuroprotectants, could not rescue these neurons (Yi et al., 2005). In this study, we compared the mechanisms by which okadaic acid induces neuronal death versus glutamate-induced neuronal cell death and the effects of estrogens against neurotoxicity.

II. METHODS

Chemicals

17β-estradiol and 17α-estradiol was purchased from Steraloids, Inc. (Wilton, NH). ZYC3 and the enantiomer of 17β-estradiol (ENT E2) were prepared as described previously (Green et al., 2001; Liu et al., 2002). All steroids were dissolved in dimethyl

sulfoxide (DMSO) at a concentration of 10 mM and diluted to appropriate concentration in culture media. Calcein AM and 2,7-dichlorofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes, Inc. (Eugene, OR). Okadaic acid, L-glutamate, trichloroacetic acid, 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, HCl and DMSO were purchased from Sigma-Aldrich (St Louise, MO). Anti-PP1, anti PP2A, and anti PP2B were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture

HT-22 and C-6 glioma cells were cultured in DMEM supplemented with 10% charcoal-stripped FBS (HyClone, Logan, UT) and gentamicin (50 μg/ml), at 37° C in an atmosphere containing 5% CO_2 and 95% air. HT-22 cells were obtained from David Schubert (Salk Institute, San Diego, CA). C6-gilioma cells were obtained from ATTC. HT-22 and C6-glioma cultures were maintained at 50% and 100% confluency, respectively, in monolayers in plastic 75 cm^2 flasks.

Culture of primary cortical neurons

Cerebral cortex rat embryo (18-day) were dissected and harvested in preparation medium (DMEM, glucose 4.5g/L, Penicillin 100 U/ml, Streptomycin 100μg/ml). The cortical tissue was treated with trypsin. The tissue was washed three times using washing medium (Hank's medium, glucose 4.5g/L, Penicillin 100 U/ml, Streptomycin 100μg/ml) and individual cells will be isolated by mechanical trituration using three different sizes of fire polished Pasteur pipettes. The cells were harvested in seeding medium (DMEM,

glucose 4.5g/L, Penicillin 100 U/ml, Streptomycin 100μg/ml, Glutamine 2mM, 19% horse serum) and filtered through 40μm filter. The cerebral cortical cells were seeded in various poly-L-lysine treated dishes and plates at various cell densities. The cells were incubated in neurobasal medium (DMEM, glucose 4.5g/L, Penicillin 100 U/ml, Streptomycin 100μg/ml, glutamine 2mM) supplemented with B-27 with antioxidants in normal cell culture condition of 37°C in a humid atmosphere of 5% CO_2. Two hours before treatment with various inhibitors and/or 17β-estradiol or ZYC3, the media was replaced with neurobasal medium supplemented with B-27 without antioxidants.

MTT Assay

Mitochondrial function was indirectly determined by MTT (Sigma Chemical Co., St. Louis, MO) reduction by mitochondrial dehydrogenases. MTT was added to each well 24 h after the beginning of the insult at a final concentration of 250 μM. After a 2-hr incubation in at 37°C, the media was removed, and cells dissolved in DMSO. The assay of the formation of formazan was performed by measuring the amount of reaction product by absorbance change (570 nM) using a microplate reader (BioTek, Highland Park, VT).

Measurement of Cytosolic ROS.

The extent of cytosolic cellular oxidative stress was estimated by monitoring the amount of ROS by the fluorescent dye DCFH-DA (Molecular Probes, Eugene, OR).

Primary cortical neurons were plated in 96-well plates at a density of 25,000 cells/well in 96-well plates. Following seven days in vitro, the neurons were loaded with DCFH-DA at a final concentration of 50 μM for 45 min at 37°C. After incubation, DCFH-DA was removed, and cells were washed twice with PBS, pH 7.4. Following treatment with various compounds for 24 hr, DCF2,7-dichlorofluorescin fluorescence was determined at an excitation of 485 nm and an emission of 538 nm using an FL600 microplate-reader (BioTek, Highland Park, VT). Values were normalized to percentage of untreated control groups.

Lipid Peroxidation Measurement.

Lipid peroxidation was monitored by measuring malondiadehyde (MDA), a stable end product of lipid peroxidation cascades using the thiobarbituric acid reactive substances (TBARS) assay. As one of the main compounds among TBARS, MDA reacts with TBA under acidic and high heat conditions and the product of this reaction can be detected spectrometrically or fluorometrically. Primary neuronal cells were plated in 100-mm dishes at the density of 500,000 cells. Cells were exposed to 50 μM glutamate for 24 hr in the presence of estrogens or vehicle. Cells were washed twice with ice-cold PBS and harvested with 0.6 ml/dish ice-cold PBS using rubber policeman. Then, cells were homogenized by sonication. To prevent sample oxidation during homogenization, 0.5 M BHT (10 μl/ml cell suspension) was added before sonication. Cell homogenates were centrifuged at 3000*g* at 4°C for 10 min. The clear supernatant was used for TBARS assay and protein determination. For MDA measurement, 100 μl of sample was added into 48-

well plate followed by addition of a solution containing 1% TBA, 12.5% trichloroacetic acid, and 0.8 N HCl. Reaction mixtures were incubated at 50°C for 60 min, and then precipitated proteins were removed by centrifuging at 12,000 rpm for 2 min. Supernatants were transferred to 96-well plates, and relative fluorescence values were determined using a BioTek FL600 plate reader (BioTek, Highland Park, VT) at an excitation wavelength of 530 ± 25 nm, emission wavelength of 590 ± 20 nm, and sensitivity of 100. External standards used in the TBARS assay were made from 1,1,3,3-tetramethoxypropane in reagent grade ethanol and diluted in 0.9% normal saline to give concentrations ranging from 0 to 20 μM.

Protein Carbonyl assay

The accumulation of oxidized proteins was evaluated by the carbonyl group content via reaction with DNPH (2,4-dinitrophenilhidrazine) at 360 nm (Fagan et al., 1999). Neurons were treated with different paradigms and cells were collected and centrifuged at 1500 x g for 10 min at 4°C. The pellet was solubilized in 1 ml of ice-cold buffer (trichloroacetic acid (TCA)) and centrifuged (1500 × *g*, 10 min). The sediments were incubated with 1 ml of 10 mM DNPH (freshly prepared in 2 M HCl, in the dark) for 1 h at room temperature, with vortex agitation every 10 min. Following this incubation, 1 ml of 20% TCA was added and samples were centrifuged at 20,000 × *g*, for 3 min. The supernatant was decanted and the pellet mixed with 1 ml of a 1:1 ethanol:ethyl acetate solution. The pellet was then incubated with 1 ml of 6 M guanidine (prepared in PBS, pH 6.5), for 15 min at 37 °C and centrifuged at 1500 × *g* for 10 min. The supernatant was

collected and protein oxidation was estimated spectrophotometrically at 360 nm in 96-well plates. For all samples a blank was prepared, which was incubated with 2 M HCl instead of DNPH. The carbonyl content was calculated using a molar extinction coefficient of 22 mM^{-1} cm^{-1} for DNPH and was expressed as nmoles DNPH/mg protein.

Caspase-3 acitivity assay

Caspase-3 enzyme activity was measured by proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AFC by counting on a microplate fluorometer (Bio-Tek FL600 Winooski, VT). After incubation with 10 mM glutamate, 10 μM 17β-estradiol, and/or 100 μM okadaic acid for 24 hr, cells was harvested and washed once with cold PBS. The cells were lysed using lysis buffer containing 10mM EDTA, 0.5% Triton X-100, and 10mM Tris–HCl (pH 8.0) at room temperature for 10 min. Then, assay buffer (100mM HEPES; pH 7.5, 10mM dithiothreitol, 10% sucrose, 0.1% CHAPS, 0.1% BSA) and substrate (50 μM) was added on ice. Fluorescence at 400 nm (excitation) and 505 nm (emission) was measured using a BioTek FL600 plate reader (BioTek, Highland Park, VT) after incubation at 37 °C for 1-3 hr.

Immunoblot analysis

Protein from whole cell lysates (25 μg) was separated by SDS-polyacrylamide gel electrophoresis and transferred to Immunobilon-P polyvinylidene difluoride (Millipore Corp., Bedford, MA) membrane. Membranes were rinsed in Tris-buffered saline (10 mM Tris-base, pH 8.0, 100 mM NaCl) containing 0.2% Tween 20 then blocked with 3%

bovine serum albumin. Blots were then incubated with primary antibodies overnight at 4°C, rinsed and incubated in the appropriate secondary antibody before detection using enhanced chemiluminescence (ECL; Pierce Biotechnology, Inc., Rockford, IL). ECL results were digitized and quantified using UVP Bioimaging System.

Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. $p < 0.05$ was considered significant for all experiments. Each set of data represents three or more individual assays performed separately, with each containing 4-8 replicate wells. The values are reported as the mean ± SEM.

III. RESULTS

Cellular markers of oxidative stress in glutamate or okadaic acid mediated cell death in primary cortical neurons

In order to determine if okadaic acid caused production of reactive oxygen species (ROS) in primary cortical neurons, we simultaneously treated 17β-estradiol, 17α-estradiol, enantiomer of 17β-estradiol (ENT E2), or ZYC3 with glutamate and/or okadaic acid. Treatment with glutamate or okadaic acid induced approximately 4.5 fold increase in hydrogen peroxide (H_2O_2) production as measure by DCFH-DA florescent dye (Fig 1A). The estrogens alone did not have any effect on ROS production. All estrogens attenuated the glutamate-induced generation of hydrogen peroxide; however, they were

ineffective against the okadaic acid-induced ROS production. In addition, the presence of okadaic acid abolished the estrogen-mediated down-regulation of glutamate induced ROS generation. Similar effects were seen with lipid peroxidation production (Fig 1B) and protein carbonylation (Fig 1C).

MTT formazen reduction and caspase 3/7 activity in glutamate or okadaic acid mediated cell death in primary cortical neurons

We examined the caspase activity in response to treatment with glutamate, okadaic acid, and/or various forms of estrogens in primary cortical neurons. Estrogen treatment alone did not affect MTT formazen reduction (Fig 2A) or caspase 3/7 activity (Fig 2B); however, the various estrogens effectively attenuated MTT reduction, an indirect indication of mitochondrial function, as well as caspase activation caused by glutamate induced oxidative/excitotoxic stress. Treatment of primary cortical neurons with okadaic acid induced MTT reduction and the activation of caspase 3/7 similar to that caused by glutamate treatment. Yet, estrogens that are effective against glutamate mediated MTT reduction and caspase activation did not significantly alter the okadaic acid induced effects.

Inhibition of MAPK pathway in glutamate or okadaic acid induced neuronal cell death

Since the MAPK kinase has been implicated in the determination of cell survival or death and estrogens are known to activate and suppress ERK 1/2 phosphorylation, we

examined whether pharmacological inhibition of MEK 1/2 using PD98059 or U0126 would be neuroprotective against glutamate and okadaic acid mediated neuronal cell death because MEK 1/2 is the upstream kinase that phosphorylates ERK 1/2. MEK 1/2 inhibition with PD98059 or U0126 was neuroprotective against glutamate toxicity but not okadaic acid toxicity (Fig 3A & 3B). In addition, the presence of non-lethal concentrations of okadaic acid attenuated the PD98059 or U0126 mediated neuroprotection against glutamate-induced neurotoxicity. Inhibition of PKC is also neuroprotective, and show similar trends as those seen with ERK 1/2 inhibition. We also assessed the ability of PKA, PI3K, and Akt inhibitors to protect cortical neurons against glutamate and okadaic acid-induced cell death. As shown in figure 3C-3E, inhibition of PKA, PI3K, or Akt was not protective against neither glutamate nor okadaic acid-mediated death.

MEK inhibition mediated neuroprotection against oxidative/excitotoxic stress

In order to determine the effects of MEK inhibition on ROS production induced by glutamate and okadaic acid treatment, we examined the cytosolic hydrogen peroxide content of primary cortical neurons following 24 hr simultaneous treatment of glutamate, OA, and/or PD98059, U0126, H-89, LY294002, or Akt inhibitor. Interestingly, the presence of a MEK inhibitor attenuated the glutamate but not okadaic acid-induced production of ROS inhibition (Fig 4). In addition the presence of okadaic acid abolished this ERK1/2 inhibition mediated down-regulation of glutamate-induced ROS production.

Interestingly, inhibition of MEK 1/2 with PD98059 or U0126 alone caused a decrease in basal ROS production. PKA, PI3K, or Akt inhibition had no effect on basal ROS production, and these inhibitors did not attenuate the ROS induction by glutamate or okadaic acid.

DISCUSSION

The present study demonstrates that okadaic acid mediated neuronal cell death is similar to glutamate induced neurotoxicity and involves the production of reactive oxygen species, lipid peroxides, protein carbonyls, activation of caspase 3/7, and mitochondrial dysfunction. We also show that while estrogen and estrogen analogues mitigate or abolish the glutamate-induced oxidative/excitotoxic stress and subsequent cell death, they are not effective against the same events produced by okadaic acid, consistant with the hypothesis that protein phosphatase activity is a necessary componenet of estrogen-mediated neuroprotection. In addition, the presence okadaic acid in non-lethal concentrations abolishes the estrogen and estrogen analogue-mediated neuroprotection against oxidative/excitotoxic stress induced by glutamate toxicity. Pharmacological inhibition of ERK1/2 phosphorylation also attenuated the ROS production mediated by glutamate but was ineffective against okadaic acid induced oxidative stress. Similarly, ERK 1/2 inhibition rescued primary cortical neurons from cell death induced by glutamate toxicity but not okadaic acid toxicity.

The okadaic acid induced oxidative stress found in our study is consistent with the results of others (Schreck and Baeuerle, 1994; Schmidt et al., 1995; Tunez et al., 2003).

The mitochondrial dysfuction and the consequential induction of caspase 3/7 is also in agreement with previous observations by other laboratories (Boe et al., 1991; Fernandez et al., 1991; Arias et al., 1993; Fernandez-Sanchez et al., 1996; Yoon et al., 2006). Yoon et al has shown mitochondrial swelling, and decreased membrane potential as well as increased protein expression of caspase-3, Bad, Bax, and Bim following okadaic acid treatment.

Protein phosphatases are important modulators of cellular function; therefore, it is not surprising that inhibition of protein phosphatases by okadaic acid leads to cellular dysfunction that ultimately ends with cell death. Interestingly, the activities of various phosphatases including PP1, PP2A, and PP2B are found to be depressed in the brains of AD patients (Gong et al., 1995; Lian et al., 2001; Vogelsberg-Ragaglia et al., 2001), and AD brains show increased oxidative stress (Butterfield, 2006), which is thought to be the mediator of AD pathology. In addition, serine/threonine phosphatases have been shown to play a role in hyperphosphorylation of tau and amyloid plaques (Tian and Wang, 2002; da Cruz e Silva et al., 2004). Therefore, it appears that they may have a multifaceted role in the pathology of neurodegenerative diseases.

We have previously shown that estrogen-mediated neuroprotection involves the maintanence of serine/threonine phosphatase protein expressions and activities (Yi et al., 2005; Yi and Simpkins, 2007). Inhibition of phosphatases aborgated the estrogen-induced attenuation of persistent ERK 1/2 phosphorylation following oxidative/excitotoxic stress (Yi and Simpkins, 2007), suggesting that estrogens influences

MAPK signaling pathway during neurotoxic events. In aggreement with this observation, we show in this study that pharmacological inhibition of ERK 1/2 with PD98059 or U0126 leads to neuroprotection against oxidative/excitotoxic stress caused by glutamate, similar to that seen with estrogen treatment. However, ERK 1/2 inhibition did not protect against okadaic acid induced cell toxicity. The expected results would have been that inhibition of phosphatases by okadaic acid would not have an inhibitory effect on the neuroprotection provided by PD98059 or U0126 since these block the phosphorylation of ERK 1/2 via MEK inhibition. Instead, non-lethal concentrations of okadaic acid was able to attenuate ERK 1/2 inhibition mediated neuroprotection. It is possible that parallel pathways are involved in the oxidative/excitotoxic stress. Indeed, it has been shown that p38 MAPK pathway, which is thought to be involved in cell death signaling, can be activated parallel to the ERK 1/2 pathway (Johnson and Lapadat, 2002; Wang et al., 2006b; Tanel and Averill-Bates, 2007). We also have unpublished data that shows that inhibition of p38 is neuroprotective against oxidative stress in HT-22 cells. Activation of the JNK, p38, and ERK 1/2 pathways have been shown in primary cortical neurons following 10 nM okadaic acid (Yoon et al., 2006). Moreover, various experimental data have shown that estrogen-mediated neuroprotection involves the different MAPK pathways (Singh et al., 2000; Wang et al., 2006a). It has also been shown that PKC is an important mediator of cell survival (Maiese and Boccone, 1995; Gressens et al., 1998; Noh et al., 2000; Jung et al., 2003) and has been shown to be involved in estrogen mediated neuroprotection (Jung et al., 2005). Therefore, it is possible that direct inhibition of death inducing kinases is sufficient for neuronal survival,

but inhibition of phosphatases leads to activation of multiple death signaling pathways that cannot be overcome by inhibition of ERK, p38, or PKC alone.

The pathology of neurodegenerative diseases is multifaceted which makes it difficult to design an effective therapy that can combat all the different aspects of neuronal degeneration. Therefore, it would be ideal to have a compound that can have an effect on many of the pathways involved in the progression of neurodegeneration. Even though the WHI-MS study have shown negative effects of estrogen replacement therapy on cognition and increased risks of stroke and heart disease (Shumaker et al., 2003; Wassertheil-Smoller et al., 2003), the vast amount of experimental, epidemiological, and clinical data show the benefits of estrogen replacement therapy in cognitive functions (Paganini-Hill et al., 1988; Henderson et al., 1994; Ohkura et al., 1994; Simpkins et al., 1994; Paganini-Hill, 1995; Paganini-Hill and Henderson, 1996). Although the latter data are, in part, confounded by a "healthy user bias," much of the inconsistency may be explained by the fact that women in the latter studies initiated hormone replacement therapy at the menopausal transition, whereas the WHI trial was conducted in older women who were approximately 12 yr postmenopausal. In addition, older trials included women on either unopposed estrogen therapy or cyclic HRT regimens. Further, a variety of other factors confound the issue of the beneficial effects of estrogen therapy, such as doses of hormones, type of hormones, the age at which HRT is administered, and a host of other factors. Estrogens have been shown to activate or inhibit different proteins that are involved in various signaling pathways such as the Akt pathway, PKC pathway, MAPK pathway, NFκB pathway, caspases, Bcl group of proteins, various other

mitochondrial related proteins, and many more (Migliaccio et al., 1996; Kelly and Wagner, 1999; Singh et al., 1999; Zhang et al., 2001; Wen et al., 2004; Yao et al., 2007). Therefore, it seems likely that estrogens are good candidates for neurodegenerative therapy for a particular population.

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FIGURES LEGENDS

Figure 5-1. Effects of glutamate, okadaic acid, 17β-estradiol, 17α-estradiol, ENT E2, and/or ZYC3 on oxidative stress markers in primary cortical neurons. Primary cortical neurons were seeded into 100 mm dishes at a density of 500,000 cells/well and various assays performed following 24 hr treatment. **A**) ROS production was measured in neurons treated simultaneously with 50 nM okadaic acid, 50 μM glutamate, and/or 100 nM 17β-estradiol, 100 nM 17α-estradiol, 100 nM ENT E2, and/or 10 nM ZYC3. **B**) Lipid peroxidation was measured in cortical neurons 24 hr following simultaneously treatment with 50 nM okadaic acid, 50 μM glutamate, and/or 100 nM 17β-estradiol, 100 nM 17α-estradiol, 100 nM ENT E2, and/or 10 nM ZYC3. **C**) Protein carbonylation was measured in cortical neurons 24 hr following simultaneously treatment with 50 nM okadaic acid, 50 μM glutamate, and/or 100 nM 17β-estradiol, 100 nM 17α-estradiol, 100 nM ENT E2, and/or 10 nM ZYC3. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for six independent experiments with two replicates per experiment for ROS production and six independent experiments each for lipid peroxidation and protein carbonylation experiments. * $P < 0.05$ vs. vehicle control.

Figure 2. Effects of glutamate, okadaic acid, 17β-estradiol, 17α-estradiol, ENT E2, and/or ZYC3 on oxidative stress markers in primary cortical neurons. Primary cortical neurons were seeded into 100 mm dishes at a density of 500,000 cells/well and various assays performed following 24 hr treatment. **A**) MTT reduction was measured in neurons treated simultaneously with 50 nM okadaic acid, 50 μM glutamate, and/or 100

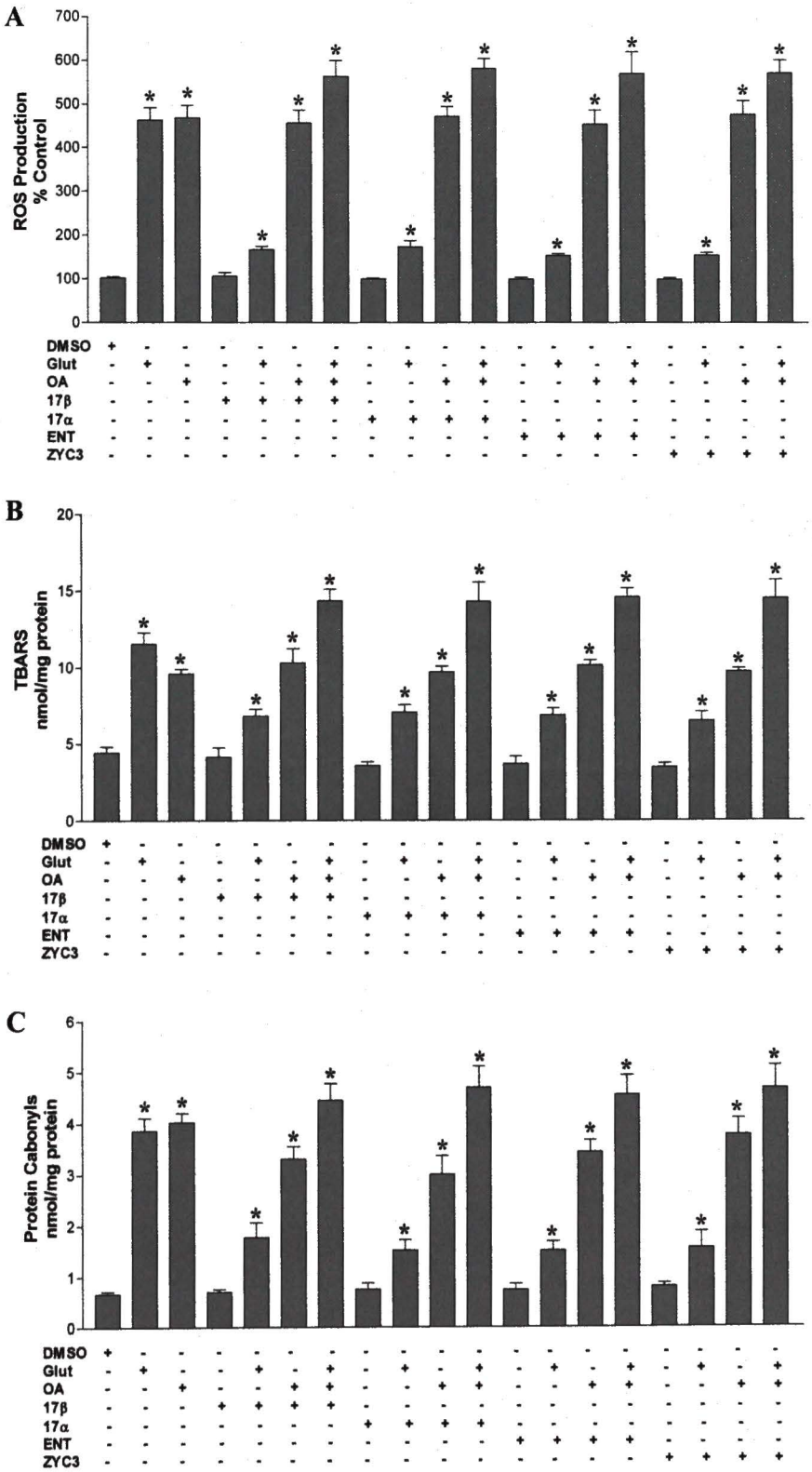
nM 17β-estradiol, 100 nM 17α-estradiol, 100 nM ENT E2, and/or 10 nM ZYC3. **B)** Caspase 3/7 activity was measured in cortical neurons 24 hr following simultaneously treatment with 50 nM okadaic acid, 50 μM glutamate, and/or 100 nM 17β-estradiol, 100 nM 17α-estradiol, 100 nM ENT E2, and/or 10 nM ZYC3. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for six independent experiments with two replicates per experiment for ROS production and six independent experiments for caspase 3/7 activity measurement. * $P < 0.05$ vs. vehicle control. † $P < 0.05$ vs. glutamate treated group.

Figure 3. Effects of glutamate, okadaic acid and/or various protein kinase inhibitors on cell viability in primary cortical neurons. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. **A)** Cells were treated simultaneously with 50 μM glutamate, 50μM PD98059 and/or varying concentrations of okadaic acid. **B)** Cells were treated simultaneously with 50 μM glutamate, 10 μM U0126 and/or varying concentrations of okadaic acid. **C)** Cells were treated simultaneously with 50 μM glutamate, 1 μM H-89 and/or varying concentrations of okadaic acid. **D)** Cells were treated simultaneously with 50 μM glutamate, 50 μM LY294002 and/or varying concentrations of okadaic acid. **E)** Cells were treated simultaneously with 50 μM glutamate, 100 μM Akt Inhibitor and/or varying concentrations of okadaic acid. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two

replicates per experiment. * P <0.05 vs. vehicle control; † P < 0.05 vs. glutamate treated group.

Figure 4. Effects of glutamate, okadaic acid and/or various protein kinase inhibitors on ROS production in primary cortical neurons. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. **A**) Cells were treated simultaneously with 50 μM glutamate, 50μM PD98059, 10 μM U0126, 1 μM H-89, 50 μM LY294002, 100 μM Akt Inhibitor, and/or 50nM of okadaic acid. ROS production was determined by DCFH-DA assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * P <0.05 vs. vehicle control.

