MECHANISMS OF NEUROPROTECTION BY ESTROGEN AND SELECTIVE ESTROGEN RECEPTOR MODULATORS

By

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This dissertation is submitted by Krishnan M Dhandapani and has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

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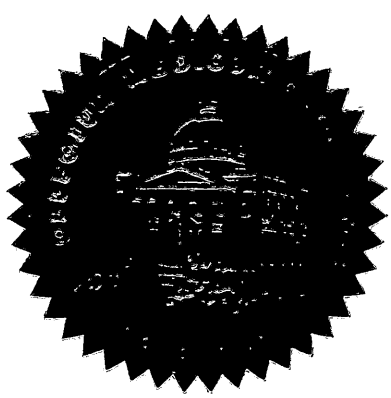

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LIST OF ABBREVIATIONS

AP-1	Activator Protein-1
bFGF	basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
C6 CM	C6 Glial Cell Conditioned Medium
CA CM	Cortical Astrocyte Conditioned Medium
CMPT	Camptothecin
CNS	Central Nervous System
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMEM-F12	Dulbecco's Modified Eagle Medium-Ham's F12
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ELISA	Enzyme Linked Immunosorbent Assay
ER-ε	Estrogen Receptor-alpha
ER-β	Estrogen Receptor-beta
ERE	Estrogen Response Element
ERK	Extracellular Regulated Kinase/Mitogen Activated Kinase
FBS	Fetal Bovine Serum
GFAP	Glial Fibrillary Acidic Protein
HRP	Horse Radish Peroxidase
ICI	ICI182,780
JNK	c-Jun N-terminal Kinase
KCN	Potassium Cyanide
LDH	Lactate Dehydrogenase
MAPK	Mitogen Activated Protein Kinase/Extracellular Regulated Kinase
MCAO	Middle Cerebral Artery Occlusion

MKK4	MAP Kinase Kinase 4
MMLV	Maloney Murine Leukemia Virus
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OVX	Ovariectomy
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PI3-K	Phosphatidylinositol 3-kinase
PVDF	Polyvinylidene Fluoride
RIA	Radioimmunoassay
RLX	Raloxifene
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SERM	Selective Estrogen Receptor Modulator
TAK1	TGF-β activated kinase 1
TGF-β	Transforming Growth Factor-β
TGF-β1	Transforming Growth Factor-β1
TGF-β2	Transforming Growth Factor-β2
TGF-β3	Transforming Growth Factor-β3
TβRI	Transforming Growth Factor Beta Type I receptor
TβRII	Transforming Growth Factor Beta Type II receptor
TβRIII	Transforming Growth Factor Beta Type III receptor
TMX	Tamoxifen
2-DG	2-deoxyglucose
4-OHT	4-hydroxytamoxifen
17β-E_2	17β-estradiol

OVERVIEW AND INTRODUCTION

1.1 Neurodegeneration and Gender

Over the past decade, evidence has emerged in support of a neuroprotective role for the ovarian steroid hormone, 17β-estradiol (17β-E_2). The risk of developing ischemic stroke is lower in females as compared to age-matched males. However, the occurrence of stroke dramatically rises in women following menopause, when endogenous 17β-E_2 levels decline to 1% of premenopausal levels. Epidemiological studies indicate postmenopausal women taking estrogen replacement therapy experience improved cognitive function and decreased incidence of neurodegenerative disease (1-10). Beneficial effects of 17β-E_2 on the mortality and morbidity associated with ischemic stroke have also been reported; however, the efficacy of estrogen replacement therapy in stroke prevention is controversial (11-19). Although postmenopausal estrogen replacement is associated with decreased cardiovascular disease (still controversial), decreased risk of osteoporosis, and a decrease in all-cause mortality, the role of 17β-E_2 in protecting the brain is less clear (20-21). Animal studies suggest a potential neuroprotective role for 17β-E_2, stimulating clinical interest in determining the effectiveness of 17β-E_2 in the prevention of neurodegenerative and cerebrovascular disease in humans. Similarly, the ability of selective estrogen receptor modulators (SERMs), clinically relevant drugs that influence estrogen receptor activity, to protect against neurodegeneration is not known. SERMs may circumvent some of the disadvantages of estrogen replacement therapy, such as the undesired stimulatory effects of estrogen on the breast and uterus.

1.2 Evidence for a Neuroprotective Role for 17β-E_2 and SERMs

1.2.1 17β-E_2 protects against cerebral ischemia-induced damage

A gender difference in stroke damage was first suggested by studies which demonstrated that female gerbils experience a lower incidence of stroke and less severe brain damage following carotid artery occlusion as compared with male gerbils (22). This

gender difference in susceptibility to ischemic stroke damage was subsequently extended to the rat animal model through studies, which showed that intact adult female rats sustain lower mortality and less neuronal damage as compared to age-matched male rats following middle cerebral artery occlusion (MCAO) (23). Evidence that a gonadal factor was responsible for the gender difference in neuroprotection was suggested from studies showing that ovariectomy (OVX) eliminated the endogenous protective effect observed in females following cerebral ischemia (23). Further evidence suggested that the critical protective gonadal factor was 17β-E_2, as exogenous administration of 17β-E_2 dramatically reduced infarct volume following MCAO in OVX female rats (24-28), in male rats (29) and in aged, reproductively senescent female rats (30), which represent a model of female menopause. The mechanism of 17β-E_2 actions in these studies was independent of effects on cerebral blood flow, implying the neuroprotective effect of 17β-E_2 occurs directly at the level of the brain rather than on the vasculature (28, 31). Furthermore, serum 17β-E_2 levels were inversely correlated with ischemic stroke damage in female rats, supporting the notion that 17β-E_2 provides the gender difference in the extent of brain injury following ischemic stroke in rodents (32).

1.2.2 Mechanisms of 17β-E_2 mediated neuroprotection in vivo

Physiological doses of 17β-E_2 exert a neuroprotective role in the rodent brain, however the mechanism(s) underlying this protection is unclear. Several studies indicated pretreatment with 17β-E_2 was necessary to reduce infarct volume following permanent MCAO in OVX female rats (25, 28, 33). Acute pretreatment or administration of physiological doses of 17β-E_2 at the time of MCAO failed to reduce brain injury, suggesting a genomic mechanism of action mediates the protective effect. 17β-E_2, at physiological doses, activates nuclear estrogen receptors and subsequently influences gene transcription (25, 33). In support of a possible genomic mechanism of action, 17β-E_2 increased the expression of the anti-apoptotic gene, *bcl-2*, in the ischemic penumbra following MCAO (33). Elevated Bcl-2

immunoreactivity was previously demonstrated on proestrous in the hypothalamus of female rats, further confirming the potentially important role for 17β-E_2 in the regulation of anti-apoptotic proteins in the brain (34). Furthermore, the bcl-2 gene promoter contains an estrogen responsive element (ERE), making it a target for transcriptional activation by an estrogen receptor dependent mechanism (35). In other brain regions, such as the hippocampus, 17β-E_2 enhanced the expression of another anti-apoptotic Bcl family member, Bcl-xL (36-37). Together, these studies support the possibility that 17β-E_2 protects the brain through a genomic mechanism, which is potentially mediated by the estrogen receptor. However, it is unknown what other genes may influence the neuroprotective ability of 17β-E_2. Further elucidation of the genes involved in estrogen and SERM neuroprotection was an important goal of our studies.

1.2.3 Neuroprotective role for estrogen receptors during brain injury

The neuroprotective effects elicited by endogenous 17β-E_2 hypothetically involve nuclear estrogen receptor activation and subsequent gene transcription (38-40). In support of this hypothesis, ICI182,780, a potent and selective estrogen receptor antagonist, exacerbated infarct volume following MCAO in intact female rats, suggesting a critical role for estrogen receptors in mediating the protection by endogenous 17β-E_2 following brain injury (41). To more precisely define the role of individual estrogen receptor subtypes involved in neuroprotection *in vivo*, isoform specific estrogen receptor knockout mice have been utilized.

Two estrogen receptor (ER) isoforms have been identified to date, ER-α and ER-β, both of which are expressed in the adult brain (42-45). Speculation currently exists as to the presence of a third estrogen receptor isoform, which may be localized to the cell membrane and mediate the rapid actions of 17β-E_2 (46-51). The generation of ER-α knockout mice (ERKO), ER-β knockout mice (βERKO) and ER-α/ER-β dual isoform knockout mice has increased the understanding of the role of individual estrogen receptors in many physiological processes (52-58). However, the use of estrogen receptor knockout mice in brain injury models has resulted in conflicting data as to the role of individual estrogen receptor isoforms

during brain injury. This data is reviewed below along with other studies concerning the putative role of ER-α and ER-β in neuroprotection.

1.2.4 Evidence supporting a neuroprotective role for ER-α

Following MCAO in rats, ER-α mRNA was dramatically up-regulated in the ischemic penumbra, implying ER-α may influence the protection of the brain during injury (33). Along these lines, 17β-E_2 protected the brain of OVX βERKO mice from stroke damage, but the protective effect of 17β-E_2 was lost in OVX ERKO mice (31). These findings suggest a critical role for ER-α in the protection of the brain by 17β-E_2. However, these findings conflict with those of Sampei et al. (59), who demonstrated that intact wild type and ERKO mice sustain similar infarct volumes following MCAO, indicating ER-α is potentially dispensable for protection by endogenous 17β-E_2. One potential explanation for the observed differences between these studies is intact ERKO mice possess up to ten-fold higher serum levels of 17β-E_2 as compared to wild type controls (60). Therefore, the elevated endogenous 17β-E_2 levels in ERKO mice may protect the brain by mechanisms that may differ from the protection observed with physiological doses of 17β-E_2.

1.2.5 Evidence in support of a neuroprotective role for ER-β

Recent work has shown that βERKO mice undergo a significant loss of neurons between the somatosensory and parietal cortex (61). For instance, the size of the brain of two year old, but not two month old βERKO mice is dramatically reduced as compared to wild type controls, suggesting neuronal cell loss occurs throughout the life of the animal rather than exclusively during development. These observations contrast with the phenotype of ERKO mice, which lack gross brain morphological changes. As a whole, ER-β appears to facilitate basal maintenance of neuronal survival and/or neuronal replacement/neurogenesis in the adult brain. However, it is unclear what role, if any, ER-β has in protecting or replacement of neurons following brain injury.

1.3 Mechanisms of 17β-E_2 mediated neuroprotection

Although the mechanisms of estrogen-mediated neuroprotection are not well defined, three possible mechanisms have emerged (see Figure 1-1): a) 17β-E_2 protects through either ER-α or ER-β isoform b) 17β-E_2 protects through a non-ER-α, non-ER-β estrogen receptor, or c) 17β-E_2 protects through an estrogen receptor independent pathway. 17β-E_2 influences many diverse cellular pathways in the body, such as the activation of estrogen receptors and subsequent gene transcription, induction of anti-apoptotic genes (25, 34), stimulation of second messengers (62), maintenance of calcium levels (63), and induction of protein kinase signaling pathways (56, 64-69). Antioxidant actions of 17β-E_2 involving a receptor-independent mechanism have also been described (70-74). To evaluate the contribution of these mechanisms toward neuroprotection, *in vitro* models of neuronal injury have been established. As a whole, these studies demonstrate 17β-E_2 protects against a wide variety of neurotoxic insults at a wide range of doses. However, interpretation of data from these *in vitro* studies and extrapolation to the *in vivo* paradigm has been hindered by the wide variety of experimental paradigms of injury used, inherent limitations due to the artificial nature of *in vitro* studies, and in many cases the non-physiological doses of 17β-E_2 used to elicit neuroprotection.

1.3.1 A role for estrogen receptor(s) during in vitro neuroprotection

The neuroprotective ability of physiological doses of 17β-E_2 *in vitro* is highly controversial with compelling data both for and against a direct neuroprotective role. Several laboratories have reported a neuroprotective effect with physiological concentrations of 17β-E_2 and this effect appears to largely be mediated by non-classical genomic signaling pathways, although many other groups have failed to document such a protective role with physiological levels of 17β-E2 (see next section). In support of a direct neuroprotective effect, several recent reports indicate 17β-E_2 protects cultured rat cortical neurons from

glutamate excitotoxicity (65,75-79). Tamoxifen, a partial estrogen receptor antagonist, attenuated this protection, implying a role for estrogen receptors (75). However, it is currently not clear whether 17β-E_2 influences gene transcription to attain the observed protective effects. In support of a genomic mechanism, 17β-E_2 induced the expression of Bcl-2 in human NT2 neurons, which correlated with protection against oxidative stress and excitotoxicity in these cultures (76). This induction was blocked by the co-addition of ICI182,780, demonstrating an important role for estrogen receptors in mediating the neuroprotection. Furthermore, pretreatment with 17β-E_2 protected rat organotypic cortical explants cultures from subsequent ischemia-induced cell death via an ICI182,780 sensitive estrogen receptor. Thus, these data suggest 17β-E_2 induces gene expression to influence the cytoprotective effects observed in culture.

In addition to the classical regulation of gene transcription via nuclear estrogen receptors, 17β-E_2 also activates 'non-genomic' signaling pathways, possibly through a membrane bound estrogen receptor. 17β-E_2 reportedly influences neuronal development and brain function via activation of signaling pathways indicating the rapid induction of cell signaling pathways is crucial for the actions of 17β-E_2 in the brain (65-67). Specifically, two signaling pathways, the mitogen activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3-K) pathway have been implicated in mediating neuronal survival (46, 56, 65-67, 77-78, 80-84).

1.3.2 MAP Kinase and Estrogen Mediated Neuroprotection

17β-E_2 activated the MAPK pathway in cultured rat cortical neurons and in rat organotypic cerebrocortical explant cultures, an effect that was correlated with neuroprotection (65-66). In cultured rat cortical neurons, MAPK phosphorylation was increased within 30 minutes of treatment via activation of an ICI182,780-sensitive estrogen receptor. Co-administration of PD98059, an inhibitor of MAPK, blocked the direct neuroprotective effect of 17β-E_2, suggesting MAPK activation by 17β-E_2 influences neuronal survival. 17β-E_2 also elicited MAPK phosphorylation within 15 minutes of treatment in rat

cortical explant cultures, an effect that was sustained for two hours (66). Interestingly, ICI182,780 failed to inhibit MAPK phosphorylation in cortical explants, suggesting a novel estrogen receptor subtype potentially mediates this effect. In support of this contention, 17β-E_2 induced MAPK phosphorylation in cortical explants derived from ERKO mice, implying ER-α was not required for this effect. Further, neither 16α-17β-iodo-17β-estradiol, a preferential ER-α ligand, nor genistein, an ER-β selective ligand, elicited MAPK phosphorylation (56). Beyond the cerebral cortex, 17β-E_2 protected hippocampal neurons from glutamate excitotoxicity via MAPK activation and activated MAPK phosphorylation in developing cerebellar neurons (68,85). These findings imply the activation of MAPK phosphorylation by 17β-E_2 occurs in numerous brain regions and is important for the regulation of cell survival and function.

1.3.3 PI3-K and Estrogen mediated neuroprotection

Treatment of cultured rat cortical neurons with 17β-E_2 resulted in the rapid activation of PI3-K (77). Phosphorylation of Akt (protein kinase B; PKB), a substrate of PI3-K, was increased as early as 15 minutes following treatment and remained elevated over basal levels for up to 24 hours, resulting in neuroprotection. However, pretreatment with 17β-E_2 for less than 24 hours failed to protect neurons from cell death, indicating PI3-K activation may be necessary, but not sufficient for neuroprotection (77). Addition of LY294002, a PI3-K inhibitor, abolished the protective effects of 17β-E_2, further implicating PI3-K in the neuroprotective actions of 17β-E_2. Intriguingly, co-addition of ICI182,780 completely blocked the neuroprotective effects of 17β-E_2, although phosphorylation of Akt was only partially attenuated (77). These findings suggest 17β-E_2 mediated neuroprotection is via an ICI182,780-sensitive estrogen receptor and that 17β-E_2 -induced activation of PI3-K may be mediated by both an ICI182,780-sensitive and insensitive estrogen receptor.

1.3.4 Evidence for estrogen receptor independent neuroprotection in vitro.

A number of neurodegenerative diseases are linked with oxidative stress and free radical-induced cell death. 17β-E_2, at pharmacological (μM) doses, is a potent electron donor and free radical scavenger which can reduce oxidative stress-induced neuronal damage (73, 86-90). 17□-E_2 attenuated lipid peroxidation induced by amyloid β protein exposure (89, 91-92), glutamate excitotoxicity (89), or $FeSO_4$ exposure (73, 88-91). The antioxidant effects are observed at the same concentrations as the neuroprotective effects when examined in identical systems (86, 88). *In vivo*, administration of supraphysiological doses of 17β-E_2 to rats as late as 90 minutes following MCAO, prevented ischemia-induced brain damage (24). However, this effect may be due to increased vasodilation and cerebral blood flow through the production of vasodilatory compounds, via an estrogen receptor independent mechanism. Furthermore, many *in vitro* studies have demonstrated supraphysiological doses of 17β-E_2 protect both primary neuronal cultures and neuronal cell lines from cell death.

17β-E_2 influenced neuronal survival against a variety of cell death paradigms, including serum deprivation (93-95), oxidative stress (73, 76, 87-89, 96-97), β-amyloid protein (Aβ)-induced toxicity (36, 86-87, 89, 91, 95-96, 98-101), and excitotoxicity (65, 75-76, 89, 97, 102-104). The demonstration of the neuroprotective effects of 17β-E_2 was independent of the cell death assay utilized, as neuroprotection was shown using morphological markers (89, 97, 100, 104-105), dye exclusion techniques (86-88, 90, 93, 98, 103, 106-107), vital dye techniques (36), lactate dehydrogenase release (65, 75-76, 101-102), and formazan dye conversion (73, 86-87, 96, 101). Doses of 17β-E_2 from a low of 0.1 nM 17β-E_2 (93, 95, 104, 106) to a high of 50 μM (73, 103) have been shown to be neuroprotective. The sensitivity of different cell types and populations to 17β-E_2, potentially due to the differential expression and/or abundance of specific estrogen receptor isoforms, contributes to the wide range of protective doses observed. Additionally, the type of insult, the severity of the insult, and the endpoint used to define viability influences the dose of 17β-E_2 necessary to achieve protection.

Despite the abundant evidence in favor of a receptor-independent mechanism of protection by 17β-E_2, this does not account for the protection observed with physiological levels of 17β-E_2 *in vivo.* The antioxidant capability of 17β-E_2 is observed following administration of high, supraphysiological doses, far exceeding the levels found under physiological conditions. Additionally, the protective effects observed with high doses of 17β-E_2 are associated with both increased blood flow and reduction in free radical induced damage. However, the protection of the brain following 17β-E_2 administration *in vivo* is independent of cerebral blood flow changes (22,25). Thus, pharmacological doses of 17β-E_2 represent an attractive clinical therapy following ischemia stroke injury, but these mechanisms of action do not explain the well-documented protection of the brain by physiological levels of 17β-E_2 *in vivo.*

1.4 Estrogen mediated neuroprotection: a role for another cell type?

The failure of physiological doses of 17β-E_2 to directly protect neurons in many studies could suggest involvement of another cell type and/or requirement of an intact architectural hierarchy in mediating the protective actions of 17β-E_2 in the brain. This hypothesis is supported by the observation that physiological doses of 17β-E_2 protect against cell death in rat organotypic cortical explant cultures, which contain various cell types with an intact cellular architecture. Several of these cell types, including endothelial cells and glia, express estrogen receptors *in vitro,* although the actions of 17β-E_2 on these cell types are poorly understood (108). Astrocytes, the most abundant type of glial cell, are located in juxtaposition to neurons and may outnumber neurons by a 10:1 ratio in some regions of the brain. Astrocytes maintain homeostasis in the brain, including the regulation of extracellular glutamate levels and the control of salt and water concentrations. More recently, astrocytes were identified as an abundant source of neurotrophic factors in the brain. However, the physiological roles of astrocytes *in vivo* are not completely understood. Furthermore, the factors regulating astrocytic function are not well defined.

Astrocytes protect neurons against numerous toxic insults, although the mechanism of protection is not established (109-112). Growth factors, such as Transforming Growth Factor-β (TGF-β), Transforming Growth Factor-α (TGF-α), Insulin-like Growth Factor-I and basic Fibroblast Growth Factor (bFGF), are produced by astrocytes and possess cytoprotective capabilities. Furthermore, astrocytes also release other potentially neuroprotective factors such as pyruvate, serine, lysine, adenosine and plasminogen activator inhibitor-1 (PAI-1) (111, 113-115). Preliminary work by our laboratory and others (116-118) implicate astrocyte-derived TGF-β as playing a pivotal role in protection of neurons. This is intriguing, as TGF-β isoforms are induced in response to brain injury *in vivo*, primarily in astrocytes and activated microglia.

1.5 Localization of estrogen receptors in astrocytes

Initial evidence that astrocytes may be targets of estrogen action was derived from studies showing that 17β-E_2 increases glial cell proliferation and regulates expression of the astrocyte specific marker, glial fibrillary acidic protein (GFAP) (119). Further work using [^{3}H]-tamoxifen, a high affinity, estrogen receptor ligand, identified specific binding sites in cultured rat glia. The presence of estrogen receptors in glia was confirmed by the immunocytochemical detection of estrogen receptor in brain sections derived from the adult guinea pig (120) and by the presence of estrogen receptor mRNA in rat astrocyte cultures *in vitro* (108). Work by our laboratory and others detected the presence of both ER-α and ER-β in cultured rat hypothalamic and cortical astrocytes (121-123). In the hippocampus, ER-β colocalized in GFAP positive cells *in vivo*, suggesting hippocampal astrocytes express ER-β (124).

A potential role for astrocytes in mediating the actions of 17β-E_2 in the brain was suggested by an injury-induced, astrocyte-specific increase in ER-α expression following fornix transection in primates (125). It is unknown whether estrogen receptors are induced following other types of brain injury, such as ischemic stroke; however, ER-α mRNA expression was shown to be elevated in the cerebral cortex following MCAO in rats (33),

although the cell type(s) involved in this increase was not determined. The injury-induced expression of ER-α may indicate a potential mechanism for 17β-E_2 to attenuate cell death.

1.6 Selective estrogen receptor modulators (SERMs)

1.6.1 Clinical use of SERMs

Although the average human lifespan continues to increase, the age of the menopause has remained constant for many years, resulting in women spending up to a third of their life in a chronic hypoestrogenic state. 17β-E_2 deficiency is associated with numerous clinical problems, such as the development of osteoporosis, heart disease, cardiovascular disease, hot flushes, cognitive deficits, and increased risk of stroke and neurodegenerative disease. To offset many of the problems associated with postmenopausal estrogen deficiency, hormone replacement therapy (HRT) is currently in widespread use. However, concern exists that HRT may increase the risk for breast and uterine cancers due to the stimulatory actions of estrogen in these tissues (126-127). This limitation of HRT has spurred the search for selective estrogen receptor modulators (SERMs), non-steroidal compounds that mimic the actions of 17β-E_2 in some tissues and antagonize the effects of 17β-E_2 in other tissues. The ideal SERM possesses agonistic activity in bone, in the cardiovascular system, in the vagina, and in the brain, and antagonistic activity in the breast and in the uterus. No ideal or perfect SERM has been identified to date, although two SERMs are approved for clinical use by the Food and Drug Administration (FDA), tamoxifen and raloxifene. Tamoxifen is used for the treatment/prevention of recurrent breast cancer whereas raloxifene is used in the treatment of osteoporosis (128). Given the support for a neuroprotective role of 17β-E_2 in rodent models of ischemic stroke, it is of interest to determine whether SERMs may similarly have a beneficial role in the brain, without the detrimental effects of 17β-E_2 on other organs.

1.6.2 SERMs and ischemic stroke damage

Several laboratories have demonstrated a neuroprotective role for SERMs in animal models of cerebral ischemia. Pretreatment with LY353381.HCl, a recently developed SERM,

protected the caudoputamen region of the brain of OVX female rats in an ischemia-reperfusion model of ischemic stroke (129). This effect was independent of cerebral blood flow changes, indicating a potential direct neuroprotective effect of this compound in the brain. Recently, tamoxifen was shown to protect the rodent brain from injury following both permanent MCAO as well as in an occlusion-reperfusion model of cerebral ischemia (130-132). Together, these findings suggest that SERMs protect the brain against injury, although it is unclear whether estrogen receptors mediate this effect of SERMs. Preliminary studies suggest high doses of tamoxifen exert protection in the brain via the inhibition of nitrosyl generation (131). However, the doses of tamoxifen utilized in this study are known to affect many cellular pathways independent of the estrogen receptor. Preliminary work from our laboratory demonstrated clinically therapeutic doses of tamoxifen (1 mg/kg/day), which induce estrogen receptor activation, attenuated acute ischemic stroke damage. In addition to preserving the cerebral cortex from ischemia induced injury, tamoxifen also protected the striatum against MPP+-induced toxicity by inhibiting dopamine efflux and subsequent hydroxyl radical generation (133). Together, these findings suggest tamoxifen possesses neuroprotective capability over a wide range of doses, although the mechanisms underlying the protection are not fully understood.

The identification of SERMs possessing neuroprotective capability, but lacking stimulatory effects on the uterus and breast, represent an attractive therapeutic intervention in the prevention/treatment of neurodegenerative disease. Furthermore, SERMs are a potential treatment for males, providing beneficial effects on brain function without undesirable estrogenic side effects.

1.7 Objectives and Specific Aims

The overall objective of this research is to elucidate mechanisms involved in 17β-E_2 and SERM-mediated neuroprotection. 17β-E_2 has a well established neuroprotective role in rodent models of brain injury. Additionally, SERMs have been proposed to similarly protect the brain against ischemic brain injury. In particular, the clinically utilized SERMs, tamoxifen

and raloxifene, have recently been shown to also protect the brain from neuronal injury. However, it is currently unclear as to the mechanism whereby 17β-E_2 and SERMs protect the brain. Although several investigators report a direct neuroprotective effect of 17β-E_2, many other researchers fail to observe this direct protection. However, in the presence of other cell types, physiological levels do protect explant cultures from cell death, suggesting another cell type may mediate this effect. In particular, astrocytes represent a potential target for estrogen actions in the brain, as several studies have demonstrated the presence of estrogen receptors on astrocytes, both *in vivo* and *in vitro*. Astrocytes are widely regarded for their ability to maintain homeostasis in the brain, as well as a proposed function in maintaining neuronal survival. To address the mechanisms of 17β-E_2/SERM mediated protection, five specific aims were proposed:

<u>Specific Aim #1.</u> To determine whether 17β-E_2 and SERMs directly influence neuronal survival.