Figure 5-1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ROS Production % Control
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B
TBARS nmol/mg protein
20
15
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C
Protein Cabonyls nmol/mg protein
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Glut
OA
17β
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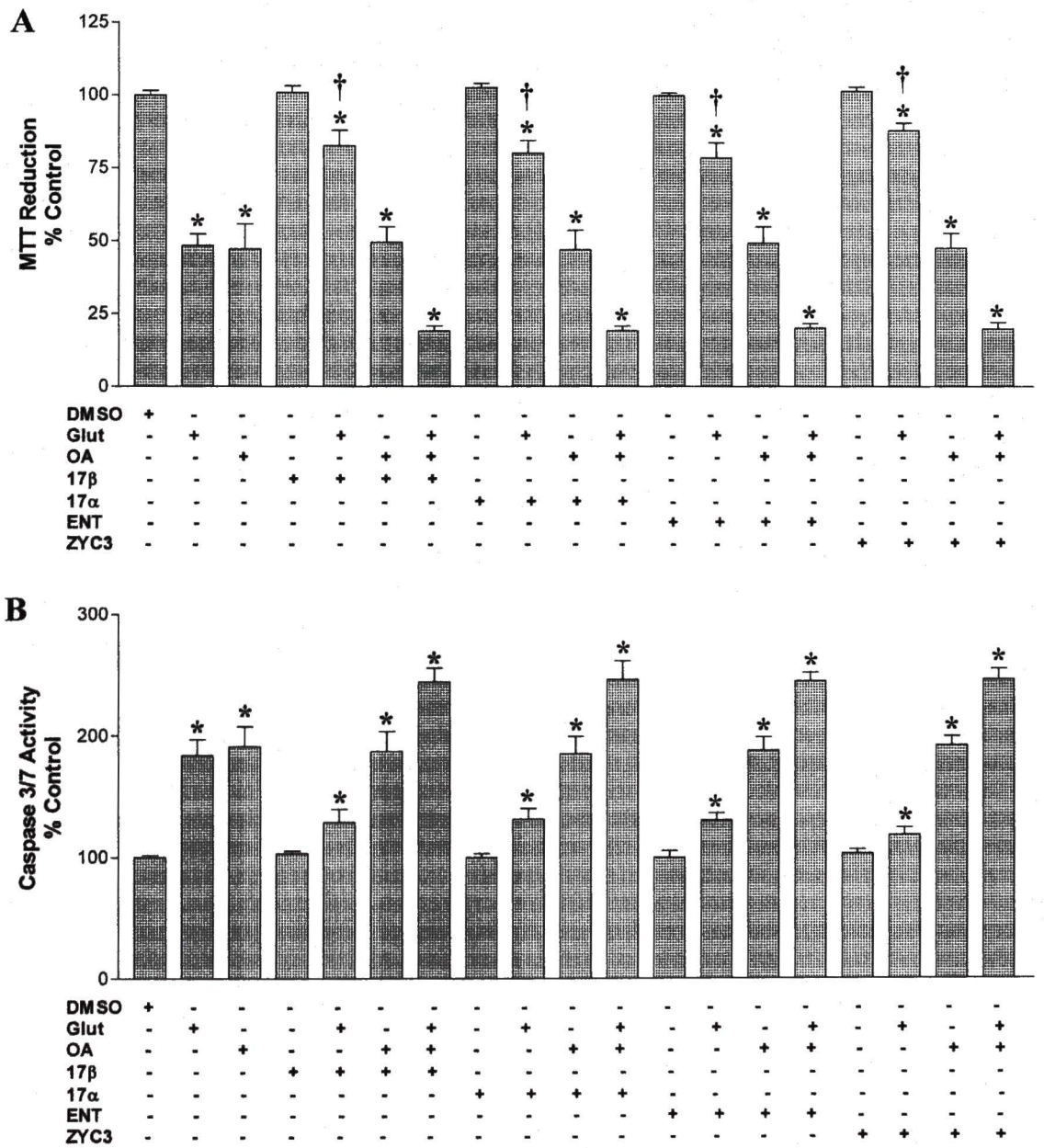
ENT
ZYC3

Figure 5-2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Glut
OA
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ENT
ZYC3
B
Caspase 3/7 Activity
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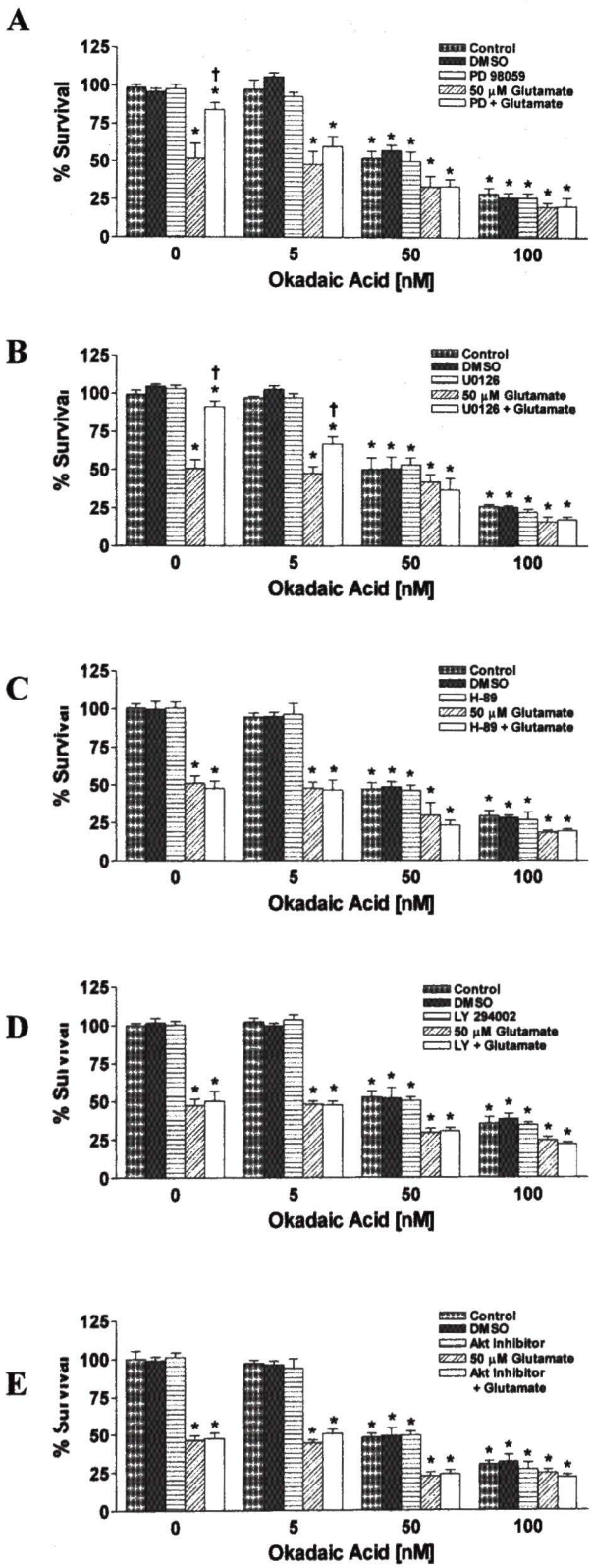
17α
ENT
ZYC3

Figure 5-3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% Survival
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DMSO
PD 98059
50 μM Glutamate
PD + Glutamate
Okadaic Acid [nM]
B
% Survival
Control
DMSO
U0126
50 μM Glutamate
U0126 + Glutamate
Okadaic Acid [nM]
C
% Survival
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DMSO
H-89
50 μM Glutamate
H-89 + Glutamate
Okadaic Acid [nM]
D
% Survival
Control
DMSO
LY 294002
50 μM Glutamate
LY + Glutamate
Okadaic Acid [nM]
E
% Survival
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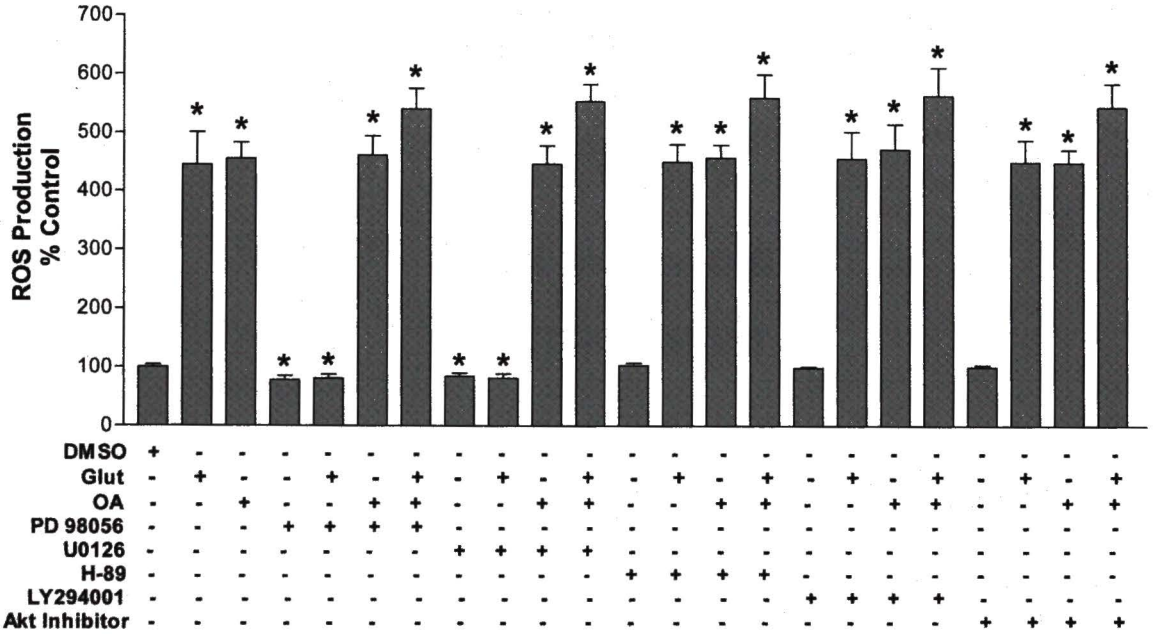
Akt Inhibitor
50 μM Glutamate
Akt Inhibitor
+ Glutamate
Okadaic Acid [nM]

Figure 5-4


ROS Production
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700
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DMSO + -
Glut - + - - + - + - + - + - + - + - + - + - + - +
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CHAPTER 6

SUMMARY AND CONCLUSIONS

Increasing numbers of people will experience age-related declines in cognition, stroke, cardiovascular diseases, and a number of neurodegenerative diseases such as AD and PD as life-spans have significantly increased in recent years. Gender differences in the incidence, symptomology, and severity of damage from neurodegenerative diseases are very evident, indicating a role for sex steroids. Interestingly, it has been shown that before the onset of menopause, women experience fewer strokes and other neurodegenerative diseases, and their prognosis and outcomes are better than that of their male counterparts (Hurn and Macrae, 2000). However, following the onset of menopause, the incidences and prognosis match, if not worsen, than that of age-matched males (Hurn and Macrae, 2000). It has been shown that estrogens are not just "female" hormones. In fact, estrogens are essential modulators of brain development, structure, function, and metabolism.

There is a large body of literature that supports the neuroprotective effects of estrogens. However, the mechanisms by which estrogens are neuroprotective are under intense debate. In addition, the WHI-MS study have shown negative effects of estrogen replacement therapy on cognition and increased risks of stroke and heart disease (Shumaker et al., 2003; Wassertheil-Smoller et al., 2003); while there is a vast amount of experimental, epidemiological, and clinical data showing the benefits of estrogen replacement therapy in cognitive functions (Fillit et al., 1986; Henderson et al., 1994; Tang et al., 1996; Costa et al., 1997; Kawas et al., 1997; Yaffe et al., 1997; Resnick et al., 1998; Slooter et al., 1999; Waring et al., 1999; Asthana et al., 2001). Although the latter data are, in part, confounded by a "healthy user bias," much of the inconsistency may be

explained by the fact that women in the latter studies initiated hormone replacement therapy at the menopausal transition, whereas the WHI trial was conducted in older women who were approximately 12 yr postmenopausal. In addition, older trials included women on either unopposed estrogen therapy or cyclic HRT regimens. Further, a variety of other factors confound the issue of the beneficial effects of estrogen therapy, such as doses of hormones, type of hormones, the age at which HRT is administered, and a host of other factors. Therefore, an understanding of the mechanism of estrogens is extremely important.

In these studies we show that one of the mechanisms of estrogen-mediated neuroprotection is via preservation of serine/threonine phosphatase expressions and activities, which in turn maintains normal cellular function. We confirmed that protein phosphatase inhibitors are potent neurotoxins in transformed neuronal and glial cell types as well as in primary cortical neurons. We also demonstrated for the first time that estrogen treatment regimens that are protective against other neurotoxic insults do not protect against the toxic effects of the phosphatase inhibitors, and the protective effects of estrogens against glutamate toxicity are antagonized by phosphatase inhibition. In addition estrogens antagonize the reduction in PP1, PP2A and PP2B induced by glutamate, but not in the presence of okadaic acid. Estrogens preserve PP expression and activity via an ER-independent pathway as demonstrated with estrogen analogues that have little or no binding to either ERα or ERβ. The ER-independent mechanism for the neuroprotective effects of estrogens is shown to be at least in part via the prevention of insult-induced decrease in phosphatase activity and the resulting neurotoxic, persistent

hyperphosphorylation of proteins in multiple signaling pathways that are detrimental to cell survival. Inhibition of individual phosphatases did not abolish estrogen-mediated neuroprotection as seen with general inhibition with okadaic acid or calyculin A. However, when the specific inhibitors of PP1, PP2A, and PP2B were combined, there was additive death inducing effects that estrogens fail to protect against. Finally, we show that while OA-induced neurotoxicity produces similar events seen with glutamate

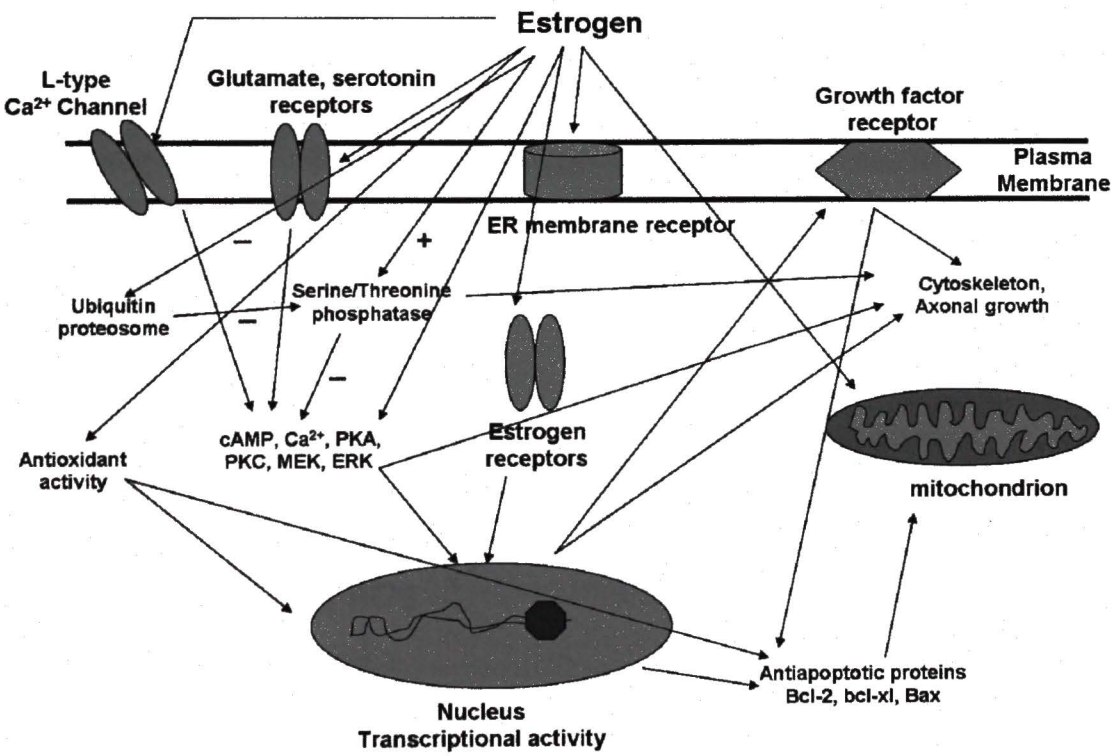

Figure 6-1. Potential mechanisms of neuroprotective effects of estrogens.

toxicity, such as increased reactive oxygen species, lipid peroxidation, protein carbonylation, mitochondrial dysfunction, and caspase 3/7 activity, estrogens and specific kinase inhibitors were not neuroprotective against OA but were effective against

glutamate. These results lead us to believe that the mechanisms of neuroprotective effects of estrogens are via multifaceted signaling pathways and that serine/threonine phosphatases are essential components of estrogen-mediated neuroprotection.

As shown in figure 6-1, estrogens' actions are multifaceted in that it has direct and indirect effects on different signaling pathways and other proteins. However, we have a simpler schematic shown in figure 6-2 which shows the major role that serine/threonine phosphatases play in estrogen-mediated neuroprotection. Glutamate toxicity causes increased ROS production, protein carbonylation, and lipid peroxidation that cause the prolonged phosphorylation of ERK. This prolonged ERK phosphorylation then exacerbate the already unfavorable condition of the cells by inducing more ROS production which then disrupts normal cellular functions and causes profound inhibition

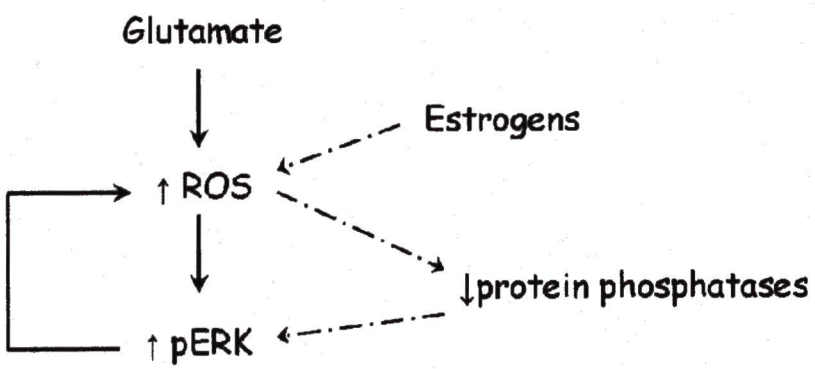

Figure 6-2. Proposed mechanism of estrogen-mediated neuroprotection involving protein phosphatases. Solid lines represent positive effects. Dashed lines represent opposite effects.

of protein phosphatases that leads to more ERK phosphorylation which ultimately leads to cell death. In the presence of estrogens, which is thought to act as an antioxidant, the

glutamate induced ROS production, protein carbonylation, and lipid peroxidation is attenuated and the normal cellular environment is maintained. However, estrogens are unable to protect against okadaic acid-induced cell death because in addition to causing similar events induced by glutamate, okadaic acid also inhibits serine/threonine phosphatases. Therefore, estrogens are not able to maintain the "normal" cellular environment and thus, cannot rescue cells from cell death induced by okadaic acid.

Interestingly, under normal cellular conditions, estrogens did not have any effect on the activity and expression of phosphatases. Only when the cells were under duress does estrogens have an effect on phosphatase; whereas, it has been demonstrated that estrogens alone induce phosphorylation of ERK within minutes and is sustained for about 2-3 hrs. The question is what causes the estrogens to respond in such a specific manner, and must be examined further. In addition, it should be noted that the results were similar in transformed neurons, glia, and primary cortical neurons (see appendix) suggesting a common mechanism by which estrogens influence protein phosphatase expression and activity. The only remarkable difference among the cell types were that PP2A activity was more profoundly affected in neuronal cell types, whereas, calcineurin appeared to play a more prominent role in glioma cells. Nonetheless, maintaining protein phosphatase activities appear to be an important aspect of estrogen neuroprotection.

It is yet unclear how estrogens influence the functions of serine/threonine phosphatases. It seems likely that estrogens are influencing the clearance of serine/threonine phosphatases since the effects of maintaining protein expressions and

activities are similar for PP1, PP2A, and PP2B. Therefore, the effects of ubiquitin and proteosome inhibition following glutamate and OA induced neurotoxicity in the presence and absence of estrogens needs to be determined. Thus far, there is no evidence to suggest that estrogens interact physically with phosphatases to alter their activity. However, this is a possibility that cannot be ruled out. Previous work has shown a direct interaction of estrogens to a variety of proteins such as GSH, which sulfhydryl group (redox sensitive cysteine groups). Estrogens are most likely to interact especially with those proteins that have sulfhydryls in their active domains, such as Na/K ATPase (Shivakumar et al., 1995), ryanodine receptors (Pessah et al., 2002), and GAPDH (Brune and Mohr, 2001). Further work needs to be done to determine if estrogens are interacting directly with the serine/threonine phosphatases as they do possess cysteine groups that are redox sensitive.

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Yaffe K, Cauley J, Sands L, Browner W (1997) Apolipoprotein E phenotype and cognitive decline in a prospective study of elderly community women. Arch Neurol 54:1110-1114.

APPENDIX

Figure 1

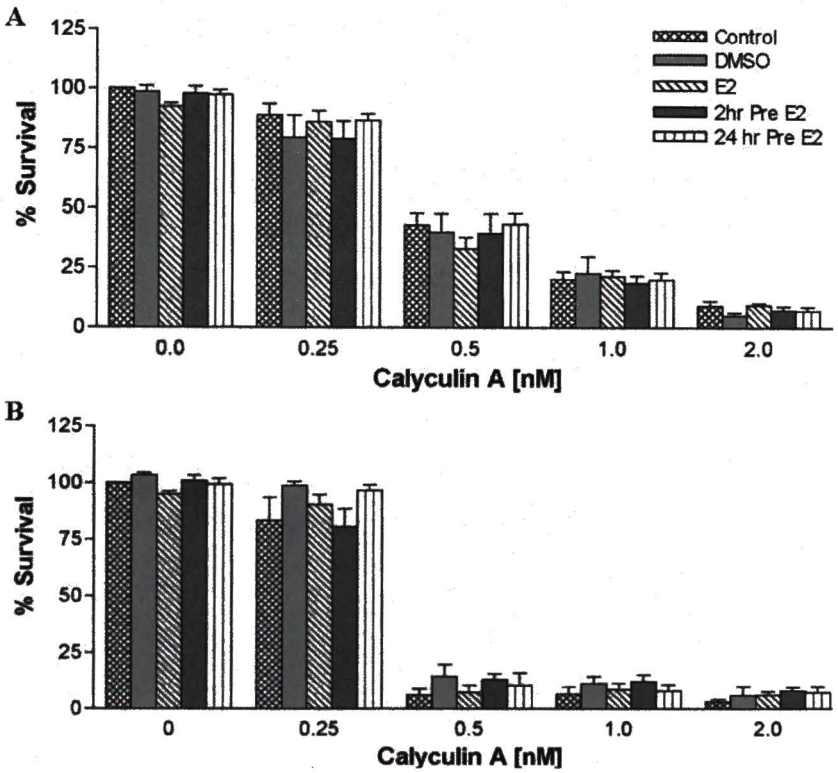

Figure 1. Effects of calyculin A on estrogen-mediated neuroprotection from glutamate neurotoxicity. HT-22 cells, C6-glioma cells, or primary rat cortical neurons were seeded into 96-well plates at a density of 3,500 or 25,000 cells per well. **A**, HT-22 cells were pretreated for 2 hr, 24 hr, or simultaneously with 10 μM 17β-estradiol; then treated with varying concentrations of calyculin A and 10 mM glutamate. **B**, C6-glioma cells were pretreated for 2 hr, 24 hr, or simultaneously with 10 μM 17β-estradiol; then treated with varying concentrations of calyculin A and 20 mM glutamate. Cell viability was determined by calcein AM assay after 24 h exposure to the various compounds. All data were normalized to percentage survival of vehicle control. Data are represented as mean ± SEM for $n = 6$. * $P < 0.05$ vs. control; † $P < 0.05$ vs. glutamate treated group.

Figure 2

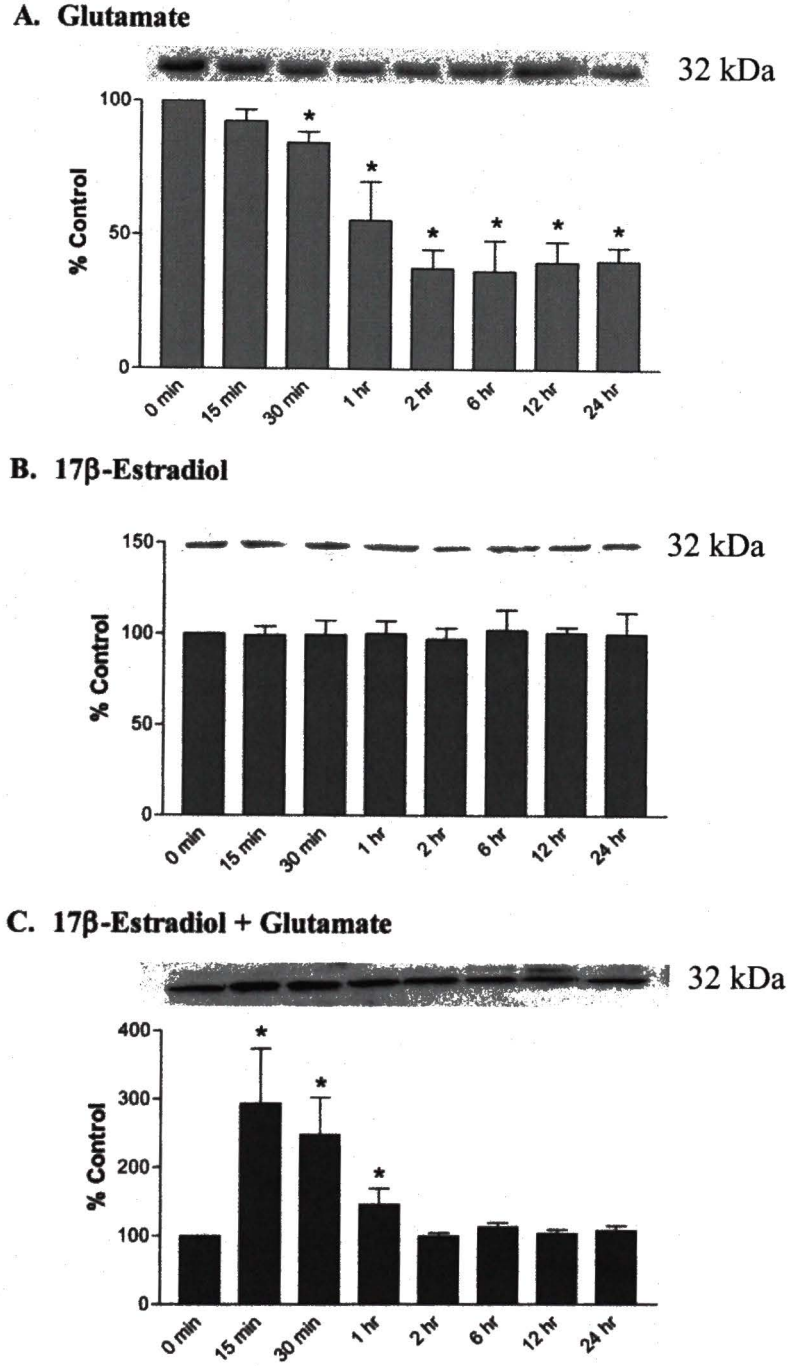

Figure 2. Time course of the effects 17β-estradiol, glutamate, and their combination on PP1 protein levels in HT-22. HT-22 cells were treated with either 10 mM glutamate (***A***) or 10 μM 17β-estradiol (***B***) and simultaneously with 10 mM glutamate and 10 μM 17β-estradiol (***C***). Cells were harvested at the times indicated for Western blot analysis of PP1. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 3

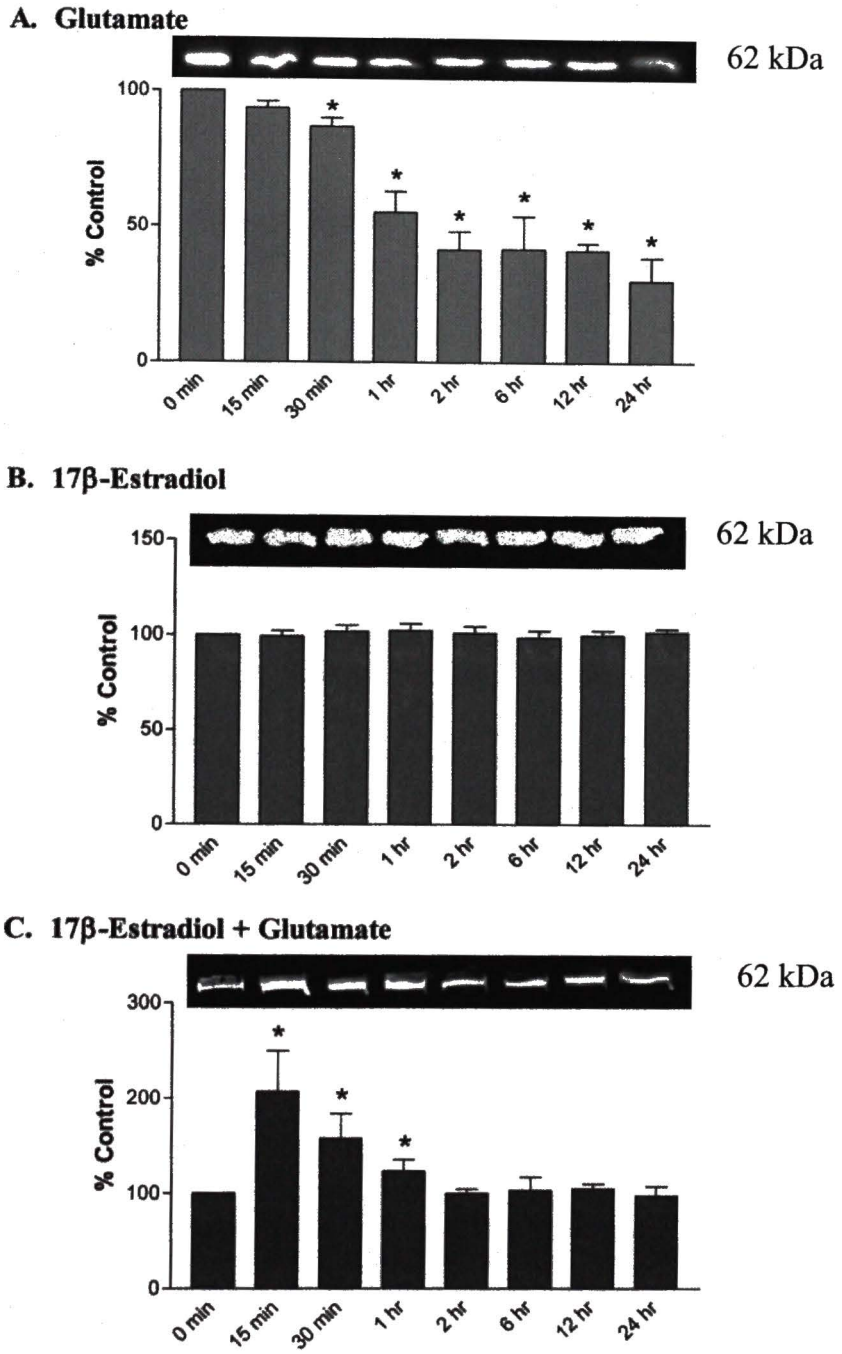

Figure 3. Time course of the effects 17β-estradiol, glutamate, and their combination on PP2B protein levels in HT-22. HT-22 cells were treated with either 10 mM glutamate (***A***) or 10 μM 17β-estradiol (***B***) and simultaneously with 10 mM glutamate and 10 μM 17β-estradiol (***C***). Cells were harvested at the times indicated for Western blot analysis of PP2B. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 4

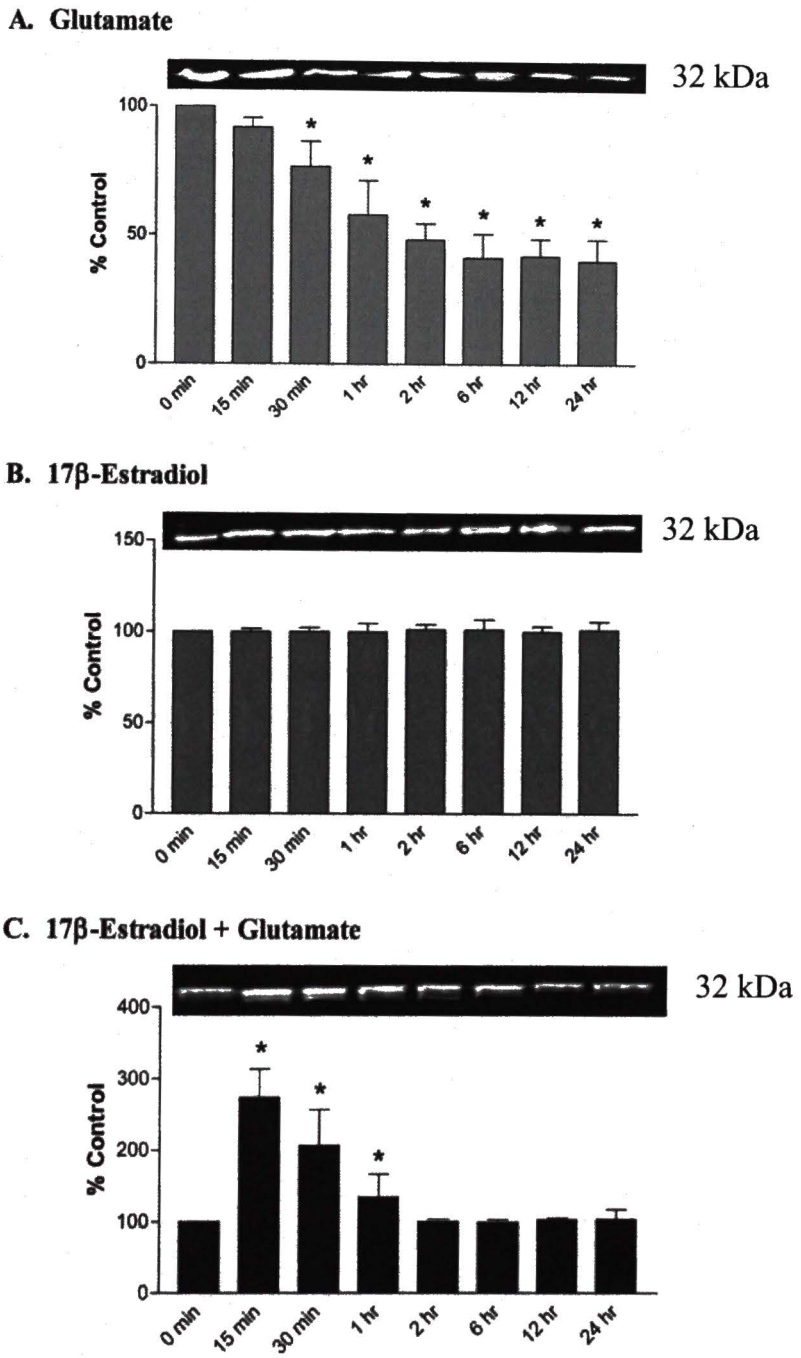

Figure 4. Time course of the effects 17β-estradiol, glutamate, and their combination on PP1 protein levels in C6-glioma. C6-glioma cells were treated with either 20 mM glutamate (***A***) or 10 μM 17β-estradiol (***B***) and simultaneously with 20 mM glutamate and 10 μM 17β-estradiol (***C***). Cells were harvested at the times indicated for Western blot analysis of PP1. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 5