Hypothesis. *17β-E_2 and SERMs directly attenuate neuronal cell death in various injury paradigms.*

Rationale. Work by several laboratories has established physiological doses of 17β-E_2 protect the rodent brain against acute ischemic stroke. Furthermore, preliminary work by our laboratory and others suggests SERMs, such as tamoxifen and raloxifene, at clinically therapeutic doses, similarly protect the brain against stroke-induced neurodegeneration. While it is known that this protective effect is not due to changes in cerebral blood flow, the precise mechanism underlying 17β-E_2 and SERM protection remains to be elucidated. To further clarify the mechanism(s) underlying this protection, the GT1-7 neuronal cell line and cultured rat cortical neurons will be utilized. The GT1-7 cell line is an immortalized neuronal cell line widely used in studies of neuroprotection due to their homogenous nature. In essence, this aim will test for a possible *direct* neuroprotective effect of physiological or

therapeutic concentration of 17β-E_2 or SERMs. Subsequent aims will test the alternative or potential parallel mechanism of an *indirect* pathway for protection (i.e. protection mediated via a second cell type – astrocytes).

Specific Aim #2. To determine whether astrocyte-derived TGF-β protects neurons from cell death.

Hypothesis. *Astrocyte-derived Transforming Growth Factor-β protects neurons from cell death.*

Rationale. Glial cells are important regulators of homeostasis in the brain and have been suggested to have a role in the process of synaptic remodeling and protecting neurons against neurotoxicity. *In vivo*, targeted ablation of astrocytes increases neuronal cell death, implicating astrocytes in the regulation of neuronal survival (109). Astrocytes reportedly release many neurotrophic factors, including growth factors, which may influence cell survival. However, the identity of the factor(s) involved in astrocyte-mediated neuroprotection is unknown. Thus, in this aim, conditioned media from rat brain astrocytes and C6 glial cells will be collected and tested for neuroprotective ability, and pharmacological and biochemical approaches would then be used to identify the factor(s) responsible for glial cell-mediated neuroprotection.

Specific Aim #3. To determine whether 17β-E_2 or SERMs regulate the release of TGF-β from astrocytes.

Hypothesis. *17β-E_2 and SERMs increase TGF-β release from astrocytes via activation of the estrogen receptor and downstream MAPK and Akt pathways.*

Rationale. Numerous laboratories have demonstrated astrocytes may be involved in the protection of neurons against cell death. However, it is controversial as to the factor(s) which

mediate the protective effect. Several factors, including TGF-α, TGF-β, plasminogen activator inhibitor (PAI-1), and basic fibroblast growth factor (bFGF) are neuroprotective and are produced by astrocytes. Preliminary work by our laboratory has implicated TGF-β as the astrocyte derived factor which protects neurons. Thus, we hypothesize 17β-E_2 and SERMs may enhance the release of astrocyte-derived neuroprotective factors, such as TGF-β. Sequence analysis of the TGF-β1 promoter reveals an estrogen response element (ERE) half site in the 5' upstream region of the TGF-β1 gene, suggesting TGF-β may be directly regulated by estrogenic compounds. Recent work by several laboratories including our own indicates the presence of estrogen receptors on rat hypothalamic, hippocampal and cortical astrocytes, further suggesting astrocytes may represent a novel target for mediating the actions of 17β-E_2.

Specific Aim #4. To elucidate the mechanism of TGF-β mediated neuroprotection in GT1-7 Neurons.

Hypothesis. *Astrocyte-derived TGF-β1 protects neurons by increasing the activity of the AP-1 mediated gene transcription.*

Rationale. Preliminary work by our laboratory and others has demonstrated a neuroprotective role for TGF-β against a wide variety of neurotoxic insults, both *in vivo* and *in vitro*. However, the cellular mechanism(s) whereby TGF-β exerts this effect is not known and is a subject of intense investigation. TGF-β influences many diverse signaling pathways, including AP-1 mediated gene transcription. AP-1, which has long been associated with promoting apoptosis, has recently been recognized to enhance cell survival following various insults in both non-neuronal and neuronal tissues. AP-1 also mediates TGFβ-1 mediated cytoprotection in several non-neuronal tissues. Furthermore, AP-1 activity is increased in surviving neurons of the ischemic penumbra following acute ischemic stroke, suggesting AP-

1 mediated transcription may be a mechanism of neuronal survival. Based on these observations, we have decided to explore the possible role of the c-Jun/AP-1 pathway in mediating TGF-β neuroprotection.

To test this aim, the GT1-7 neuronal serum deprivation cell death model will be utilized. The activation of c-Jun and upstream kinases will be determined following TGF-β treatment. Further, the ability of TGF-β1 to protect neurons will be assessed after specific inhibitors of the c-Jun signaling pathway are added to cultures.

Specific Aim #5. To identify genes potentially mediating the neuroprotective effects of 17β-E_2 and/or tamoxifen through the use of high density gene chip arrays.

Hypothesis. *17β-E_2 and tamoxifen influence gene expression, which influences neuronal survival*

Rationale. Work from several laboratories suggests physiological doses of 17β-E_2 protect the brain via estrogen receptor activation, which is associated with increased gene transcription. Along these lines, 17β-E_2 increased the expression of bcl-2, an anti-apoptotic protein, in both the hypothalamus and cerebral cortex. We speculate 17β-E_2 may similarly regulate other genes involved in protecting the brain from injury. Preliminary work demonstrated tamoxifen, at clinically therapeutic doses, which are known to influence estrogen receptor activity, dramatically attenuated ischemic stroke damage in rats. Thus, tamoxifen may protect the brain via similar mechanisms as 17β-E_2. The identification of the specific genes regulated by 17β-E_2 and SERMs would provide important new insights into how these factors exert their neurotrophic and neuroprotective actions upon the brain.

Figure 1-1: Potential mechanisms of 17β-E_2 and SERM-mediated neuroprotection in the brain. Three mechanisms of action have been proposed to account for the neuroprotective ability of 17β-estradiol and the SERMs. These compounds may a. directly protect neurons from cell death, b. indirectly protect neurons by acting on an intermediary cell type, such as astrocytes, or c. exert actions independent of receptor binding, such as via free radical scavenging.

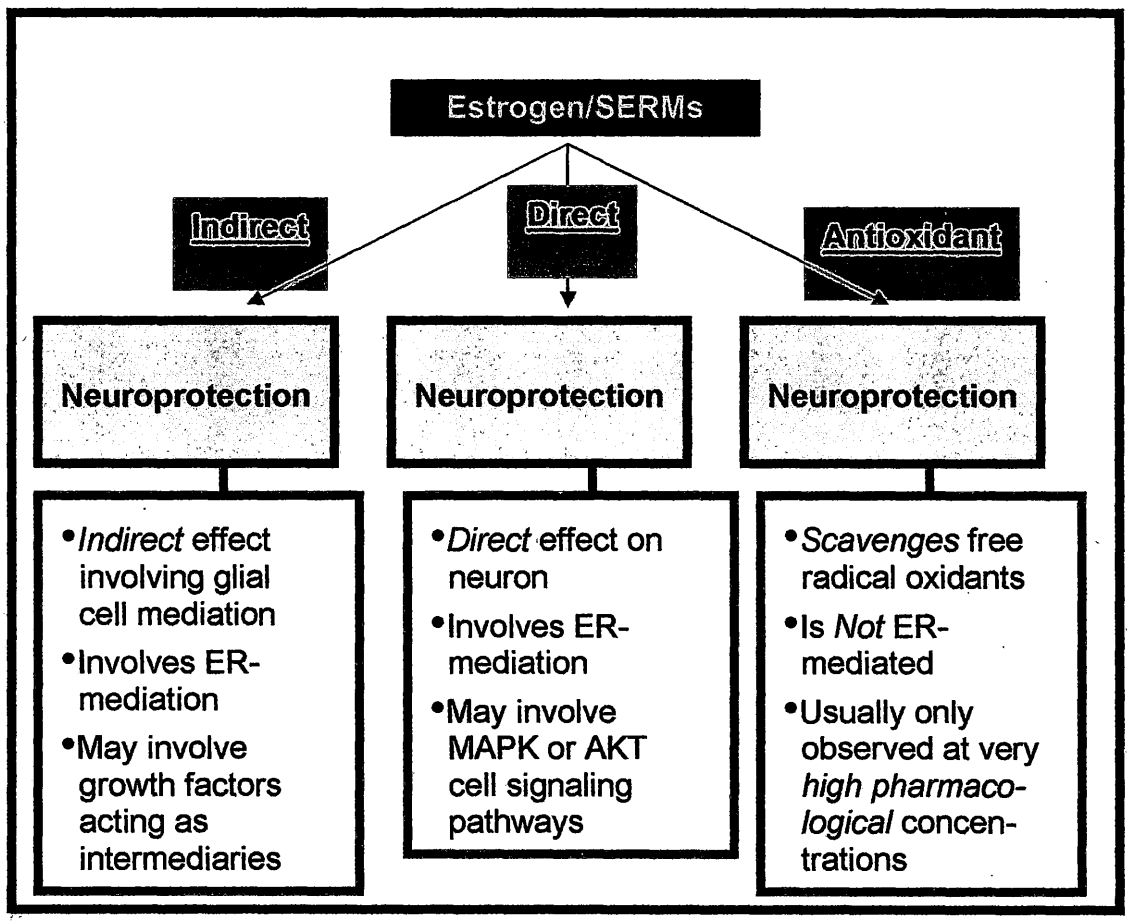
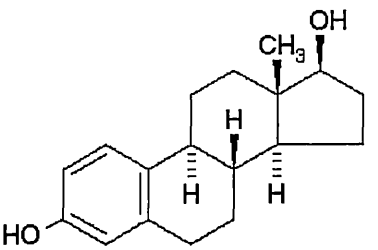
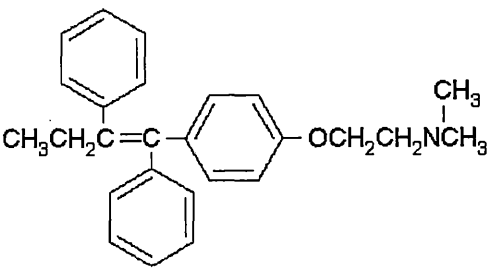

Estrogen/SERMs
Indirect
Direct
Antioxidant
Neuroprotection
Neuroprotection
Neuroprotection
•*Indirect* effect involving glial cell mediation
•Involves ER-mediation
•May involve growth factors acting as intermediaries
•*Direct* effect on neuron
•Involves ER-mediation
•May involve MAPK or AKT cell signaling pathways
•*Scavenges* free radical oxidants
•Is *Not* ER-mediated
•Usually only observed at very *high pharmaco-logical* concen-trations

Figure 1-2: Chemical structures of 17β-Estradiol and SERMs. A. Chemical structure of 17β-Estradiol. B. Chemical structure of tamoxifen. C. Chemical structure of raloxifene hydrochloride.

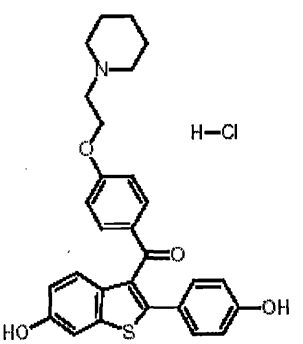
A.



B.



C.



2

INFLUENCE OF 17β-ESTRADIOL AND SELECTIVE ESTROGEN RECEPTOR MODULATORS (SERMS) ON NEURONAL CELL DEATH

2.1 17β-Estradiol – Mechanisms of Neuroprotection

Numerous studies to date have demonstrated a neuroprotective effect of 17β-E_2 in rodent models of acute ischemic stroke. Although controversy exists as to the efficacy of 17β-E_2 in the prevention of neurodegenerative diseases in humans, there is some promise that 17β-E_2 may reduce the severity of ischemic stroke damage in patients. Given the correlation between the occurrence of stroke and Alzheimer's disease following the menopause, when endogenous 17β-E_2 levels decline to 1% of the levels observed during the normal reproductive years, it is important to understand the mechanisms of protection by 17β-E_2. It is also important to characterize the effects of selective estrogen receptor modulators (SERMs) upon the brain and the underlying mechanisms of such effects. The sections below outline the rationale for our studies to address the mechanisms of protection of the brain by 17β-E_2 and SERMs.

2.1.2 Is 17β-E_2 mediated neuroprotection mediated by the estrogen receptor?

The physiological actions of 17β-E_2 are largely exerted by the activation of nuclear estrogen receptors, which stimulate gene transcription. To date, two estrogen receptors, estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β) have been identified. Both ER-α and ER-β are expressed in neurons, thus either/both estrogen receptor isoforms potentially mediate the reported neuroprotective effect. Cell culture models of neuronal cell death following estrogen treatment have produced conflicting results, although several studies have demonstrated 17β-E_2 directly protects rat cortical neurons from glutamate excitotoxicity and β-amyloid toxicity via estrogen receptor activation. Together, these studies suggest estrogen receptors mediate the neuroprotective effect of 17β-E_2 directly at the level of the neuron.

However, an equally substantial body of evidence fails to observe a direct neuroprotective effect of physiological effects of 17β-E_2 on cultured neurons. In several of these studies, 17β-E_2 mediated neuroprotection was only achieved at pharmacological doses, which may be due to the anti-oxidant properties of 17β-E_2. Thus, there is considerable controversy in the literature regarding the mechanism of protection exerted by physiological doses of 17β-E_2.

2.1.2 A Neuroprotective Role for SERMs in the brain

While the protective effects of SERMs on breast and bone are well documented, virtually nothing is known concerning whether SERMS can protect the CNS from cell death. As recent work has shown 17β-E_2 protects the brain from ischemic stroke injury, it is tempting to speculate a SERM may act in a similar manner. Indeed, both tamoxifen and raloxifene have been demonstrated to protect the rodent brain against ischemic stroke damage, although the mechanism of this protection is unexplored (129-132). It is apparent however that the neuroprotective effect of both SERMs is independent of cerebral blood flow suggesting SERMs protect the brain by some direct action of these compounds in the CNS (129-130, 132). As SERMs are mixed estrogen receptor agonists, the neuroprotective functions may be via a mechanism similar to that of 17β-E_2. Understanding the mechanism of protection by FDA-approved SERMS, such as tamoxifen and raloxifene, would aid in the design of better compounds, maximizing the neuroprotective abilities of these compounds, while minimizing unwanted side effects outside of the brain. Recent studies *in vivo* suggest the ability of SERMs to protect against cerebral ischemic involves a genomic mechanism of action. Twenty-four hour pretreatment with LY353381.HCl, a raloxifene analogue, was necessary for protection of the brain of OVX female rats from ischemic damage, suggesting gene regulation may be involved in this process (129). Similarly, one week pretreatment, but not acute treatment, of OVX female animals with therapeutic doses of tamoxifen was necessary for neuroprotection following permanent middle cerebral artery occlusion (132). However, it is unknown whether these compounds may directly influence neuronal survival.

Thus, the goal of this aim is to address the issue of whether 17β-E_2 and the SERMs, raloxifene and tamoxifen, can directly influence neuronal cell survival.

Specific Aim #1. To determine whether 17β-E_2 and SERMs directly influence neuronal survival. In an attempt to resolve the discrepancies regarding the ability of physiological doses of 17β-E_2 to protect neurons from cell death, neuronal cell and tissue culture models will be utilized. Additionally, the direct neuroprotective ability of clinically relevant SERMs will be assessed in these models.

2.2 Methods and Materials

2.2.1 Cell culture models

Supplies. Unless otherwise specified, all cell culture reagents, sera and media were purchased from Gibco BRL (Invitrogen, Grand Island, NY). 17β-Estradiol (17β-E_2) and Tamoxifen were purchased from Sigma (St. Louis, MO). Raloxifene was a generous gift from Eli Lilly (Indianapolis, IN).

GT1-7 Neurons. GT1-7 neurons (a gift from Dr. Pamela Mellon, University of California, San Diego) were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Upon reaching 80% confluency, cells were recovered using 0.05% trypsin-0.53 mM EDTA and replated in 24-well plates at $1x10^5$ cells/well. Cells were cultured until reaching 60% confluency, at which time they were used for treatments.

Primary Rat Cortical Neurons. Primary cultures of rat cerebrocortical neurons were isolated from rat pups at gestational day 18 (E18) (Holtzman, Sprague Dawley, Madison, WI). Pregnant female rats were sacrificed on day 18 gestation and pups were collected by Caesarian section. Embryos were decapitated, the brains were carefully removed and the cerebral cortex was dissected into ice cold PBS. Cerebral cortices were then mechanically

dissociated by trituration using a fire polished glass Pasteur pipet. Following centrifugation at 1000 rpm for 10 minutes, cells were resuspended in Neurobasal medium and plated at 6 x 10^5 cells/well in poly-D-lysine coated 24-well plates. Neurons were cultured in Neurobasal media containing 2% B27 supplement, 0.5 mM glutamine, 25 μM glutamic acid, and 1% penicillin-streptomycin at 37°C in a humidified 95%O_2-5% CO_2 atmosphere. Neurobasal is a specialty formulation which has specifically been developed to support the long term viability of neuronal cells without the need for a glial cell monolayer (134-135). B27 supplement consists of several potent antioxidants, which reduce the incidence of oxidative stress-induced cell death (Brewer et al., 1993). Media was partially replenished twice per week with plating medium lacking glutamic acid. All studies were performed between days 8-10 *in vitro*. This protocol routinely yields cultures that are greater than 95% pure neurons, as assessed by neuron specific enolase immunofluorescence (134).

2.2.2 Treatments

GT1-7 Neurons. Upon reaching 60% confluence, media was removed and cells were gently washed in PBS. Neurons were treated in complete culture medium (serum), serum free DMEM, or serum-free DMEM containing 17β-E_2, tamoxifen, or raloxifene. Doses of 17β-E_2 used were between 10 nM (physiological range) – 1 μM (pharmacological range). The SERMs, tamoxifen and raloxifene were used at a dose of 1 μM, which corresponds to a clinically therapeutic dose of ~0.8 mg/kg/day. Treatments were for 24h, 48h or 72h at which time cell viability was assessed using various methods as described below. Neuronal morphology was also assessed with a light microscope equipped with a digital camera.

Primary Cortical Neurons For all models of cell death, culture medium was removed on day 8 *in vitro* (DIV8) and cells pretreated for 24h with culture medium containing vehicle, 17β-E_2, tamoxifen, or raloxifene. Doses of 17β-E_2 used were between 10 nM (physiological range) – 1 μM (pharmacological range). The SERMs, tamoxifen and raloxifene were used at a dose of 1 μM, which corresponds to a clinically therapeutic dose of ~0.8 mg/kg. Following

pretreatment, media was removed and cells exposed to the appropriate cell death paradigm, as described below.

The first cell death model used was the glutamate excitotoxicity cell death model, which is neurotoxic via a mixture of both necrotic and apoptotic mechanisms and mimics the neuronal death which occurs in numerous neurodegenerative diseases. Glutamate exposure (300 μM) was carried out for 15 minutes at room temperature in buffer containing 2 mM KCl, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 4.2 mM $NaHCO_3$, 12.5 mM HEPES, 10 mM glucose, and 0.1 M NaCl, as previously described (75). Cultures were then gently washed in PBS and returned to culture medium for an additional 24 hours, at which time cell death was measured.

A negative protective effect of the compounds in the glutamate excitotoxicity model could be interpreted to mean that the compounds do not protect directly or that they do not protect from this particular type of cell death pathway. Stroke is a mixture of ischemia, hypoxia, apoptosis and glutamate excitotoxicity. Therefore, to draw meaningful interpretations, we felt it critical that we also assess the protective ability of 17β-E_2 and SERMs in an apoptotic cell death model and in a model for ischemia/ hypoxia-induced cell death. To model a pure apoptotic mechanism of cell death, neurons were exposed to 10 μM camptothecin for 24 hours, following pretreatment with appropriate treatments. Camptothecin, a DNA topoisomerase I inhibitor, induces reproducible neuronal cell death by forming DNA strand breaks (136-138). Following exposure to camptothecin, cell death was assessed by cell death assays as described below.

To reproduce the neuronal death that occurs following hypoxia-ischemia *in vivo*, a chemical hypoxia-ischemia model was utilized, as previously described (139). Following pretreatment, media was replaced with medium containing 1 mM KCN (potassium cyanide) and 2 mM 2-deoxyglucose for 24h. KCN uncouples the mitochondrial respiratory chain and induces chemical hypoxia in a neuron. 2-deoxyglucose cannot be entered into the Krebs cycle and instead prohibits the cellular metabolism of glucose, resulting in chemical ischemia. Following treatments, cell viability was assessed by cell death assays as described below.

2.2.3 Cell Viability/Death Measurements

Cell viability/death was assessed using several methods. Data shown is based on MTT assays, but LDH assay and trypan blue exclusion assays were also performed to confirm cell viability results.

MTT Reduction Assay. To confirm the results of the LDH assay, an MTT assay was utilized to estimate cell viability. Following treatments, 50 µl of 5 mg/ml MTT in phenol red free RPMI-1640 medium (Sigma, St. Louis, MO) was added to each culture well and incubated for 4 hours at 37°C. MTT is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not cleave significant amounts of MTT, making it a sensitive assay of cell viability. Following incubation, formazan crystals were solubilized by the addition of 500 µl 0.04 M HCl in isopropanol to each well. 200 µl cell suspension/sample was transferred to a 96 well plate and absorbance was determined using a plate reader (Labsystems Multiskan MCC/340) at 540 nm using a reference wavelength of 690 nm. All readings were standardized to the control treatment group, which represented 100% viability.

2.2.4 Western blotting

Western blot analysis was performed for protein detection of estrogen receptor isoforms in neurons. GT1-7 neurons or cultured rat cortical neurons were plated in 100 mm cell culture dishes or 35 mm cell culture dishes, respectively. GT1-7 cells were grown to ~70% confluency and rat cortical neurons were plated at 1 x 10^6 cells/dish and cultured for 9 days *in vitro*. Cells were washed with PBS, then 200 µl of ice cold RIPA buffer (1X PBS, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (10 µl of 10 mg/ml PMSF, 30 µl/ml aprotinin) and a phosphatase inhibitor (10 µl/ml sodium orthovanadate) was added to each dish. Cells lysates were transferred to a microcentrifuge tube on ice, and passed through a 21 gauge needle several times to shear DNA. 10 µl of 10 mg/ml PMSF was added to each tube and incubated for an additional 30 minutes on ice.

Tubes were centrifuged at 10,000x*g* for 10 minutes at 4°C. The resulting supernatant (total cell lysate) was transferred to a clean microcentrifuge tube and stored at –70°C until quantification. To quantify protein content in cell lysates, a modified Lowry assay was performed, as recommended by the manufacturer (Sigma, St. Louis, MO).

Twenty-five μg of total cell lysate was dissolved in loading buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol), boiled for 5 minutes, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (0.45 μm pore size, Immobilon-P, Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membranes were washed in T-TBS (20 mM Tris, 137 mM NaCl, 0.1% Tween 20), blocked in 5% non-fat dry milk for 1 hour, and primary antibody was added at a 1:1000 final concentration in T-TBS overnight at 4°C (Santa Cruz Biotechnology). A horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) was used to detect the presence of primary antibody and enhanced chemiluminescence system was used to visualize the proteins (ECLPlus, Amersham Pharmacia Biotechnology). Blots were exposed and molecular weight determination was determined using a digital imaging system (IS-1000, Alpha Innotech, San Leandro, CA).

2.2.5 RNA Isolation

Total RNA was isolated from GT1-7 cells and from cultured rat cortical neurons using TriZOL (Life Technologies, Grand Island, NY) according to manufacturer's recommendations. Samples were stored at room temperature for 5 minutes to allow the dissociation of nucleoprotein complexes, and then transferred to sterile Eppendorf tubes. Chloroform was added (0.2 ml/1ml TriZOL), tubes were vortexed, and then samples were incubated at room temperature for 15 minutes. Samples were centrifuged at 12,000x*g* for 15 minutes at 4°C causing the mixture to separate into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA-containing aqueous phase was transferred to a sterile microcentrifuge tube, and RNA was precipitated by the addition of isopropanol at

room temperature for 15 minutes. RNA was pelleted by centrifugation at 12,000xg for 10 minutes at 4°C, and pellets were washed with 75% ethanol (1 ml/1 ml TriZOL used in initial step). Following air drying for 5 minutes, RNA pellets were resuspended in sterile distilled water. Aliquots of 5 µl were used for spectrophotometric analysis to determine RNA concentrations. The integrity of the total RNA was visualized on a 1.5% agarose gel. Samples were stored at –80°C until required for RT-PCR.

2.2.6 Real Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Real time RT-PCR was performed in a Cepheid SmartCycler (Cepheid, Sunnyvale, CA) using the RNA Amplication Kit – SYBR Green I (Roche, Indianapolis, IN), according to manufacturer's recommendations. Briefly, each reaction tube containing the following components: 5 µl Light Cycler Reaction Mix (containing polymerase, RT enzymes, dNTPs, and SYBR green), 2.5 µl resolution solution, 2.5 µl sense primer, 2.5 µl antisense primer, 0.5 µg total RNA, and 5 µl stdH_2O. Additionally, $MgCl_2$ (8 mM final concentration for ER-α and 16 mM final concentration for ER-β) was added to each tube following optimization. Final volume of each reaction was 25 µl. Following reverse transcription of RNA, cDNA was amplified with the following cycling parameters: denaturation at 94°C for 1 minute, followed by annealing (50°C), and extension at 72°C for 2 minutes. Thirty-five cycles were performed and a single, clean peak following melting curve analysis confirmed product specificity. Additionally, PCR products were resolved in a 1.5% agarose gel and visualized by ethidium bromide fluorescence using a digital imaging system (IS-1000, Alpha Innotech, San Leandro, CA)

For detection of ER-α and ER-β isoforms, the following primers were utilized, as previously described (Buchanan et al., 2000): ER-α: FP 5'-AGTCCTGGACAAGATCAACGA-3' and RP 5'-ATGAAGACGATGAGCATCCAG-3' Product Length 220 bp, ER-β: FP 5'-AATGCTCACACGCTTCGAG-3' and RP 5'-AACTTGGCATTCGGTGGTAC-3' Product Length 292 bp.

2.2.7 Statistical Analysis

The effect of different treatments was analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keul's test. For all groups, $n \geq 5$ and experiments were repeated in triplicate for verification of results. The results were expressed as means ± standard error of the mean. P values < 0.05 were considered significant.

2.3 Results

2.3.1 Effects of 17β-E_2 and SERMs on serum deprivation induced cell death of GT1-7 neurons

To determine whether 17β-E_2 or the SERMs, tamoxifen and raloxifene, directly protect neurons, a serum deprivation model of cell death was utilized in GT1-7 neurons. Cell death was determined following 24h, 48h, and 72h of treatment with either serum free medium or serum free medium containing investigated compounds. As shown in Figure 2-1, neither 17β-E_2 nor SERMs at the doses tested, rescued GT1-7 neurons from serum deprivation-induced cell death. Interestingly, tamoxifen slightly potentiated serum deprivation-induced cell death in GT1-7 neurons at a 1 μM dose.

2.3.2 Effects of 17β-E_2 and SERMs on cell death in Rat Cortical Neurons

Effect of 17β-E_2 and SERMs in an Excitotoxicity Model of Cell Death

To test for the neuroprotective effects of 17β-E_2 and SERMs in non-transformed neurons, primary cultures of rat cortical neurons were exposed to glutamate at a concentration which induces excitotoxicity. This model mimics the neuronal loss occurring in the ischemic core following acute ischemic stroke and is reported to induce neuronal cell death via a mixture of apoptosis and necrosis. As shown in Figure 2-2a, 17β-E_2 failed to protect against glutamate excitotoxicity at doses ranging from physiological levels to pharmacological doses. Furthermore, the SERMs were unable to rescue neurons when treated at clinically therapeutic doses.

Effect of 17β-E_2 and SERMs in an Apoptotic Model of Cell Death

To test the ability of 17β-E_2 and SERMs to protect against an apoptotic inducer, a camptothecin-induced cell death paradigm was utilized. Unlike the glutamate excitotoxicity model, which may involve a necrotic mechanism, camptothecin-induced neuronal cell death has been reported to be purely apoptotic. As shown in Figure 2-2b, similar to the observations in the glutamate excitotoxic model of cell death, neither 17β-E_2 nor the SERMs rescued neurons from subsequent camptothecin exposure.

Effect of 17β-E_2 and SERMs in an Ischemia/Chemical Hypoxia Model of Cell Death

To reproduce hypoxia-ischemia, such as that which occurs following cerebral ischemia, an established chemical hypoxia-ischemia model was utilized using potassium cyanide and 2-deoxyglucose. Cell death in this model reportedly involves a combination of necrosis and apoptosis. As shown in Figure 2-2c, 17β-E_2 and SERMs failed to protect cortical neurons from chemical hypoxia-induced neuronal death.

2.3.2 Expression of Estrogen Receptor Isoform Transcripts in Neurons

To determine whether the failure of 17β-E_2 and SERMs to protect neurons was due to a lack of neuronal estrogen receptors, we utilized real time RT-PCR to investigate the presence of both known estrogen receptor isoforms in GT1-7 neurons and in cultured cortical neurons. As shown in Figure 2-5, both ER-α and ER-β transcripts were expressed in GT1-7 neurons. Similarly, the expression of estrogen receptor isoforms was investigated in primary rat cortical neuronal cultures. Like GT1-7 neurons, ER-α mRNA was abundantly expressed; however, ER-β transcript was not detected in these cultures (Figure 2-3).

2.3.3 Expression of Estrogen Receptor Protein in Neurons

In order to confirm the RT-PCR data, estrogen receptor isoform protein expression was studied using Western blotting. As shown in Figure 2-3, both ER-α and ER-β protein expression was detectable in GT1-7 neurons. However, in contrast, ER-β protein expression

was weakly expressed in cultured rat cortical neurons. Conversely, ER-α protein showed a robust expression in cortical neurons.

2.3.4 Effects of 17β-E_2 and SERMs on cell death in Mixed Neuronal-Glial Cultures

The lack of a significant protective effect in GT1-7 neurons and purified rat cortical neurons by 17β-E2 and tamoxifen, while they are protective *in vivo*, could suggest a role for another cell type in mediating the protective effects. Thus, we next examined whether 17β-E2 or tamoxifen would be protective in mixed glial/neuronal cortical cultures, which may better mimic the *in vivo* situation due to the presence of multiple cell types. Cultures of rat cortical cells containing both neurons and glial cells were therefore pretreated for 24 hours with either 10 nM 17β-E2 or 1 μM tamoxifen, and then exposed to camptothecin (10 μM) for a further 24 hours. Camptothecin was chosen as the cell death agent because it has been shown to induce cell death in neurons, while glial cells are resistant. As shown in Figure 2-4, both 17β-E2 and tamoxifen exerted a significant protective effect against camptothecin-induced cell death in the mixed cortical cultures ($p<0.01$ versus vehicle control). These findings contrast the results obtained in pure neuronal cultures, where no significant protective effect of 17β-E2 or tamoxifen was observed against camptothecin- induced cell death (see Figure 2-2).

2.4 Discussion and Significance

In the present aim, no direct neuroprotective effect of 17β-E_2 or the SERMs, tamoxifen and raloxifene, were observed against a range of death inducing agents in two neuronal models. 17β-E_2, at physiological or pharmacological ranges, was unable to rescue GT1-7 neurons from serum deprivation-induced cell death although a modest protective effect was observed with high pharmacological doses (10 μM) of 17β-E_2 (data not shown). The protective effect by the 10 μM dose of 17β-E_2 may be explained by the well-documented anti-oxidant properties of high doses of 17β-E_2, rather than via estrogen receptor activation (73).

Nonetheless, physiological levels of 17β-E_2, which are well documented to protect *in vivo*, failed to protect GT1-7 neurons from cell death. Interestingly, tamoxifen, at a dose which approximates that used in the treatment of breast cancer patients, modestly exacerbated neuronal injury following serum deprivation in GT1-7 neurons. This finding was unexpected as tamoxifen exerts a significant protective effect on the brain following middle cerebral artery occlusion (130-132). Raloxifene, another class of SERM, did not influence the survival of serum deprived GT1-7 neurons although an increase in neurite outgrowth was observed following treatment (data not shown). A similar increase in neurite outgrowth was observed following treatment of PC12 neurons with 0.1 μM raloxifene (140). Together these observations suggest raloxifene, at clinically therapeutic doses, may exert a direct neurotrophic effect at the level of the neuron, despite a lack of protection at these same doses. In summary, none of the estrogenic compounds tested in this aim were capable of rescuing GT1-7 neurons from cell death.

To determine whether the lack of a direct neuroprotective effect on GT1-7 neurons was due to the immortalized nature of these cells, we next sought to determine whether 17β-E_2 or SERMs would protect primary rat neuronal cultures from cell death. The ability of 17β-E_2 and SERMs to protect against three widely used cell death paradigms, which mimic the neuronal injury which occurs following ischemic stroke, was tested in primary cultures of rat cortical neurons. Interestingly, neither 17β-E_2, nor the SERMs, protected against the cell death induced by any of the three paradigms. The lack of protection was similar to that observed in the immortalized neuronal cell line, GT1-7.

As 17β-E_2 and SERMS exert many of their actions by activating the estrogen receptor, the expression of estrogen receptors in neurons was investigated. ER-α transcript and protein was present in both GT1-7 neurons and in primary rat cortical neuronal cultures, while ER-β was only expressed in GT1-7 neurons, with little to no expression in rat cortical neurons. The high expression of ER-α and low to no expression of ER-β in rat embryonic cortical neurons *in vitro* is exactly opposite of the ER expression pattern reported *in vivo* in the adult rat, i.e. ER-β is highly expressed in the adult rat cerebral cortex, while expression of

ER-α is low (141). This developmental difference in expression of ER may explain the lack of a significant protective effect in the *in vitro* rat cortical neuronal cultures as compared to the strong protection observed with the compounds *in vivo*. An additional important caveat concerning interpretation of the *in vitro* results is that the cultures are highly purified dissociated neuronal cultures, which is unlike the *in vivo* situation where many cell types are present and the cellular and tissue architecture is preserved. Thus, the lack of a protective effect in the highly purified neuron cultures could suggest the need for another cell type for mediation of the protective effects of 17β-E_2 and SERMs and/or the need for intact cellular/tissue architecture. The present data have demonstrated a neuroprotective effect in the presence of glial-neuronal mixed cultures against camptothecin-induced cell death. As camptothecin is a neuronal specific cell death inducer, the protective effect of both 17β-E_2 and tamoxifen, which failed to protect purified neuronal cultures from camptothecin, is likely due to neuronal survival. In further support of a glial intermediary role in estrogen-medaited neuroprotection, 17β-E_2 has been shown to protect rat cerebrocortical tissue slice explants from chemical/hypoxia-induced cell death, a model that maintains an intact cellular architecture and the presence of several different cell types (143-144). Together, these findings suggest the presence of other cell types, such as glial cells, may be important in mediating the neuroprotective effects of estrogens. Finally, we cannot exclude the possibility that 17β-E_2-mediated neuroprotection may not require either ER-α or ER-β and may rather act in an estrogen receptor independent manner or may act via a yet unidentified estrogen receptor subtype. In support of this possibility, Toran-Allerand and colleagues (51) recently reported the possibility of a novel estrogen receptor, ER-X, in mammals, which is membrane-associated, expressed in the adult brain, and is suggested to influence neuronal differentiation, survival, and plasticity. However, ER-X has not fully been cloned to date, thus the presence of ER-X in our cultures could not be determined. Furthermore, a third estrogen receptor has recently been cloned from the teleost, suggesting that more than two mammalian estrogen receptor isoforms may exist (145). As a whole, our studies did not reveal a significant protective effect of 17β-E_2 or SERMs in either GT1-7 neurons or highly

purified rat embryonic cortical neurons in culture. This is true despite using several cell death-inducing agents that model excitotoxic, apoptotic and ischemia/hypoxia cell death. Conversely, both physiological levels of 17β-E_2 and therapeutic concentrations of tamoxifen are neuroprotective in a glial-neuronal mixed culture. Subsequent aims of this proposal thus explored the role of other cell types, particularly astrocytes (the major subtype of glial cells), in exerting neuroprotection and as a potential target for estrogen/SERM action. Gene chip arrays were also employed *in vivo* in order to circumvent the *in vitro* limitations and to help identify target genes that could mediate the neuroprotective and neurotrophic effects of 17β-E_2 and SERMs.

Figure 2-1: 17β-E_2 and SERMs fail to rescue GT1-7 neurons from serum deprivation. Neither 17β-E_2 (10 nM; closed triangles) nor the SERMs (1 μM), tamoxifen (open triangles) and raloxifene (closed squares), rescued GT1-7 neurons from serum deprivation induced cell death. Serum controls = closed circles; serum deprived = open circles. * = Significantly different from all other treatment groups ($p<0.05$).

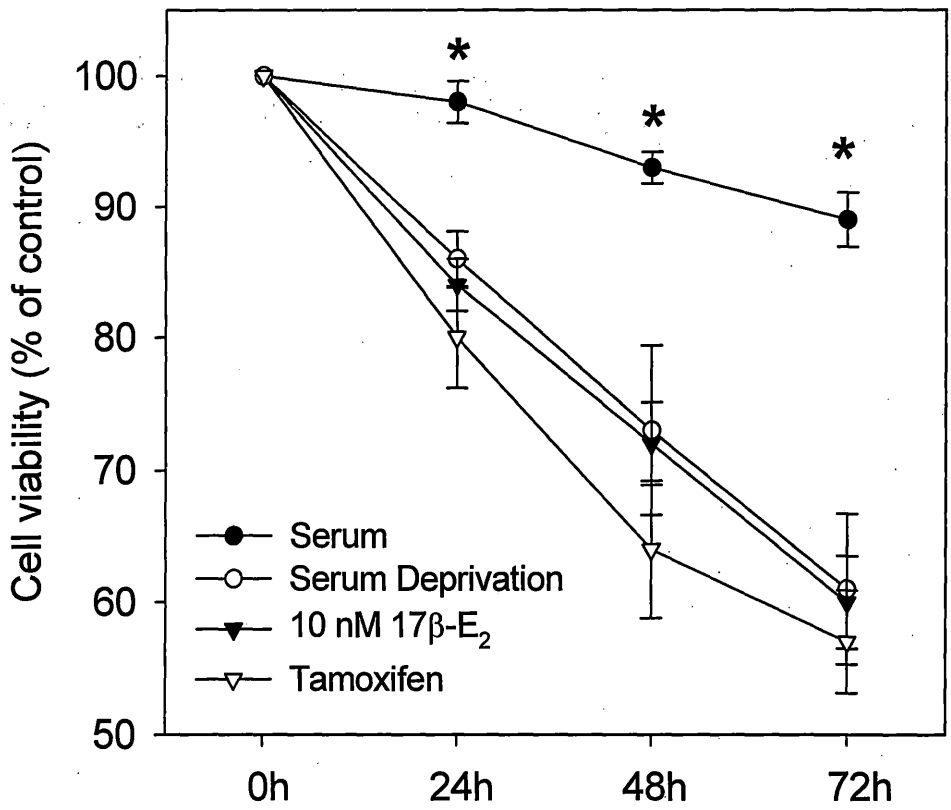

Cell viability (% of control)
100
90
80
70
60
50
0h
24h
48h
72h
*
*
*
Serum
Serum Deprivation
10 nM 17β-E2
Tamoxifen

Figure 2-2. Effect of 17β-E_2 and tamoxifen on cell death induced by camptothecin (A), glutamate (B) and chemical hypoxia (C) in purified primary rat embryonic cortical neurons in culture. Rat cortical neurons were pretreated with vehicle (Veh), 17β-E2 or tamoxifen (TMX) 24 hours prior to either application of camptothecin (10 μM), glutamate (300 μM), or chemical hypoxia (potassium cyanide and 2-deoxyglucose, 1 mM and 2 mM, respectively)). Cell viability was assessed 24 hours following addition of cell death inducers. * = Significantly different from all other treatment groups ($p<0.05$).

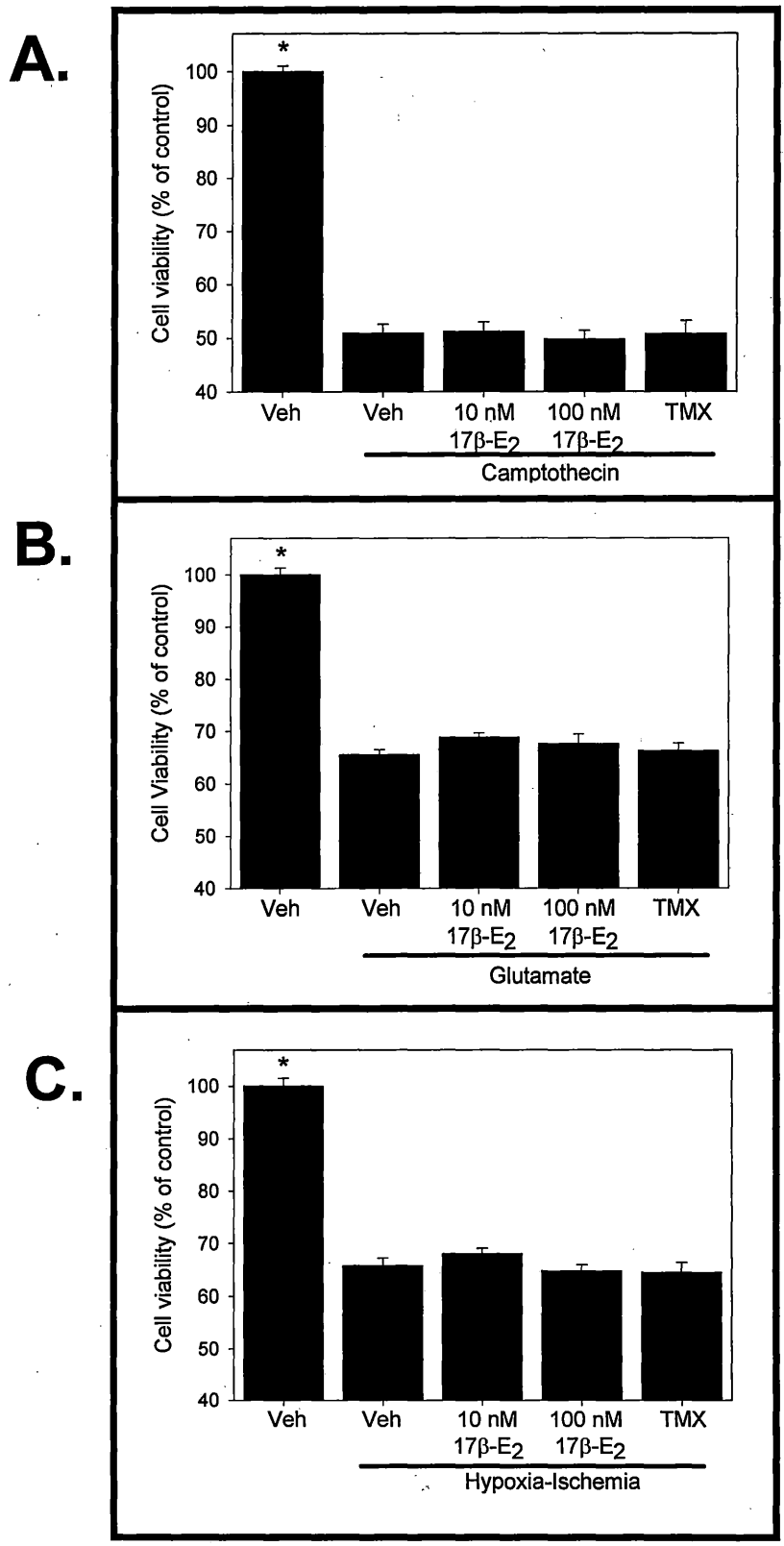
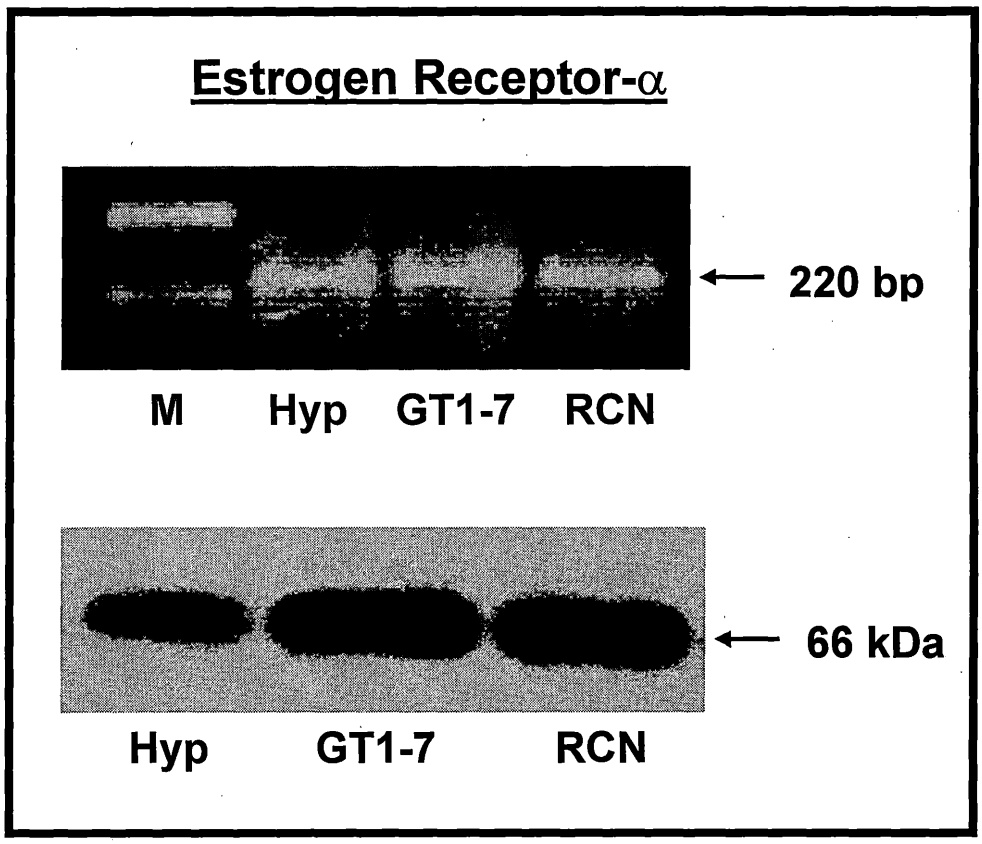

A.
Cell viability (% of control)
100
90
80
70
60
50
40
*
Veh
Veh
10 nM 17β-E2
100 nM 17β-E2
TMX
Camptothecin
B.
Cell Viability (% of control)
100
90
80
70
60
50
40
*
Veh
Veh
10 nM 17β-E2
100 nM 17β-E2
TMX
Glutamate
C.
Cell viability (% of control)
100
90
80
70
60
50
40
*
Veh
Veh
10 nM 17β-E2
100 nM 17β-E2
TMX
Hypoxia-Ischemia

Figure 2-3. RT-PCR (A) and Western blot (B) analysis of estrogen receptor expression in GT1-7 neurons and in purified primary rat embryonic cortical neurons. Rat hypothalamus was included as positive controls for estrogen receptor-α and estrogen receptor-β. Hyp = rat hypothalamus, GT1-7 = immortalized GnRH (GT1-7) neuronal cell line, RCN = purified primary rat embryonic cortical neurons, M = 100 base pair marker.

A.


Estrogen Receptor-α
220 bp
M
Hyp
GT1-7
RCN
66 kDa
Hyp
GT1-7
RCN

B.

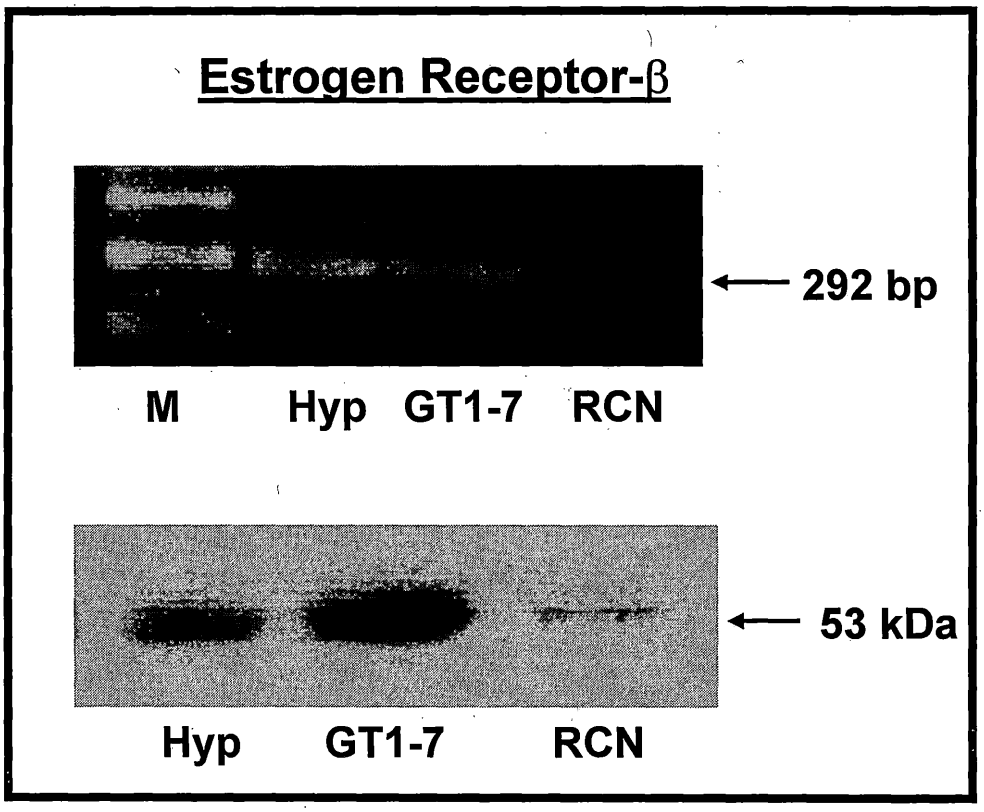
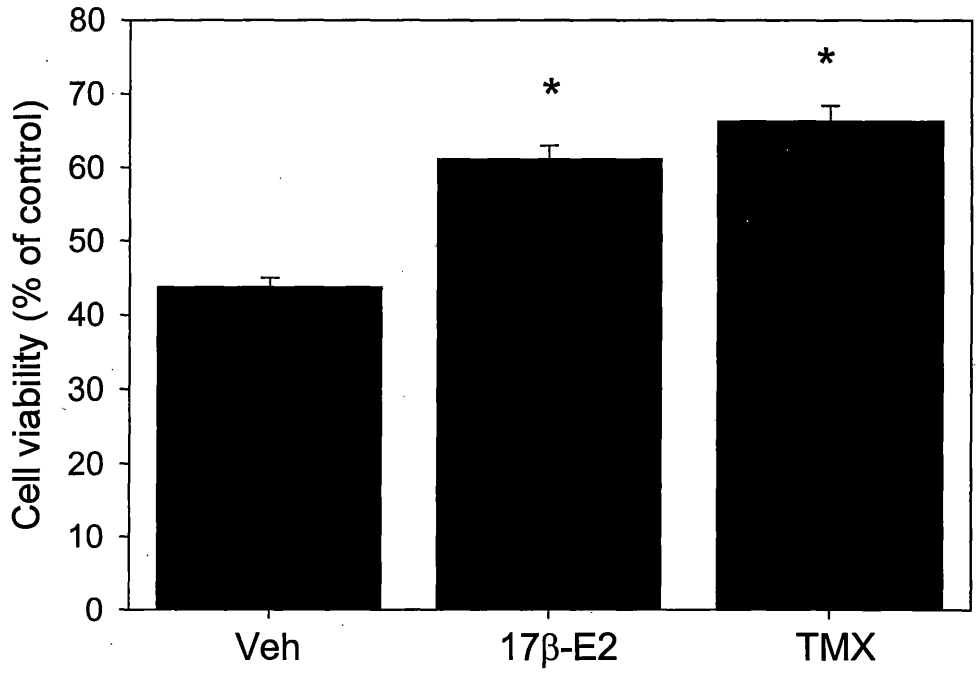

Estrogen Receptor-β
292 bp
M
Hyp
GT1-7
RCN
53 kDa
Hyp
GT1-7
RCN

Figure 2-4. **Effect of 17β-E_2 and tamoxifen on cell death induced by camptothecin in mixed glial-neuronal cultures.** Mixed cultures were pretreated for 24 hours with 10 nM 17β-E2 or 1 μM tamoxifen, and then exposed to 10 μM camptothecin for another 24 hours prior to cell viability determination. * = Significantly different from all other treatment groups ($p<0.01$).