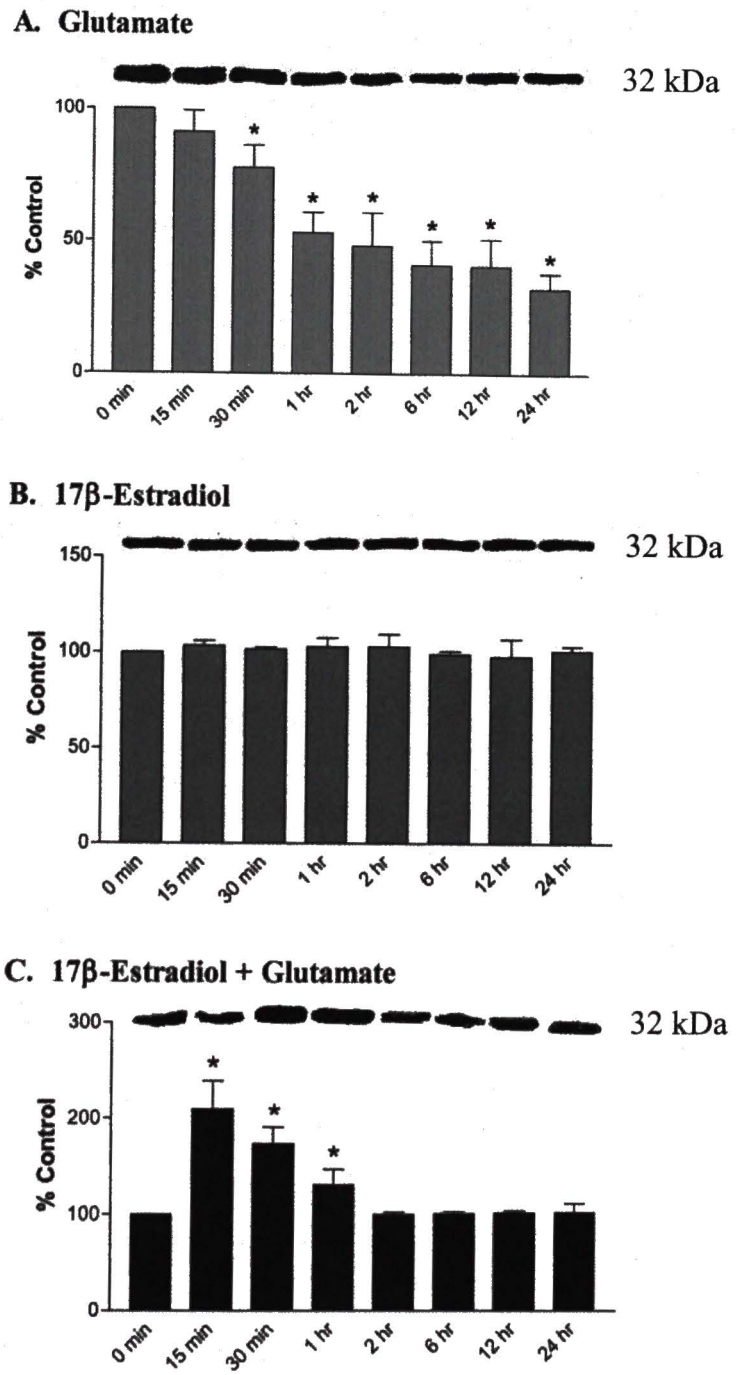

Figure 5. Time course of the effects 17β-estradiol, glutamate, and their combination on PP2A protein levels in C6-glioma. C6-glioma cells were treated with either 20 mM glutamate (*A*) or 10 μM 17β-estradiol (*B*) and simultaneously with 20 mM glutamate and 10 μM 17β-estradiol (*C*). Cells were harvested at the times indicated for Western blot analysis of PP2A. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 6

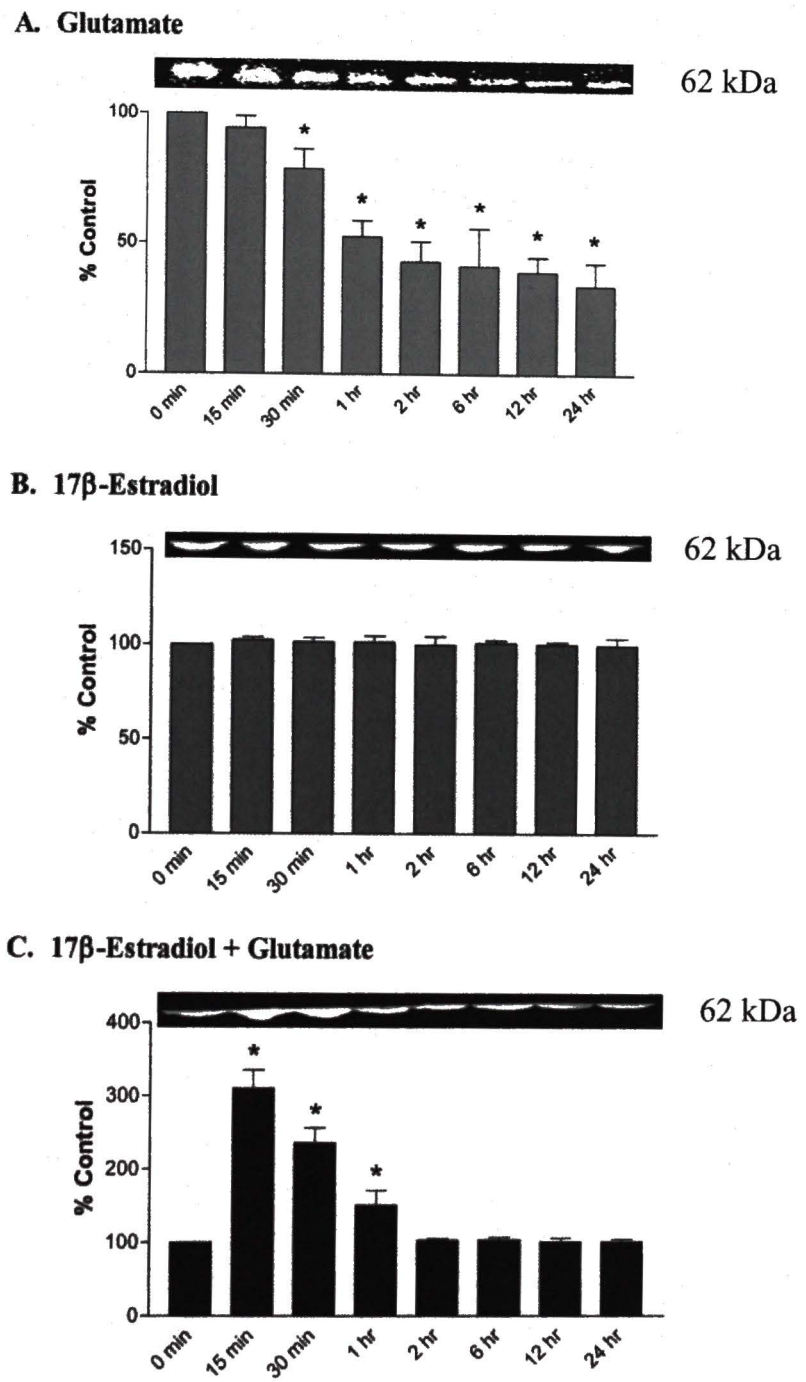

Figure 6. Time course of the effects 17β-estradiol, glutamate, and their combination on PP2B protein levels in C6-glioma. C6-glioma cells were treated with either 20 mM glutamate (***A***) or 10 μM 17β-estradiol (***B***) and simultaneously with 20 mM glutamate and 10 μM 17β-estradiol (***C***). Cells were harvested at the times indicated for Western blot analysis of PP2B. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 7

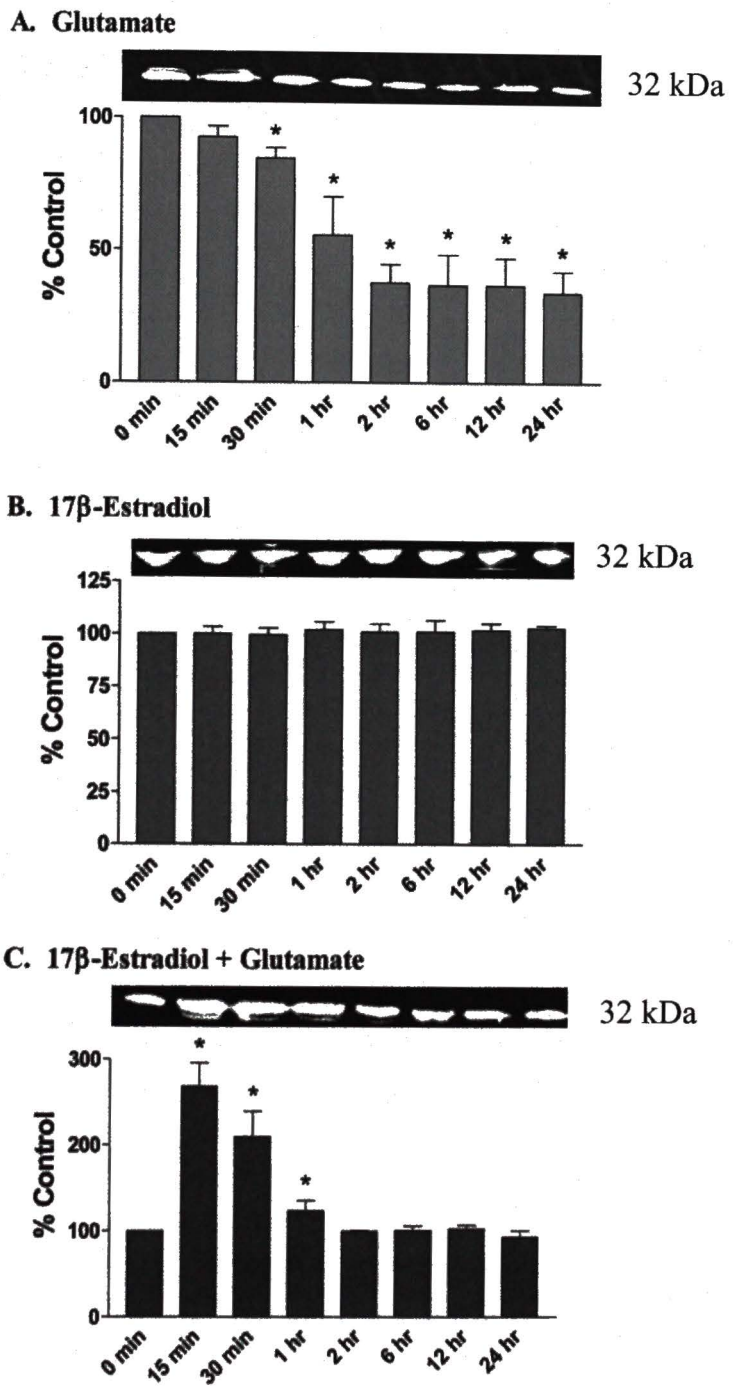

Figure 7. Time course of the effects 17β-estradiol, glutamate, and their combination on PP1 protein levels in primary cortical neurons. Primary cortical neurons were treated with either 50 μM glutamate (*A*) or 100 nM 17β-estradiol (*B*) and simultaneously with 50 μM glutamate and 100 nM 17β-estradiol (*C*). Cells were harvested at the times indicated for Western blot analysis of PP1. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 8

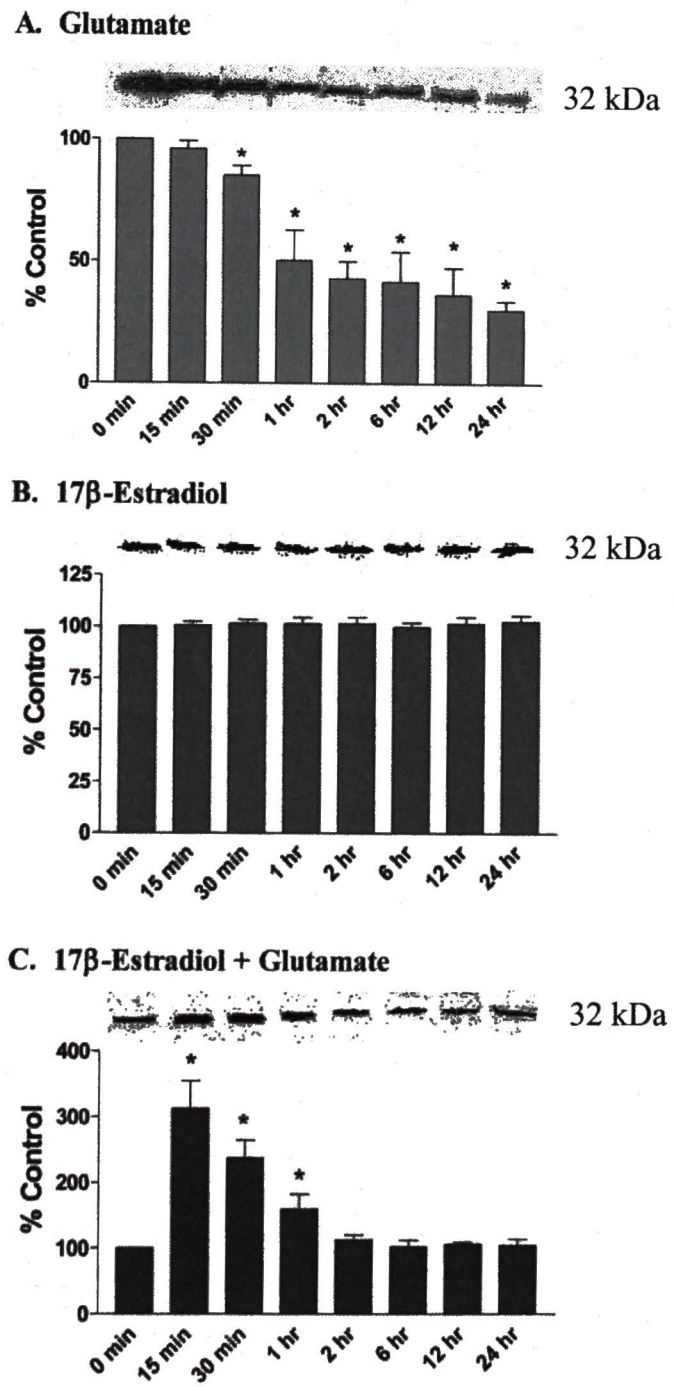

Figure 8. Time course of the effects 17β-estradiol, glutamate, and their combination on PP2A protein levels in primary cortical neurons. Primary cortical neurons were treated with either 50 μM glutamate (***A***) or 100 nM 17β-estradiol (***B***) and simultaneously with 50 μM glutamate and 100 nM 17β-estradiol (***C***). Cells were harvested at the times indicated for Western blot analysis of PP2A. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 9

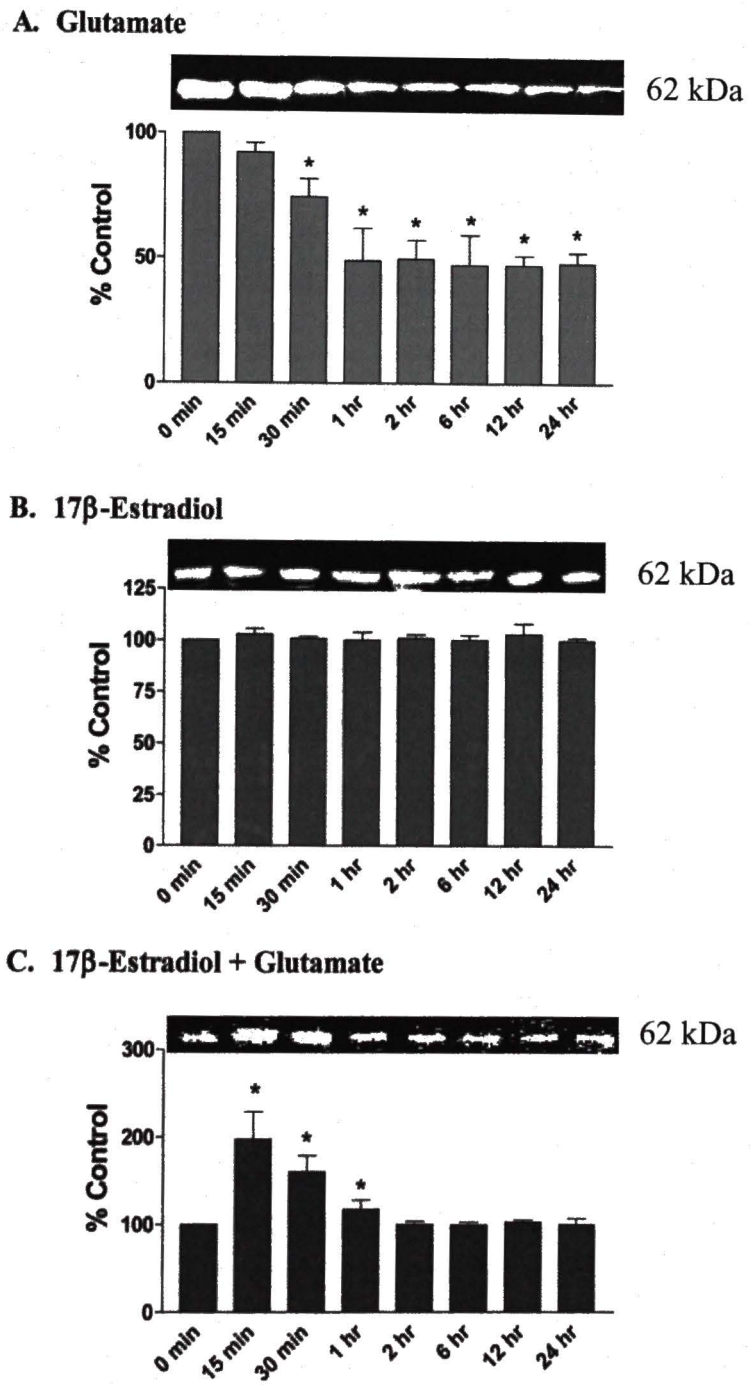

Figure 9. Time course of the effects 17β-estradiol, glutamate, and their combination on PP2B protein levels in primary cortical neurons. Primary cortical neurons were treated with either 50 μM glutamate (***A***) or 100 nM 17β-estradiol (***B***) and simultaneously with 50 μM glutamate and 100 nM 17β-estradiol (***C***). Cells were harvested at the times indicated for Western blot analysis of PP2B. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 10

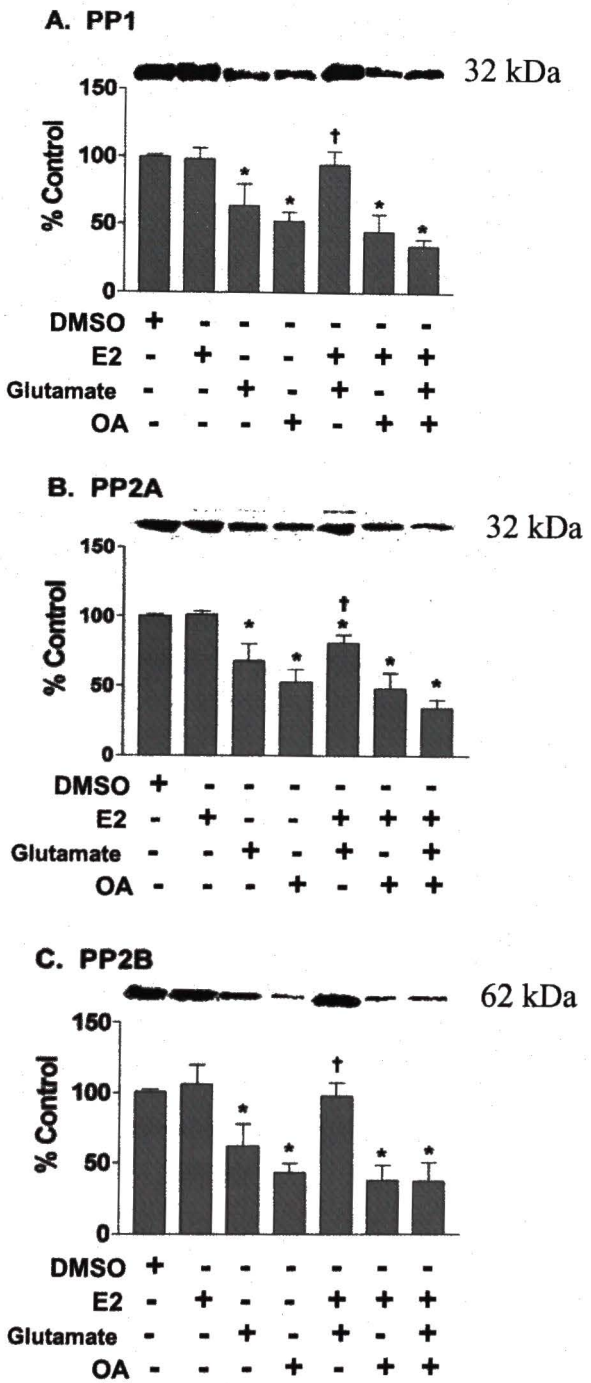

Figure 10. PP1, PP2A, and PP2B protein levels in response to 17β-estradiol in the presence and absence glutamate and/or okadaic acid in C6-glioma cells. HT-22 cells were treated with 100 nM okadaic acid, 10 mM glutamate, and/or 10 μM 17β-estradiol. Cells were harvested after 24 hr of treatment for western blot analysis of PP1, PP2A, and PP2B. Graphs are represented as mean ± SEM for n=3, and werstern blots were normalized to β-actin (not shown). * $P < 0.05$ vs. control; † $P < 0.05$ vs. glutamate treated group.

Figure 11

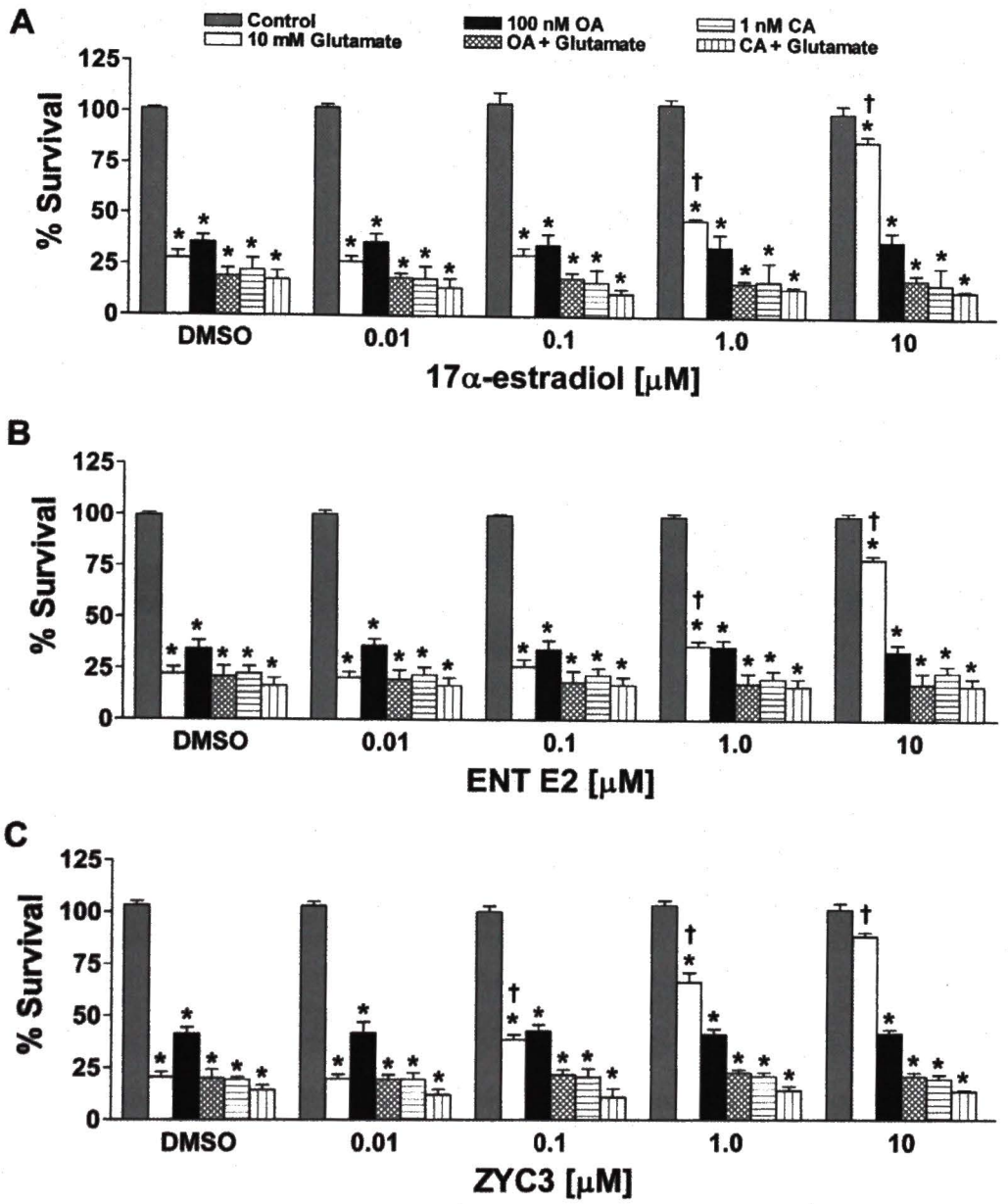

Figure 11. Effects of okadaic acid and calyculin A on 17α-estradiol, ENT E2, or ZYC3 mediated neuroprotection in HT-22. HT-22 cells were seeded into 96-well plates at a density of 3,500 cells/well. **A)** Cells were treated simultaneously with 100 nM okadaic acid, 1 nM calyculin A, 10 mM glutamate, and/or varying concentrations of 17α-estradiol. **B)** Cells were treated simultaneously with 100 nM okadaic acid, 1 nM calyculin A, 10 mM glutamate, and/or varying concentrations of ENT E2. **C)** Cells were treated simultaneously with 100 nM okadaic acid, 1 nM calyculin A, 10 mM glutamate, and/or varying concentrations of ZYC3. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 12

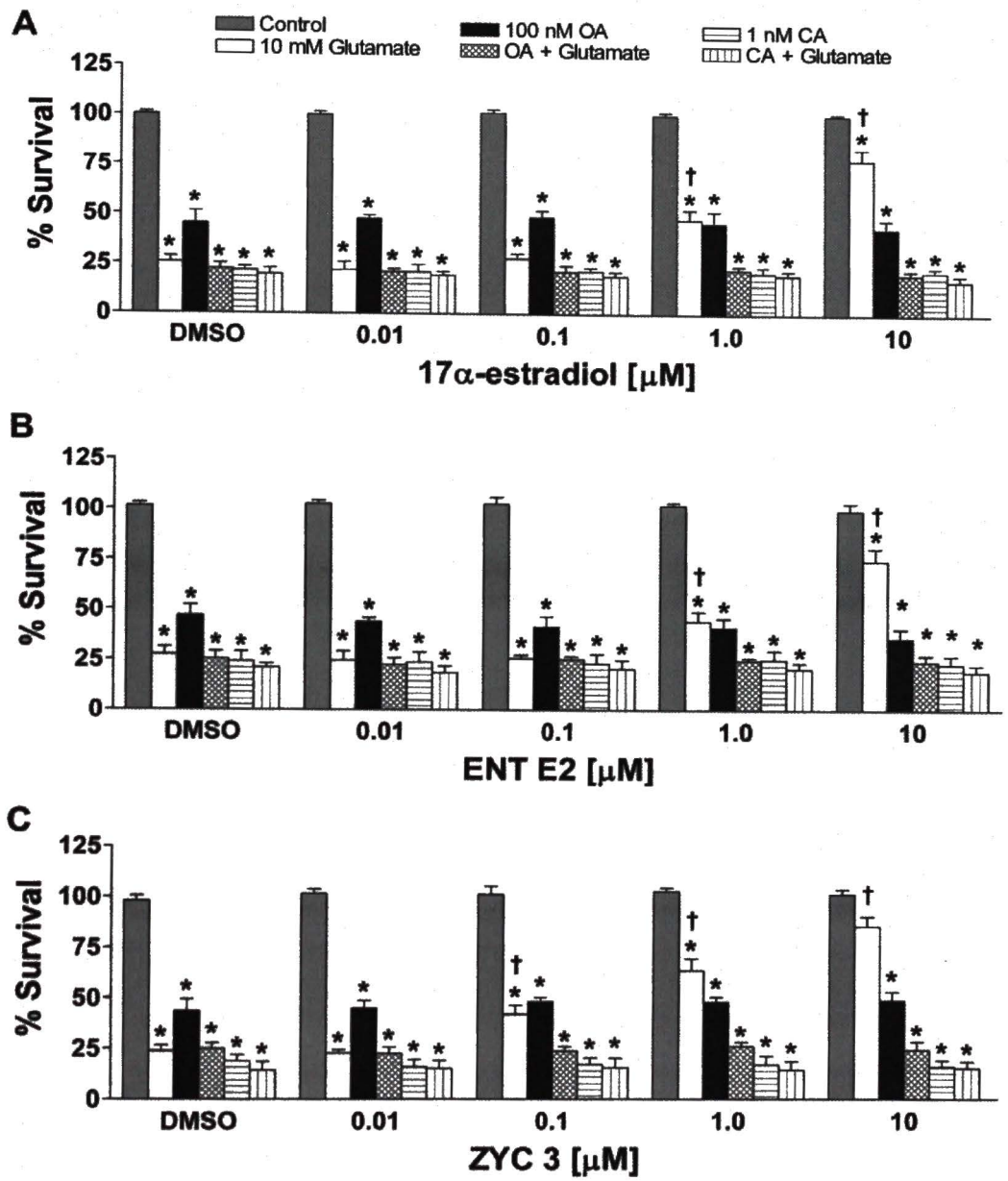

Figure 12. Two hour pretreatment with 17α-estradiol, ENT E2, or ZYC3 does not attenuate calyculin A mediated abolishment of estrogen-mediated neuroprotection against glutamate toxicity in HT-22. HT-22 cells were seeded into 96-well plates at a density of 3,500 cells/well. **A)** Cells were pretreated for 2 hr with varying concentrations of 17α-estradiol then treated with 100 nM OA, 1 nM CA, and/or 10 mM glutamate. **B)** Cells were pretreated for 2 hr with varying concentrations of ENT E2 then treated with 100 nM OA, 1 nM CA, and/or 10 mM glutamate. **C)** Cells were pretreated for 2 hr with varying concentrations of ZYC3 then treated with 100 nM OA, 1 nM CA, and/or 10 mM glutamate. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 13