Cell viability (% of control)
80
70
60
50
40
30
20
10
0
*
*
Veh
17β-E2
TMX

3

ROLE OF ASTROCYTE-DERIVED FACTORS IN THE PROTECTION OF NEURONS AGAINST CELL DEATH

3.1 Astrocyte-neuron interactions during Neuroprotection

The lack of protection of estrogen and SERMs in purified embryonic rat cortical neurons *in vitro*, while clearly protective *in vivo*, sparked our interest in the potential role of other brain cell types in neuroprotection and as targets for estrogen/SERM action. In particular, the potential role of *astrocytes* as neuroprotective mediators and possible targets for estrogen/SERM action was targeted for further study. Astrocytes are the most abundant cell type in the brain, outnumbering neurons by a 9:1 ratio (145). Astrocytes maintain homeostasis in the brain, regulating glutamate concentration, ionic concentration, and water balance. For these reasons, astrocytes have traditionally been associated with a structural and supportive role in the brain. However, over the past several years, astrocytes have been implicated in diverse processes such as synaptogenesis, neurogenesis, regulation of neurotransmission, brain development, control of hormone release, and neuroprotection (110-111, 116-118, 121, 146-161). These recent findings suggest that astrocytes have a much more diverse, multi-functional role in the brain than previously envisioned.

With regard to neuroprotection, astrocytes have been shown to protect neurons against a wide range of toxic insults *in vitro*, although the mechanism(s) of astrocyte-mediated neuroprotection is not well established (109-112). Astrocytes may protect against neuronal injury, at least in part, via the release of soluble factors (118). In support of this contention, astrocytes are an abundant source of neurotrophic growth factors, such as Transforming Growth Factors and Fibroblast Growth Factors. Furthermore, astrocytes are also known to release pyruvate, serine, lysine, adenosine and plasminogen activator inhibitor-1 (PAI-1), all of which possess some neuroprotective capability (113-115). Thus, the goal of Aim #2 was to further clarify the mechanism of neuroprotection by astrocytes.

Specific Aim #2. Astrocyte-derived Transforming Growth Factor-β protects neurons from cell death. The primary purpose of the present aim was to determine whether astrocytes could protect neurons from cell death in a variety of culture models by releasing soluble factors. It is hypothesized soluble factors, such as TGF-β family members, which are contained within astrocyte-conditioned media, will protect neurons against cell death.

3.2 Methods and Materials

3.2.1 Cell culture models

Supplies. Unless otherwise specified, all cell culture reagents, sera and media were purchased from Gibco BRL (Invitrogen, Grand Island, NY).

C6 glial cells. *C6* glial cells (American Type Cell Culture, Manassas, VA) were cultured in 75 cm^2 flasks in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Upon reaching 80% confluency, cells were recovered using 0.05% trypsin-0.53 mM EDTA and replated in 6 well plates at 2 x 10^5 cells/well. Cells were grown to ~80% confluency at which time media was removed, cells washed with Dulbecco's Phosphate Buffered Saline (PBS), and 1 mL serum free DMEM was added to each well for 18 hours. Following incubation, media was pooled and stored at –80°C until use. This media will hereafter be referred to as C6 18h conditioned media (C6 CM).

For immunoneutralization studies, C6 CM was incubated with 15 μg/ml of anti-TGF-β pan specific polyclonal antibody (R&D Systems, Minneapolis, MN) for 30 minutes at room temperature. According to the manufacturer, at this dose, the antibody neutralizes the biological activity of all three mammalian TGF-β isoforms, TGF-β1, TGF-β2 and TGF-β3. Immediately following incubation on a rotating shaker, the TGF-β-immunoneutralized C6 CM was used for treatments.

Primary Rat Astrocyte Cultures. Primary astrocyte cultures were obtained from the hypothalamus and cerebral cortex of 2-3 day old rats (Holtzman, Sprague Dawley, Madison,

WI). Tissue blocks (of either hypothalami or cerebral cortices) were dissected and immersed in ice cold Dulbecco's Phosphate Buffered Saline. Tissue was mechanically disrupted by mincing in a sterile Petri dish, followed by chemical disruption using a 25 minute exposure to trypsin (1:250; final concentration 0.1%; Difco, Detroit, MI) neutralized to pH 7.0 in saline solution at 37°C. Trypsinization was terminated by the addition of an equal volume of complete culture medium (DMEM-Ham's F-12 Medium; DMEM-F12, 1:1, vol:vol) containing 10% fetal bovine serum and 1% penicillin-streptomycin (referred to hereafter as complete astrocyte culture media). Suspended cells were filtered through a 100 μM nylon mesh filter to remove debris, then centrifuged at 1200 rpm for 10 minutes. Cell pellets were resuspended in complete culture medium, filtered through a 40 μM nylon mesh filter and plated at high density in 75 cm^2 tissue culture flasks. Astrocytes were grown in a humidified cell culture incubator under an atmosphere of 5% CO_2-95% O_2 at 37°C for 10 days, at which point cultures were 100% confluent. Cell cultures were shaken at 220 rpm on an orbital shaker (stroke diameter = 1.5 inches) for 18 hours under an atmosphere of 5% CO_2-95% O_2 at 37°C to remove contaminating oligodendrocytes, microglia, and neurons. Astrocytes were recovered using 0.1 M EDTA, replated at one-third of their confluent density, and grown to ~90% confluency. Astrocytes were then seeded in six-well plates at 4 x 10^5 cells/well and grown to ~80% confluency. For collection of conditioned media, 1 mL of serum free media (either DMEM for GT1-7 treatments or Neurobasal for use with primary neuronal cultures) was added to each well and incubated for 24 hours. Conditioned medium was then removed and stored at –80°C until neuroprotection studies. This medium will be referred to hereafter as hypothalamic astrocyte 24h conditioned medium (HA-CM) or cortical astrocyte 24h conditioned medium (CA-CM). These cultures routinely are >95% pure astrocytes, as assessed by immunostaining with the glial specific marker, glial fibrillary acidic protein (GFAP).

GT1-7 Neurons and Primary Rat Cortical Neuronal Cultures. GT1-7 neurons and embryonic rat cortical neurons were cultured as described previously in the dissertation.

3.2.2 Treatments

GT1-7 Neurons. Upon reaching 60% confluence, media was removed and cells were gently washed in PBS. Neurons were then treated in complete culture medium ("serum"), serum free DMEM, astrocyte conditioned media (which was collected in serum free DMEM), or serum-free DMEM containing exogenous TGF-β. Treatments were for 24h, 48h or 72h at which time cell viability was assessed. Cultures were also photographed using a light microscope to assess the morphology of neurons following treatments.

Primary Cortical Neurons. For all models of cell death (glutamate excitotoxicity, apoptotic, and chemical hypoxia), culture medium was removed on day 8 *in vitro* and cells pretreated for 24 hours with culture medium containing vehicle, CA CM, or TGF-β. Following pretreatment, medium was removed and cells exposed to the appropriate cell death paradigm, as described previously in the dissertation. Cell viability was assessed by the MTT assay as described previously. Additionally, results were confirmed using the LDH cell death assay and trypan blue exclusion assay (data not shown).

3.2.3 Statistical Analysis

The effect of different treatments was analyzed using a one-way analysis of variance (ANOVA) followed by the appropriate multiple comparison test (Tukey's test or Student-Newman-Keul's method). For all groups, $n \geq 5$. The studies were repeated at least three times for verification of results. The results were expressed as means ± standard error of the mean. P values < 0.05 were considered significant.

3.3 Results

3.3.1 Effects of C6 glial cell conditioned media on GT1-7 neuronal survival

C6 glial cell conditioned media (C6 CM) (media exposed to C6 glial cells for 18h) significantly protected GT1-7 neurons from serum deprivation-induced cell death at all time points analyzed (Figure 3-1). Cells treated with C6 CM in serum free media had a survival rate comparable to control

cultures maintained in serum supplemented culture medium. Additionally, C6 CM treated neurons exhibited increased neurite outgrowth as compared to control cultures (data not shown).

3.3.2 Effects of TGF-β immunoneutralization of C6 CM on GT1-7 neuronal protection

We previously had shown that C6 glial cells release TGF-β1 and thus wanted to examine whether TGF-β1 in C6 CM could mediate its protective effects. To determine this, we used an immunoneutralization approach utilizing a pan-specific TGF-β antibody. As shown in Figure 3-2, immunoneutralization of C6 CM with the pan specific TGF-β antibody completely reversed the neuroprotective ability of C6 CM against serum deprivation induced cell death of GT1-7 neurons (Figure 3-2). TGF-β immunoneutralization attenuated C6 CM neuroprotection at all times examined and morphologically, the cells exhibited neurite retraction and rounding, following by detachment from the plate following serum deprivation or TGF-β immunoneutralized C6 CM treatment.

3.3.3 Effects of astrocyte conditioned medium on GT1-7 neuronal survival

To determine that the neuroprotective properties of C6 CM was not due to the transformed nature of C6 cells and that non-transformed astrocytes can equally protect, the ability of primary (non-transformed) hypothalamic astrocytes to protect neurons was assessed. Similar to that observed for C6 CM, HA-CM significantly protected GT1-7 neurons from serum deprivation induced cell death at all time points analyzed (Figure 3-3). Cells treated with HA-CM in serum free media had a survival rate comparable to control cultures maintained in serum supplemented culture medium.

3.3.4 Effects of TGF-β Immunoneutralization on HA CM mediated neuroprotection in GT1-7 neurons

Immunoneutralization of HA-CM with a pan specific TGF-β neutralizing antibody completely blocked the neuroprotective ability of HA-CM in serum deprived GT1-7 neurons. As shown in Figure 3-4, immunoneutralized HA-CM was not significantly different from serum free media in the amount of cell death observed following 48 hours and 72 hours of culture.

3.3.5 Effects of exogenous TGF-β isoforms on survival following serum deprivation in GT1-7 neurons

To further demonstrate the neuroprotective capacity of TGF-β, GT1-7 cells were treated with exogenous TGF-β1 or TGF-β2 during serum deprivation. Both TGF-β1 (Figure 3-5a) and TGF-β2 (Figure 3-5b) significantly protected GT1-7 neurons from serum deprivation-induced cell death, with TGF-β1 maintaining slightly higher potency than TGF-β2. In both cases, a maximal effect was demonstrated with 1 ng/ml TGF-β, which approximates the levels found in HA CM. Furthermore, both HA CM and exogenous TGF-β preserved the morphology of GT1-7 neurons under serum deprivation-induced stress and preserved/enhanced neurite outgrowth (Figure 3-6).

3.3.6 Effects of cortical astrocytes and TGF-β1 on excitotoxic, apoptotic and chemical hypoxia-induced cell death in primary neuronal cultures

To determine whether astrocyte-derived TGF-β could similarly protect primary *non-transformed* neurons from cell death, primary cultures of embryonic rat cortical neurons were utilized. Additionally, cortical astrocyte conditioned media (CA CM) was also used to determine the protective effects of astrocytes in a region where cell death occurs during cerebral ischemia (i.e. cerebral cortex). As shown in Figure 3-7, 24-hour pretreatment with CA CM significantly protected primary cultures of rat cerebrocortical neurons against glutamate excitotoxicity, as assessed 24 hours following the insult. Similarly, exogenous TGF-β1 significantly protected neurons against glutamate excitotoxicity-induced cell death, with a maximal effect observed at a dose of 10 ng/ml (Figure 3-7). As observed in Figure 3-7, 24h pretreatment with either CA CM or 10 ng/ml TGF-β1 also yielded significant protection of cultured rat cortical neurons against camptothecin-induced apoptosis, as assessed 24 hours following exposure to camptothecin. Finally, as observed in Figure 3-7, 24h pretreatment with either CA-CM or TGF-β1 was also found to protect cultured rat cortical neurons against chemical hypoxia-ischemia induced neuronal cell death.

3.4 Discussion and Significance

3.4.1 Astrocytes release factors which are neuroprotective

The work completed in this aim has filled some important deficits in our knowledge concerning the potential mechanisms of astrocyte neuroprotection. Along these lines, the present study demonstrated that soluble factors, released by an immortalized astrocyte cell line, C6, and by primary astrocyte cultures possess neuroprotective ability in a variety of neuronal cell culture injury models. The protection against cell death was observed in an immortalized murine cell line, GT1-7, as well as in primary neuronal cultures derived from the embryonic rat cerebral cortex. Addition of conditioned media from C6 glial cells significantly protected GT1-7 neurons from cell death induced by serum deprivation. C6 CM also induced dramatic neurite outgrowth (data not shown), suggesting glia-derived factors may also influence the neurotrophic ability of neurons, indicating a potential role for astrocyte in synaptic plasticity. Although C6 are a convenient model of astrocyte function, the ability of natural astrocytes to protect neurons was characterized to rule out the possibility that immortalized astrocytes possess unique neuroprotective properties not observed in non-transformed glia cells. Astrocyte conditioned media (from either hypothalamus or cerebral cortex astrocytes) rescued GT1-7 neurons in a similar manner to C6 CM and also provided neurotrophic support. Additionally, neuroprotection by astrocyte conditioned media was also observed in primary embryonic rat cortical neuronal cultures from a variety of cell death inducers. Together, these studies suggest astrocytes possess neuroprotective ability by releasing soluble factors, which exert protective effects upon neurons. Our findings suggesting that astrocytes can protect neurons from excitotoxic, serum deprivation, apoptotic and chemical hypoxia-induced cell death extends the work by others that implicated astrocyte-neuron interactions as critical for the reduction of neuronal cell death following oxidative stress (163-165). Furthermore, during acute ischemic stroke, neuronal cell death rapidly progresses following the loss of astrocytes, suggesting astrocytes support neuronal survival *in vivo* (166). Administration of a gliotoxin, which abolishes astrocytic function, increases the sensitivity of neurons to cellular stressors (167). Additionally, targeted ablation of astrocytic function *in vivo* resulted in increased neuronal cell death, as well as increased susceptibility to ischemic stroke damage, further supporting an important role for astrocytes in the regulation of neuronal survival (109,

168). These observations all point to a potential important role for astrocytes in neuronal survival and work by our laboratory presented herein implicates soluble factors released by astrocytes as potentially critical mediators for the neuroprotective effects.

3.4.2 Astrocyte derived Transforming Growth Factor-β protects neurons from cell death

With respect to the identity of the "active" soluble factor in astrocyte conditioned media, the present study provided evidence that astrocyte release of the neuroactive growth factors, TGF-β1 and TGF-β2 plays an important mediatory role in astrocyte conditioned media-induced neuroprotection. Previous work by our laboratory had shown that astrocytes release significant amounts of TGF-β1 and TGF-β2, which can exert neurosecretory effects on neurons (121). The present study extended this observation by showing that TGF-β in the astrocyte conditioned media also exerts neuroprotective and neurotrophic effects on transformed and non-transformed neurons. Multiple lines of investigation were used to reach this conclusion – including exogenous application of TGF-β1 and TGF-β2 in transformed and non-transformed neuronal cultures, immunoneutralization with a pan-specific TGF-β antibody, and utilization of several widely-used models of cell death that reproduce excitotoxic, apoptotic and hypoxia/ischemia-induced cell death.

Application of exogenous TGF-β1 and TGF-β2, at doses approximating levels found within astrocyte conditioned media (1 ng/ml), significantly protected GT1-7 neurons from serum deprivation induced cell death. Although TGF-β2 significantly protected neurons from cell death, it was slightly less potent as compared to TGF-β1 in the GT1-7 neuronal cell model. Differential effects of TGF-β isoforms have previously been reported in many systems, including the central nervous system. We were unable to explore what role, if any TGF-β3 may have in astrocyte-mediated protection of neurons, as no commercial assay was available at the time the studies were conducted. Nevertheless, TGF-β1 and TGF-β2 are the primary isoforms expressed in the CNS *in vivo* and most likely constitute the majority of the TGF-β activity of astrocyte-conditioned media, although a role for TGF-β3 cannot be entirely excluded. Morphologically, TGF-β treated neurons maintained a healthy phenotype and did not undergo the neurite retraction and rounding up that were characteristic of

serum deprived cells. In contrast, TGF-β treated GT1-7 exhibited elonged neurites, similar to the effect observed with astrocyte conditioned media treatment.

3.4.3 Effects of astrocyte derived factors on neuronal survival in primary rat cortical neurons

Although GT1-7 neuronal cells are a convenient model of neuronal function, it is not known if GT1-7 neurons retain all of the characteristics of natural, non-transformed neurons. Thus, the issue of astrocyte-mediated neuroprotection was next addressed using primary neuronal cultures derived from rat cerebral cortex. Unfortunately, a direct comparison to the GT1-7 serum deprivation model could not be performed as primary cortical neurons are cultured in serum free media. Thus, we determined the effect of astrocyte conditioned media and TGF-β isoforms on several established models of neuronal cell death, which mimic the neuronal death which occurs following acute ischemic stroke.

Addition of CA CM (cortical astrocyte conditioned media) significantly protected rat cortical neurons against all forms of cell death paradigms tested (glutamate excitotoxicity, camptothecin-induced apoptosis, and chemical hypoxia. TGF–β1 similarly protected neurons against cell death. These findings suggest both CA CM and TGF-β isoforms can rescue neurons from cell death caused by both apoptosis (camptothecin, glutamate) and from necrosis (glutamate, chemical hypoxia). Together, these studies suggest astrocytes and astrocyte-derived TGF-β isoforms are capable of rescuing neurons from the various mechanisms of cell death (excitotoxicity, apoptosis and hypoxia) that are known to occur in neurodegenerative disease. An interesting corollary to this point is that TGF-β has been shown to be increased in cerebral ischemia *in vivo*, and a correlation has been established to neuronal survival in brain regions of enhanced TGF-β expression (169-170). Furthermore, acute elevation of TGF-β1 by exogenous central administration or a more prolonged adenoviral-driven over-expression of TGF-β1 has been shown to result in a significant reduction of infarct size and improved neurological outcome in rodent animal models of cerebral ischemia (171-174). Thus, there is already a case for TGF-β being neuroprotective *in vivo*. Our work adds a potentially new conceptual layer to this framework by elaborating that astrocytes can utilize a soluble

factor, TGF-β, to exert neuroprotection, which may be relevant to the postulated critical neuroprotective role that astrocytes purportedly fulfill *in vivo*.

Figure 3-1: Effects of C6 Glial Cell Conditioned Media on GT1-7 Neuronal Cell Viability Following Serum Deprivation. C6 glial cell conditioned media (C6 CM) protected GT1-7 neurons from serum deprivation-induced cell death. Cell viability was assessed 24h, 48h and 72h following serum deprivation or following treatment with C6 CM in serum free conditions. Complete culture medium was used as a control. a = significantly different from all other values at the same time point; * = $p<0.05$, ** = $p<0.01$ as determined by One Way ANOVA and Student-Newman-Keul's Test.

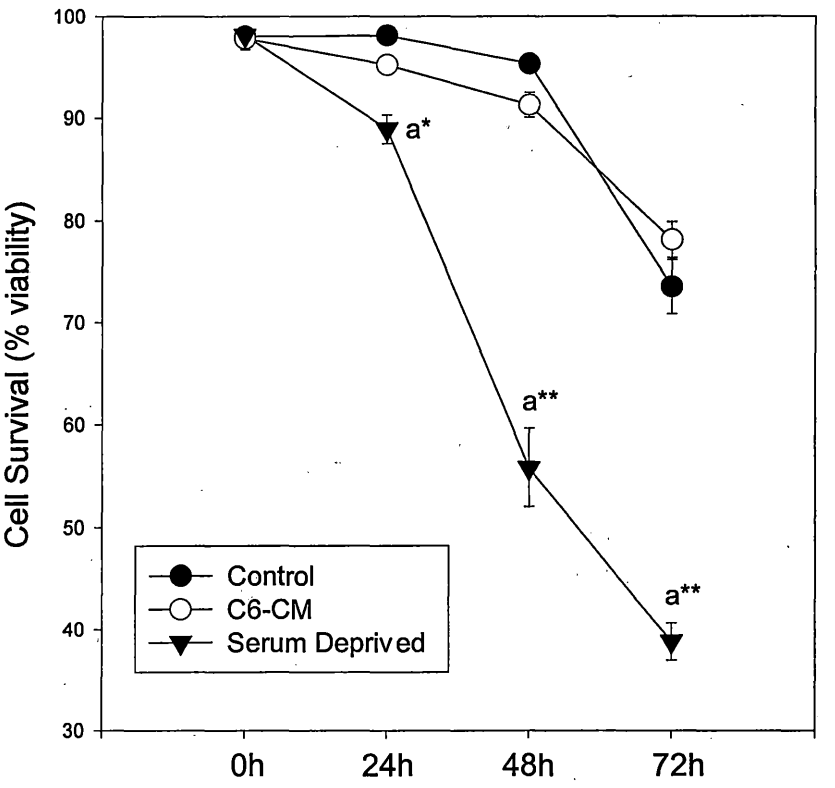

Cell Survival (% viability)
100
90
80
70
60
50
40
30
0h
24h
48h
72h
a*
a**
a**
Control
C6-CM
Serum Deprived

Figure 3-2: Effects of TGF-β Immunoneutralization of C6 Glial Cell Conditioned Media on GT1-7 Neuroprotection. Immunoneutralization of C6 CM with a pan specific TGF-β neutralization antibody (15 μg/ml) completed blocked the neuroprotective effect previously demonstrated with C6 CM. a = significantly different from control at the same time point; * = $p<0.05$, ** = $p<0.01$ as determined by One Way ANOVA and Student-Newman-Keuls's Test.

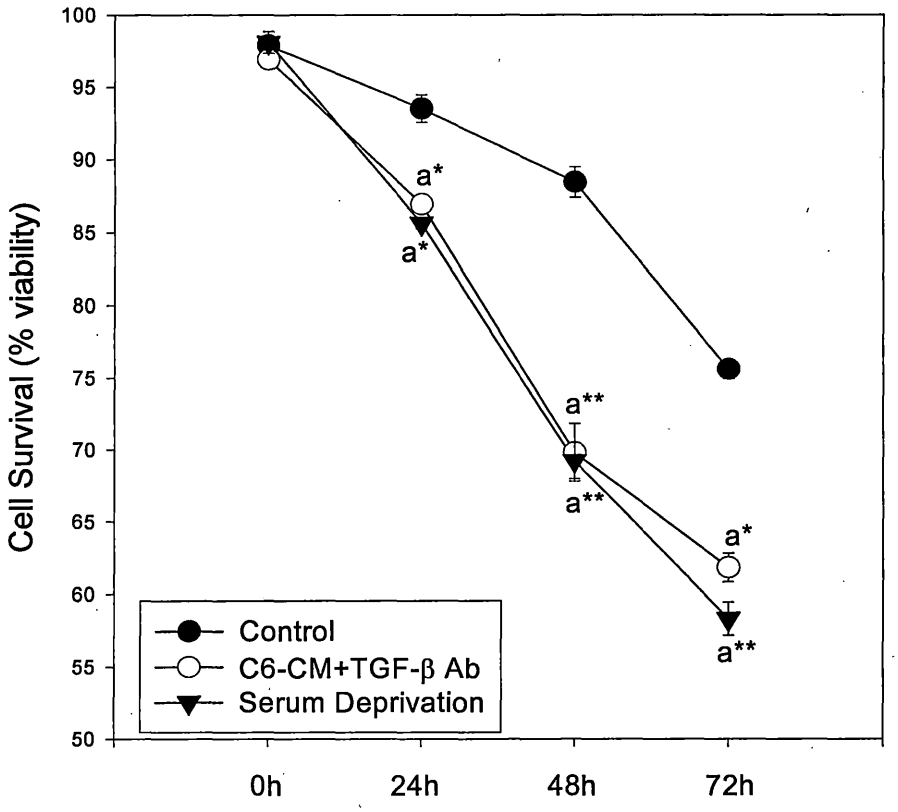

Cell Survival (% viability)
100
95
90
85
80
75
70
65
60
55
50
0h
24h
48h
72h
a*
a*
a**
a**
a*
a**
Control
C6-CM+TGF-β Ab
Serum Deprivation

Figure 3-3: Effects of Rat Astrocyte Conditioned Media on GT1-7 Neuronal Cell Viability Following Serum Deprivation. Rat astrocyte conditioned media (ACM) protected GT1-7 neurons from serum deprivation-induced cell death. Cell viability was assessed 24h, 48h and 72h following serum deprivation or following treatment with ACM in serum free conditions. Complete culture medium was used as a control. a = significantly different from all other values at the same time point; * =$p<0.05$, ** = $p<0.01$ as determined by One Way ANOVA and Student-Newman-Keul's Test.

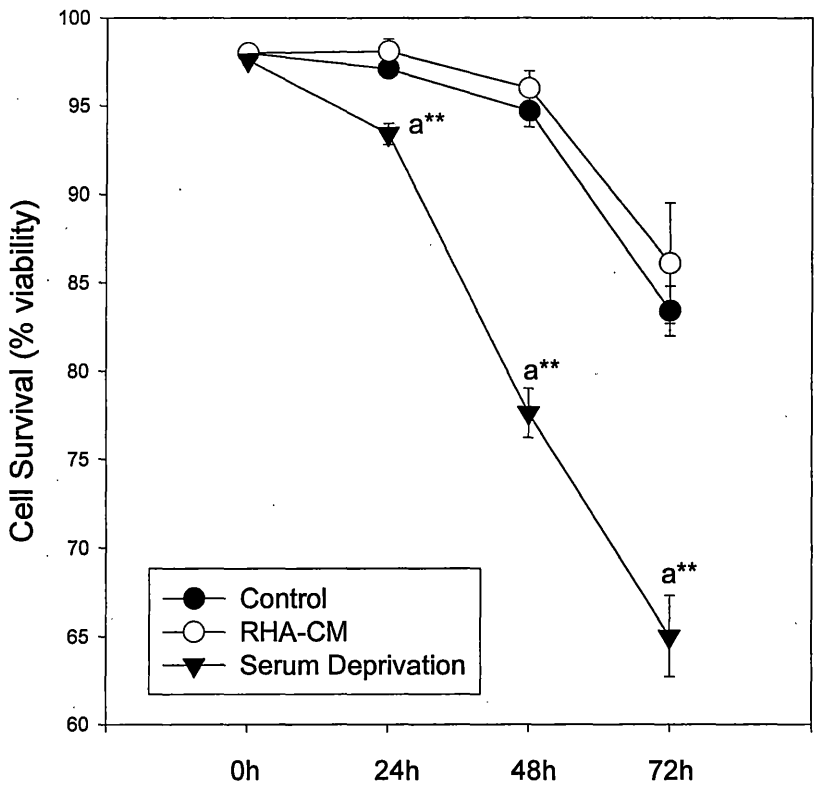

100
95
90
85
80
75
70
65
60
Cell Survival (% viability)
a**
a**
a**
Control
RHA-CM
Serum Deprivation
0h
24h
48h
72h

Figure 3-4: TGF-β Immunoneutralization Attenuates the Survival Promoting Ability of Astrocyte Conditioned Media and Serum in GT1-7 Neurons. Immunoneutralization of either (A) Astrocyte Conditioned Media (ACM) or (B) serum containing culture media reversed the neuroprotective ability of each treatment. Following immunoneutralization, cell viability was not significantly different from serum deprived GT1-7 neurons. * Significantly different from all other treatment groups ($p<0.05$).

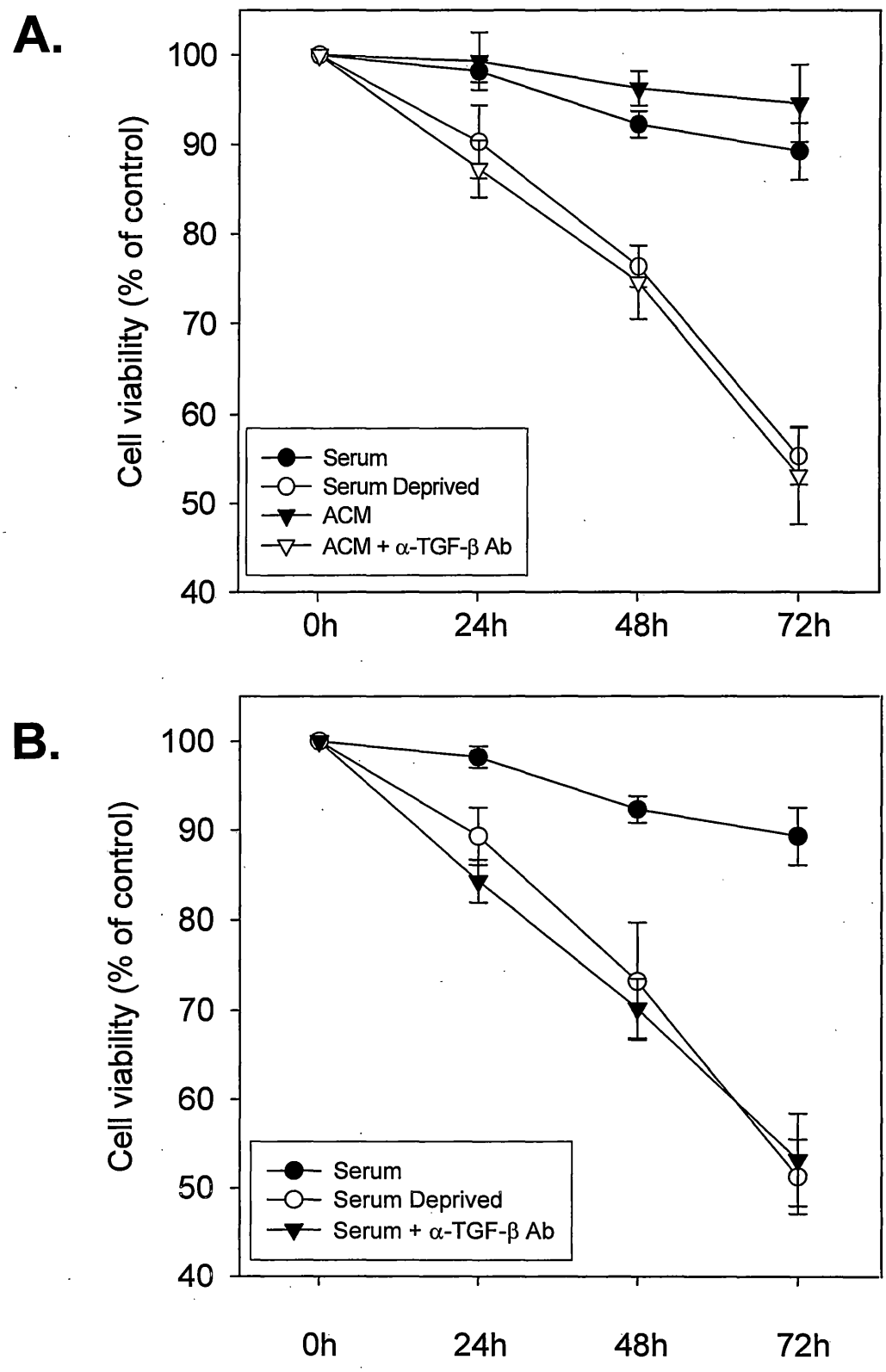
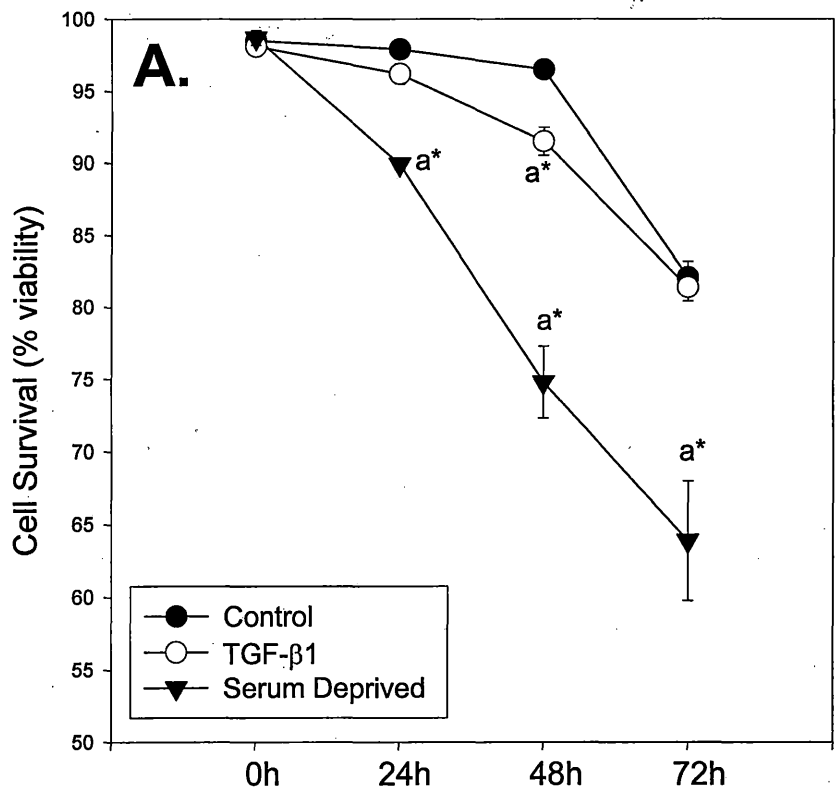

A.
Cell viability (% of control)
100
90
80
70
60
50
40
0h
24h
48h
72h
Serum
Serum Deprived
ACM
ACM + α-TGF-β Ab
B.
Cell viability (% of control)
100
90
80
70
60
50
40
0h
24h
48h
72h
Serum
Serum Deprived
Serum + α-TGF-β Ab

Figure 3-5: Exogenous TGF-β protects GT1-7 Neurons from Serum Deprivation induced Cell Death. Exogenous (A) TGF-β1 or (B) TGF-β2 (1 ng/ml) rescues GT1-7 neurons from serum deprivation-induced Cell Death. TGF-β treatment was performed in serum free medium. Control represents complete culture medium. a,b = significantly different from all other values at the same time point * = $p<0.05$ as assessed by One Way ANOVA and Student-Newman-Keul's Test.


A.
Cell Survival (% viability)
100
95
90
85
80
75
70
65
60
55
50
0h
24h
48h
72h
a*
a*
a*
a*
Control
TGF-β1
Serum Deprived

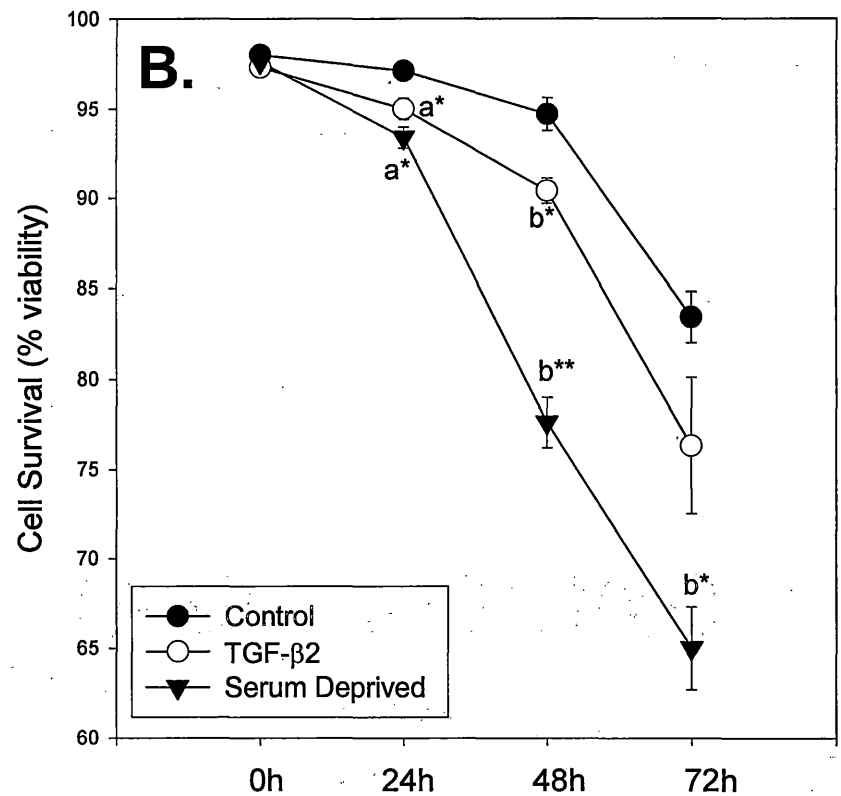

B.
Cell Survival (% viability)
100
95
90
85
80
75
70
65
60
0h
24h
48h
72h
a*
a*
b*
b**
b*
Control
TGF-β2
Serum Deprived

Figure 3-6: Morphology of GT1-7 following serum deprivation. GT1-7 neurons were treated in serum free medium, or serum free medium containing astrocyte conditioned media, 1 ng/ml TGFβ-1, or 1 ng/ml TGF-β2. Cells treated with astrocyte conditioned media or with TGF-β isoforms had an intact morphology and increased numbers of neurites, as compared to serum free controls.

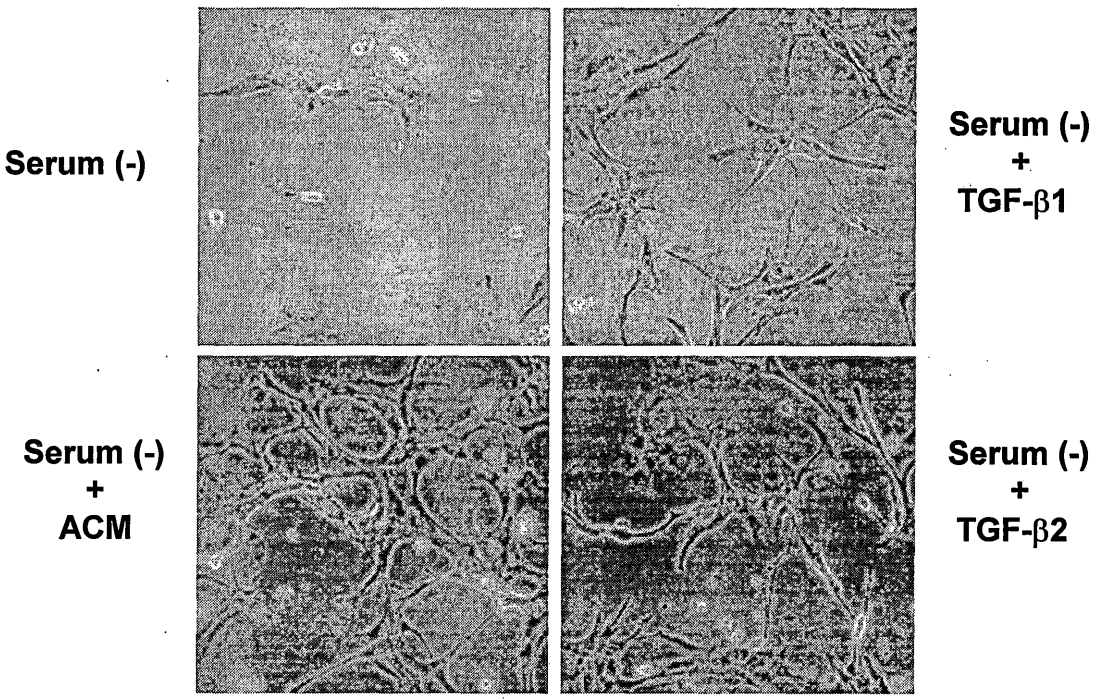
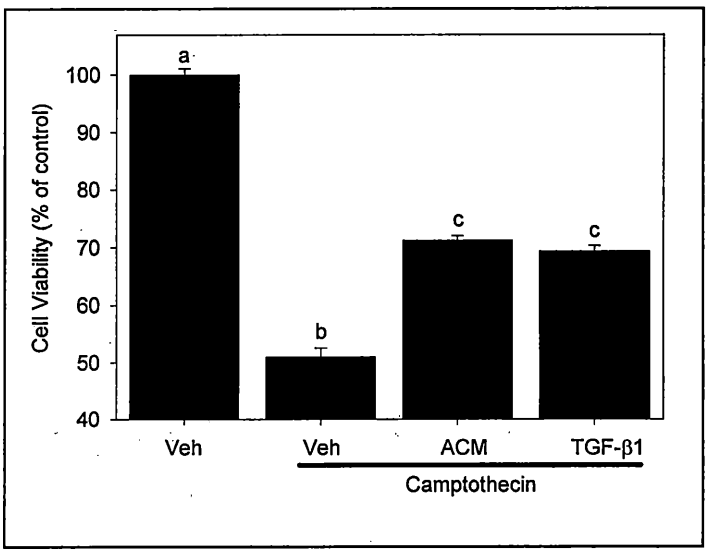
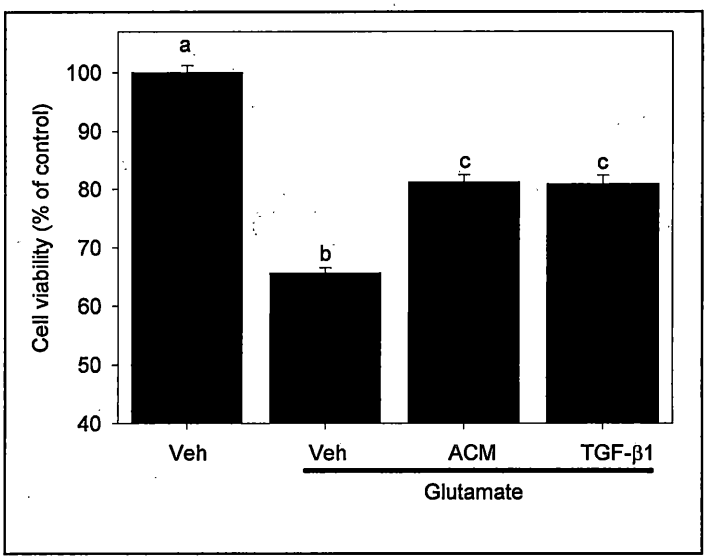

Serum (-)
Serum (-)
+
TGF-β1
Serum (-)
+
ACM
Serum (-)
+
TGF-β2

Figure 3-7. **Effect of astrocyte conditioned media and TGF-β1 on cell death induced by camptothecin (A), glutamate (B) and chemical hypoxia (C) in purified primary rat embryonic cortical neurons.** Rat cortical neurons were pretreated with vehicle (Veh), astrocyte conditioned media, or 10 ng/ml TGF-β1 24 hours prior to either application of camptothecin (10 μM), glutamate (300 μM), and chemical hypoxia (potassium cyanide and 2-deoxyglucose, 1 mM and 2 mM, respectively. Cell viability was assessed 24 hours following addition of cell death inducers. Groups with different subscripts are significantly different from each other ($p<0.05$).

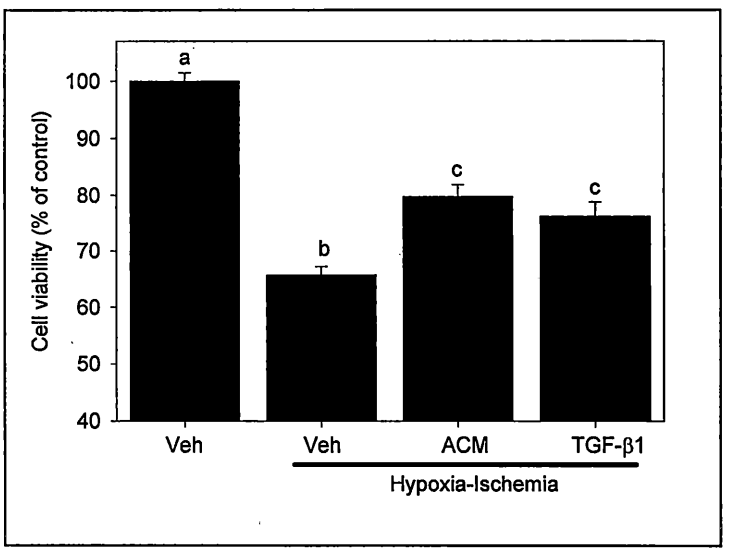
A.


Cell Viability (% of control)
100
90
80
70
60
50
40
a
b
c
c
Veh
Veh
ACM
TGF-β1
Camptothecin

B.


Cell viability (% of control)
100
90
80
70
60
50
40
a
b
c
c
Veh
Veh
ACM
TGF-β1
Glutamate

C.


Cell viability (% of control)
100
90
80
70
60
50
40
a
b
c
c
Veh
Veh
ACM
TGF-β1
Hypoxia-Ischemia

4

MECHANISMS OF TRANSFORMING GROWTH FACTOR-β REGULATION IN RAT CORTICAL ASTROCYTES BY ESTROGEN AND SERMS

4.1 Estrogen and SERM Action on Astrocytes

The results in the preceding section demonstrated that low dose 17β-E2 and SERMs did not induce significant neuroprotection in GT1-7 neurons or purified cortical neurons *in vitro*. This finding agrees with the results of several other groups in the field who likewise failed to observe a significant protective effect of low dose estrogen in purified cortical neuronal cultures (86-89, 102). The lack of an effect could be due the failure of this *in vitro* model to fully reproduce the *in vivo* situation, i.e. the lack of multiple cell types, loss of tissue and cellular architecture and possible developmental differences of embryonic cells versus adult in vivo cells. Along these lines, it is interesting to note that low dose estrogen has been shown to exert protection in cortical slice cultures, which has multiple cell types and preserved cellular/tissue architecture (143-144) as well as in mixed neuronal-glial cultures (Aim 1). We thus next examined whether estrogen and SERMs could stimulate release of the neuroprotective factor, TGF-β from astrocytes.

Specific Aim #3. To determine whether 17β-E_2 or SERMs can protect in mixed cortical cultures and whether they can regulate the release of TGF-β from astrocytes. To assess the possible role of astrocytes as intermediary cells for estrogen/SERM protective effects, we examined the ability of estrogen and SERMs to exert protection in mixed cortical cultures and their ability to regulate the release of the astrocyte-derived neuroprotective factor, TGF-β from purified astrocyte cultures.

4.2 Methods and Materials

4.2.1 Cell Cultures Models

Supplies. Unless otherwise specified, all cell culture reagents, sera and media were purchased from Gibco BRL (Invitrogen, Grand Island, NY). 17β-E_2, tamoxifen, and 4-hydroxytamoxifen were purchased from Sigma (St. Louis, MO). ICI182,780 was from Tocris (Ballwin, MO). LY294002 and PD98059 were supplied by Promega (Madison, WI). Antibodies were purchased from New England Biolabs (Beverly, MA).

Primary Rat Astrocyte Cultures. Primary astrocyte cultures were cultured as described previously in the dissertation.

4.2.2 Treatments

Following the second passage, astrocytes were plated at 4 x 10^5 cells/well in 6-well plates. Cell were cultured in complete culture medium until reaching ~70% confluency. At this time, media was removed, cells gently washed in Dulbecco's PBS, then cells were treated in Opti-MEM I Reduced Serum Medium or Opti-MEM I containing combinations of 10 nM 17β-E2, SERMs (1 μM), or inhibitors (20 μM LY294002 or 30 μM PD98059, 1 μM ICI182,780). Cells were cultured for 6-72h, then supernatants were collected in microcentrifuge tubes and stored at –80°C until growth factor assays.

4.2.3 *RNA Isolation*

Total RNA was isolated from GT1-7 cells and from cultured rat cortical neurons using TriZOL (Life Technologies, Grand Island, NY) according to manufacturer's recommendations. Samples were stored at room temperature for 5 minutes to allow the dissociation of nucleoprotein complexes, and then transferred to sterile Eppendorf tubes. Chloroform was added (0.2 ml/1ml TriZOL), tubes were vortexed, and then samples were incubated at room temperature for 15 minutes. Samples were centrifuged at 12,000x*g* for 15 minutes at 4°C causing the mixture to separate into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA-containing aqueous phase was transferred

to a sterile microcentrifuge tube, and RNA was precipitated by the addition of isopropanol at room temperature for 15 minutes. RNA was pelleted by centrifugation at 12,000xg for 10 minutes at 4°C, and pellets were washed with 75% ethanol (1 ml/1 ml TriZOL used in initial step). Following air drying for 5 minutes, RNA pellets were resuspended in sterile distilled water. Aliquots of 5 µl were used for spectrophotometric analysis to determine RNA concentrations. The integrity of the total RNA was visualized on a 1.5% agarose gel. Samples were stored at –80°C until required for RT-PCR.

4.2.4 *Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)*

RT-PCR of estrogen receptors was performed as described previously in the dissertation.

4.2.5 Western blotting

Western blotting of estrogen receptors was performed as described previously in the dissertation.

4.2.6 TGF-β measurements

TGF-β levels in astrocyte conditioned medium were determined by enzyme linked immunosorbent assay using commercially available kits (Promega, Madison, WI). The system detects biologically active TGF-β1 (or TGF-β2) in an antibody sandwich format. 96 well plates were coated with monoclonal TGF-β1 (or TGF-β2) antibody, which binds soluble TGF-β1 (or TGF-β2) from solution. Captured TGF-β1 (or TGF-β2) was bound by a polyclonal antibody specific for TGF-β1 (or TGF-β2) and following washing was detected using anti-rabbit IgG conjugated to horseradish peroxidase (HRP) and a chromogenic substrate for HRP. Absorbance of samples was read within one hour of stopping the reaction at 450-nm using plate reader (Labsystems Multiskan MCC/340). TGF-β1 (or TGF-β2) was measured in 100 µl samples with a sensitivity of 32 pg/ml. To determine total TGF-β1 (or TGF-β2) levels, additional 100 µl samples were treated with 1.0 N HCl to activate latent TGF-β1 (or TGF-β2).

Active or total TGF-β1 (or TGF-β2) levels were expressed as pg/ml, as indicated in the figure legends.

4.2.7 Plasminogen Activator Inhibitor-1 (PAI-1) measurements

PAI-1 levels in astrocyte conditioned medium were determined by enzyme linked immunosorbent assay using a commercially available kit (American Diagnostica Inc., Greenwich, CT). The system detects PAI-1 in an antibody sandwich format. 96 well plates were coated with a monoclonal PAI-1 antibody, which specifically binds rat PAI-1 from solution. Captured PAI-1 was bound by another monoclonal antibody coupled to horseradish peroxidase (HRP). The antibody binds to its corresponding free epitope on the immobilized rat PAI-1 protein. PAI-1 levels were visualized by treatment of the wells with Ortho-Phenylene-Diamine (OPD) in the presence of hydrogen peroxide, which produced an orange-brown color. A plate reader (Labsystems Multiskan MCC/340) was used to quantitate the absorbance of the reaction at 492 nm. PAI-1 was measured in 100 μl samples with a sensitivity of 1 ng/ml.

4.2.8 Statistical Analysis

The effect of different treatments was analyzed using a one-way analysis of variance (ANOVA) followed by the appropriate multiple comparison test (Tukey's test or Student-Newman-Keul's method). For all groups, $n \geq 5$ and experiments were repeated in triplicate for verification of results. The results were expressed as means ± standard error of the mean. P values < 0.05 were considered significant.

4.3 Results

Cultured rat cortical astrocytes express both estrogen receptor isoforms. To determine whether astrocytes express the transcript for estrogen receptor isoforms, we utilized RT-PCR. Analysis of estrogen receptor expression in rat cortical astrocytes revealed the expression of both estrogen receptor-alpha (ER-α) and estrogen receptor-beta (ER-β)

mRNA, a finding which was corroborated by Western blotting, which revealed both isoforms are also present at the protein level (Figure 4-1).

Estrogen and SERMs increase the release of TGF-β isoforms from cultured rat cortical astrocytes. Incubation of cultured rat cortical astrocytes with 17β-E_2 or tamoxifen induced the release of TGF-β1 and TGF-β2, as determined by a specific immunoassay. This effect was observed as early as 6h following treatment and persisted for 36 hours (Figure 4-2 – Figure 4-4). However, by 48h and 72h, the stimulatory effect was lost (data not shown). Interestingly, the estrogenic effect of 17β-E_2 and tamoxifen was not observed for TGF-β2 following a 36h treatment. In these same samples, TGF-β1 regulation was still observed, suggesting a divergent mechanism of regulation between TGF-β1 and TGF-β2 by estrogen and tamoxifen. Importantly, we observed a similar stimulatory effect of 4-hydroxytamoxifen, which represents the active tamoxifen metabolite, *in vivo*. Tamoxifen appeared to be slightly more potent than 17β-E_2 in the stimulation of TGF-β release.

Estrogenic stimulation of PAI-1 occurs following 36 hour treatment. To determine whether 17β-E2 and tamoxifen have a similar stimulatory effect on another rat cortical astrocyte-derived neuroprotective factor, we measured the release of PAI-1 from astrocytes using a specific immunoassay. No increase in PAI-1 release was observed during early timepoints (6h, 18h) as was observed for TGF-β. However, a significant increase in PAI-1 release was observed following a 36 hour treatment, suggesting the possibility of a genomic regulation (Figure 4-5).

Estrogenic induction of TGF-β is blocked by co-addition of ICI182,780, a specific estrogen receptor antagonist. To determine whether the estrogen and tamoxifen increase in TGF-β release was due to estrogen receptor activation, we co-added ICI182,780, a potent and specific estrogen receptor antagonist, to cultures during treatment. Pretreatment with 1

μM ICI182,780 completely blocked the estrogenic stimulation of TGF-β release from astrocytes following an 18-hour exposure (Figure 4-6).

TGF-β release is blocked by PI3-K inhibitor, LY294002, but not by a MAPK inhibitor, PD98059. To determine whether the 17β-E_2-induced stimulation of TGF-β release is due to activation of the phosphoinositol-3-kinase pathway, we co-added LY294002, a specific inhibitor of PI3-K to cultures with estrogen and tamoxifen. Indeed we found at 20 μM LY294002, a dose widely shown to block PI3-K activity, blocked TGF-β release following an 18h treatment (Figure 4-7a). Assays of cell death revealed no decrease in cell viability due to LY294002 treatment at this time (data not shown). For specificity, no inhibitory effect was observed when the MAPK pathway inhibitor, PD98059 (30 μM) was added (Figure 4-8a). These findings suggest PI3K, but not MAPK influence the release of TGF-β by 17β-E_2 and tamoxifen in cultured rat cortical astrocytes.