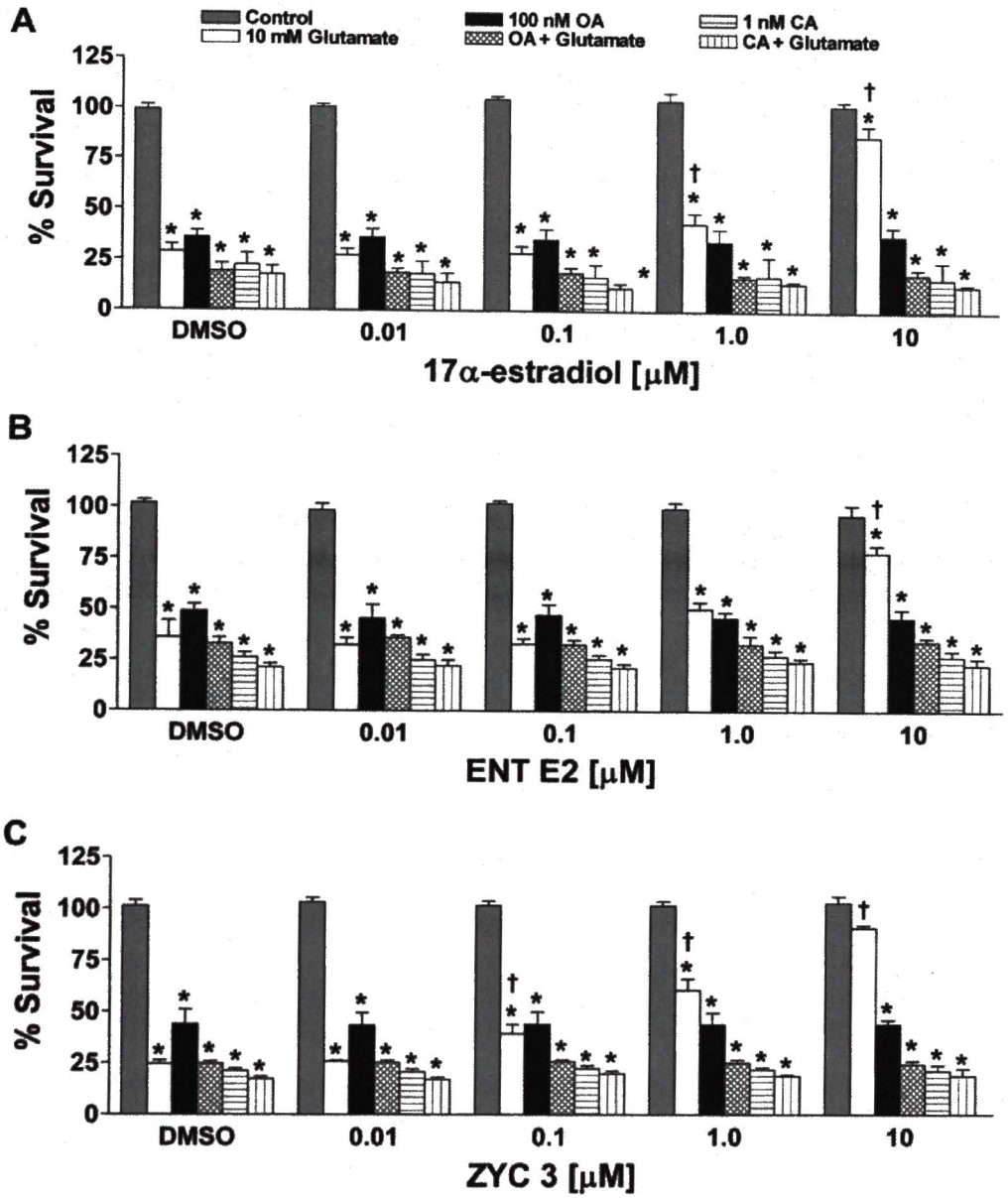

Figure 13. Twenty-four hour pretreatment with 17α-estradiol, ENT E2, or ZYC3 does not attenuate calyculin A mediated abolishment of estrogen-mediated neuroprotection against glutamate toxicity in HT-22. HT-22 cells were seeded into 96-well plates at a density of 3,500 cells/well. **A**) Cells were pretreated for 24 hr with varying concentrations of 17α-estradiol then treated with 100 nM OA, 1 nM CA, and/or 10 mM glutamate. **B**) Cells were pretreated for 24 hr with varying concentrations of ENT E2 then treated with 100 nM OA, 1 nM CA, and/or 10 mM glutamate. **C**) Cells were pretreated for 24 hr with varying concentrations of ZYC3 then treated with 100 nM OA, 1 nM CA, and/or 10 mM glutamate. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 14

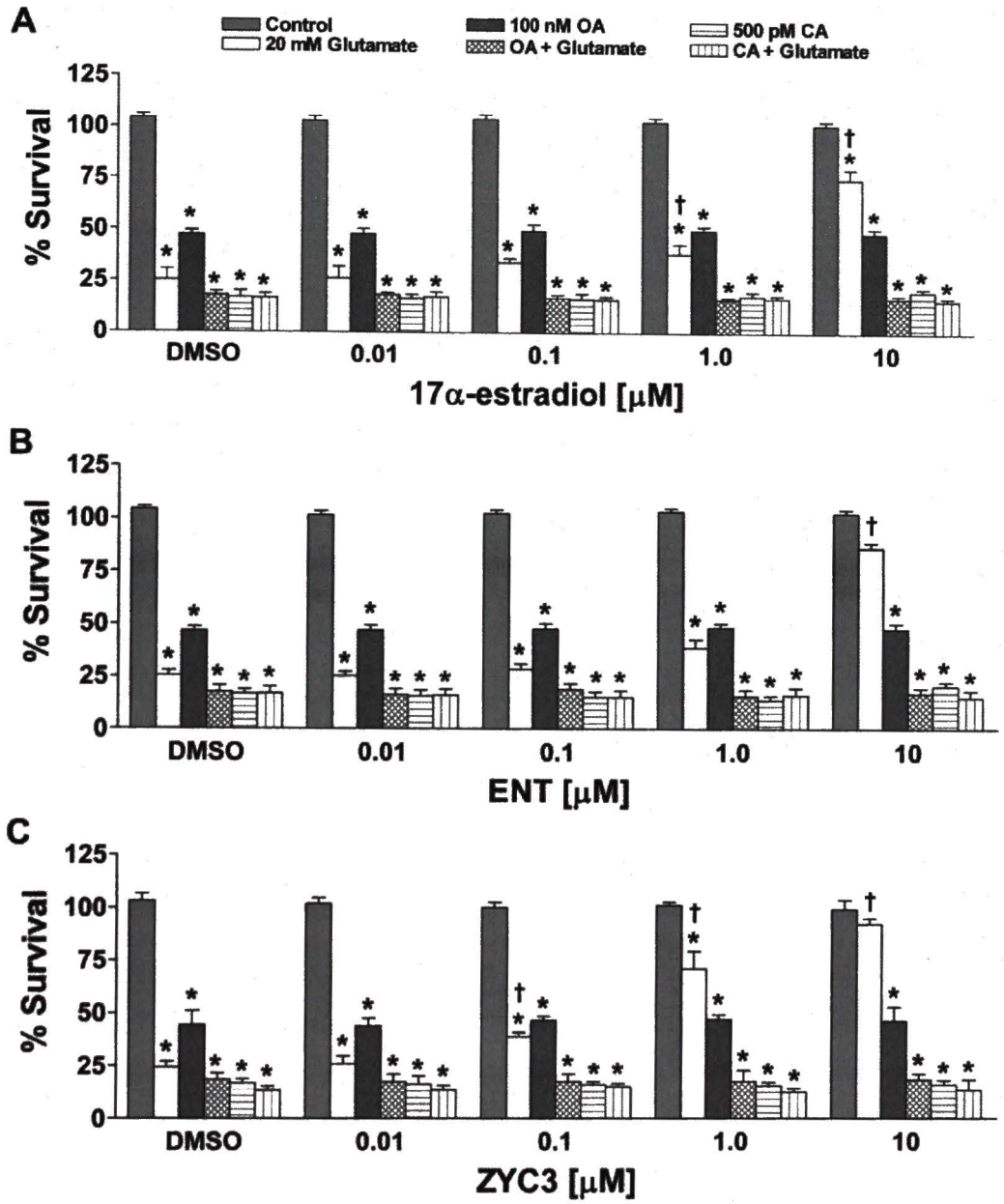

Figure 14. Effects of okadaic acid and calyculin A on 17α-estradiol, ENT E2, or ZYC3 mediated neuroprotection in C6-glioma cells. C6-glioma cells were seeded into 96-well plates at a density of 3,500 cells/well. **A**) Cells were treated simultaneously for 24 hr with varying concentrations of 17α-estradiol, 100 nM OA, 500 pM CA, and/or 20 mM glutamate. **B**) Cells were treated simultaneously for 24 hr with varying concentrations of ENT E2, 100 nM OA, 500 pM CA, and/or 20 mM glutamate. **C**) Cells were treated simultaneously for 24 hr with varying concentrations of ZYC3, 100 nM OA, 500 pM CA, and/or 20 mM glutamate. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 15

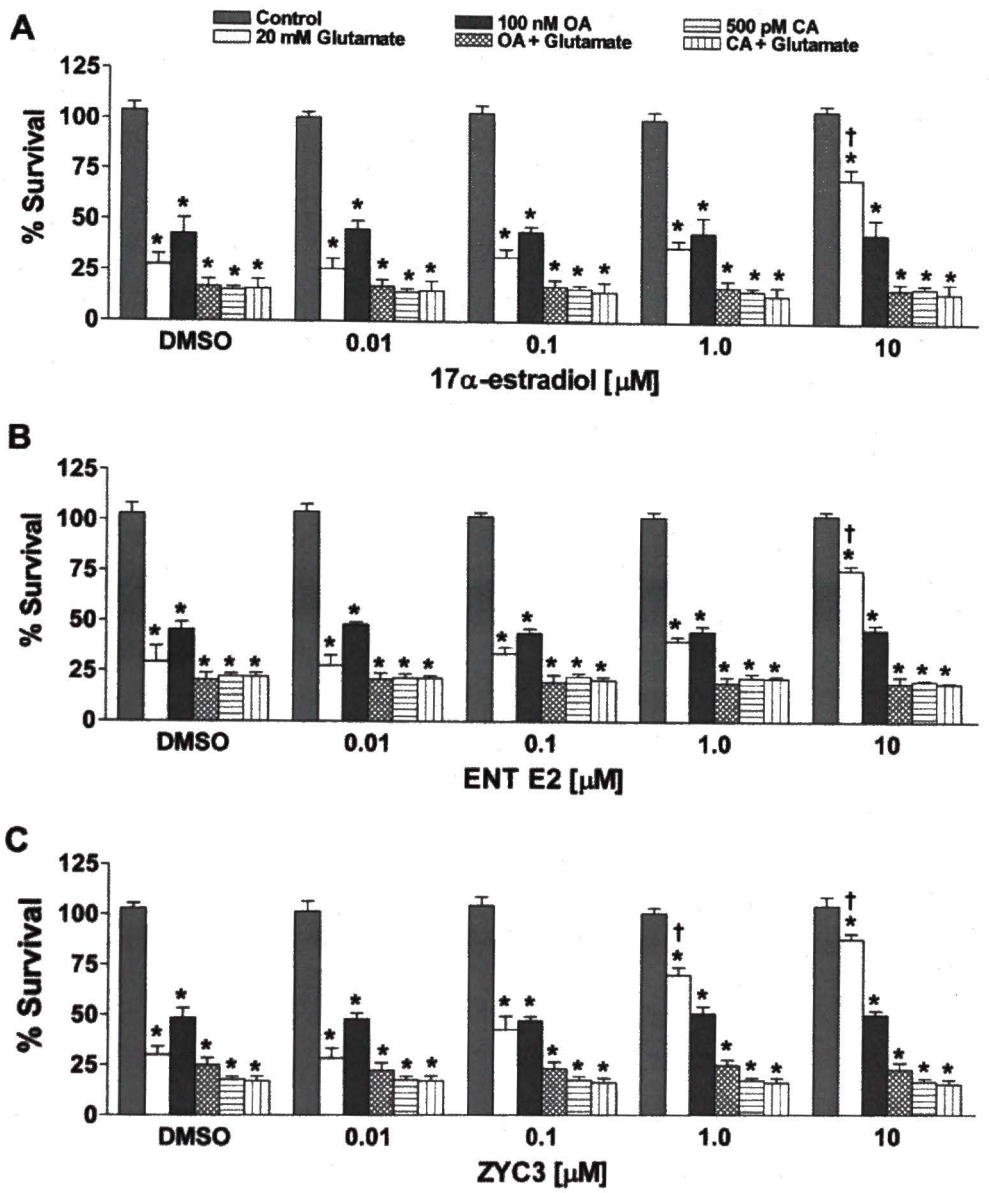

Figure 15. Two hour pretreatment with 17α-estradiol, ENT E2, or ZYC3 does not attenuate calyculin A mediated abolishment of estrogen-mediated neuroprotection against glutamate toxicity in C6-glioma. C6-glioma cells were seeded into 96-well plates at a density of 3,000 cells/well. **A**) Cells were pretreated for 2 hr with varying concentrations of 17α-estradiol then treated with 100 nM OA, 500 pM CA, and/or 10 mM glutamate. **B**) Cells were pretreated for 2 hr with varying concentrations of ENT E2 then treated with 100 nM OA, 500 pM CA, and/or 10 mM glutamate. **C**) Cells were pretreated for 2 hr with varying concentrations of ZYC3 then treated with 100 nM OA, 500 pM CA, and/or 10 mM glutamate. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 16

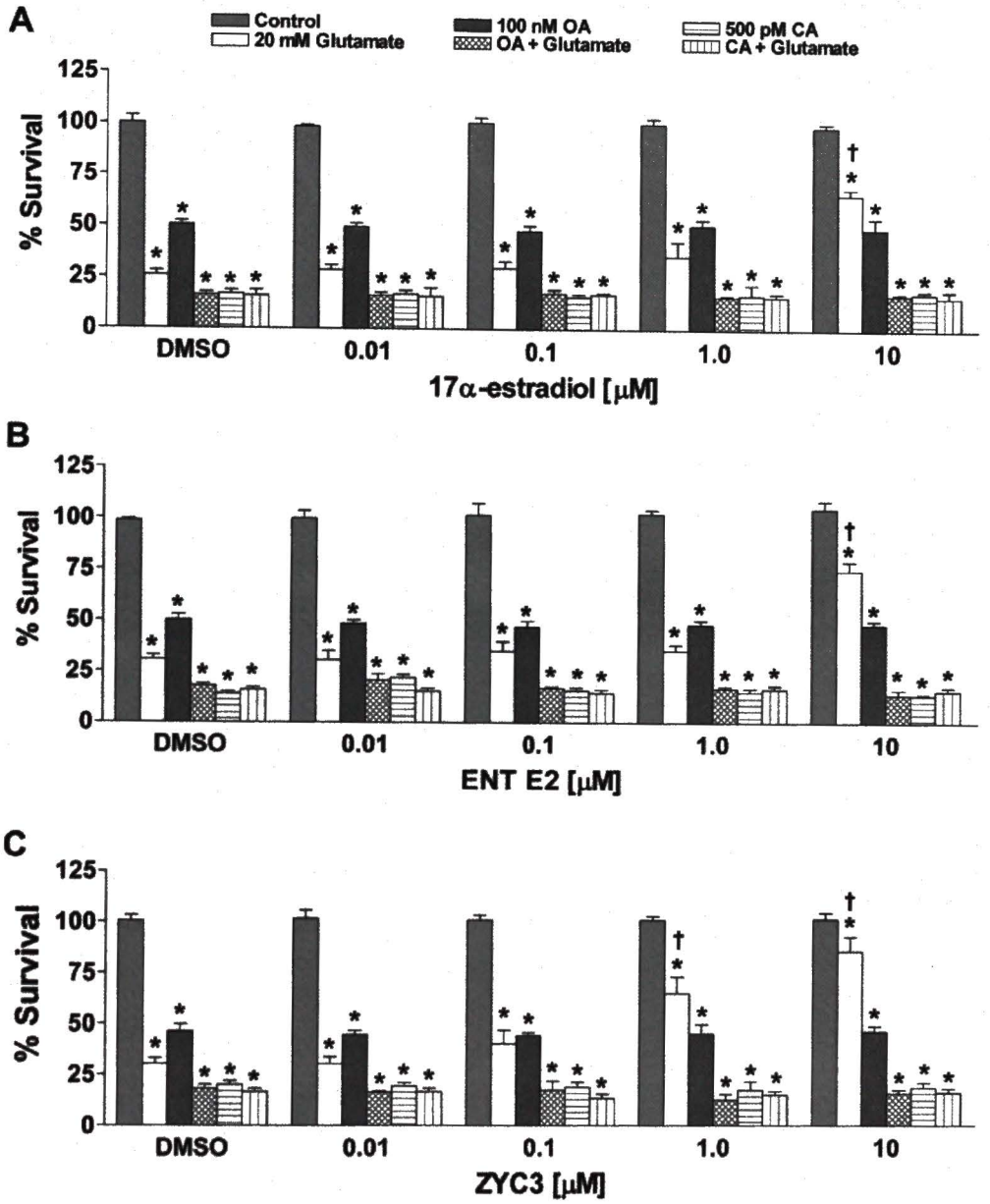

Figure 16. Twenty-four hour pretreatment with 17α-estradiol, ENT E2, or ZYC3 does not attenuate calyculin A mediated abolishment of estrogen-mediated neuroprotection against glutamate toxicity in C6-glioma. C6-glioma cells were seeded into 96-well plates at a density of 3,500 cells/well. **A**) Cells were pretreated for 24 hr with varying concentrations of 17α-estradiol then treated with 100 nM OA, 500 pM CA, and/or 10 mM glutamate. **B**) Cells were pretreated for 24 hr with varying concentrations of ENT E2 then treated with 100 nM OA, 500 pM CA, and/or 10 mM glutamate. **C**) Cells were pretreated for 24 hr with varying concentrations of ZYC3 then treated with 100 nM OA, 500 pM CA, and/or 10 mM glutamate. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 17

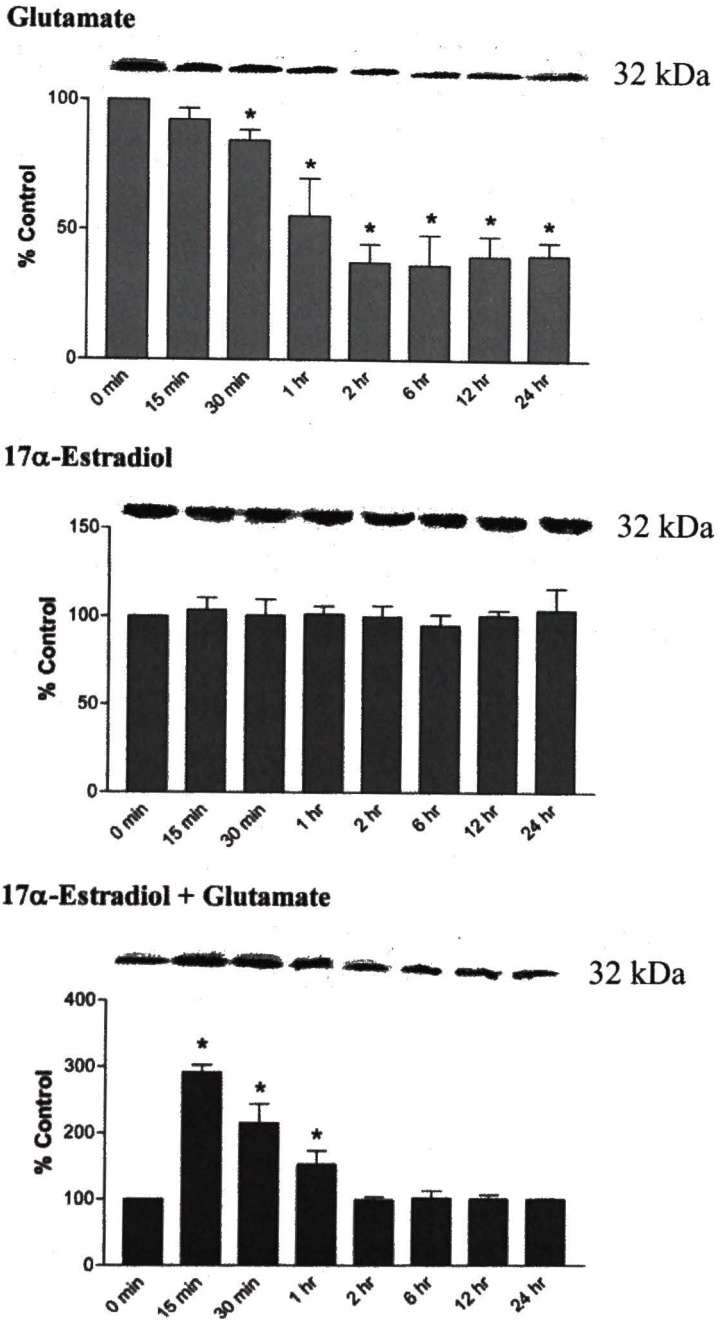

Figure 17. Time course of the effects 17α-estradiol, glutamate, and their combination on PP1 protein levels in primary cortical neurons. Primary cortical neurons were treated with either 50 μM glutamate (*A*) or 100 nM 17α-estradiol (*B*) and simultaneously with 50 μM glutamate and 100 nM 17α-estradiol (*C*). Cells were harvested at the times indicated for Western blot analysis of PP1. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 18

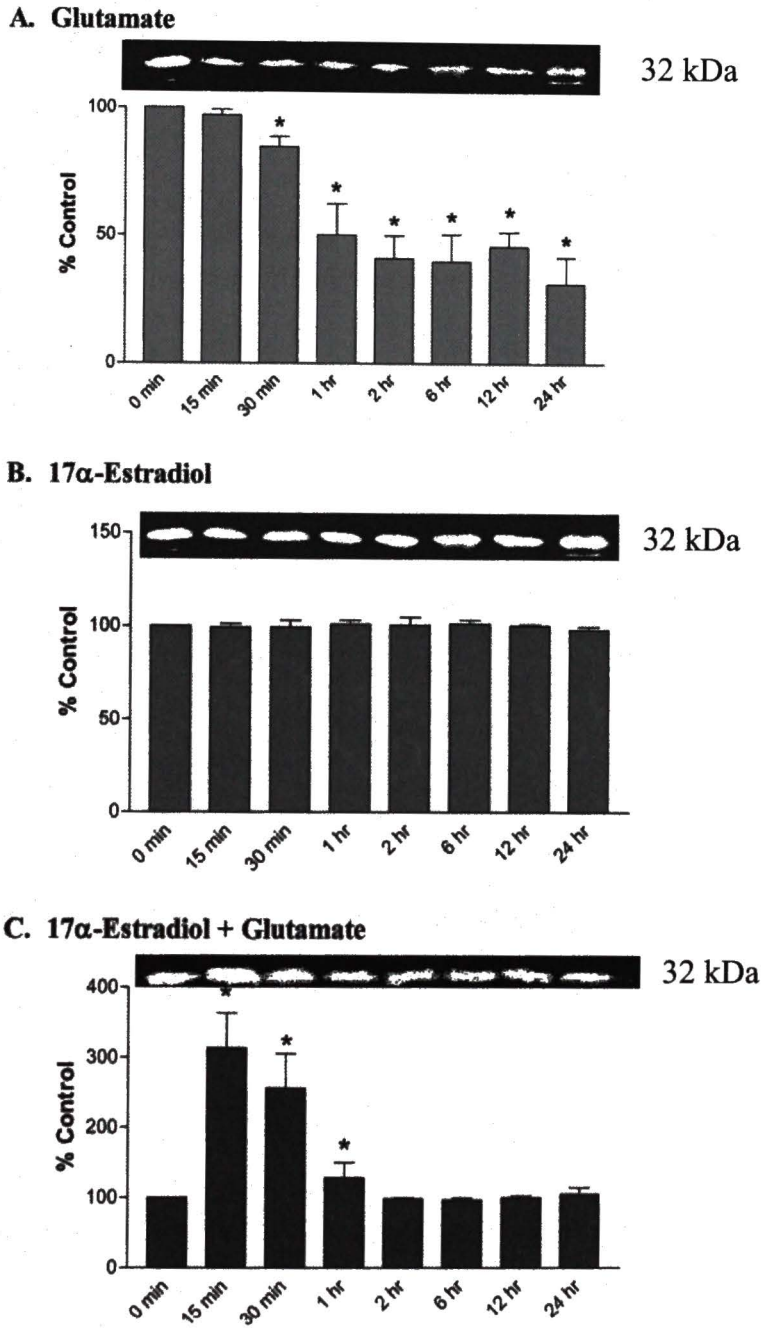

Figure 18. Time course of the effects 17α-estradiol, glutamate, and their combination on PP2A protein levels in primary cortical neurons. Primary cortical neurons were treated with either 50 μM glutamate (*A*) or 100 nM 17α-estradiol (*B*) and simultaneously with 50 μM glutamate and 100 nM 17α-estradiol (*C*). Cells were harvested at the times indicated for Western blot analysis of PP2A. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 19

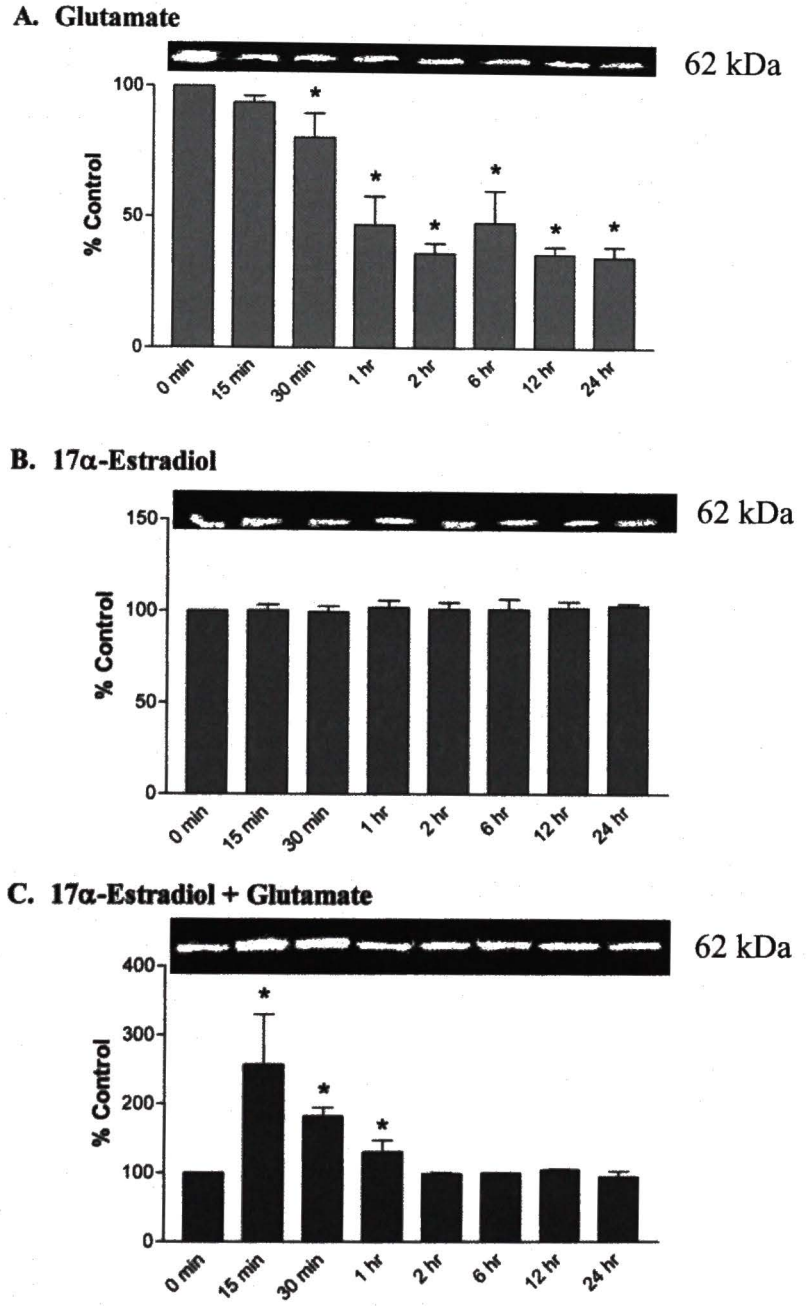

Figure 19. Time course of the effects 17α-estradiol, glutamate, and their combination on PP2B protein levels in primary cortical neurons. Primary cortical neurons were treated with either 50 μM glutamate (*A*) or 100 nM 17α-estradiol (*B*) and simultaneously with 50 μM glutamate and 100 nM 17α-estradiol (*C*). Cells were harvested at the times indicated for Western blot analysis of PP2B. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 20