Estrogen and tamoxifen induce the rapid activation of Akt, but not MAPK. Although LY294002 blocked the estrogenic stimulation of TGF-β, we next sought to determine whether 17β-E_2 and tamoxifen activated the phosphorylation of Akt. A rapid and transient activation of Akt was observed, beginning as early as 5 minutes following treatment and was not different beyond 30 minutes post-treatment (Figure 4-7b). Interestingly, this effect was not influenced by the co-addition of ICI182,780, suggesting an ICI182,780-sensitive estrogen receptor does not mediate the effect of 17β-E_2 and tamoxifen on Akt phosphorylation. In contrast, no effect of 17β-E_2 or tamoxifen treatment was observed on MAPK phosphorylation at any timepoint analyzed (data not shown).

4.4 Discussion and Significance

Work by our laboratory (Aim 1) and others have failed to demonstrate a direct neuroprotective effect of physiological doses of 17β-E_2 or therapeutic concentrations of tamoxifen in purified cortical neuronal cultures. Interestingly, Wise and coworkers

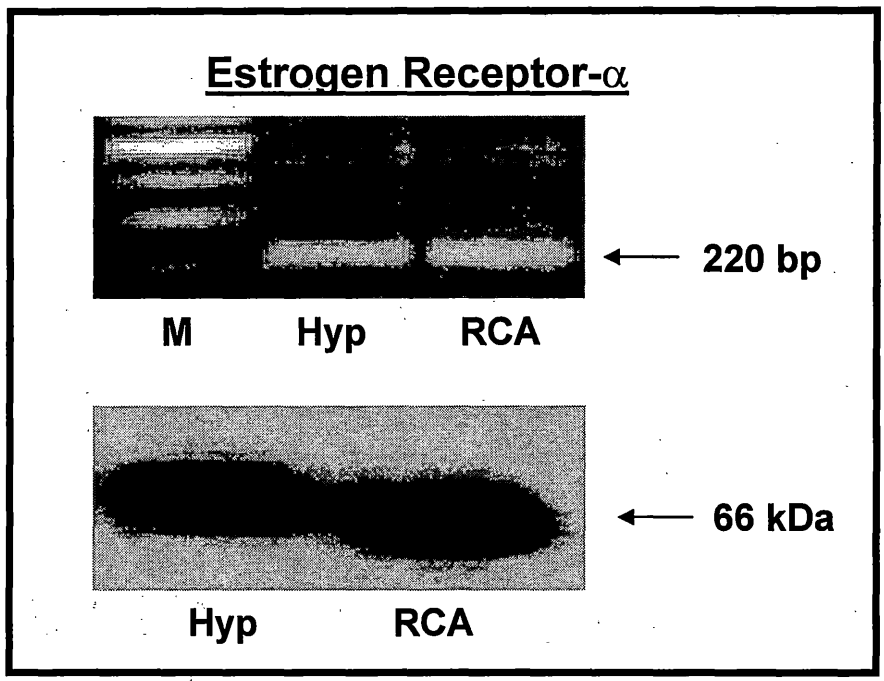
demonstrated a neuroprotective effect of physiological doses of 17β-E_2 in organotypic cortical explant cultures, which contains numerous different cell types, such as glia (142-143). Together, these findings suggest the well-documented neuroprotective effects of estrogen and tamoxifen are not directly at the level of the neuron and may be mediated by another cell type, such as glial cells. Work by our laboratory has demonstrated a protective effect of 17β-E_2 in glial-neuronal co-cultures, but not in pure neuronal cultures. Our work also showed that cortical astrocytes express both ER-α and ER-β transcript and protein. This suggests astrocytes potentially represent physiological targets of estrogen action in the brain.

To elucidate the factor(s) that may be responsible for the neuroprotective effect of 17β-E_2, several growth factors with neuroprotective ability were measured in astrocyte-conditioned media following estrogenic stimulation. Estrogen and tamoxifen both enhanced the release of TGF-β1 and TGF-β2 from rat cortical astrocytes at numerous time points investigated, extending our previous observations that estrogen regulates the release of TGF-β1 from cultured hypothalamic astrocytes (121). In contrast, both 17β-E_2 and tamoxifen stimulated the release of PAI-1 following a 36 hour exposure, but not following a 6 or 18 hour treatment, suggesting differential regulation as compared to TGF-β release. Given the temporal pattern of release of PAI-1, it is conceivable that 17β-E_2 enhances an early (6h) release of TGF-β from astrocytes, which acts in an autocrine/paracrine manner to increase PAI-1 levels. This possibility is supported by the observation that TGF-β is a potent inducer of astrocytic PAI-1 mRNA levels (175-176). The release of TGF-β by cortical astrocytes was abolished by pretreatment with ICI182,780, an estrogen receptor antagonist, suggesting the involvement of estrogen receptors. Although protein release was measured in the current studies, this effect is likely mediated at the level of gene expression based on the temporal pattern of cytokine release. Interestingly, neither the TGF-β1 nor the TGF-β2 promoter possesses a consensus estrogen responsive element (ERE), raising the possibility that estrogen influences the expression of TGF-β in a manner independent of the ERE. This possibility is supported by the recent observation that 17β-E_2 and SERMs activate several

membrane bound signaling pathways, such as Akt and MAPK (64-67, 144, 176-180). These non-genomic signaling pathways have also been shown to mediate the effects of 17β-E_2 in numerous tissues, including the central nervous system. In support of a role for nongenomic signaling pathways, Wilson et al. (143), demonstrated an important role for Akt in mediating the neuroprotective effect of physiological doses of 17β-E_2 in organotypic cortical explant cultures. Furthermore, Dorsa and colleagues suggested MAPK signaling mediates the effects of 17β-E_2 during neuroprotection (65). Based on these findings and the hypothesis that estrogen-mediated neuroprotection may involve astrocyte intermediacy as one its components, we postulated that Akt and MAPK might regulate TGF-β release. Indeed, D'Onofrio et al. (110) demonstrated these two signaling pathways control TGF-β release in cultured rat cortical astrocytes in response to metabotropic glutamate agonists, further indicating the potential for these pathways to influence TGF-β expression.

In the current studies, TGF-β release was completely inhibited by LY294002, a potent inhibitor of Akt signaling, but not by PD98059, a MAPK signaling pathway inhibitor, suggesting a role for Akt in this process. In support of this possibility, both 17β-E_2 and tamoxifen rapidly induced the activation of Akt in cortical astrocytes, as reflected by increased phosphorylation of Akt on serine-473. Interestingly, ICI182,780 blocked the release of TGF-β from astrocytes, yet did not block the activation of Akt, which appears to be important for 17β-E_2 induced TGF-β release. A similar phenomenon was observed by Honda et al. (77), who demonstrated ICI182,780 blocked 17β-E_2-mediated neuroprotection, but did not block Akt activation, which mediated the neuroprotection. Together, these findings indicate that 17β-E2 and tamoxifen are capable of inducing release of the neuroprotective factor, TGF-β from astrocytes and this effect likely involves mediation by the PI3-kinase-Akt signaling pathway.

Figure 4-1. RT-PCR (A) and Western blot (B) analysis of estrogen receptor expression in cultured rat cortical astrocytes. Rat hypothalamus was included as positive controls for estrogen receptor-α and estrogen receptor-β. Hyp = rat hypothalamus, RCA = rat cortical astrocytes.


Estrogen Receptor-α
220 bp
M
Hyp
RCA
66 kDa
Hyp
RCA

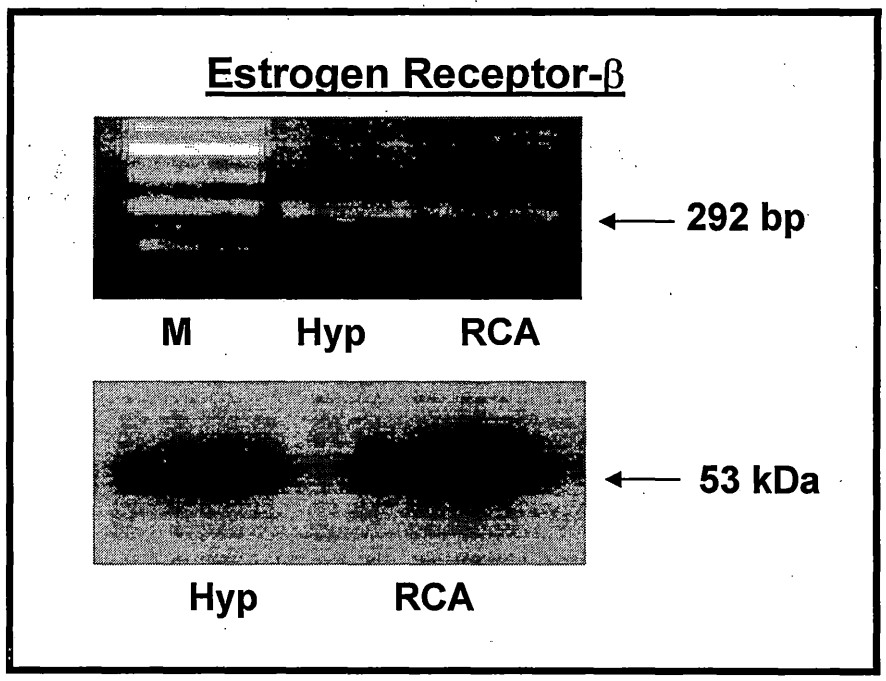
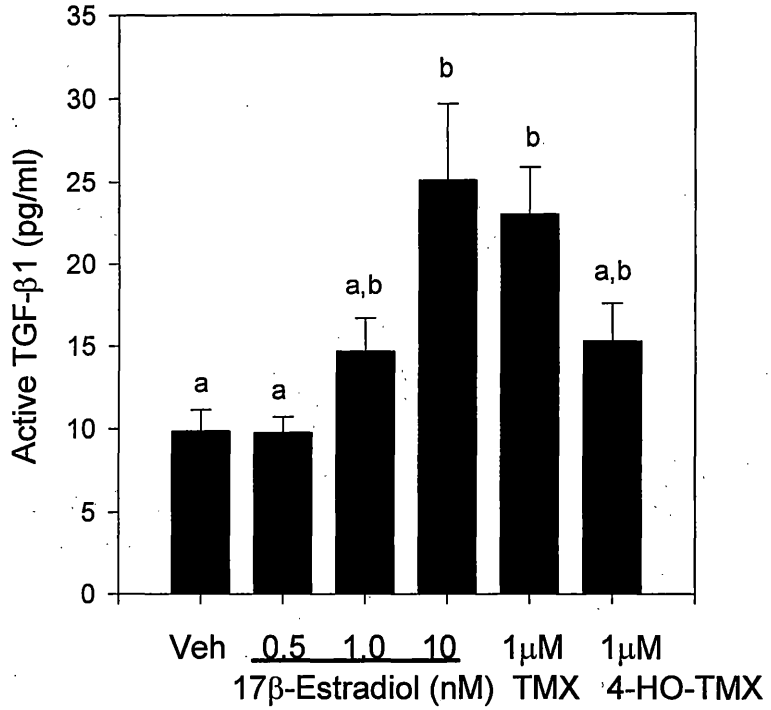

Estrogen Receptor-β
292 bp
M
Hyp
RCA
53 kDa
Hyp
RCA

Figure 4-2: Effects of 17β-Estradiol and Tamoxifen on TGF-β release from cultured rat cortical astrocytes. 17β-Estradiol, tamoxifen and 4-hydroxytamoxifen stimulate the release of active TGF-β1 and active TGF-β2 from cultured rat cortical astrocytes following a 6-hour exposure. Different subscripts denote significant differences between treatment groups ($p<0.05$).


Active TGF-β1 (pg/ml)
35
30
25
20
15
10
5
0
a
a
a,b
b
b
a,b
Veh
0.5
1.0
10
1μM
1μM
17β-Estradiol (nM)
TMX
4-HO-TMX

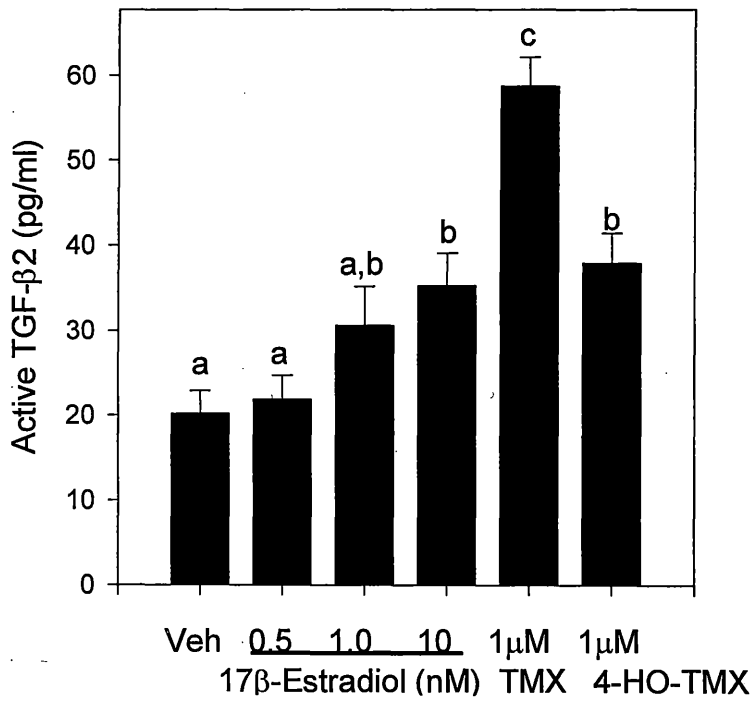
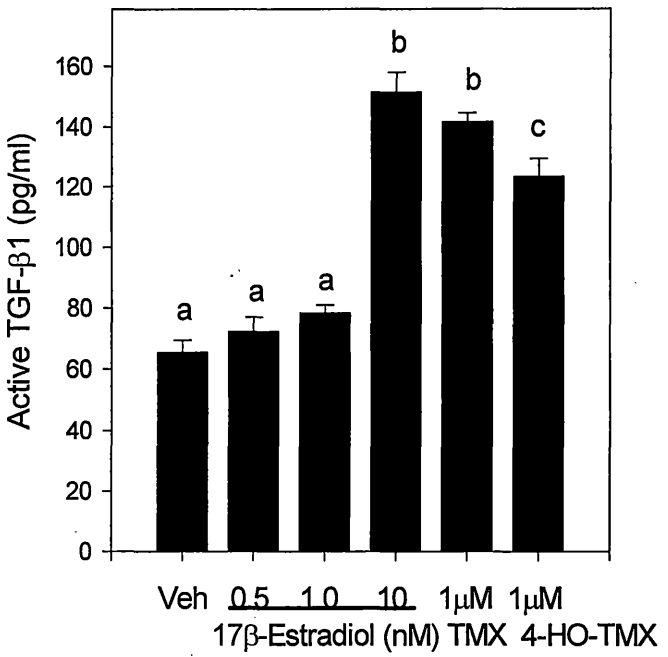

Active TGF-β2 (pg/ml)
60
50
40
30
20
10
0
a
a
a,b
b
c
b
Veh
0.5
1.0
10
1μM
1μM
17β-Estradiol (nM)
TMX
4-HO-TMX

Figure 4-3: **Effects of 17β-Estradiol and Tamoxifen on TGF-β release from cultured rat cortical astrocytes.** 17β-Estradiol, tamoxifen and 4-hydroxytamoxifen stimulate the release of active TGF-β1 and active TGF-β2 from cultured rat cortical astrocytes following an 18-hour exposure. Different subscripts denote significant differences between treatment groups ($p<0.05$).


Active TGF-β1 (pg/ml)
160
140
120
100
80
60
40
20
0
a
a
a
b
b
c
Veh
0.5
1.0
10
1μM
1μM
17β-Estradiol (nM)
TMX
4-HO-TMX

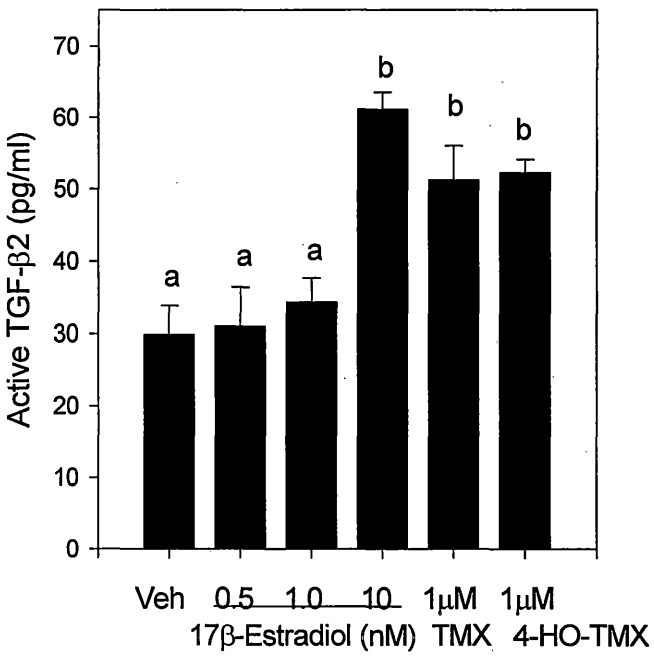
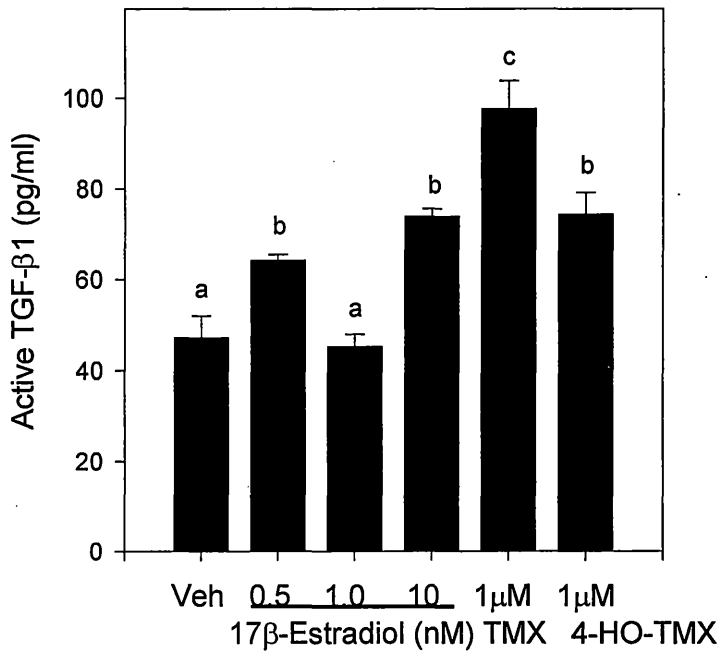

Active TGF-β2 (pg/ml)
70
60
50
40
30
20
10
0
a
a
a
b
b
b
Veh
0.5
1.0
10
1μM
1μM
17β-Estradiol (nM)
TMX
4-HO-TMX

Figure 4-4: Effects of 17β-Estradiol and Tamoxifen on TGF-β release from cultured rat cortical astrocytes. 17β-Estradiol, tamoxifen, and 4-hydroxytamoxifen stimulate the release of active TGF-β1 and TGF-β2 from cultured rat cortical astrocytes following a 36-hour exposure. No significant effect of any treatment was observed on active TGF-β2 levels. Different subscripts denote significant differences between treatment groups ($p<0.05$).


Active TGF-β1 (pg/ml)
0
20
40
60
80
100
a
b
a
b
c
b
Veh
0.5
1.0
10
1μM
1μM
17β-Estradiol (nM)
TMX
4-HO-TMX

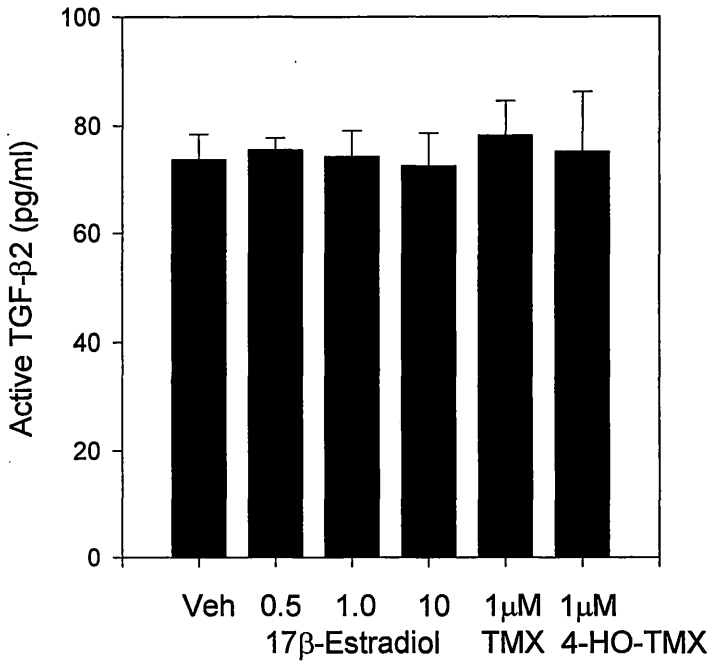

Active TGF-β2 (pg/ml)
0
20
40
60
80
100
Veh
0.5
1.0
10
1μM
1μM
17β-Estradiol
TMX
4-HO-TMX

Figure 4-5: **Effects of 17β-E_2 and tamoxifen on PAI-1 release from cultured rat cortical astrocytes.** Treatment of cultured rat cortical astrocytes with 17β-E2 or tamoxifen significantly increased the release of PAI-1 following a 36 hour treatment. Different letters denote significant differences within the 36 hour timepoint ($p<0.05$). No significant differences were observed at either the 6 hour or 18 hour timepoints.

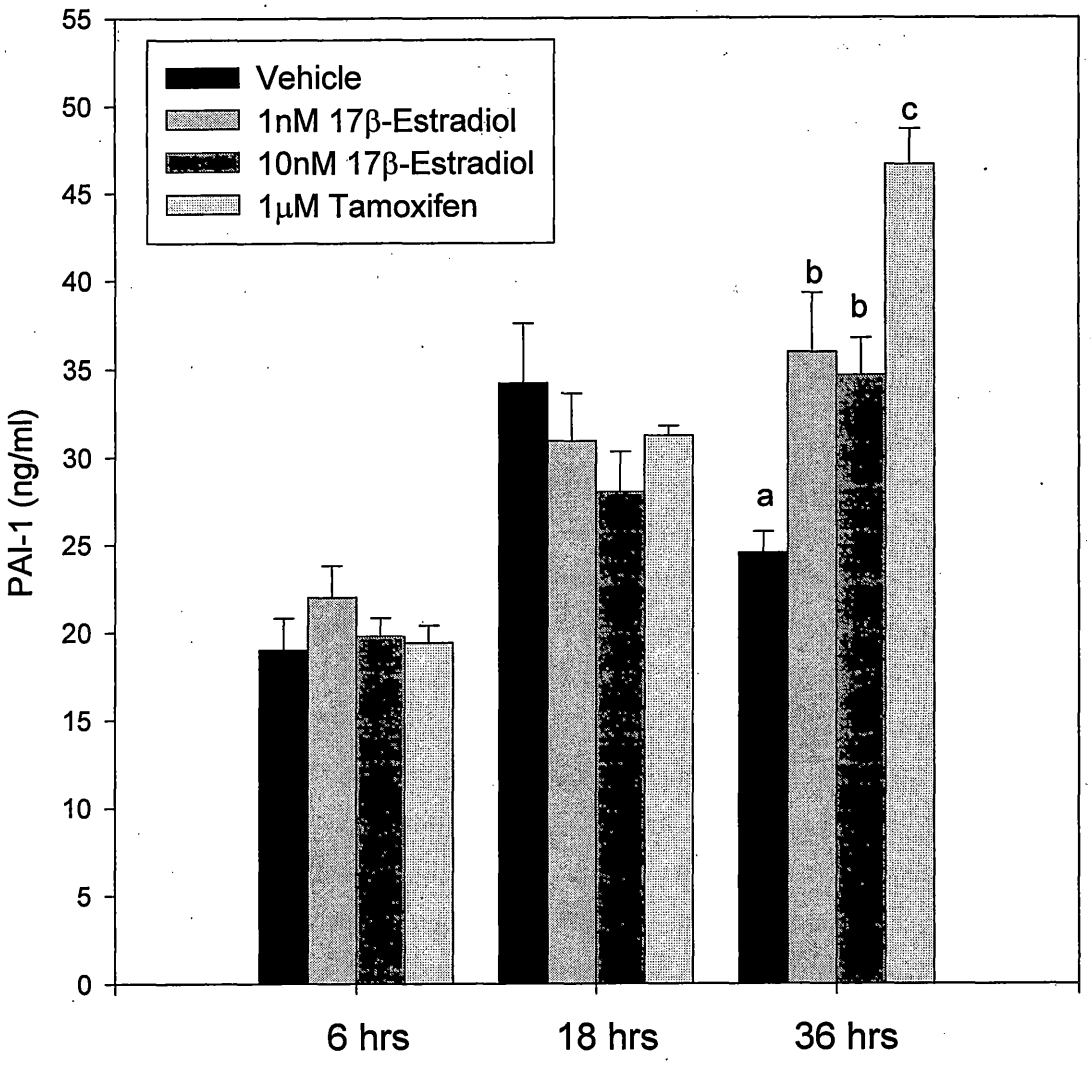

Vehicle
1nM 17β-Estradiol
10nM 17β-Estradiol
1μM Tamoxifen
PAI-1 (ng/ml)
55
50
45
40
35
30
25
20
15
10
5
0
a
b
b
c
6 hrs
18 hrs
36 hrs

Figure 4-6: Effects of the estrogen receptor antagonist, ICI182,780, on 17β-E2 stimulated TGF-β1 release in cultured cortical astrocytes. 17β-E_2 (10 nM) stimulated the release of TGF-β1 from cultured rat cortical astrocytes following an 18-hour exposure. ICI182,780 (1 μM) blocked the 17β-E_2 mediated stimulation following a one hour pretreatment. * Significantly different from vehicle control ($p<0.05$).