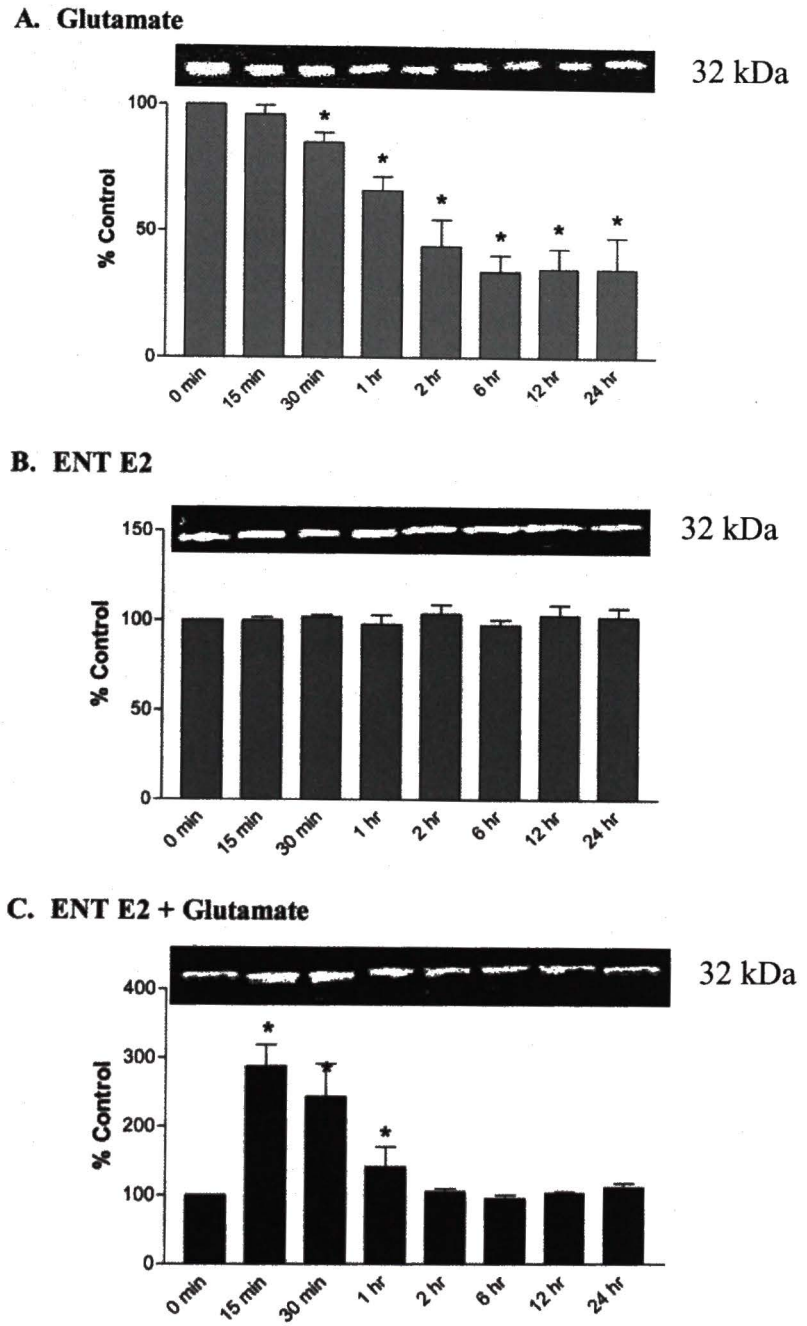

Figure 20. Time course of the effects ENT E2, glutamate, and their combination on PP1 protein levels in primary cortical neurons. Primary cortical neurons were treated with either 50 μM glutamate (***A***) or 100 nM ENT E2 (***B***) and simultaneously with 50 μM glutamate and 100 nM ENT E2 (***C***). Cells were harvested at the times indicated for Western blot analysis of PP1. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 21

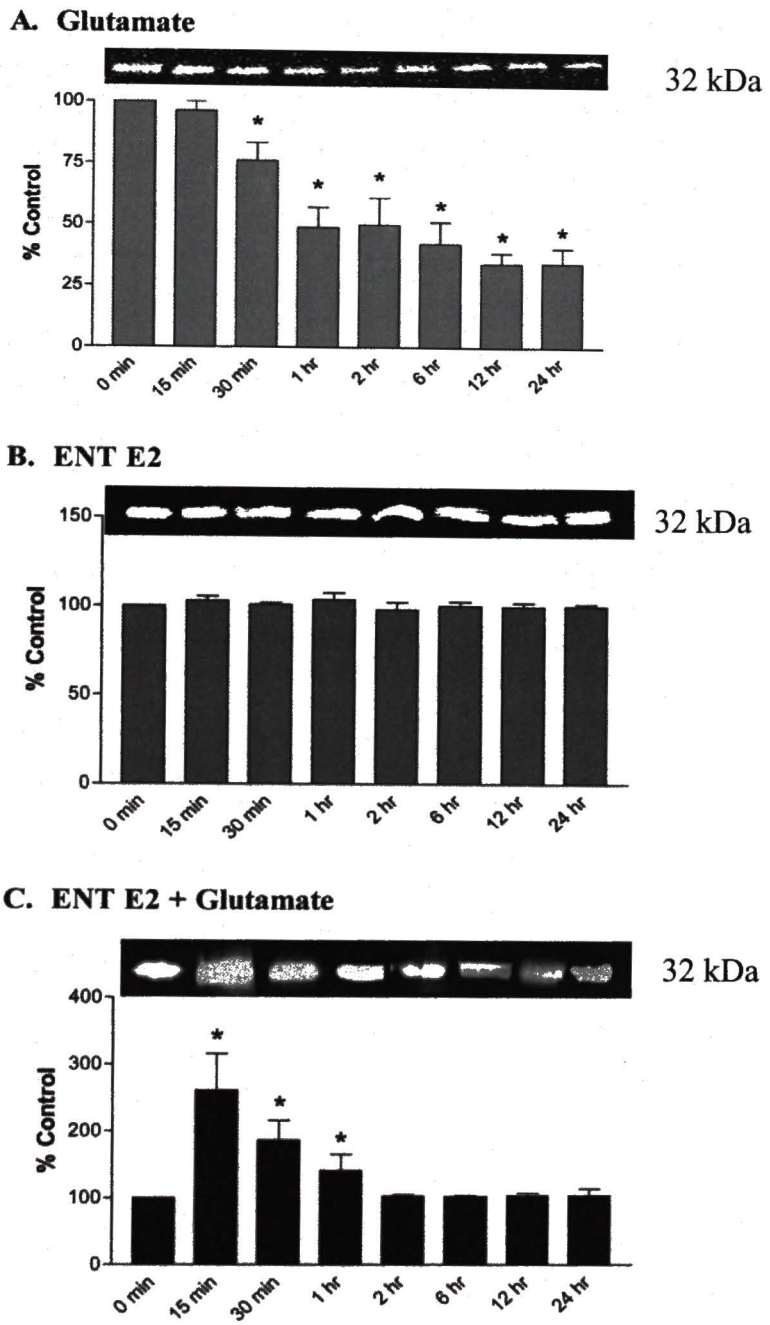

Figure 21. Time course of the effects ENT E2, glutamate, and their combination on PP2A protein levels in primary cortical neurons. Primary cortical neurons were treated with either 50 μM glutamate (***A***) or 100 nM ENT E2 (***B***) and simultaneously with 50 μM glutamate and 100 nM ENT E2 (***C***). Cells were harvested at the times indicated for Western blot analysis of PP2A. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 22

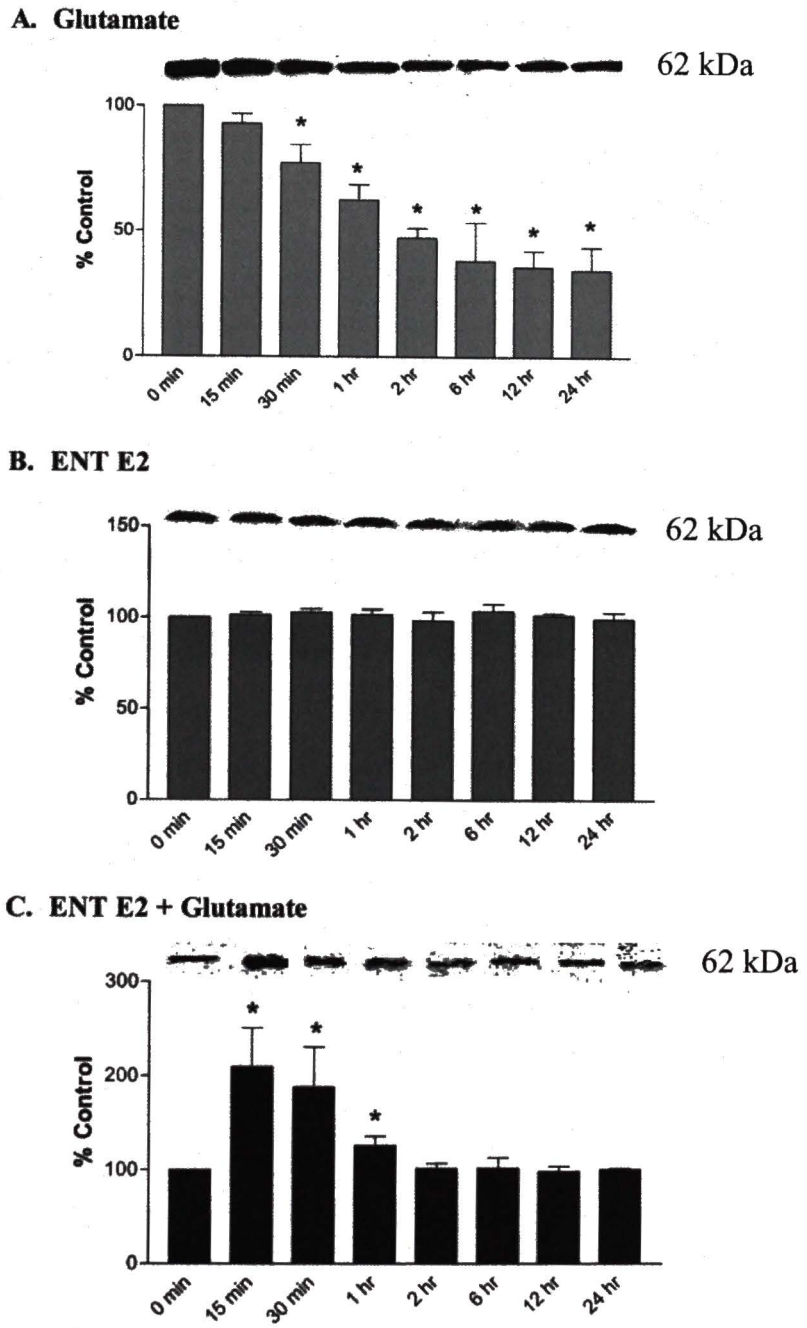

Figure 22. Time course of the effects ENT E2, glutamate, and their combination on PP2B protein levels in primary cortical neurons. Primary cortical neurons were treated with either 50 μM glutamate (***A***) or 100 nM ENT E2 (***B***) and simultaneously with 50 μM glutamate and 100 nM ENT E2 (***C***). Cells were harvested at the times indicated for Western blot analysis of PP2B. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 23

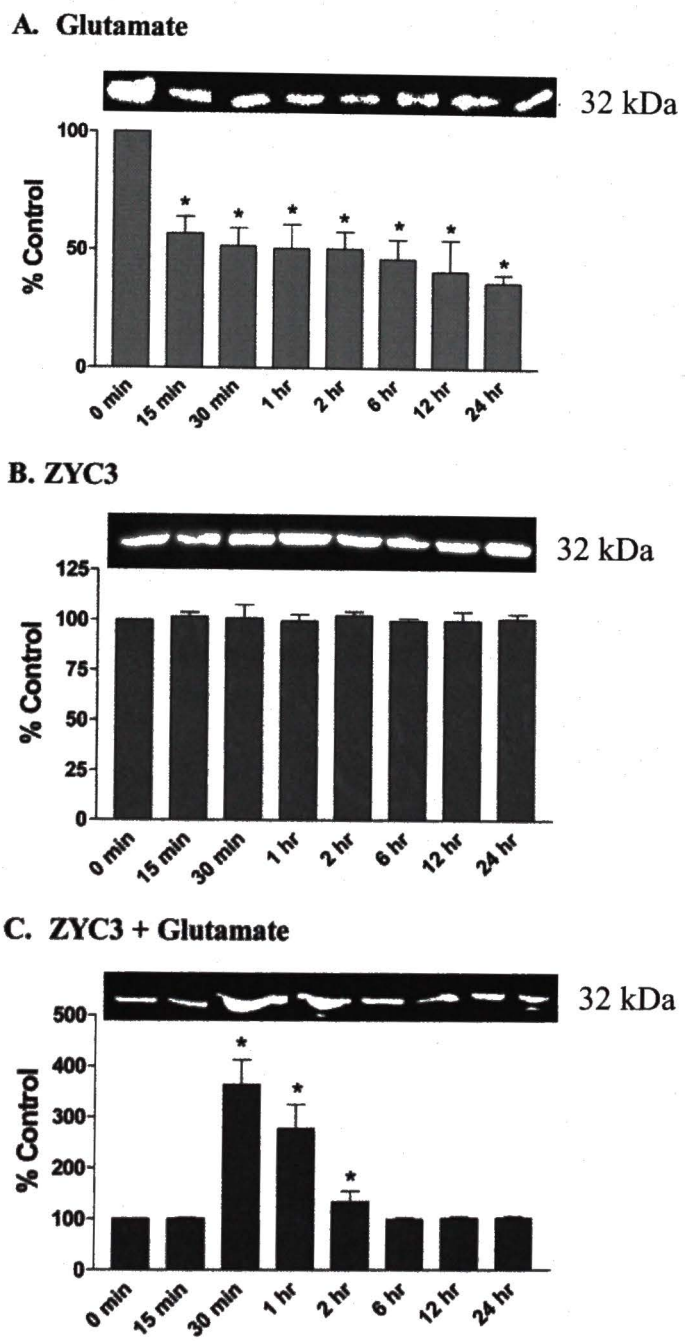

Figure 23. Time course of the effects ZYC3, glutamate, and their combination on PP2A protein levels in primary cortical neurons. Primary cortical neurons were treated with either 50 μM glutamate (***A***) or 10 nM ZYC3 (***B***) and simultaneously with 50 μM glutamate and 10 nM ZYC3 (***C***). Cells were harvested at the times indicated for Western blot analysis of PP2A. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 24

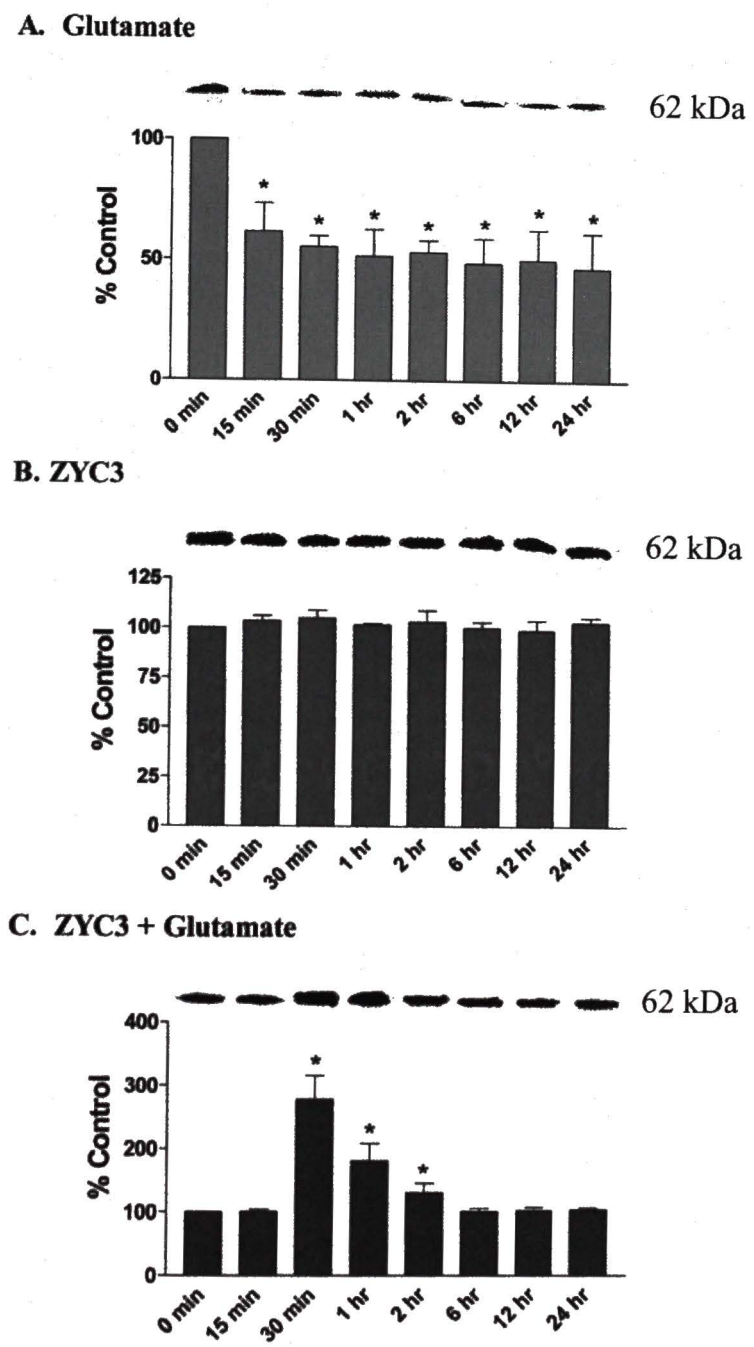

Figure 24. Time course of the effects ZYC3, glutamate, and their combination on PP2B protein levels in primary cortical neurons. Primary cortical neurons were treated with either 50 μM glutamate (***A***) or 10 nM ZYC3 (***B***) and simultaneously with 50 μM glutamate and 10 nM ZYC3 (***C***). Cells were harvested at the times indicated for Western blot analysis of PP2B. The graphs represent relative OD as a percentage of time 0 control and were normalized to β-actin (not shown). Data are represented as mean ± SEM for $n = 3$. $^{*}p < 0.05$ versus time 0 control.

Figure 25

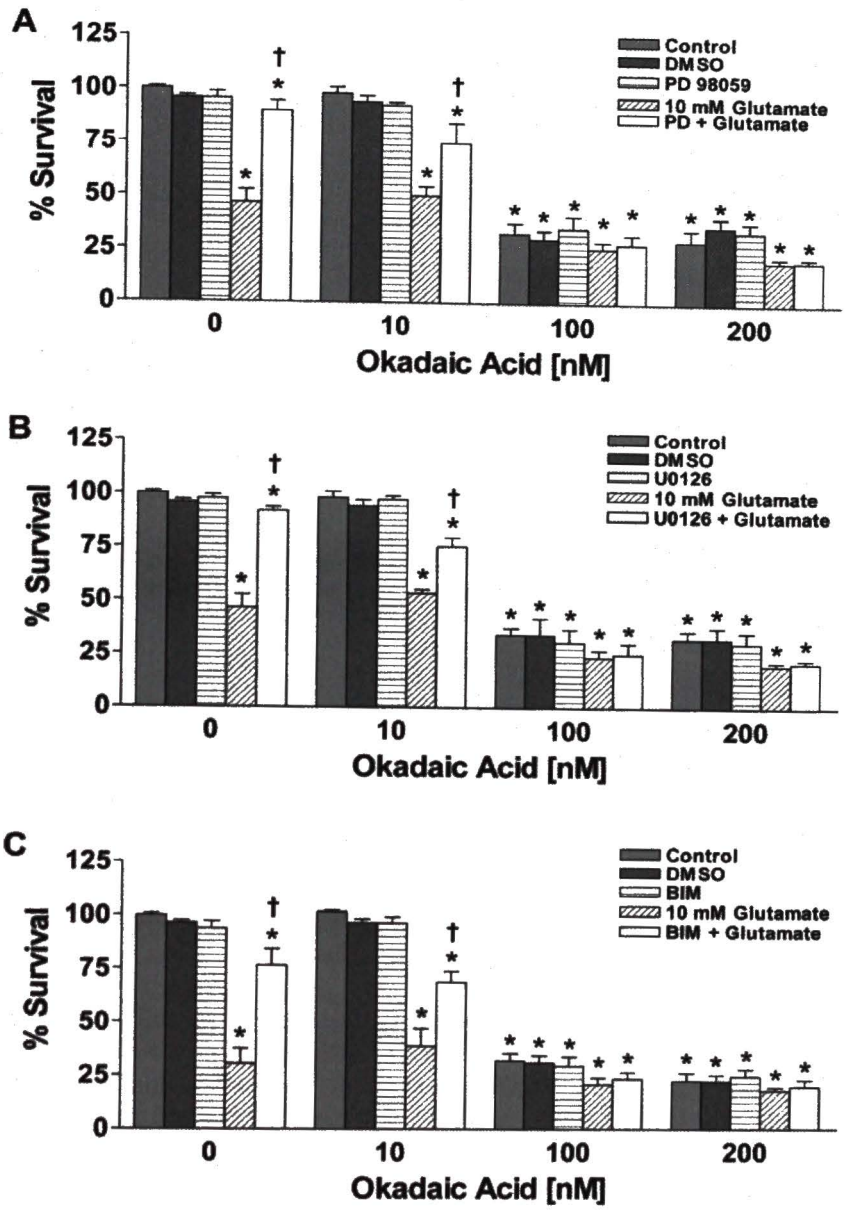

Figure 25. Effects of glutamate, okadaic acid and/or various protein kinase inhibitors on cell viability in primary cortical neurons. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. **A**) Cells were treated simultaneously with 50μM glutamate, 50μM PD98059 and/or varying concentrations of okadaic acid. **B**) Cells were treated simultaneously with 50μM, 10 μM U0126 and/or varying concentrations of okadaic acid. **C**) Cells were treated simultaneously with 50μM glutamate, 1 μM BIM and/or varying concentrations of okadaic acid. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 26

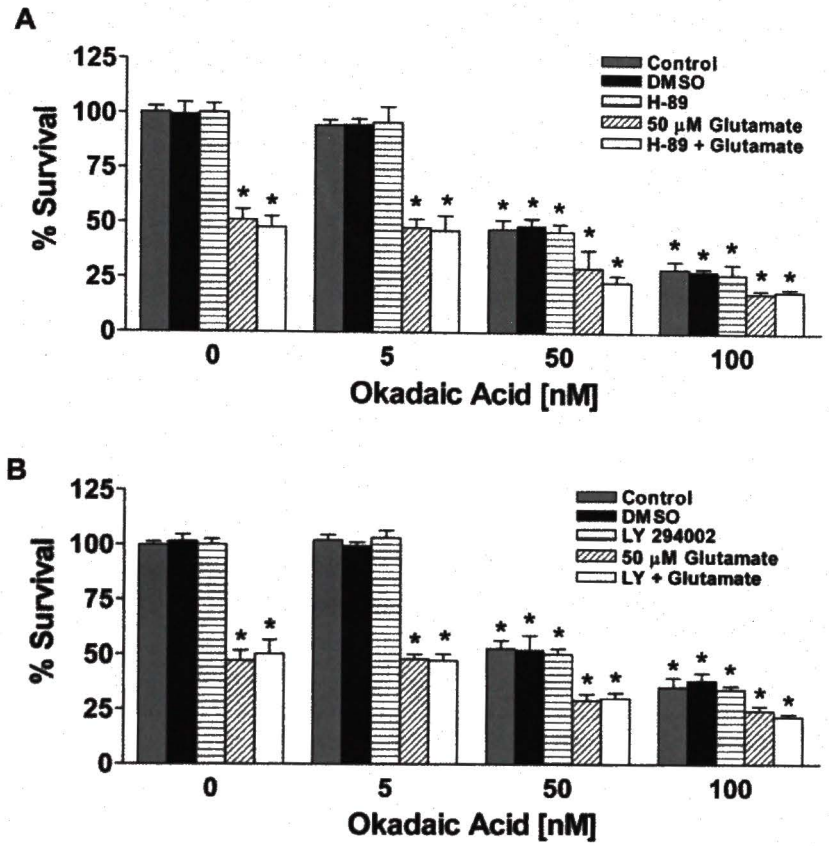

Figure 26. Effects of glutamate, okadaic acid and/or various protein kinase inhibitors on cell viability in primary cortical neurons. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. **A**) Cells were treated simultaneously with 50µM glutamate, 50µM H-89 and/or varying concentrations of okadaic acid. **B**) Cells were treated simultaneously with 50µM, 10 µM LY294002 and/or varying concentrations of okadaic acidCell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 27

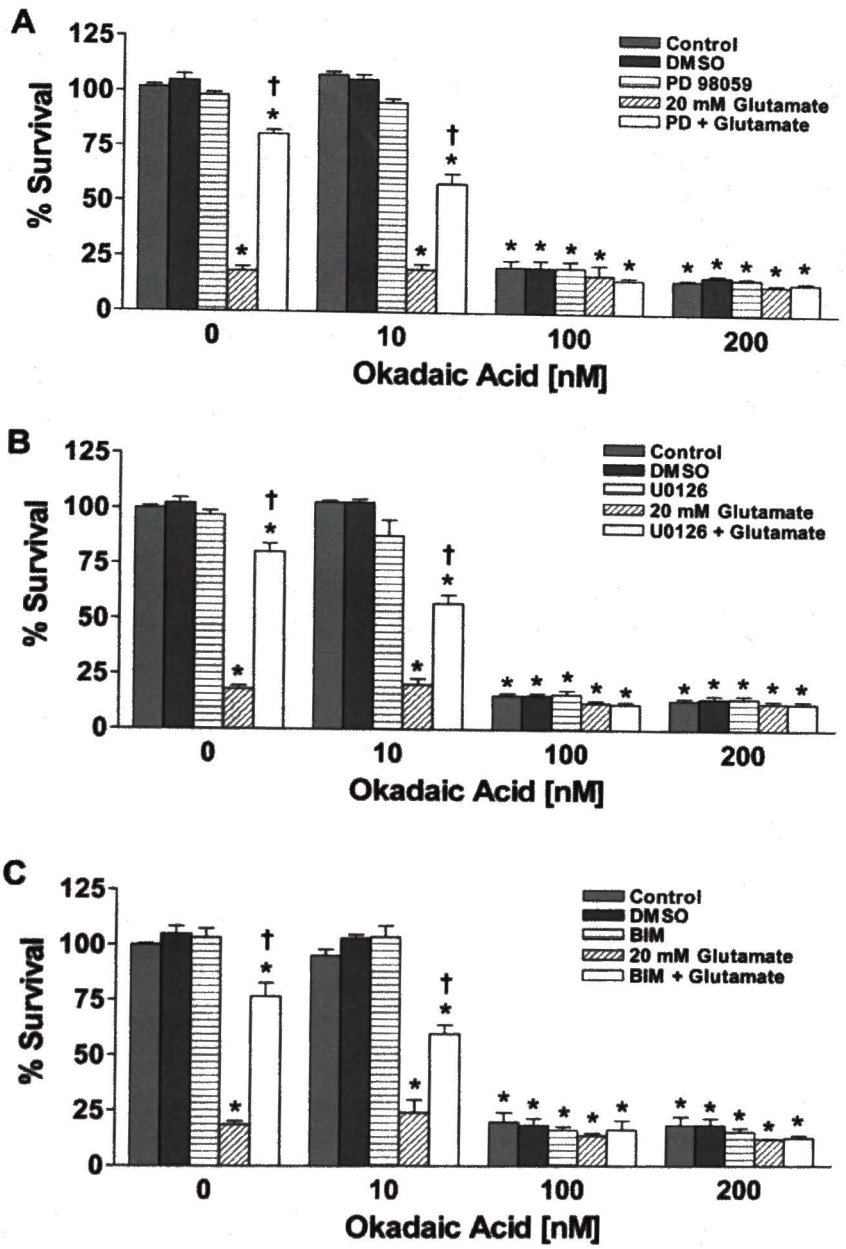

Figure 27. Effects of glutamate, okadaic acid and/or various protein kinase inhibitors on cell viability in HT-22. HT-22 cells were seeded into 96-well plates at a density of 3,500 cells/well. **A**) Cells were treated simultaneously with 10 mM glutamate, 50μM PD98059 and/or varying concentrations of okadaic acid. **B**) Cells were treated simultaneously with 10 mM glutamate, 10 μM U0126 and/or varying concentrations of okadaic acid. **C**) Cells were treated simultaneously with 10 mM glutamate, 1 μM BIM and/or varying concentrations of okadaic acid. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 28

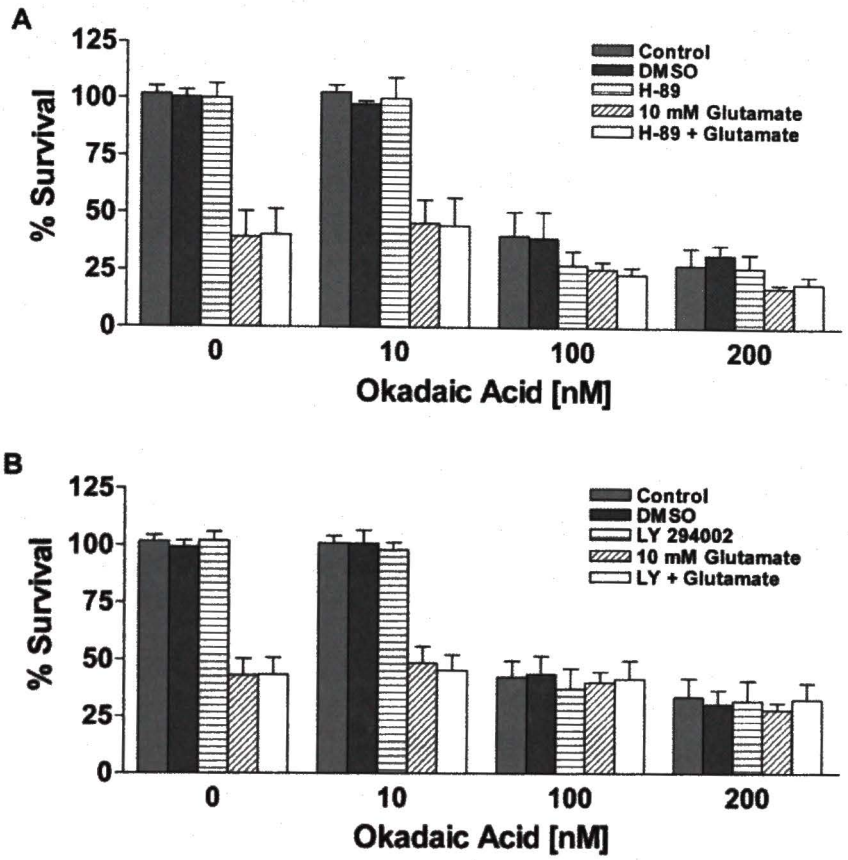

Figure 28. Effects of glutamate, okadaic acid and/or various protein kinase inhibitors on cell viability in HT-22. HT-22 cells were seeded into 96-well plates at a density of 3,500 cells/well. **A**) Cells were treated simultaneously with 10 mM glutamate, 50μH-89 and/or varying concentrations of okadaic acid. **B**) Cells were treated simultaneously with 10 mM glutamate, 10 μM LY294002 and/or varying concentrations of okadaic acid. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 29

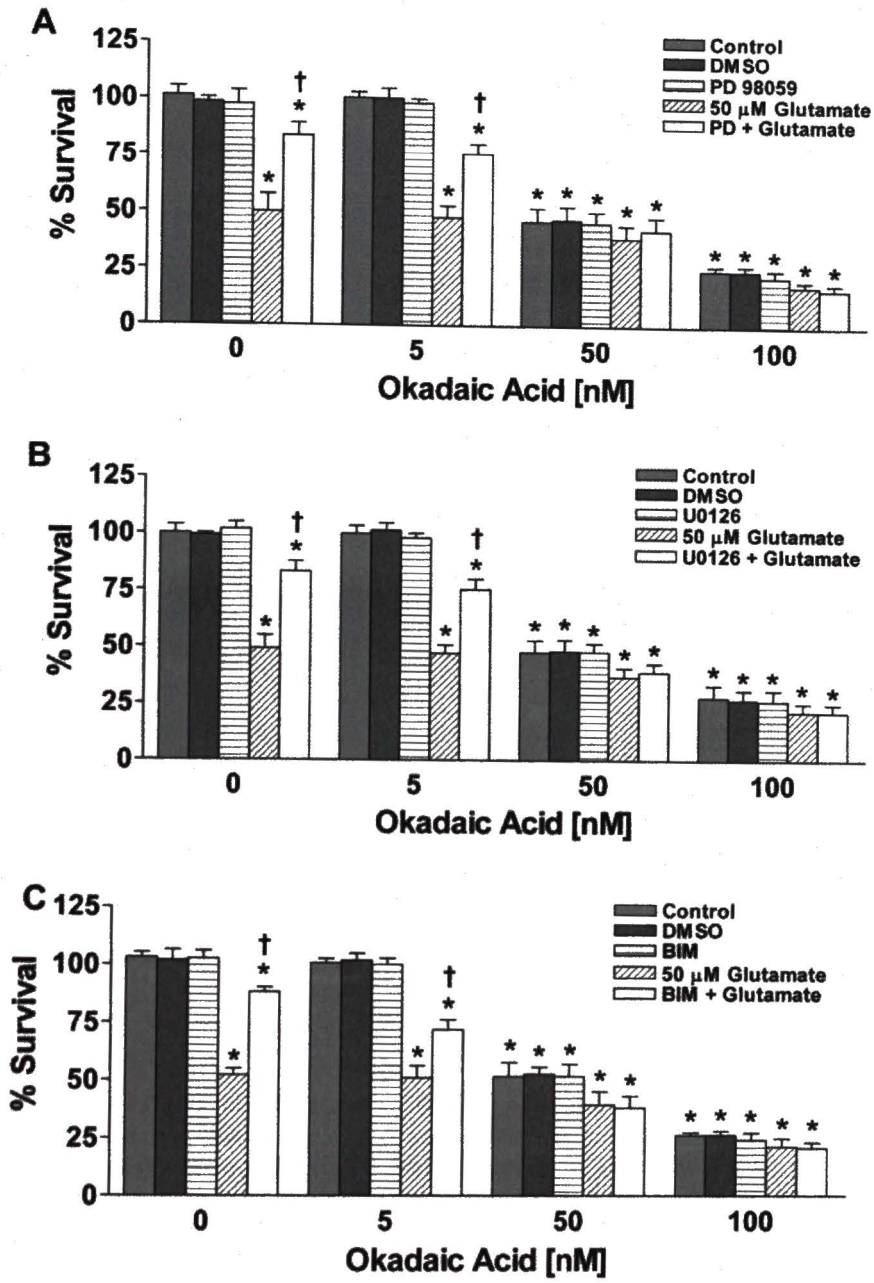

Figure 29. Effects of glutamate, okadaic acid and/or various protein kinase inhibitors on cell viability in C6. C6-glioma cells were seeded into 96-well plates at a density of 3,500 cells/well. **A**) Cells were treated simultaneously with 20 mM glutamate, 50μM PD98059 and/or varying concentrations of okadaic acid. **B**) Cells were treated simultaneously with 20 mM glutamate, 10 μM U0126 and/or varying concentrations of okadaic acid. **C**) Cells were treated simultaneously with 20 mM glutamate, 1 μM BIM and/or varying concentrations of okadaic acid. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 30

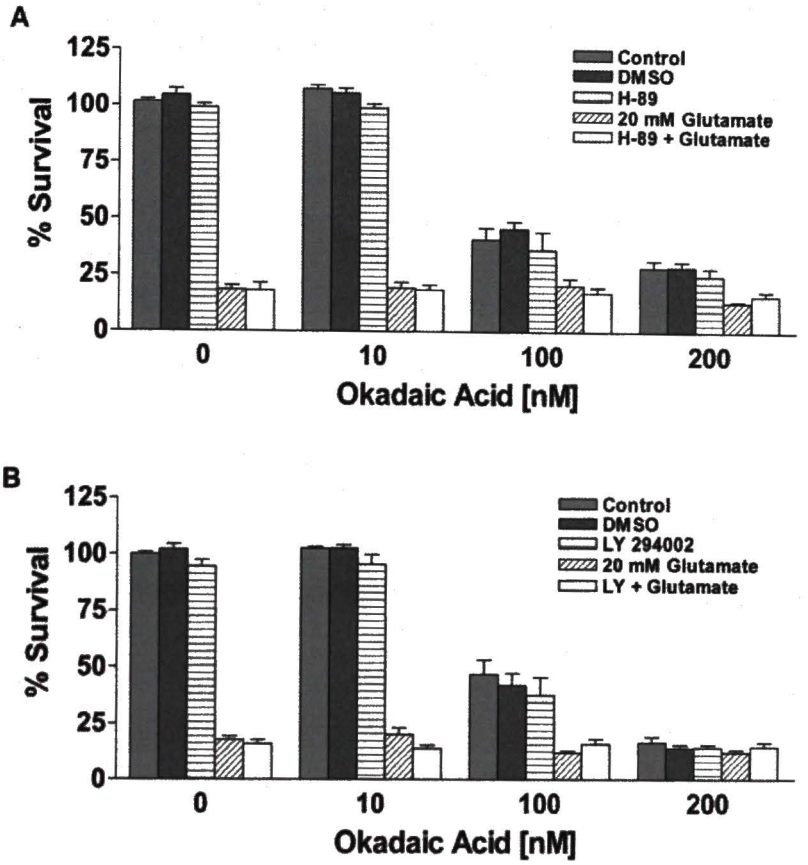

Figure 30. Effects of glutamate, okadaic acid and/or various protein kinase inhibitors on cell viability in C6. C6-glioma cells were seeded into 96-well plates at a density of 3,500 cells/well. **A**) Cells were treated simultaneously with 20 mM glutamate, 50μM H-89 and/or varying concentrations of okadaic acid. **B**) Cells were treated simultaneously with 20 mM glutamate, 10 μM LY294002 and/or varying concentrations of okadaic acid. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 31

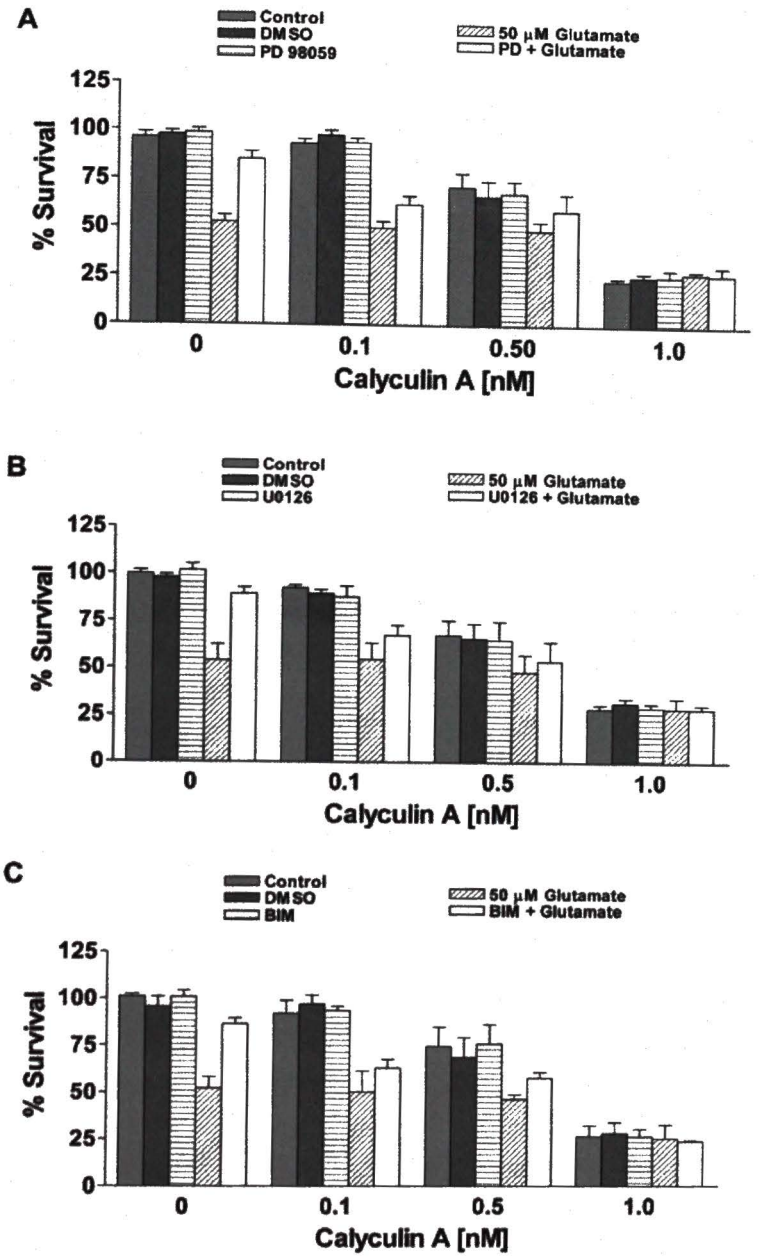

Figure 31. Effects of glutamate, caylculin A and/or various protein kinase inhibitors on cell viability in primary cortical neurons. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. **A**) Cells were treated simultaneously with 50 μM glutamate, 50 μM PD98059 and/or varying concentrations of calyculin A. **B**) Cells were treated simultaneously with 50 μM glutamate, 10 μM U0126 and/or varying concentrations of calyculin A. **C**) Cells were treated simultaneously with 50 μM glutamate, 1 μM BIM and/or varying concentrations of calyculin A. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 32

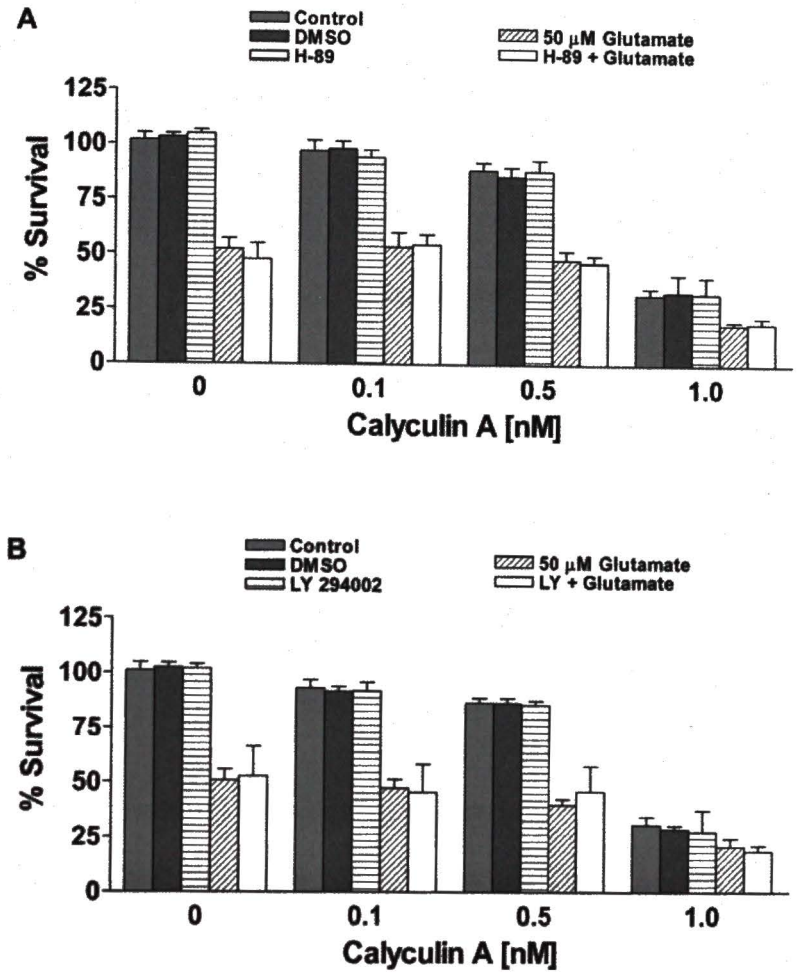

Figure 32. Effects of glutamate, calyculin A and/or various protein kinase inhibitors on cell viability primary cortical neurons. Primary cortical neurons were seeded into 96-well plates at a density of 25,000 cells/well. **A**) Cells were treated simultaneously with 50 µM glutamate, 1 µM H-89 and/or varying concentrations of calyculin A. **B**) Cells were treated simultaneously with 50 µM glutamate, 50 µM LY294002 and/or varying concentrations of okadaic acid. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 33

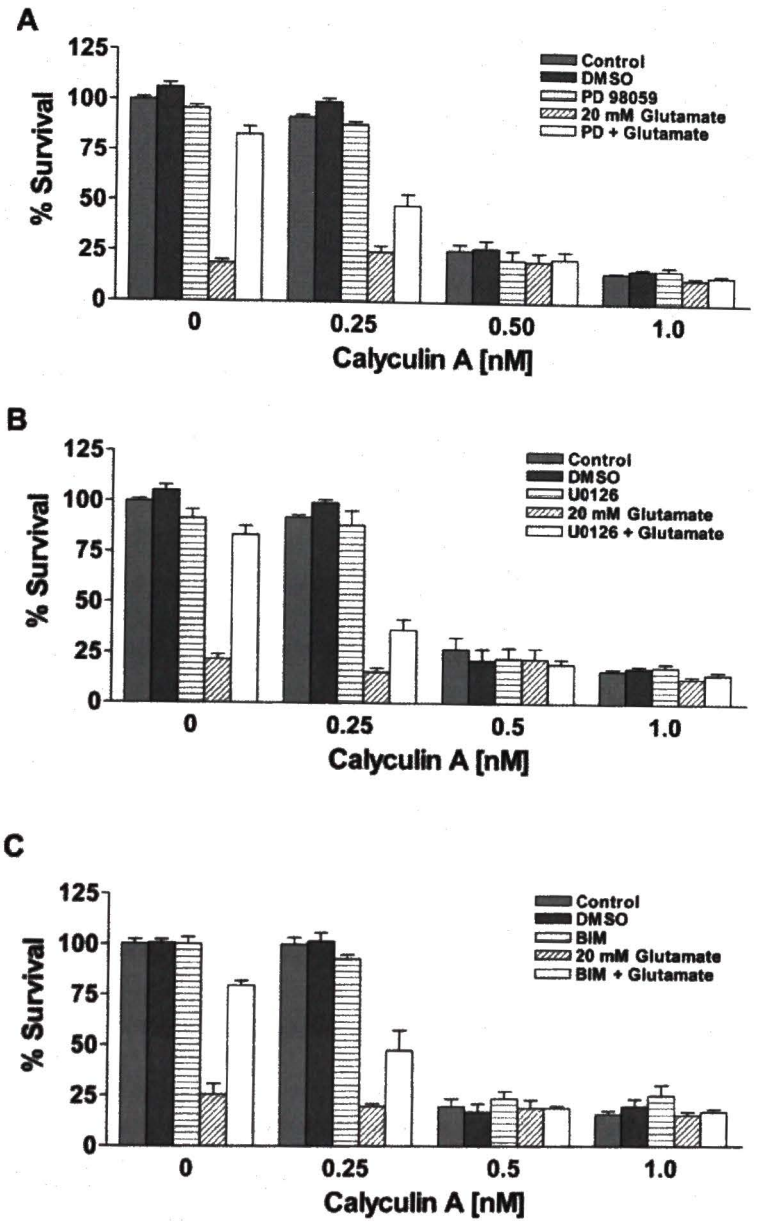

Figure 33. Effects of glutamate, calyculin A and/or various protein kinase inhibitors on cell viability in HT-22 cells. HT-22 cells were seeded into 96-well plates at a density of 3,500 cells/well. **A)** Cells were treated simultaneously with 10 mM glutamate, 50 μM PD98059 and/or varying concentrations of calyculin A. **B)** Cells were treated simultaneously with 10 mM glutamate, 10 μM U0126 and/or varying concentrations of calyculin A. **C)** Cells were treated simultaneously with 10 mM glutamate, 1 μM BIM and/or varying concentrations of calyculin A. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 34

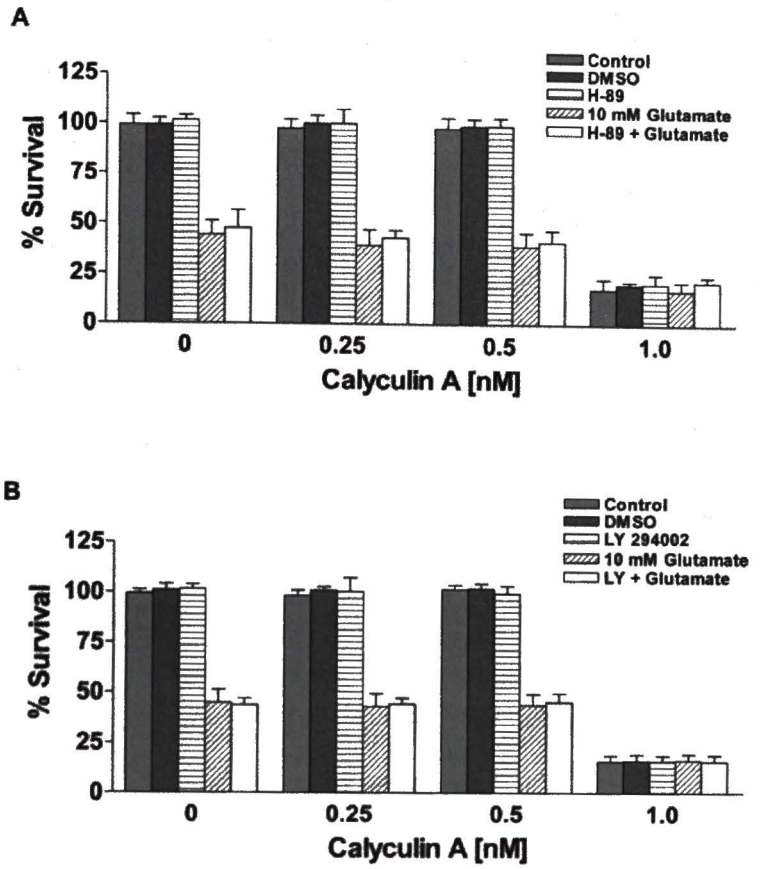

Figure 34. Effects of glutamate, okadaic acid and/or various protein kinase inhibitors on cell viability in HT-22. HT-22 cells were seeded into 96-well plates at a density of 3,500 cells/well. **A**) Cells were treated simultaneously with 10 mM glutamate 1μM H-89 and/or varying concentrations of okadaic acid. **B**) Cells were treated simultaneously with 10 mM glutamate, 50 μM LY294002 and/or varying concentrations of okadaic acid. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 35

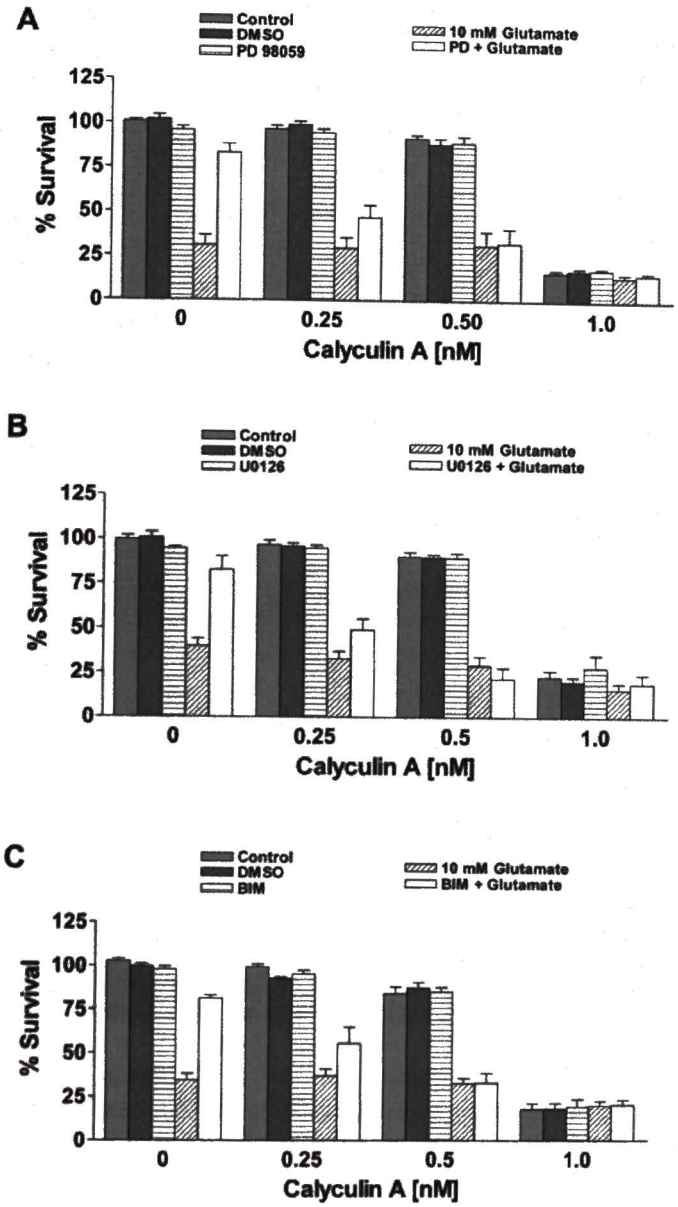

Figure 35. Effects of glutamate, calyculin A and/or various protein kinase inhibitors on cell viability in C6-glioma cells. C6-glioma cells were seeded into 96-well plates at a density of 3,500 cells/well. **A)** Cells were treated simultaneously with 20 mM glutamate, 50μM PD98059 and/or varying concentrations of calyculin A. **B)** Cells were treated simultaneously with 20 mM glutamate, 10 μM U0126 and/or varying concentrations of calyculin A. **C)** Cells were treated simultaneously with 20 mM glutamate, 1 μM BIM and/or varying concentrations of calyculin A. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 36

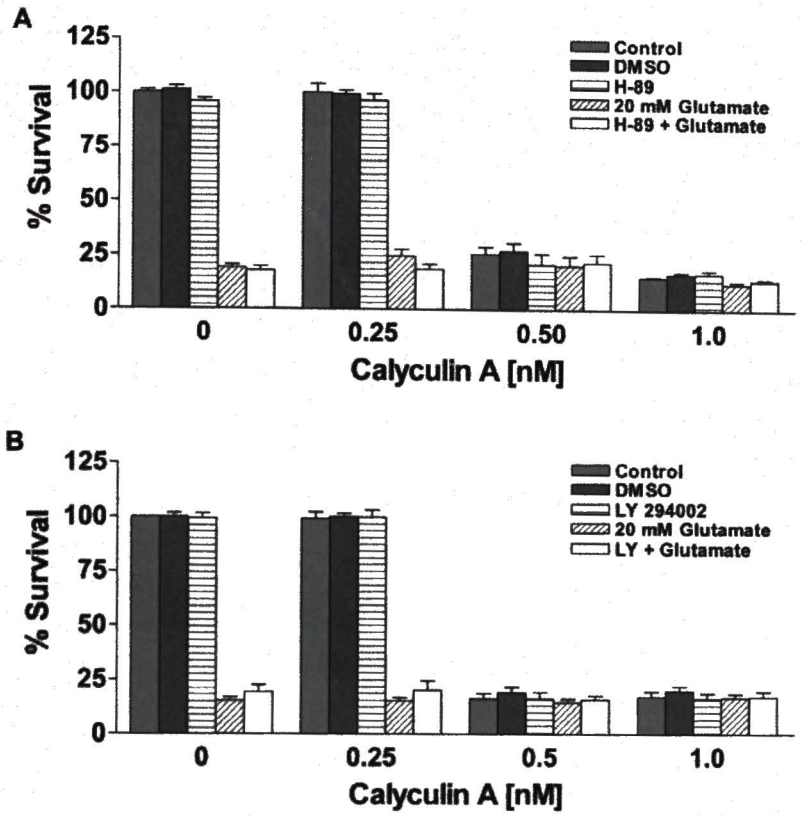

Figure 36. Effects of glutamate, calyculin A and/or various protein kinase inhibitors on cell viability in C6-glioma cells. C6-glioma cells were seeded into 96-well plates at a density of 3,500 cells/well. **A)** Cells were treated simultaneously with 20 mM glutamate, 1 μM H-89 and/or varying concentrations of calyculin A. **B)** Cells were treated simultaneously with 20 mM glutamate, 50 μM LY294002 and/or varying concentrations calyculin A. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 37

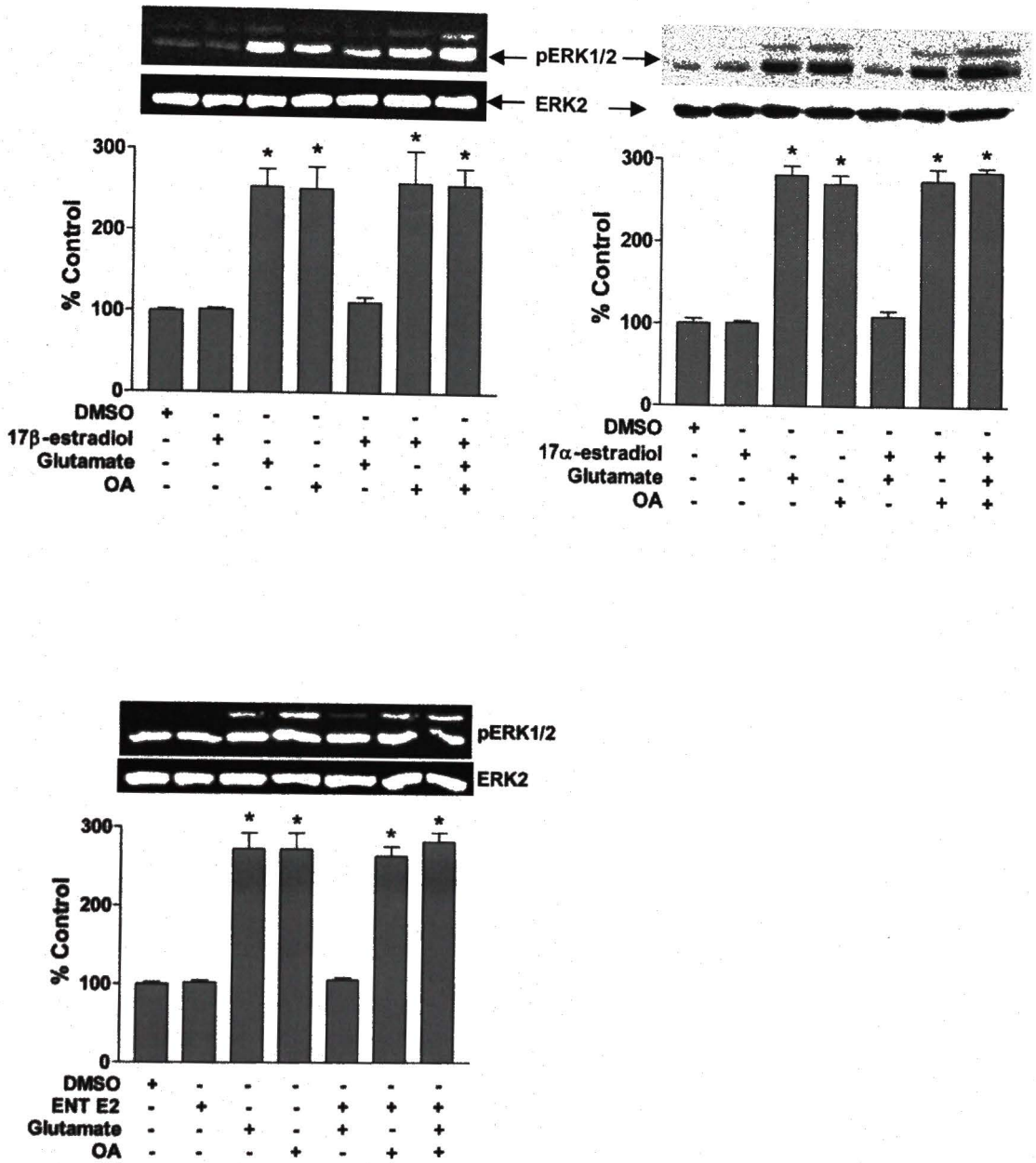

Figure 37. Phosphorylation of ERK following glutamate treatment in the presence and absence of various estrogens and/or OA. **(A)** Primary cortical neurons were treated with 50 nM okadaic acid, (OA) 50 μM glutamate (Glut), and/or 100 nM 17β-estradiol. **(B)** Primary cortical neurons were treated with 50 nM okadaic acid, (OA) 50 μM glutamate (Glut), and/or 100 nM 17α-estradiol. **(C)** Primary cortical neurons were treated with 50 nM okadaic acid, (OA) 50 μM glutamate (Glut), and/or 100 nM ENT E2. Cells were harvested after 24 hr of treatment for western blot analysis of pERK. Depicted graphs are mean ± SEM for n = 5 with a representative blot. * $P < 0.05$ vs. control.