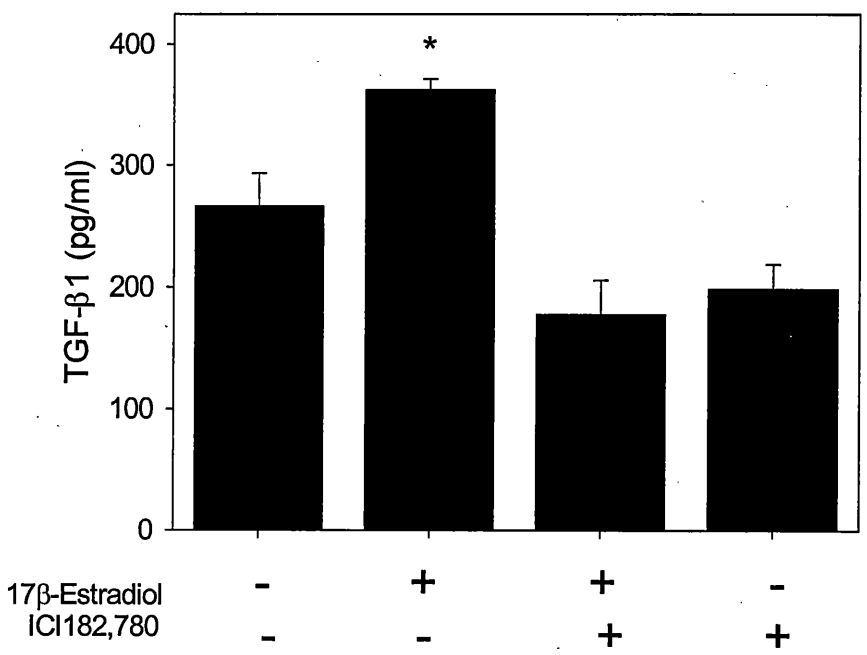
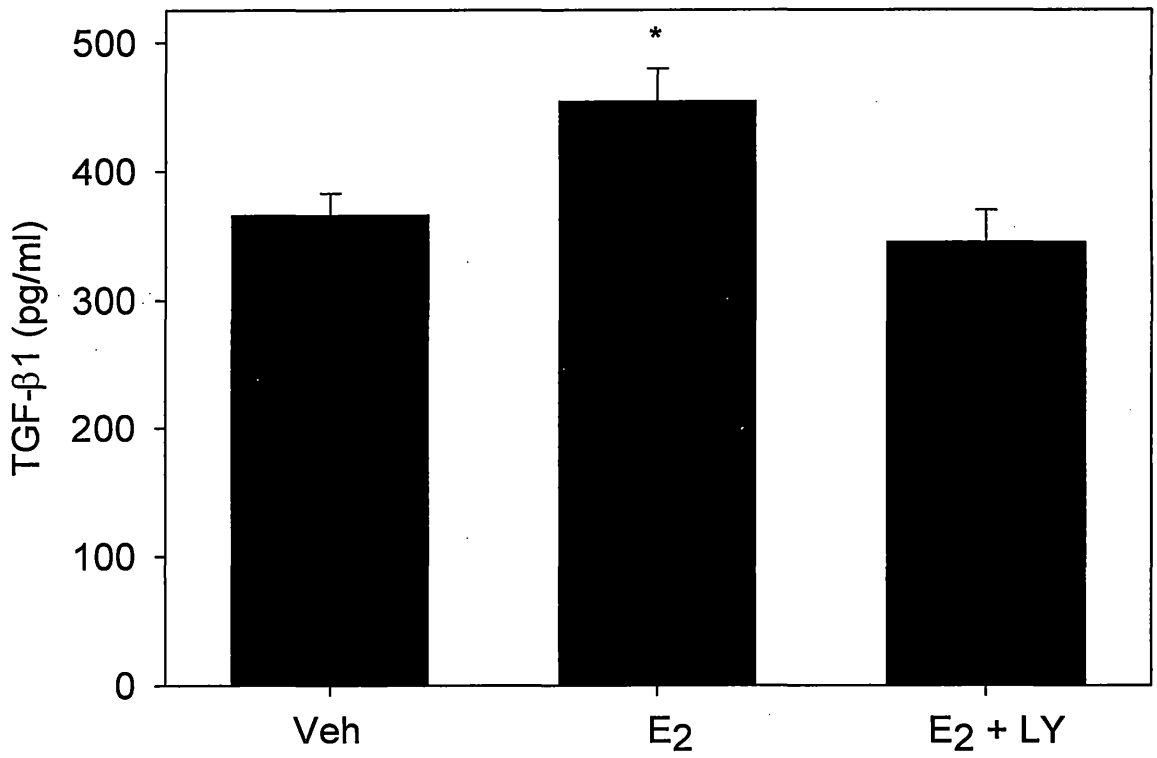

400
300
200
100
0
TGF-β1 (pg/ml)
*
17β-Estradiol
ICI182,780
-
+
+
-
-
-
+
+

Figure 4-7: Effect of PI3-K inhibitor, LY294002 on 17β-E_2 stimulated TGF-β1 release in cultured rat cortical astrocytes. (A.)17β-E_2 (10 nM) stimulated the release of TGF-β1 from cultured rat cortical astrocytes following an 18-hour incubation. LY294002 (20 μM) completely blocked the stimulatory effect of 17β-E_2. * Significantly different from vehicle control ($p<0.05$) (B.) 10 nM 17β-E_2 (E_2) and tamoxifen (TMX) increase phosphorylation of Akt on serine -473 in cultured rat cortical astrocytes.

A.


500
400
300
200
100
0
TGF-β1 (pg/ml)
*
Veh
E2
E2 + LY

B.

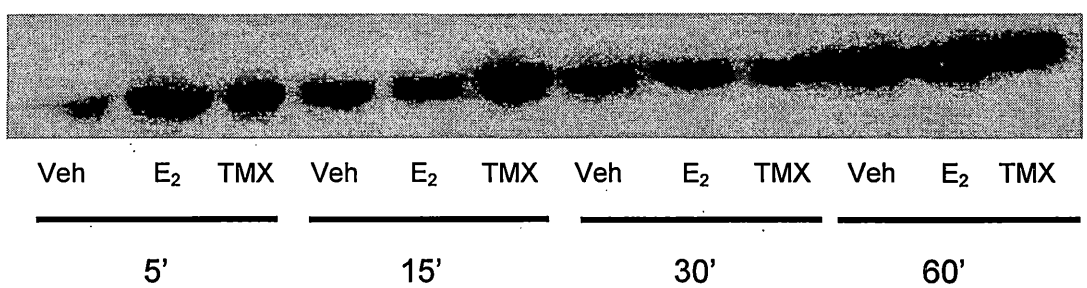
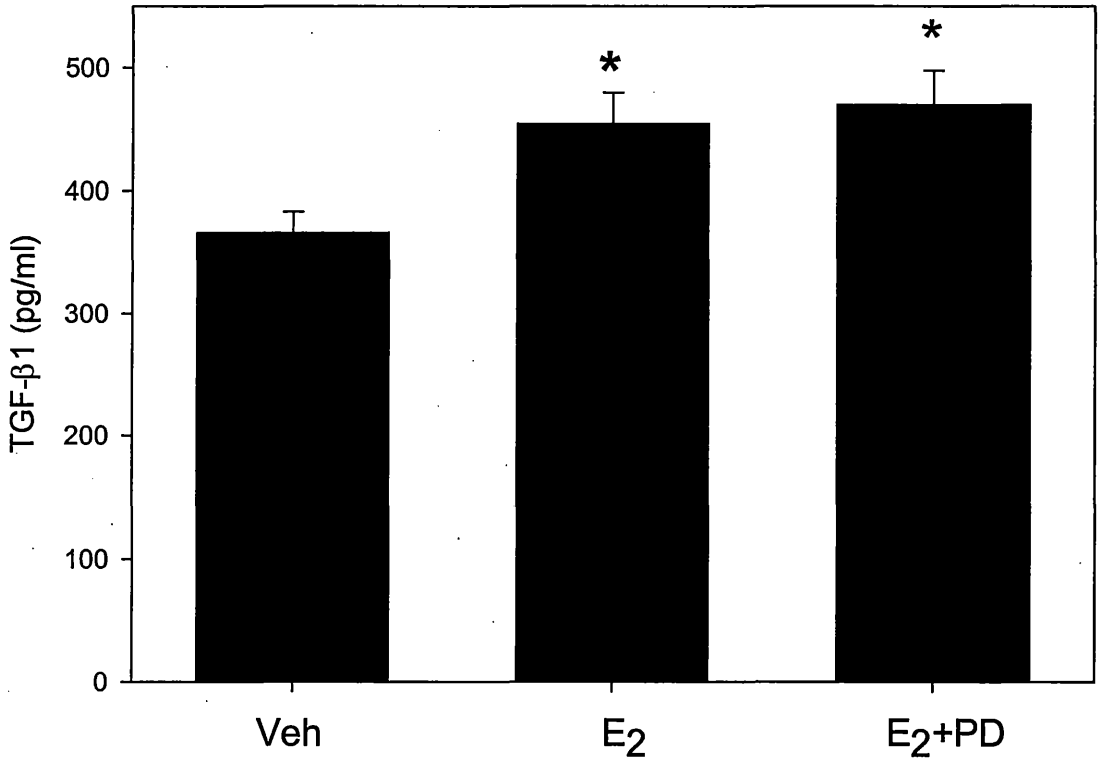

Veh
E2
TMX
Veh
E2
TMX
Veh
E2
TMX
Veh
E2
TMX
5'
15'
30'
60'

Figure 4-8: Effects of the MAP kinase inhibitor, PD98059, on 17β-E_2 stimulated TGF-β1 release in rat cultured cortical astrocytes. 17β-E_2 (10 nM) stimulated the release of TGF-β1 from cultured rat cortical astrocytes following an 18-hour exposure. PD98059 failed to inhibit 17β-E_2 stimulation of TGF-β1. * Significantly different from vehicle control ($p<0.05$)


TGF-β1 (pg/ml)
500
400
300
200
100
0
*
*
Veh
E2
E2+PD

5

MECHANISMS OF NEUROPROTECTION BY ASTROCYTES AND TRANSFORMING GROWTH FACTOR-β1

5.1 Role of AP-1 Transcription Factors During Neuronal Injury

The results described in the previous sections showed that astrocyte-derived TGF-β plays a role in mediating the ability of astrocyte-conditioned media to protect neurons from cell death, and that estrogen can stimulate TGF-β release from astrocytes. The question remained as to how astrocyte-derived TGF-β might elicit neuroprotection. Depending on the cell and tissue type, TGF-β has been shown to activate a wide variety of cell signaling pathways culminating in the activation of a transcription response. One such pathway results in the induction of AP-1 transcription factor-mediated gene expression, which is implicated in the regulation of cellular viability and apoptosis (181-183). Thus, TGF-β activation of AP-1 transcription factors could potentially underlie TGF-β-mediated neuroprotection.

AP-1 transcription factors have been associated with promoting neuronal cell death under certain conditions, however recent evidence suggests AP-1 may also promote neuronal survival following an injury (184-187). Along these lines, AP-1 binding was increased in the ischemic penumbra, which represents the area of healthy tissue surrounding an ischemic core following cerebral ischemic injury (188-191). Furthermore, neurons with increased AP-1 binding do not succumb to cell death, despite the highly vulnerable localization of these cells in proximity to the ischemic core. These findings were interpreted to suggest AP-1 factors potentially mediate ischemic tolerance in the brain and thus represent an adaptive survival mechanism. The *in vivo* findings were confirmed by Dragunow et al., (192), who demonstrated that AP-1 transcription factors are critical for the maintenance of neuronal survival and for the induction of neurite outgrowth. Furthermore, a prolonged increased in the AP-1 transcription factor c-Jun was observed in regenerating neurons, including retinal ganglion neurons and thalamic neurons (193). The potential for AP-1 to participate in recovery following neuronal injury was subsequently reported by Houle

et al. (194), who demonstrated re-induction of c-Jun following spinal cord transection up to months after injury promoted regeneration. Together, these findings suggest AP-1 transcription factors may participate in neuronal survival, neuritogenesis, and functional recovery following neuronal injury.

5.2 AP-1 Transcription Factors and Regulatory Signaling Pathway

AP-1, a major target of cell growth, differentiation, apoptosis and stress signaling (195-196), was first identified as a TPA-inducible transcription factor. AP-1, which is the name for both the transcription protein complex and the region of binding on DNA promoters, binds to a specific target DNA sequence called the Tetradecanoyl phorbol acetate (TPA)-response element (TRE) (TGAC/GTCA). The binding affinity for a given TRE is determined by the composition of AP-1 factors and the context of the surrounding sequences (197-199). AP-1 is comprised of a dimer between members of the Jun (c-Jun, JunB, JunD) and Fos (c-Fos, Fra-1, Fra-2) families of protooncogenes. Additionally, dimers can also be formed with ATF-2 (Activation Transcription Factor-2), although ATF-2 dimers prefer the CRE (cAMP response element) over AP-1 sites. Jun homodimers may also form, although heterodimers with Fos family members are more stable and have a higher affinity for the DNA target sequence (200-203). Transactivation of the principal AP-1 binding partner, c-Jun, occurs following phosphorylation on two N-terminal serines (Ser-63 and Ser-73) by an upstream kinase, c-Jun N-terminal kinase (JNK; also referred to as stress activation protein kinases (SAPK)). JNK, a member of the mitogen-activation protein kinase (MAPK) family of proteins, is implicated in a variety of processes including embryonic development, cellular proliferation, and transformation, as well as the regulation of apoptosis (204-208). JNK is activated, via phosphorylation on Thr and Tyr residues, by various cytokines or environmental stresses, which results in an association with the N-terminal domain of c-Jun (209-210). This interaction culminates in the phosphorylation of c-Jun at Ser-63 and Ser-73, which is required for dimerization and a full transcriptional response.

The physiological and pathological roles of JNKs are controversial, with conflicting reports regarding the pro- and anti-apoptotic roles during cell death. Although many studies to date implicate JNK in the induction of apoptosis, several recent studies have challenged this notion, and suggested JNK may also exert a cytoprotective function. This finding is supported by the observation that forebrain neuronal cells undergo apoptosis more frequently in *jnk1* -/- and *jnk2* -/- mice than in wild type controls (211-212). Thus, depending on the biological context, JNK exerts differing actions of the control of cell death.

Recent work by our laboratory has demonstrated astrocyte-derived TGF-β influences neuroprotection in cell culture. However, the molecular mechanisms underlying this effect are not well established. Thus, the goal of this study was to determine the cellular pathway which underlies astrocyte-derived TGF-β mediated neuroprotection. Toward this end, we examined astrocyte/TGF-β induction of the c-Jun/AP-1 signaling pathway.

Specific Aim #4. To elucidate the mechanism of TGF-β1 mediated neuroprotection in GT1-7 Neurons. Preliminary work by our laboratory and others has demonstrated a neuroprotective role for TGF-β against a wide variety of neurotoxic insults, both *in vivo* and *in vitro*. However, the cellular mechanism(s) whereby TGF-β exerts this effect is not known and is a subject of intense investigation. We hypothesize astrocyte-derived TGF-β1 protects neurons by increasing the activity of the AP-1 mediated gene transcription.

5.3 Methods and Materials

5.3.1 *Cell culture models*

Supplies. Unless otherwise specified, all cell culture reagents, sera and media were purchased from Gibco BRL (Invitrogen, Grand Island, NY). TGF-β isoforms and pan specific TGF-β neutralizing antibody were purchased from R&D Systems. All antibodies for Western blotting were obtained from New England Biolabs.

GT1-7 Neurons. GT1-7 neurons were cultured as previously described in the dissertation.

5.3.2 Treatments

GT1-7 Neurons. Upon reaching 60% confluence, media was removed and cells were gently washed in Dulbecco's Phosphate Buffered Saline. Neurons were then treated with complete culture medium (serum), serum free medium, astrocyte conditioned media, exogenous TGF-β1, or these treatments in combination with curcumin (25 μM) or dicoumarol (50 μM). The doses of curcumin and dicoumarol are within the range which is reported to inhibitor AP-1 mediated transcription and JNK activation, respectively. Treatments were for 48h at which time cell viability was assessed.

5.3.3 Cell Viability/Death Measurements.

Cell viability was determined using the MTT assay and LDH assay (data not shown), as previously described in the dissertation.

5.3.4 *Western blotting.*

Western blotting was performed as described previously in the dissertation.

Preparation of Nuclear Extracts

GT1-7 neurons, following a one hour treatment, were washed with ice-cold PBS and resuspended in hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT). Cells were allowed to swell for 10 minutes, and homogenized in a Dounce homogenizer. The suspension was centrifuged at 5000xg and the nuclei were extracted by resuspending in low salt buffer (20 mM HEPES, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 M KCL, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) and quickly mixed with high salt buffer (same as low salt buffer except 1.2 M KCl). Following a 20 minute incubation on ice,

lysates were centrifuged at 14000xg and the supernatants were aliquoted and stored at –70°C.

Electrophoretic Mobility Shift Assay (EMSA)

DNA binding reactions were performed by incubation of 1-5 μg of nuclear extract with 2 x 104 cpm of ^{32}P-labeled oligonucleotide probe for 15 minutes at room temperature in binding buffer containing 10 mM HEPES pH 8.0, 0.1 mM EDTA, 2 mM DTT, 2 mg poly(dIzdC), 4 mM KCl, 0.1% Nonidet P-40, 2 mM spermidine, and 8% glycerol. Complexes were separated on 5% nondenaturing polyacrylamide gels in 0.5X TBE. Gels were dried and visualized by autoradiography. The probe used to analyze AP-1 DNA binding contains the consensus AP-1 binding site (CGCTTGATGACTCAGCCGGAA) (Santa Cruz Biotechnology; Santa Cruz, CA).

5.3 Statistical Analysis

The effect of different treatments was analyzed using a one-way analysis of variance (ANOVA) followed by the appropriate multiple comparison test (Tukey's test or Student-Newman-Keul's method). For all groups, $n \geq 5$. The results were expressed as means ± standard error of the mean. P values < 0.05 were considered significant.

5.4 Results

Effect of Astrocyte-Conditioned Media Upon Phosphorylation of c-Jun, JNK and MKK4. Treatment of GT1-7 neurons with ACM induced a rapid increase in the phosphorylation of c-Jun on Serine-63. The effect was observed as early as 30 minutes and persisted until 12h following treatment (Figure 5-1a,b). By 24 hours following treatment, c-Jun phosphorylation levels returned to control levels. ACM also induced an increase in AP-1 binding by 1.5 hours, as assessed by electromobility gel shift assay (Figure 5-1c). Treatment of GT1-7 neurons with ACM also induced a rapid increase in the phosphorylation of MKK4, a kinase upstream of c-Jun, beginning at 30 minutes (Figure 5-2a). This increase persisted beyond 24 hours,

which was the last time point investigated. Furthermore, ACM induced the activation of both the 46 kD JNK1 isoform, as well as the 54 kDa JNK2 isoform (Figure 5-2b). This increase also persisted beyond 24 hours, which was the last time point investigated.

Immunoneutralization of ACM with a pan specific TGF-β antibody reverses AP-1 binding. To determine whether TGF-β isoforms mediated the increase in AP-1 binding by ACM, we utilized electromobility shift assays. Treatment of serum deprived GT1-7 neurons with ACM elicited a large increase in AP-1 binding following 4.5 hours of treatment (Figure 5-3). The effect of ACM was attenuated by prior incubation of ACM with a pan specific TGF-β immunoneutralizing antibody.

AP-1 pathway inhibitors prevent the neuroprotective effect of ACM and TGF-β1. To further determine the functional importance of the activation of the AP-1 transcription pathway, we utilized a JNK inhibitor, dicoumarol, and an AP-1 binding inhibitor, curcumin. As shown in Figure 5-4a,b, treatment of GT1-7 neurons with 50 μM dicoumarol, an inhibitor of JNK activity, reversed the neuroprotective ability of both astrocyte conditioned media and TGF-β1 in serum deprived cells. The ability of dicoumarol to specifically inhibit c-Jun phosphorylation was further demonstrated by reduced c-Jun phosphorylation following a 30 minute ACM treatment (Figure 5-4c). Similarly, rescue of serum deprived GT1-7 neurons with ACM or TGF-β1 was blocked by 25 μM curcumin, an inhibitor of AP-1 binding and transcription activation (Figure 5-5).

5.4 Discussion and Significance

Astrocytes have recently been identified as neuroprotective cells in the injured CNS. However, the molecular mechanisms underlying astrocyte-mediated neuroprotection are unknown. Previous work (Aim #2) implicated TGF-β isoforms as the mediators of astrocyte neuroprotection. Although well established as a neuroprotective cytokine, the mechanism of TGF-β mediated neuroprotection is unknown. The role of AP-1 transcription factors was

initially investigated, as AP-1 transcription factors protect various brain regions from ischemia-induced cell death (188, 190-191, 213-217). Serum deprivation is a cell culture model of ischemia, thus it was hypothesized a similar mechanism may protect against this form of cell death. Furthermore, numerous genes that are regulated by TGF-β require AP-1 mediated transcription to elicit a full biological action. In support of a role for AP-1 transcription factors in astrocyte-medaited neuroprotection, it was demonstrated that astrocyte conditioned media induced a rapid increase in c-Jun phosphorylation, a crucial step in AP-1 transactivation (Figure 5-1). Treatment of GT1-7 neurons with ACM was also associated with the activation of the upstream c-Jun kinases, JNK and MKK4 (Figure 5-2). Thus, it may be postulated that ACM can induce c-Jun phosphorylation by activating MKK4 and JNK. Likewise, exogenous TGF-β1 also enhanced the phosphorylation of c-Jun further supporting a role for astrocyte-derived TGF-β in mediating the neuroprotective actions of astrocyte conditioned media (data not shown). To better understand the consequences of c-Jun activation following ACM treatment, electromobility shift assay were employed to study the ability of transcription factors to bind specific AP-1 consensus sequences on DNA. Binding of transcription factors to DNA is a mandatory event in the initiation of gene transcription. Accordingly, ACM was found to enhance DNA binding of AP-1 transcription factors to consensus AP-1 sequences, as compared to cells in serum free conditions (Figure 5-1c). Immunoneutralization of ACM with a pan specific TGF-β antibody greatly attenuated the ability of ACM to induce AP-1 binding (Figure 5-3). This finding further suggests astrocyte-derived TGF-β is responsible for the ability of ACM to induce AP-1 transcription factors.

To demonstrate a functional role for AP-1 activation in mediating cell survival, inhibitors of the JNK-Jun pathway were utilized in the context of ACM and TGF-β1 mediated rescue of serum deprived GT1-7 neurons. Co-addition of dicoumarol, a specific inhibitor of JNK activation (218-219) completely blocked the rescue of serum deprived GT1-7 neurons following treatment with either ACM (Figure 5-4a) or TGF-β1 (Figure 5-4b). This effect of dicoumarol was specific for JNK as phosphorylation of c-Jun following treatment with ACM

was attenuated by co-addition of dicoumarol to cultures (Figure 5-4c). Similarly, co-addition of curcumin, an AP-1 transcriptional inhibitor (220), reversed the ability of ACM and TGF-β1 mediated rescue following serum deprivation (Figure 5-5). The effect of curcumin was observed in a dose range that is widely reported to specifically block AP-1 activation. Together, these findings indicate astrocyte-derived TGF-β1, which protects GT1-7 neurons from serum deprivation induced cell death, may do so via the activation of AP-1 transcription factors.

Figure 5-1: Effects of astrocyte conditioned media (ACM) on c-Jun/AP-1 activation. (A) ACM induction of c-Jun Ser-63 phosphorylation in GT1-7 neurons. Blots are representative of at least 4 independent experiments (B) Quantification of phosphorylated c-Jun in GT1-7 neurons following treatment with ACM, as compared to serum deprived treated neurons. (C) ACM induces AP-1-DNA complex formation as assessed by gel shift assay (arrow). S = serum, SD = serum deprivation, CM = conditioned media, R= recombinant Jun.