Figure 38

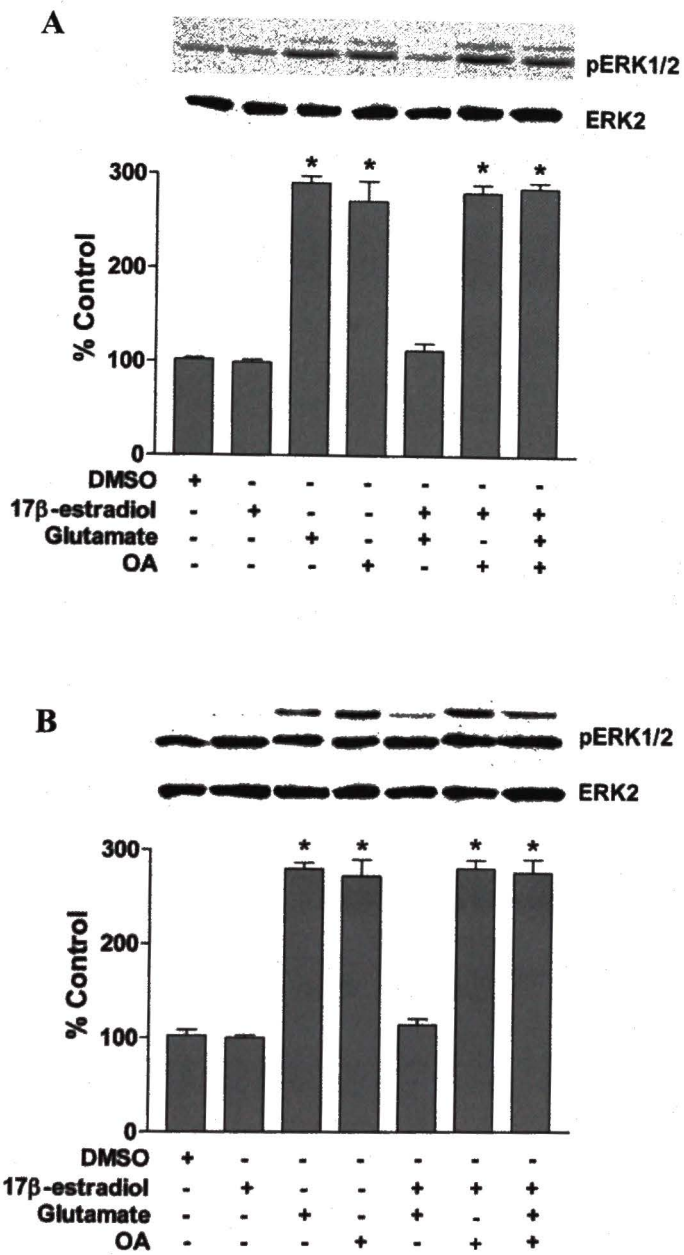

Figure 38. Phosphorylation of ERK following glutamate treatment in the presence and absence of various estrogens and/or OA. **(A)** HT-22 cells were treated with 100 nM okadaic acid, 10 mM glutamate (Glut), and/or 10 μM 17β-estradiol. **(B)** C6-glioma cells were treated with 100 nM okadaic acid, 20 mM glutamate (Glut), and/or 10 μM 17β-estradiol. Cells were harvested after 24 hr of treatment for western blot analysis of pERK. Depicted graphs are mean ± SEM for n = 5 with a representative blot. * P <0.05 vs. control.

Figure 39

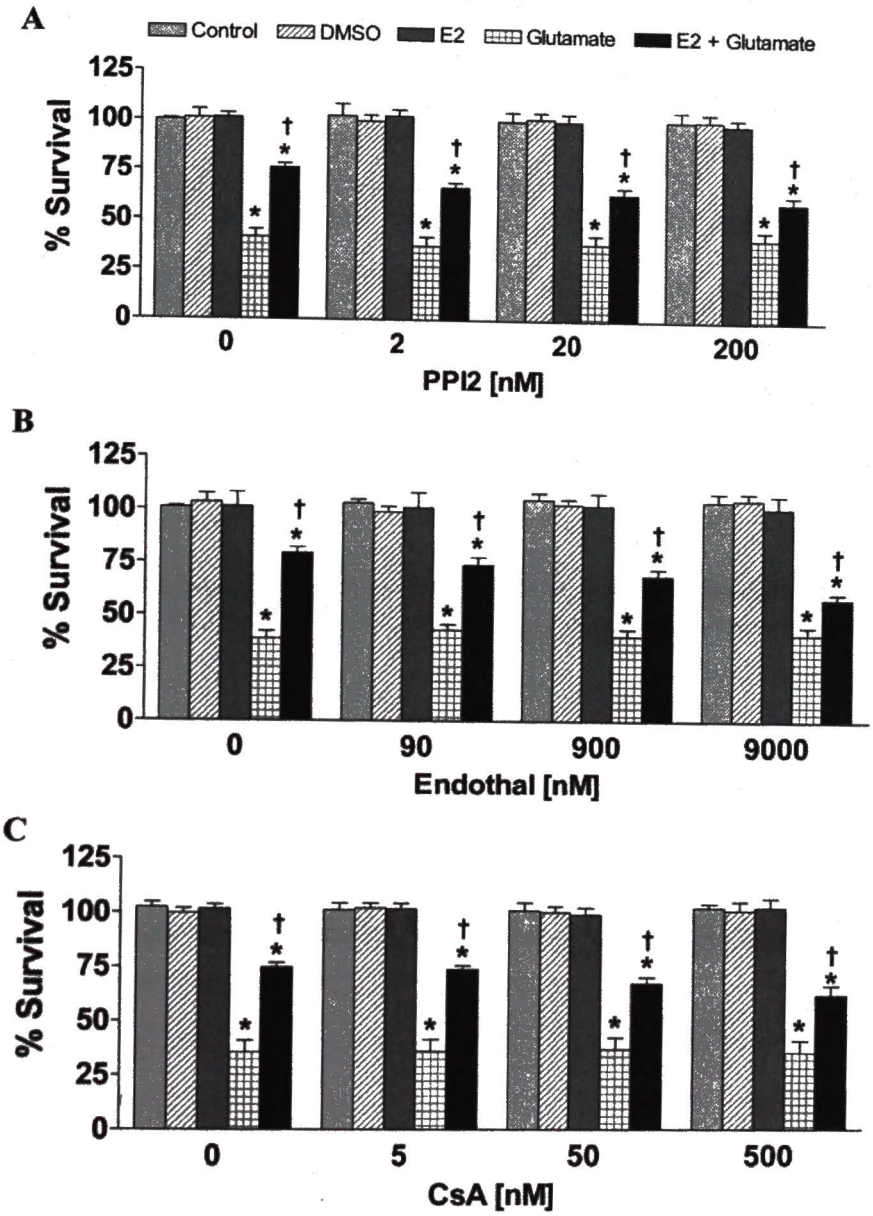

Figure 39. Effects of PPI2, endothall, or cyclosporine A on 17β-estradiol mediated neuroprotection inHT -22 cells. HT-22 cells were seeded into 96-well plates at a density of 3,500 cells/well. **A**) Cells were treated simultaneously with with 200 nM PPI2, 10 mM glutamate, and/or 10 μM 17β-estradiol. **B**) Cells were treated simultaneously with 9 μM endothall, 10 mM glutamate, and/or 10 μM 17β-estradiol. **C**) Cells were treated simultaneously with 500 nM CsA, 10 mM glutamate, and/or 10 μM 17β-estradiol. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 40

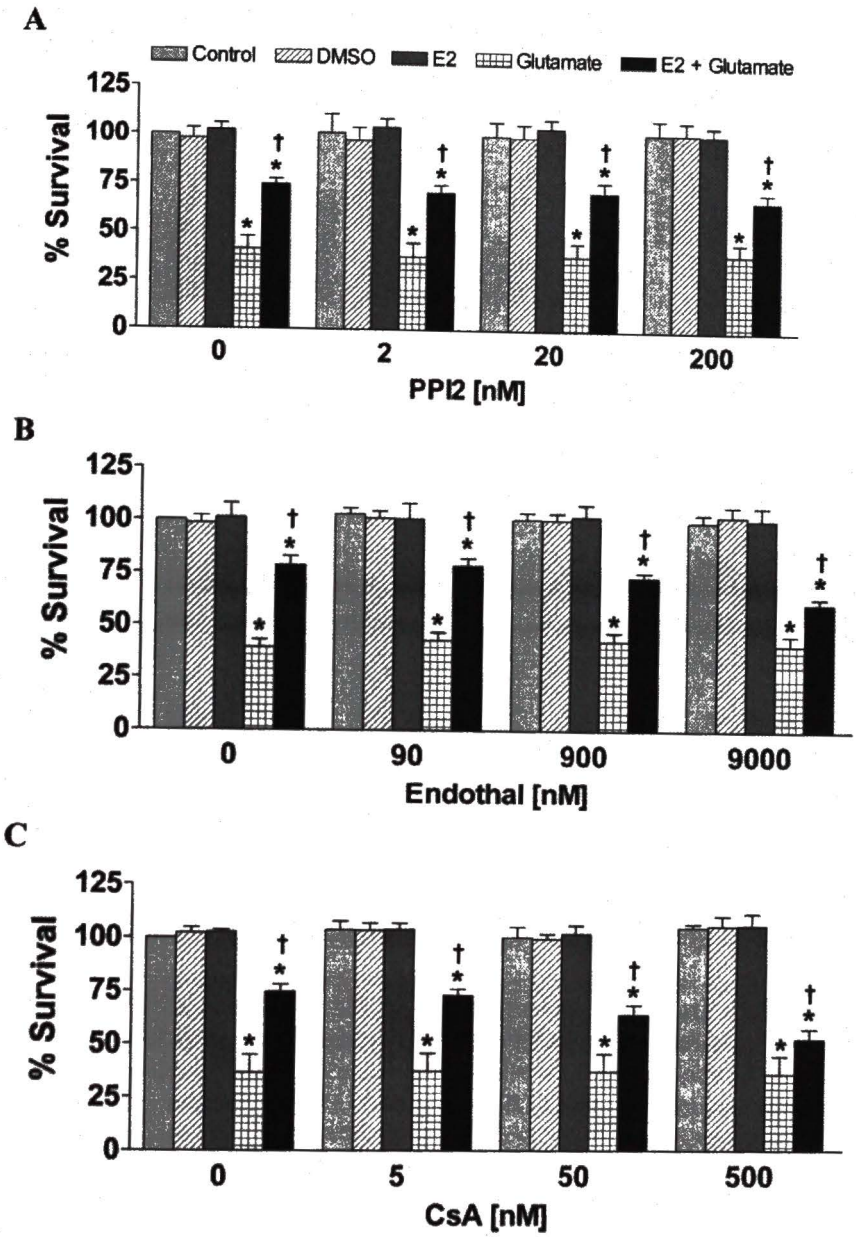

Figure 40. Effects of PPI2, endothall, or cyclosporine A on 17β-estradiol mediated neuroprotection in C6-glioma cells. C6-glioma cells were seeded into 96-well plates at a density of 3,500 cells/well. **A)** Cells were treated simultaneously with with 200 nM PPI2, 20 mM glutamate, and/or 10 μM 17β-estradiol. **B)** Cells were treated simultaneously with 9 μM endothall, 20 mM glutamate, and/or 10 μM 17β-estradiol. **C)** Cells were treated simultaneously with 500 nM CsA, 20 mM glutamate, and/or 10 μM 17β-estradiol.. Cell viability was determined by calcein AM assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control; † $P < 0.05$ vs. glutamate treated group.

Figure 41

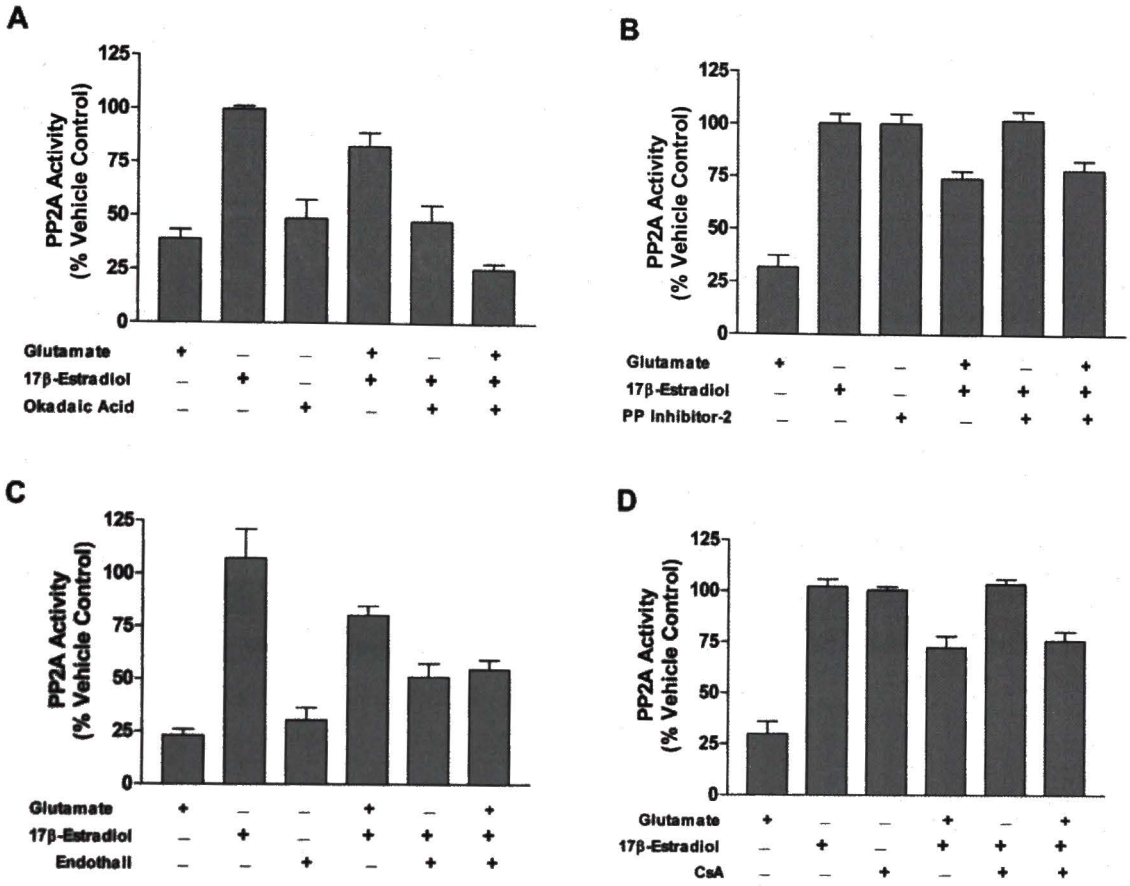

Figure 41. PP2A activity in HT-22 cells following treatment with glutamate and/or 17β-estradiol in the presence of specific inhibitors of PP1, PP2A, or calcineurin. HT-22 cells were seeded in 100 mm dishes at a density of 250,000 cells/ml. **A)** Cells were treated simultaneously with 100 nM okadaic acid, 10 mM glutamate, and/or 10 μM 17β-estradiol. **B)** Cells were treated simultaneously with 200 nM PPI2, 10 mM glutamate, and/or 10 μM 17β-estradiol. **C)** Cells were treated simultaneously with 9 μM endothall, 10 mM glutamate, and/or 10 μM 17β-estradiol. **D)** Cells were treated simultaneously with 500 nM CsA, 10 mM glutamate, and/or 10 μM 17β-estradiol. PP2A activity was determined using a serine/threonine phosphatase activity assay (Promega, Madison, WI) after 24 hr exposure to the various compounds. All data were normalized to % survival of vehicle treated control. Depicted are mean ± SEM for six independent experiments with triplicates per experiment. * $P < 0.05$ vs. glutamate treated group.

Figure 42

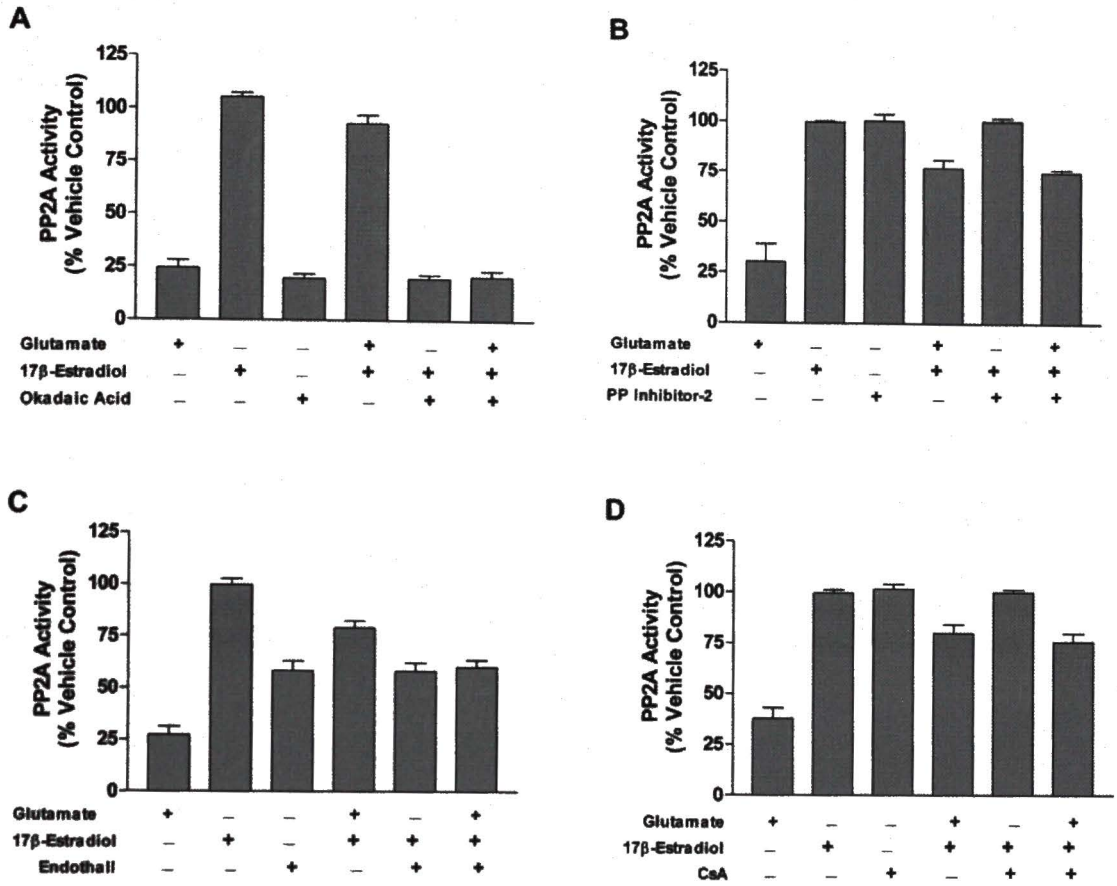

Figure 42. PP2A activity in C6-glioma cells following treatment with glutamate and/or 17β-estradiol in the presence of specific inhibitors of PP1, PP2A, or calcineurin. C6-glioma cells were seeded in 100 mm dishes at a density of 250,000 cells/ml. **A**) Cells were treated simultaneously with 100 nM okadaic acid, 20 mM glutamate, and/or 10 μM 17β-estradiol. **B**) Cells were treated simultaneously with 200 nM PPI2, 20 mM glutamate, and/or 10 μM 17β-estradiol. **C**) Cells were treated simultaneously with 9 μM endothall, 20 mM glutamate, and/or 10 μM 17β-estradiol. **D**) Cells were treated simultaneously with 500 nM CsA, 20 mM glutamate, and/or 10 μM 17β-estradiol. PP2A activity was determined using a serine/threonine phosphatase activity assay (Promega, Madison, WI) after 24 hr exposure to the various compounds. All data were normalized to % survival of vehicle treated control. Depicted are mean ± SEM for six independent experiments with triplicates per experiment. * $P < 0.05$ vs. glutamate treated group.

Figure 43

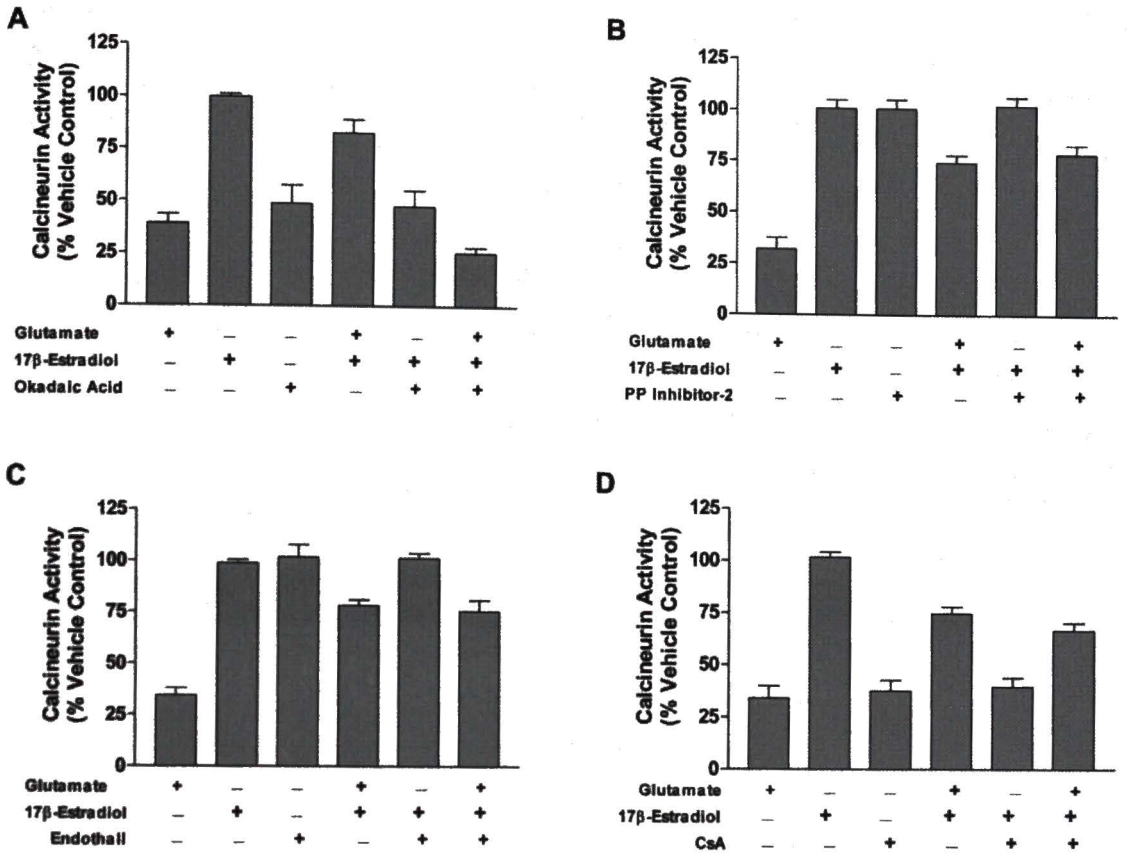

Figure 43. PP2B activity in HT-22 cells following treatment with glutamate and/or 17β-estradiol in the presence of specific inhibitors of PP1, PP2A, or calcineurin. HT-22 cells were seeded in 100 mm dishes at a density of 250,000 cells/ml. **A**) Cells were treated simultaneously with 100 nM okadaic acid, 10 mM glutamate, and/or 10 μM 17β-estradiol. **B**) Cells were treated simultaneously with 200 nM PPI2, 10 mM glutamate, and/or 10 μM 17β-estradiol. **C**) Cells were treated simultaneously with 9 μM endothall, 10 mM glutamate, and/or 10 μM 17β-estradiol. **D**) Cells were treated simultaneously with 500 nM CsA, 10 mM glutamate, and/or 10 μM 17β-estradiol. PP2B activity was determined using a serine/threonine phosphatase activity assay (Promega, Madison, WI) after 24 hr exposure to the various compounds. All data were normalized to % survival of vehicle treated control. Depicted are mean ± SEM for six independent experiments with triplicates per experiment. * $P < 0.05$ vs. glutamate treated group.

Figure 44

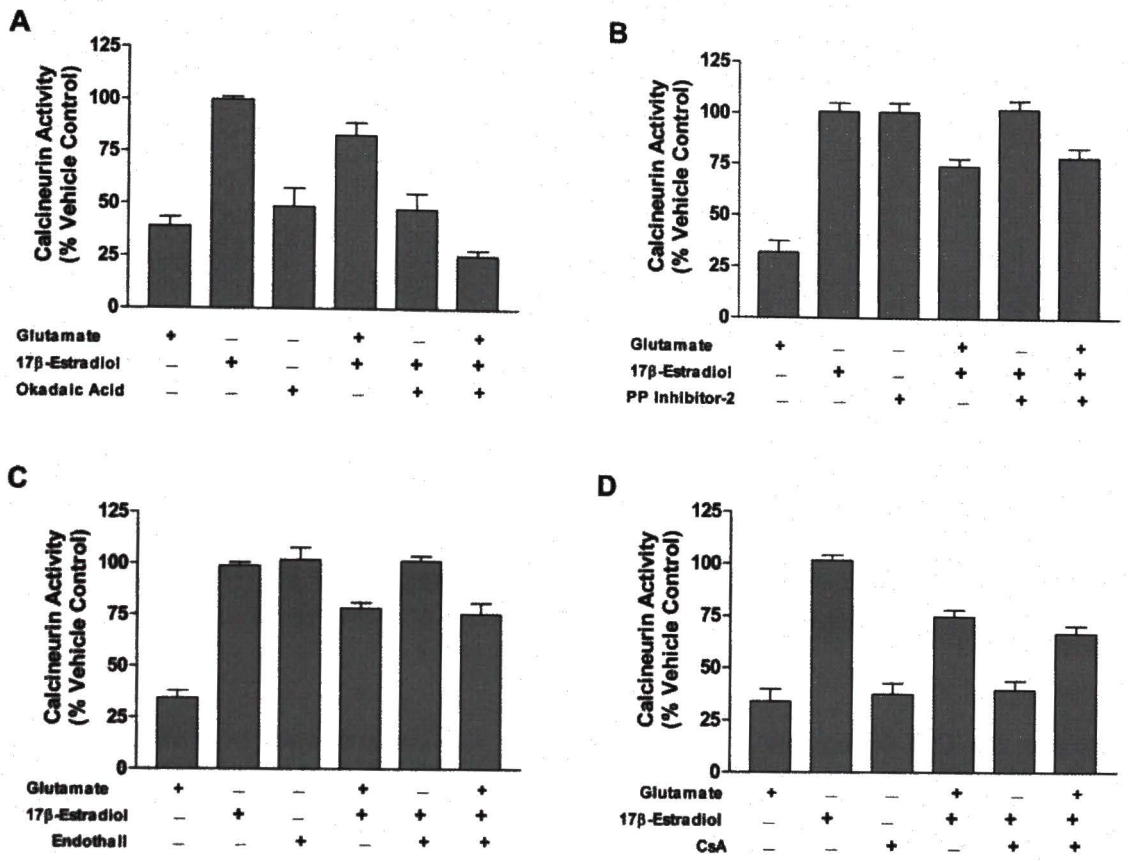

Figure 44. PP2B activity in C6-glioma cells following treatment with glutamate and/or 17β-estradiol in the presence of specific inhibitors of PP1, PP2A, or calcineurin. C6-glioma cells were seeded in 100 mm dishes at a density of 250,000 cells/ml. **A**) Cells were treated simultaneously with 100 nM okadaic acid, 20 mM glutamate, and/or 10 μM 17β-estradiol. **B**) Cells were treated simultaneously with 200 nM PPI2, 20 mM glutamate, and/or 10 μM 17β-estradiol. **C**) Cells were treated simultaneously with 9 μM endothall, 20 mM glutamate, and/or 10 μM 17β-estradiol. **D**) Cells were treated simultaneously with 500 nM CsA, 20 mM glutamate, and/or 10 μM 17β-estradiol. PP2B activity was determined using a serine/threonine phosphatase activity assay (Promega, Madison, WI) after 24 hr exposure to the various compounds. All data were normalized to % survival of vehicle treated control. Depicted are mean ± SEM for six independent experiments with triplicates per experiment. * $P < 0.05$ vs. glutamate treated group.

Figure 45

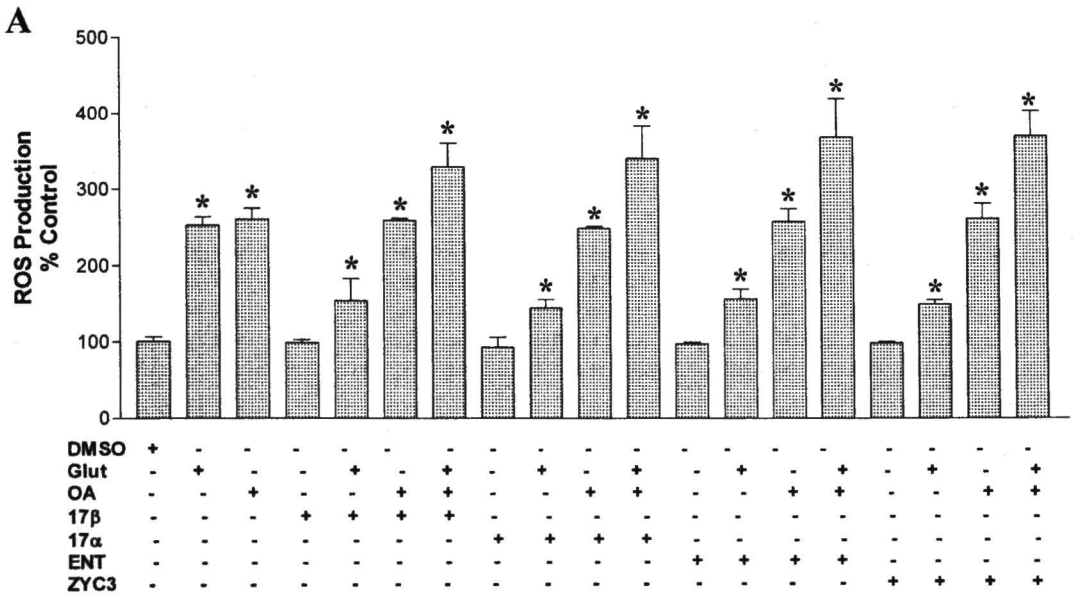

Figure 45A. Effects of glutamate, okadaic acid, 17β-estradiol, 17α-estradiol, ENT E2, and/or ZYC3 on oxidative stress markers in HT-22. HT-22 cells were seeded into 100 mm dishes at a density of 250,000 cells/well and various assays performed following 24 hr treatment. **A)** ROS production was measured in neurons treated simultaneously with 100 nM okadaic acid, 10 mM glutamate, and/or 10 mM 17β-estradiol, 10 mM 17α-estradiol, 10 mM ENT E2, and/or 1 mM ZYC3. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for six independent experiments with two replicates per experiment for ROS production and six independent experiments each for lipid peroxidation and protein carbonylation experiments. * $P < 0.05$ vs. vehicle control.

Figure 45

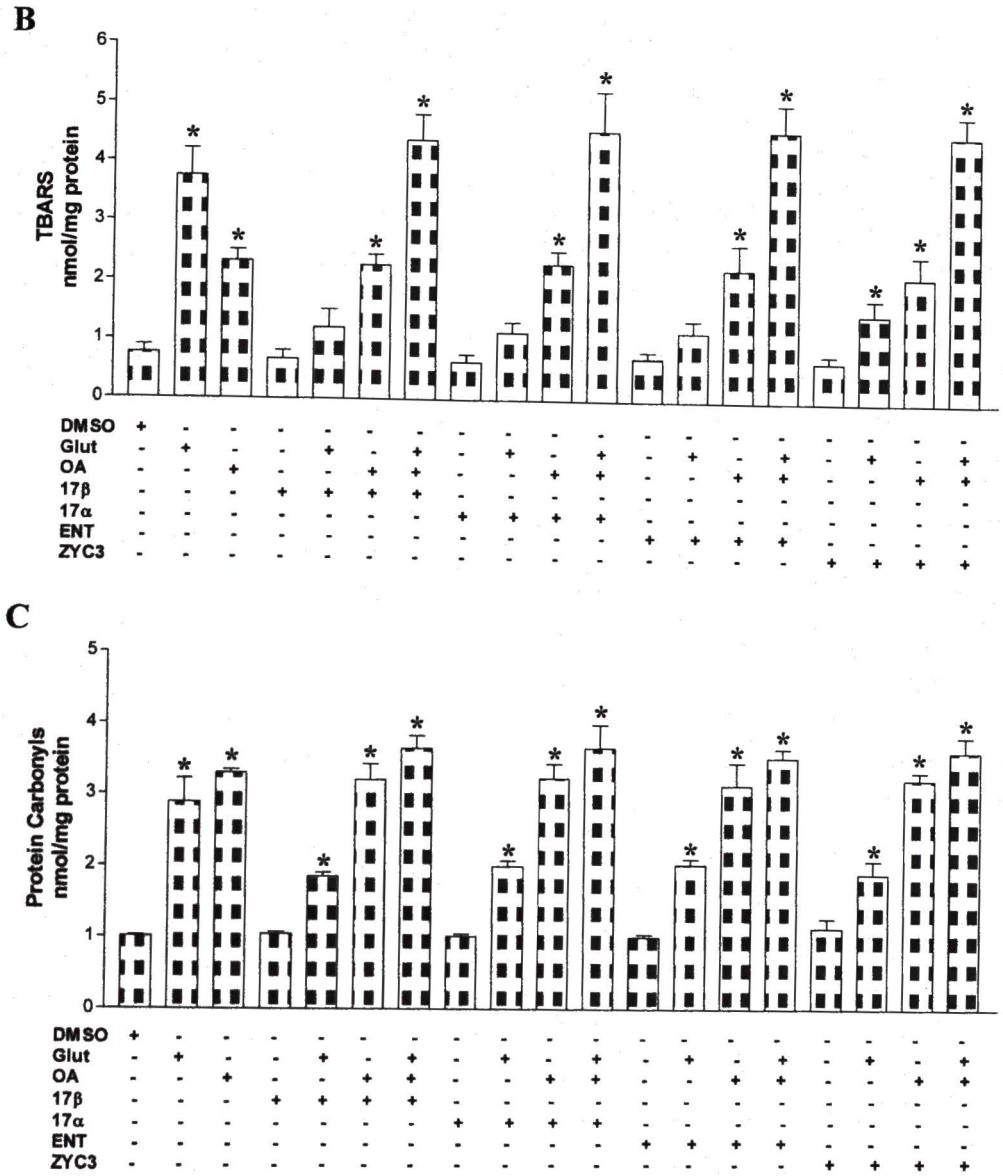

Figure 45. Effects of glutamate, okadaic acid, 17β-estradiol, 17α-estradiol, ENT E2, and/or ZYC3 on oxidative stress markers in HT-22. HT-22 cells were seeded into 100 mm dishes at a density of 250,000 cells/well and various assays performed following 24 hr treatment. **B**) Lipid peroxidation was measured in cortical neurons 24 hr following simultaneously treatment with 100 nM okadaic acid, 10 mM glutamate, and/or 10 mM 17β-estradiol, 10 mM 17α-estradiol, 10 mM ENT E2, and/or 1 mM ZYC3. **C**) Protein carbonylation was measured in cortical neurons 24 hr following simultaneously treatment with 100 nM okadaic acid, 10 mM glutamate, and/or 10 mM 17β-estradiol, 10 mM 17α-estradiol, 10 mM ENT E2, and/or 1 mM ZYC3. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for six independent experiments with two replicates per experiment for ROS production and six independent experiments each for lipid peroxidation and protein carbonylation experiments. * $P < 0.05$ vs. vehicle control.

Figure 46A

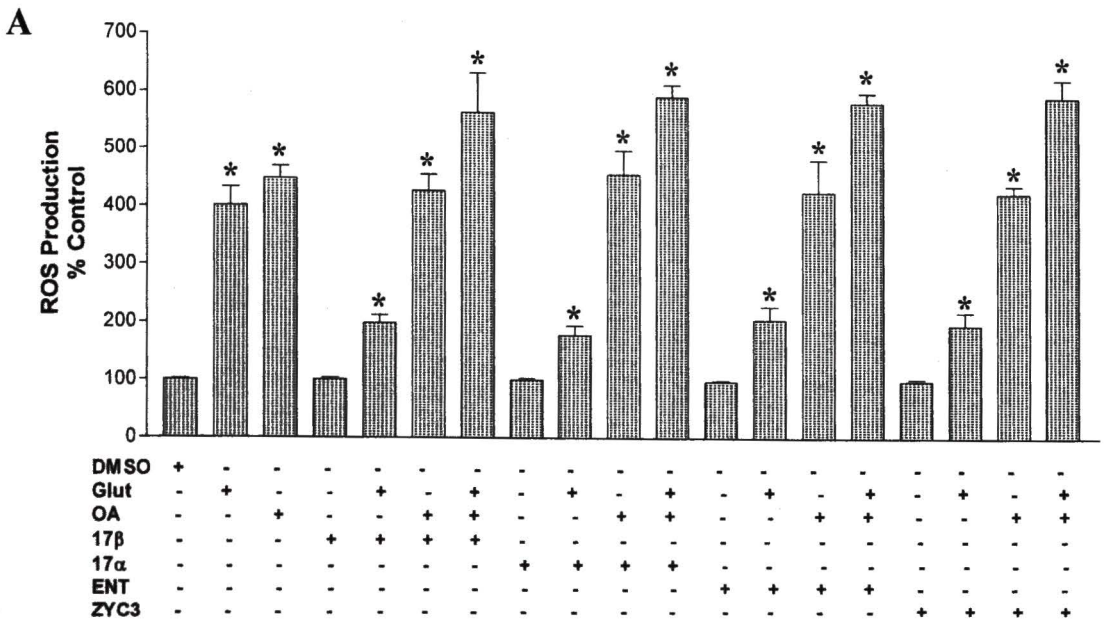

Figure 46A. Effects of glutamate, okadaic acid, 17β-estradiol, 17α-estradiol, ENT E2, and/or ZYC3 on oxidative stress markers in C6. C6-glioma cells were seeded into 100 mm dishes at a density of 250,000 cells/well and various assays performed following 24 hr treatment. **A)** ROS production was measured in neurons treated simultaneously with 100 nM okadaic acid, 20 mM glutamate, and/or 10 mM 17β-estradiol, 10 mM 17α-estradiol, 10 mM ENT E2, and/or 1 mM ZYC3All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for six independent experiments with two replicates per experiment for ROS production and six independent experiments each for lipid peroxidation and protein carbonylation experiments. * $P < 0.05$ vs. vehicle control.

Figure 46

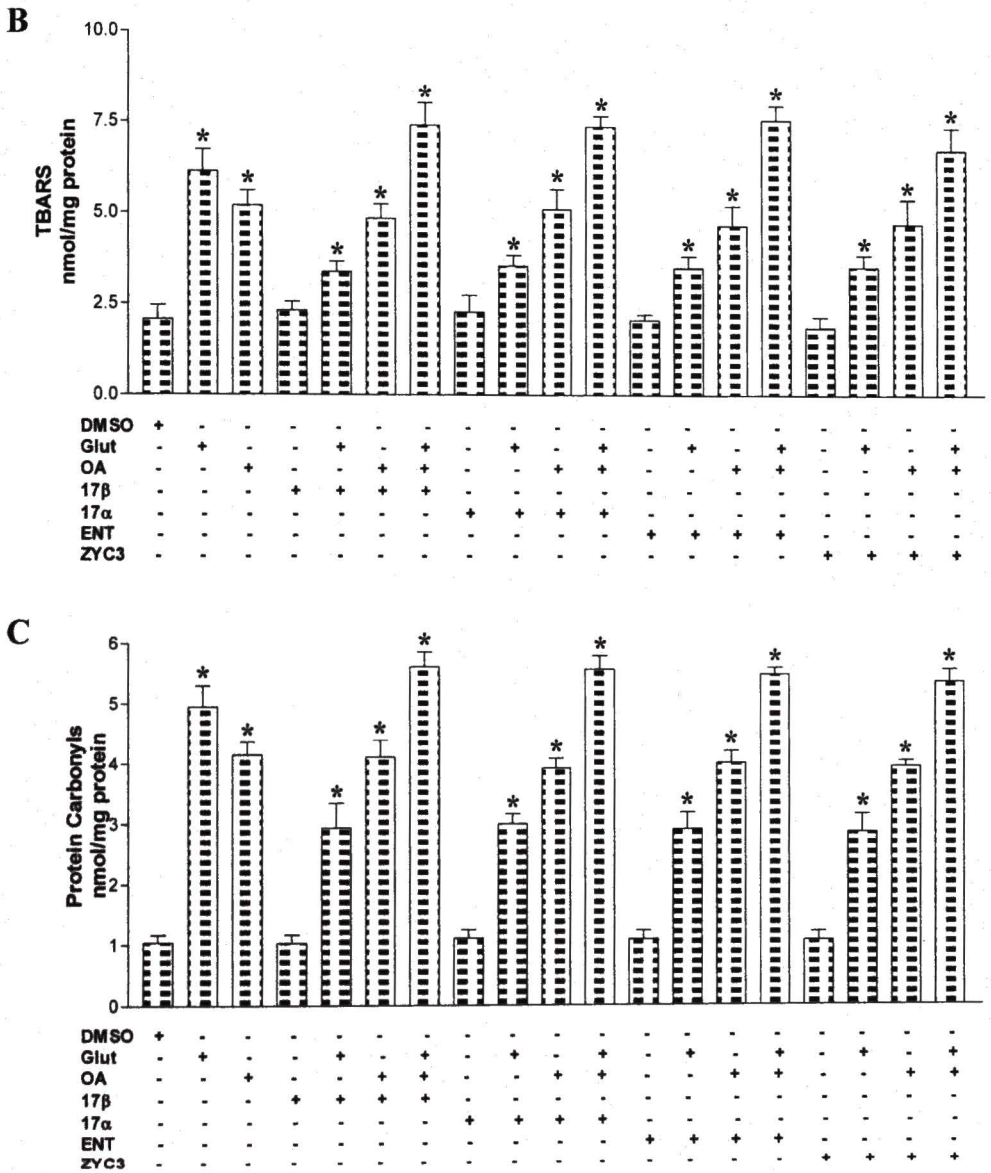

Figure 46. Effects of glutamate, okadaic acid, 17β-estradiol, 17α-estradiol, ENT E2, and/or ZYC3 on oxidative stress markers in C6. C6-glioma cells were seeded into 100 mm dishes at a density of 250,000 cells/well and various assays performed following 24 hr treatment. **B**) Lipid peroxidation was measured in cortical neurons 24 hr following simultaneously treatment with 100 nM okadaic acid, 20 mM glutamate, and/or 10 mM 17β-estradiol, 10 mM 17α-estradiol, 10 mM ENT E2, and/or 1 mM ZYC3. **C**) Protein carbonylation was measured in cortical neurons 24 hr following simultaneously treatment with 100 nM okadaic acid, 20 mM glutamate, and/or 10 mM 17β-estradiol, 10 mM 17α-estradiol, 10 mM ENT E2, and/or 1 mM ZYC3. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for six independent experiments with two replicates per experiment for ROS production and six independent experiments each for lipid peroxidation and protein carbonylation experiments. * $P < 0.05$ vs. vehicle control.

Figure 47

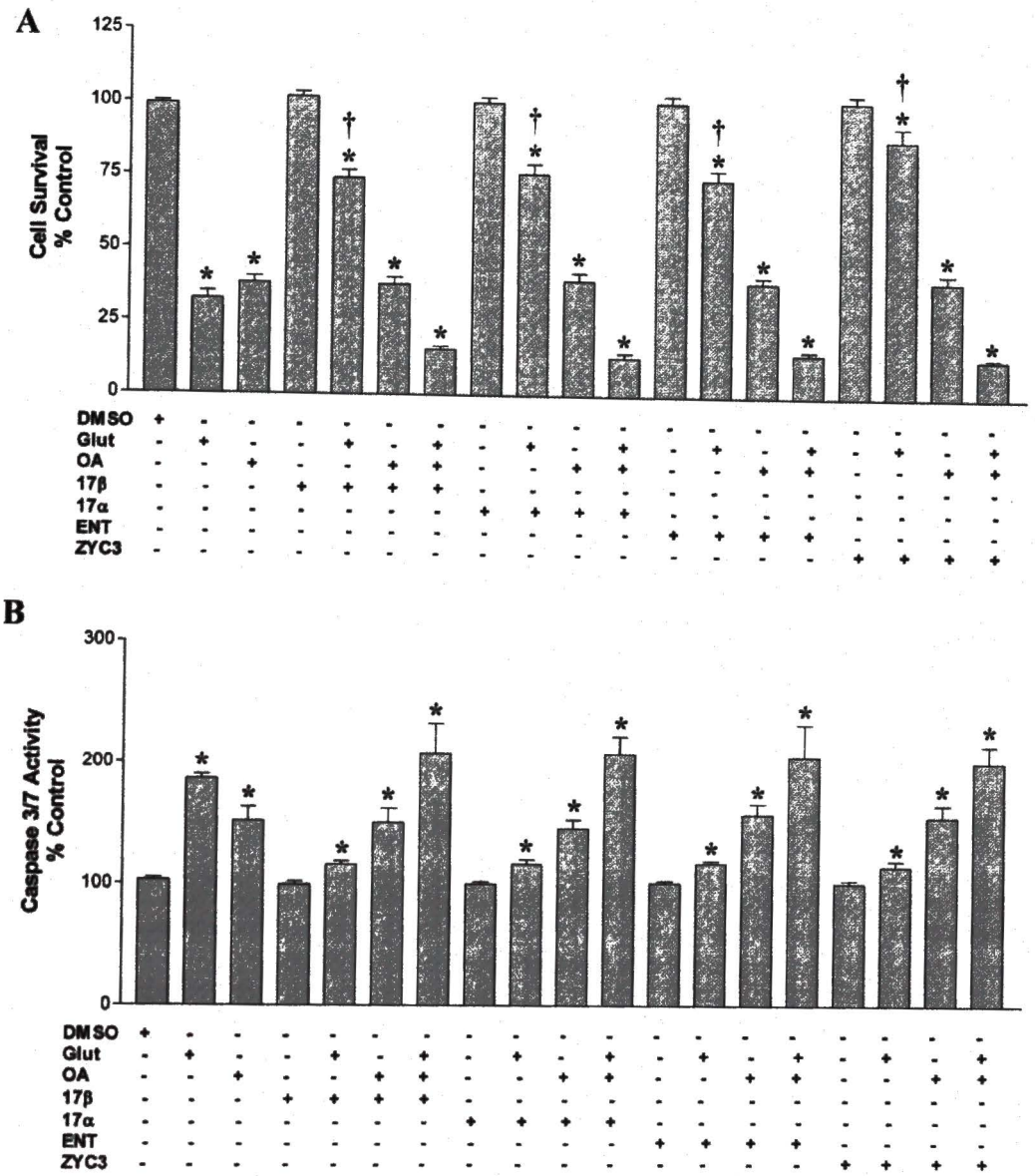

Figure 47. Effects of glutamate, okadaic acid, 17β-estradiol, 17α-estradiol, ENT E2, and/or ZYC3 on oxidative stress markers in HT-22 cells. HT-22 cells were seeded into 100 mm dishes at a density of 250,000 cells/well and various assays performed following 24 hr treatment. **A**) MTT reduction was measured in neurons treated simultaneously with 100 nM okadaic acid, 10 mM glutamate, and/or 10 mM 17β-estradiol, 10 mM 17α-estradiol, 10 mM ENT E2, and/or 1 mM ZYC3. **B**) Caspase 3/7 activity was measured in cortical neurons 24 hr following simultaneously treatment with 100 nM okadaic acid, 10 mM glutamate, and/or 10 mM 17β-estradiol, 10 mM 17α-estradiol, 10 mM ENT E2, and/or 1 mM ZYC3. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for six independent experiments with two replicates per experiment for ROS production and six independent experiments for caspase 3/7 activity measurement. * $P < 0.05$ vs. vehicle control. † $P < 0.05$ vs. glutamate treated group.

Figure 48

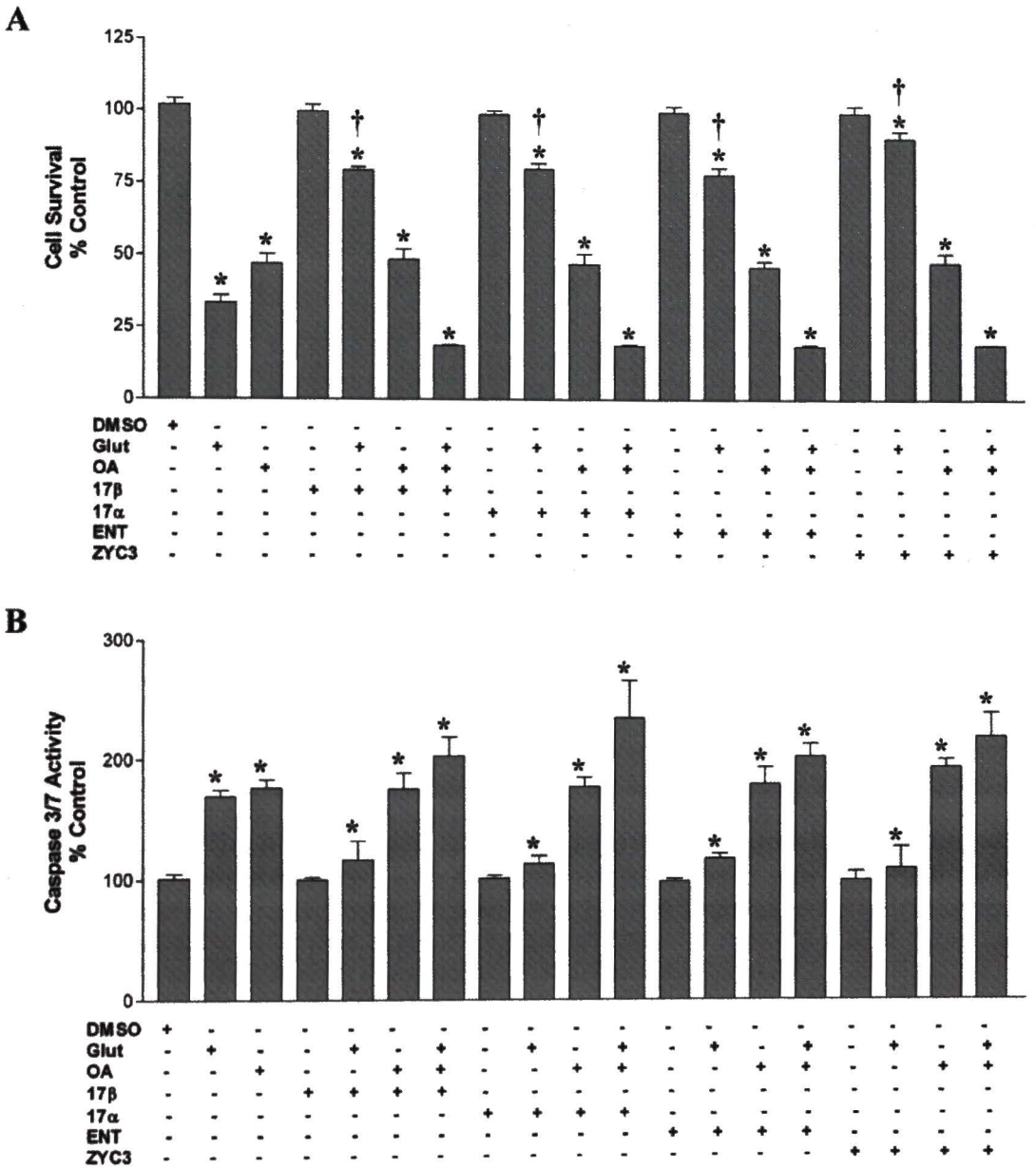

Figure 48. Effects of glutamate, okadaic acid, 17β-estradiol, 17α-estradiol, ENT E2, and/or ZYC3 on oxidative stress markers in C6 cells. C6-glioma cells were seeded into 100 mm dishes at a density of 250,000 cells/well and various assays performed following 24 hr treatment. **A**) MTT reduction was measured in neurons treated simultaneously with 100 nM okadaic acid, 20 mM glutamate, and/or 10 mM 17β-estradiol, 10 mM 17α-estradiol, 10m M ENT E2, and/or 1 mM ZYC3. **B**) Caspase 3/7 activity was measured in cortical neurons 24 hr following simultaneously treatment with 100 nM okadaic acid, 20 mM glutamate, and/or 10 mM 17β-estradiol, 10 mM 17α-estradiol, 10 mM ENT E2, and/or 1 mM ZYC3. . All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for six independent experiments with two replicates per experiment for ROS production and six independent experiments for caspase 3/7 activity measurement. * $P < 0.05$ vs. vehicle control. † $P < 0.05$ vs. glutamate treated group.

Figure 49

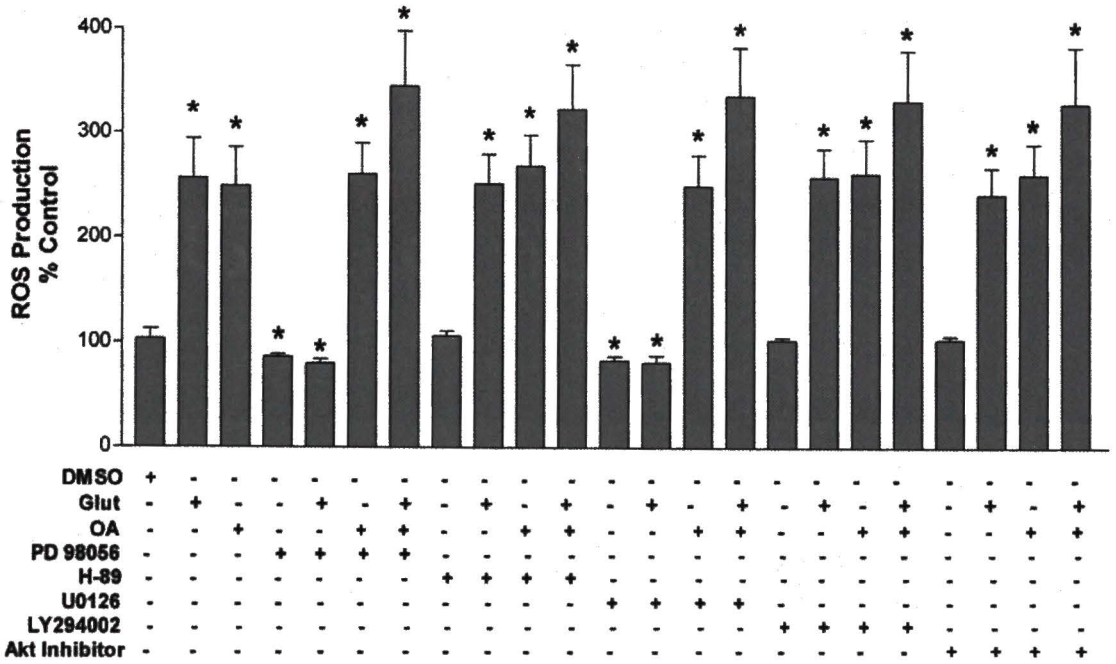

Figure 49. Effects of glutamate, okadaic acid and/or various protein kinase inhibitors on ROS production in HT-22 cells. HT-22 cells were seeded into 96-well plates at a density of 3,500 cells/well. **A)** Cells were treated simultaneously with 10 mM glutamate, 50μM PD98059, 10 μM U0126, 1 μM H-89, 50 μM LY294002, 100 μM Akt Inhibitor, and/or 100 nM of okadaic acid. ROS production was determined by DCFH-DA assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * P <0.05 vs. vehicle control.

Figure 50

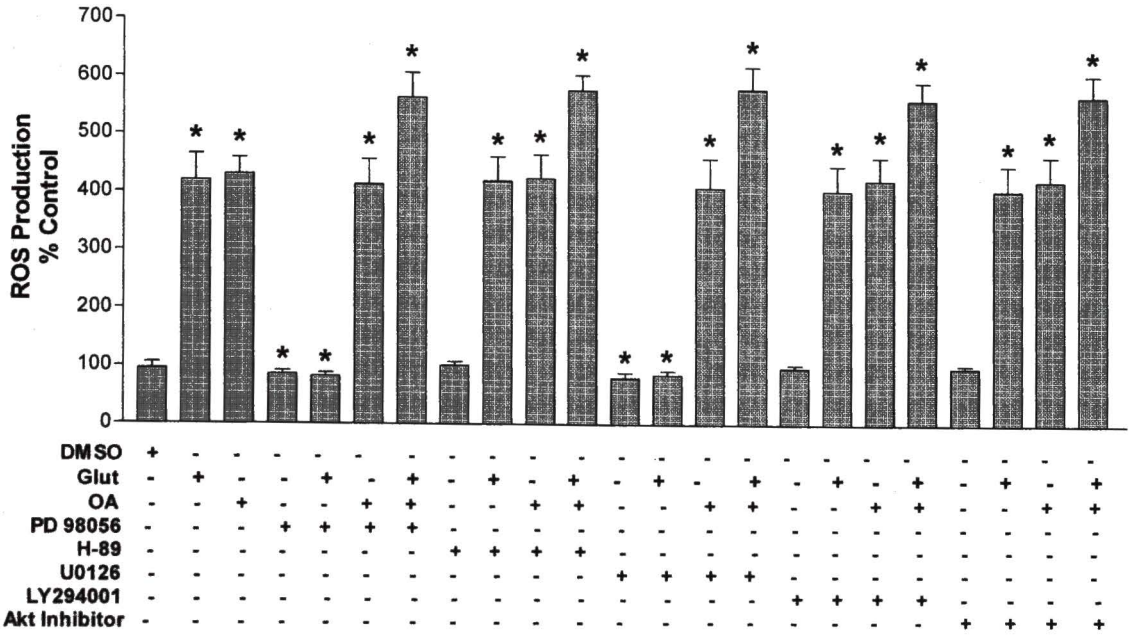

Figure 50. Effects of glutamate, okadaic acid and/or various protein kinase inhibitors on ROS production in C6-glioma cells. C6-glioma cells were seeded into 96-well plates at a density of 3,500 cells/well. **A)** Cells were treated simultaneously with 20 mM glutamate, 50μM PD98059, 10 μM U0126, 1 μM H-89, 50 μM LY294002, 100 μM Akt Inhibitor, and/or 100nM of okadaic acid. ROS production was determined by DCFH-DA assay (Molecular Probes, Eugene, OR) after 24 hr exposure to the various compounds. All data were normalized to % survival of non-treated control. Depicted are mean ± SEM for ten independent experiments with two replicates per experiment. * $P < 0.05$ vs. vehicle control.