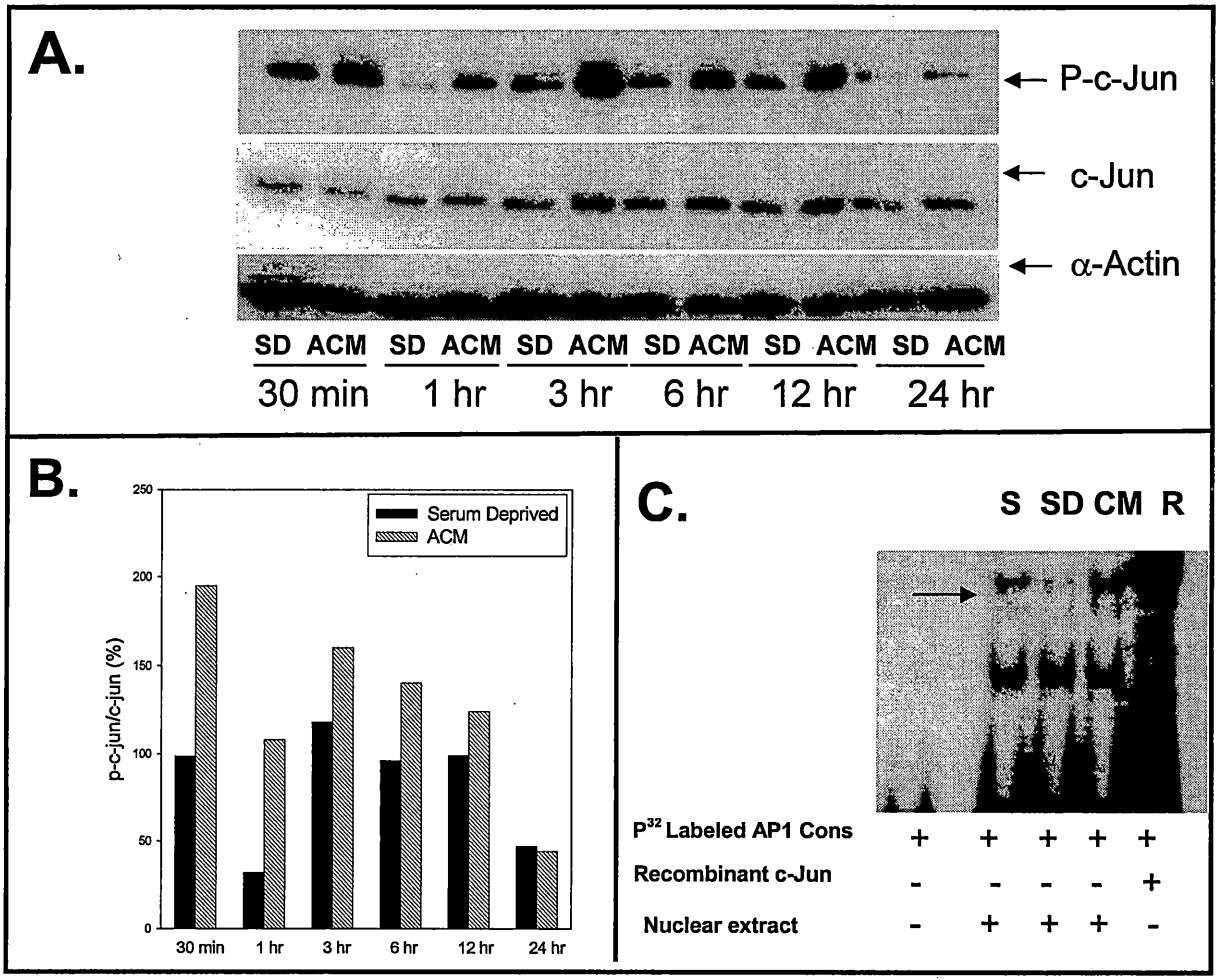
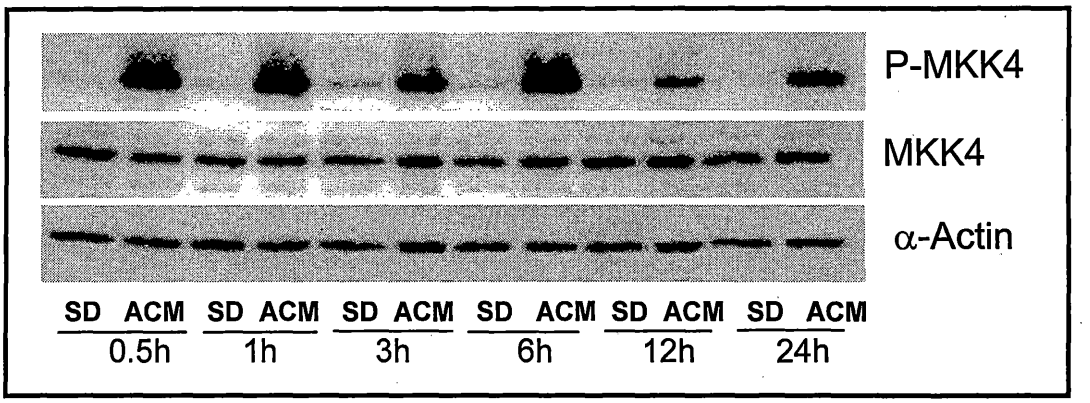

A.
P-c-Jun
c-Jun
α-Actin
SD ACM SD ACM SD ACM SD ACM SD ACM SD ACM
30 min 1 hr 3 hr 6 hr 12 hr 24 hr
B.
Serum Deprived
ACM
p-c-jun/c-jun (%)
250
200
150
100
50
0
30 min 1 hr 3 hr 6 hr 12 hr 24 hr
C.
S SD CM R
P32 Labeled AP1 Cons + + + + +
Recombinant c-Jun - - - - +
Nuclear extract - + + + -

Figure 5-2: Effects of astrocyte conditioned media (ACM) on (A) MKK4 and (B) JNK phosphorylation in GT1-7 neurons. Serum deprived GT1-7 neurons were treated with ACM or vehicle for various timepoints. Western blot analysis of phosphorylated protein was compared to non-phosphorylated protein. α-actin was used as a control for equal protein loading.

A.


P-MKK4
MKK4
α-Actin
SD ACM SD ACM SD ACM SD ACM SD ACM SD ACM
0.5h 1h 3h 6h 12h 24h

B.

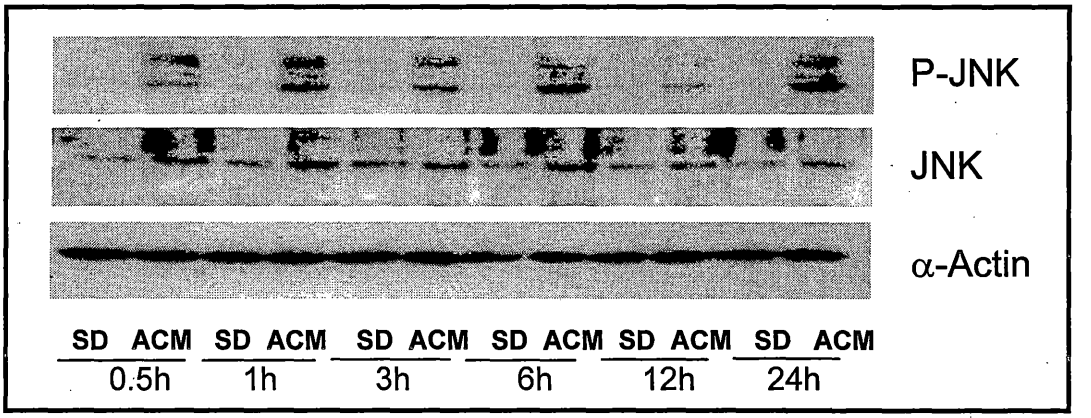

P-JNK
JNK
α-Actin
SD ACM SD ACM SD ACM SD ACM SD ACM SD ACM
0.5h 1h 3h 6h 12h 24h

Figure 5-3: Effects of TGF-β immunoneutralization on astrocyte conditioned media-induced AP-1 binding. GT1-7 neurons were treated with either serum (S), serum deprived (SD), astrocyte conditioned media (in serum deprived conditions)(ACM) or ACM which had previously been immunoneutralized with a pan specific TGF-β antibody (ACM+Ab). Nuclear lysates were collected following a one hour treatment and processed for AP-1 binding (arrow) utilizing gel shift analysis.

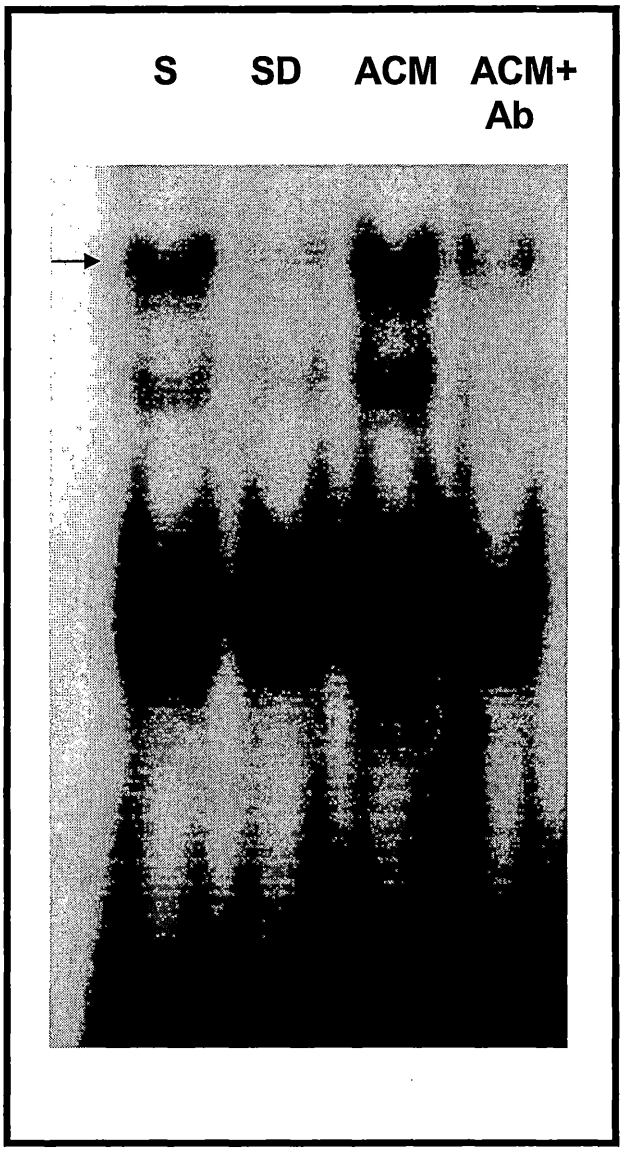
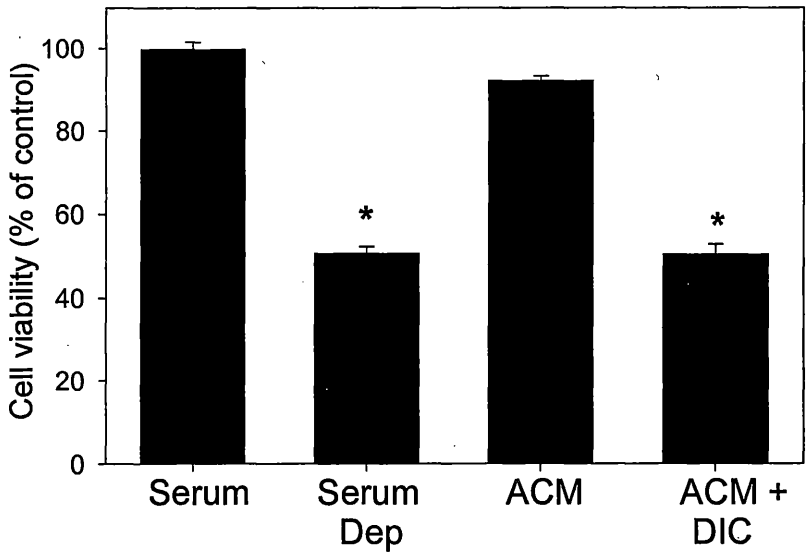
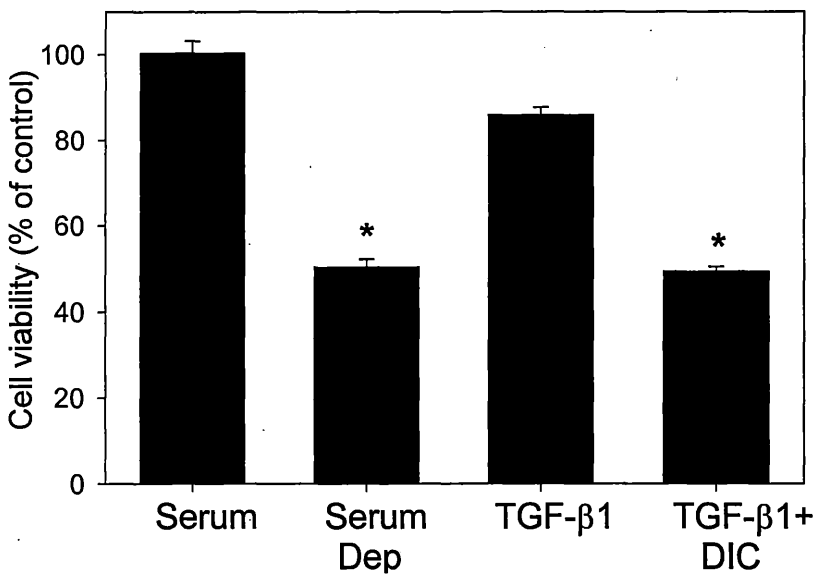

S
SD
ACM
ACM+
Ab

Figure 5-4: **Effects of dicoumarol, a JNK inhibitor, on (A) astrocyte conditioned media and (B) TGF-β1-mediated rescue of serum deprived GT1-7 neurons.** Co-treatment with 25 μM dicoumarol attenuated the neuroprotective effect of ACM and TGF-β1 in serum deprived GT1-7 neurons. (C) Dicoumarol decreased the activation of c-Jun phosphorylation following ACM treatment in serum deprived GT1-7 neurons following a 30 minute treatment. * = significantly different from serum control ($p<0.05$)

A.


Cell viability (% of control)
100
80
60
40
20
0
*
*
Serum
Serum Dep
ACM
ACM + DIC

B.


Cell viability (% of control)
100
80
60
40
20
0
*
*
Serum
Serum Dep
TGF-β1
TGF-β1+ DIC

C.

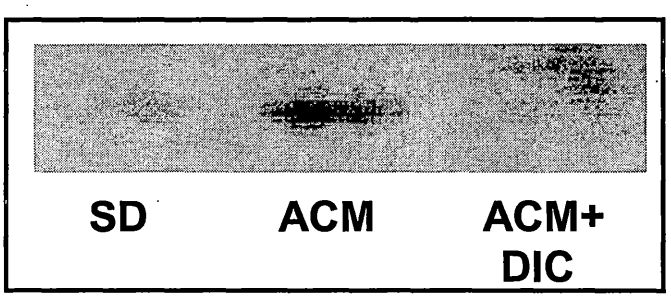
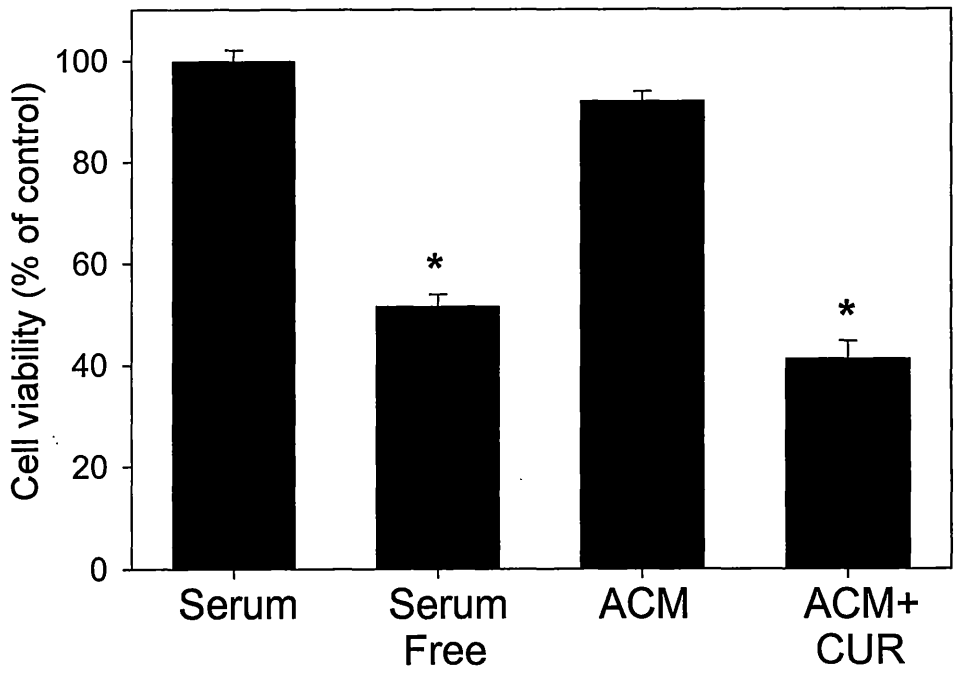
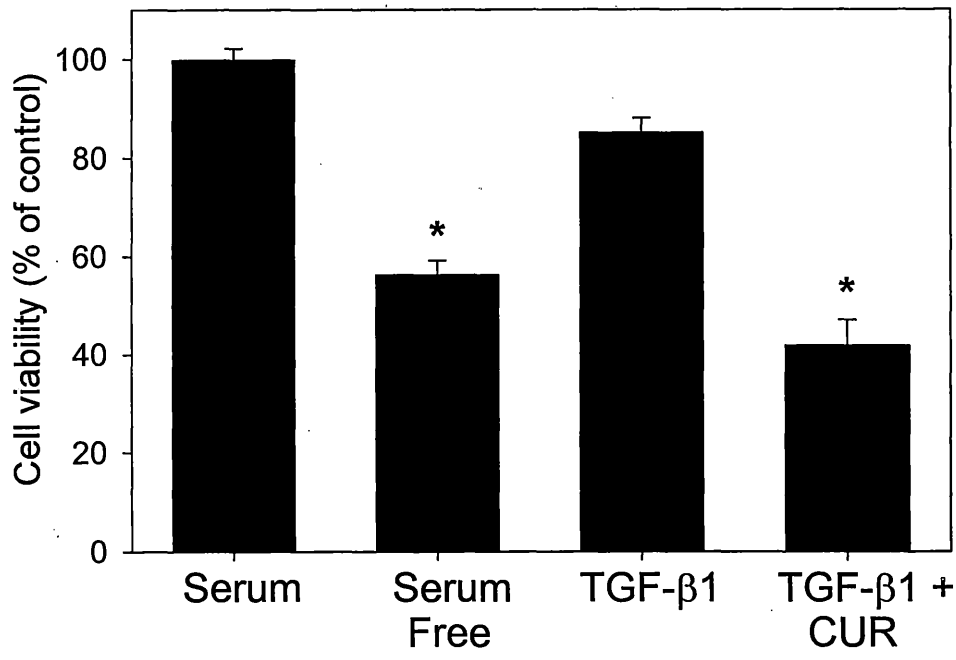

SD
ACM
ACM+ DIC

Figure 5-5: Effects of curcumin, an AP-1 inhibitor, on (A) astrocyte conditioned media and (B) TGF-β1-mediated rescue of serum deprived GT1-7 neurons. Co-treatment with 25 μM curcumin attenuated the neuroprotective effect of ACM and TGF-β1 in serum deprived GT1-7 neurons. * = significantly different from serum control ($p<0.05$)

A.


Cell viability (% of control)
100
80
60
40
20
0
*
*
Serum
Serum Free
ACM
ACM+ CUR

B.


Cell viability (% of control)
100
80
60
40
20
0
*
*
Serum
Serum Free
TGF-β1
TGF-β1 + CUR

6

HIGH DENSITY MICROARRAY ANALYSIS OF GENES REGULATED BY ESTROGEN AND TAMOXIFEN IN THE RAT CEREBRAL CORTEX

6.1 Use of Gene Arrays to Provide Novel Insights Into Mechanisms of Estrogen/SERM Action *In Vivo* in the Cerebral Cortex

Work in the previous sections used *in vitro* models, which while being excellent tools cannot fully mimic or recreate the natural, more complex *in vivo* situation. Furthermore, while they suggest a potential estrogen-astrocyte-TGF-β pathway for protection, the protective effects *in vitro* were modest, which is in contrast to the robust neuroprotection observed for estrogen and tamoxifen *in vivo* in ischemic stroke animal models. This could suggest that *in vivo,* multiple mechanisms may exist for neuroprotection by estrogen and tamoxifen. Estrogen and SERMs can also enhance synaptic plasticity in the brain and the precise mechanisms underlying this effect is also poorly understood (69, 221-223). Since recent work has demonstrated that estrogen and SERM-mediated neuroprotection requires pretreatment and involves the estrogen receptor (25, 33, 132, 143), which suggests a genomic mechanism, we decided to utilize high-density microarray analysis in order to identify genes regulated by estrogen and tamoxifen in the cerebral cortex of ovariectomized rats. Such an approach could provide new insights into the mechanisms underlying the reported neuroprotective and synaptic plasticity-regulating effects of estrogen and SERMs *in vivo.*

Specific Aim #5. To utilize high-density microarray analysis to identify genes regulated by 17β-E_2 and/or tamoxifen *in vivo* in the cerebral cortex. Work from several laboratories suggests physiological doses of 17β-E_2 and therapeutic doses of tamoxifen are neuroprotective and regulate synaptic plasticity via estrogen receptor activation. This effect has been associated with gene transcription. Thus, the identification of the specific genes regulated by 17β-E_2 and SERMs would be a significant advance to the field and could provide much needed insight into how these factors exert their neurotrophic and neuroprotective actions upon the brain.

6.2 Methods and Materials

6.2.1 Animals

Sixty day old female animals (Holtzman Sprague Dawley, Harlan, Indianapolis, IN) were bilaterally ovariectomized (OVX) under light ether anesthesia. Animals (n=5/treatment group) were treated immediately as follows: Group I – Placebo pellet (5 mg/pellet, 21 day release); Group II -17β-E_2 pellet(5 mg/pellet, 60 day release pellet); Group III –Tamoxifen pellet (15 mg/pellet, 60 day release pellet) using timed release pellets (Innovative Research of America, Sarasota, FL). Both 17β-E_2 and tamoxifen are lipophilic and readily cross the blood-brain-barrier. Animals were placed in individual cages with free access to food and water in an environmentally controlled room. Seven days following OVX, animals were sacrificed by decapitation. Cerebral cortices were rapidly dissected and homogenized in TriZOL (Invitrogen, Carlsbad, CA). Homogenates were stored at –80°C until RNA isolations. All studies received prior approval from the Institutional Committee for Animal Use in Research and Education (CAURE) and were conducted in accordance with the guidelines of the National Institutes of Health and the United States Department of Agriculture (USDA).

6.2.2 Preparation of RNA

RNA was isolated as previously described in the dissertation. Aliquots of 5 μL were used for spectrophotometric analysis to determine RNA concentrations. The integrity of the total RNA was assessed by visualization of the 28S and 18S ribosomal RNA bands in a 1.5% agarose gel stained with ethidium bromide. A total of 10 μg of RNA/group was sent to the Molecular Biology Core Facility at the Medical College of Georgia for Affymetrix gene chip analysis. The RNA was used to generate fluorescently labeled targets for hybridization to microarrays. The experiment and subsequent gene chip analysis was performed in duplicate (including independent RNA samples collected from two separate pools of animals/treatment group) to ensure reproducibility of results.

6.2.3 Affymetrix Oligonucleotide Microarray

Oligonucleotide microarrays comprising over 8,800 rat genes and expressed sequence tags (ESTs) was purchased from Affymetrix (Rat Genome U34A set). The U34A set includes all rat sequence clusters from Build #34 of the UniGene database (created from GenBank 107/dbEST 11/18/98) and supplemented with additional annotated gene sequences from GenBank110. Each gene or EST is presented on the GeneChip by 16 non-overlapping "probe" sequences, each 25 nucleotides in length. Each probe is located above a control probe containing a single-base mismatch. A score termed the "average difference" is assigned to each gene, calculated as the average signal from the twenty perfect match probes minus the average signal from the twenty corresponding mismatch probes.

6.2.4 Gene normalization

Gene normalization was performed as recommended by Affymetrix. Briefly, the 50th percentile of all measurements was used as a positive control for each sample; each measurement for each gene was divided by this synthetic positive control, assuming that this was at least 10. The bottom tenth percentile was used as a test for correct background subtraction. This was never less than the negative of the synthetic positive control. Each gene was normalized to itself by making a synthetic positive control for that gene, and dividing all measurements for that gene by this positive control, assuming it was at least 0.01. This synthetic control was the median of the gene's expression values over all the samples. Lastly, normalized values below 0 were set to 0.

6.2.5 K-means cluster analysis

To analyze differential gene expression between treatment groups, high-density microarray data was assessed using GeneSpring software Version 4.1 (Silicon Genetics, Redwood, CA). GeneSpring is a powerful analysis tool, which allows large volume, microarray data to be filtered and analyzed. Furthermore, unique gene clustering profiles can be established using the k-means clustering tool, permitting global gene analysis following treatment. K-means

clustering divides genes into distinct groups based on expression patterns. Genes are initially divided into a number (k) of user-defined and equally-sized groups (i.e. k=50). Centroids are calculated from each group corresponding to the average of the expression profiles. Individual genes are then reassigned to the group in which the centroid is the most similar to the gene. Group centroids are then recalculated, and the process is repeated until the group compositions converge.

6.2.6 Real Time Reverse Transcriptase Polymerase Chain Reaction

To confirm the changes observed following high density gene chip analysis, real time RT-PCR was performed on a Cepheid Smart Cycler (Cepheid, Sunnyvale, CA) utilizing the RNA Amplification SYBR Green I kit (Roche, Indianapolis, IN), according to the manufacturer's protocol. Candidate genes were randomly selected for real time RT-PCR analysis (Table 6-1). This was the third confirmation as the hybridization was performed twice using independent experiments, giving an n=3/gene change.

6.3 Results

6.3.1 Differential gene regulation in the cerebral cortex

Analysis of gene expression using scatterplots (Figure 6-1) and Venn diagrams (Figure 6-2) revealed a total of 858 genes which were regulated in a two-fold or greater manner by 17β-E_2 and/or tamoxifen. Of these 858 genes, 15 genes (1.7% of total genes regulated) were similarly up regulated by both treatments whereas 24 genes (2.8% of total genes regulated) were similarly down regulated. 194 genes (22.6%) were increased by 17β-E_2, but not by tamoxifen, and 182 genes (21.2%) were increased by tamoxifen, but not 17β-E_2. Conversely, 268 genes (31.2%) were down regulated greater than two-fold by 17β-E_2, but not tamoxifen; whereas, 175 genes (20.4%) were down regulated by tamoxifen, but not 17β-E_2.

6.3.2 Patterns of gene expression

To explore patterns of gene expression, a 50 k-means cluster analysis was performed (Figure 6-3). Of these 50 clusters, 15 clusters fell below the arbitrary 2-fold or less cutoff for gene expression changes. Further analysis of microarray data revealed distinct differences in gene expression between all three treatment groups. Hierarchal experiment tree analysis (Figure 6-4) demonstrated each treatment group regulated a distinct subset of genes that was generally not shared by the other two treatment conditions. The region of overlap between any two treatment groups was generally small.

6.3.3 Genes regulated by treatment groups

Tables 6-2 – 6-7 list all genes regulated in a two-fold or greater manner between treatment groups. The genes listed reproduced in a two-fold or greater manner between both experiments, which were performed with independent RNA samples. These genes fell into several categories of function, including growth factors and receptors, cell adhesion/signaling, energy and metabolism, neurogenesis, neuroprotection, and synapse-related/neurotransmission (Table 6-8).

6.3.4 Real Time RT-PCR Confirmation of Regulated Genes

To confirm gene changes predicted by the microarray, real time RT-PCR was utilized. Ten candidate genes were chosen at random from the various lists of genes that were changed two-fold or greater. Primers were designed to specifically detect these gene products (Figure 6-5). Real time RT-PCR was then performed on these genes using the samples which were previously hybridized to microarrays. Using real time RT-PCR, all ten gene changes analyzed were confirmed in a manner nearly exactly as predicted by microarray analysis. In several cases, RT-PCR found the microarray underestimated the magnitude of the change. The results of RT-PCR confirmations of eight randomly selected genes, as compared to the results predicted from gene chip analysis, are shown in Figures 6-6 – 6-15).

6.4 Discussion

Gene expression profiling using high density microarrays is a novel technology, allowing the simultaneous measurement of thousands of genes in single RNA samples. This type of analysis permits sophisticated comparisons between different treatment groups. Using a commercially available microarray and real time RT-PCR, the expression profile of cerebral cortical genes regulated by 17β-E_2 and the selective estrogen receptor modulator, tamoxifen, was assessed. The results of this study implicate several classes of genes, many of which may influence the known functions of 17β-E_2/SERMs in the brain, such as neuroprotection and synaptic plasticity.

It is important to note that the results presented in the figures are of genes that changed two-fold or greater in two separate experiments, thus enhancing confidence in the data. Furthermore, random selection of genes shown to be regulated by 17β-E_2 or tamoxifen by the gene chip were confirmed using a separate technique, real time RT-PCR. All genes tested confirmed the gene chip results, further enhancing confidence in the validity of the changes. Intriguingly, experimental tree analysis suggested only a small population of genes were regulated in a similar manner by both physiological levels of 17β-E_2 and therapeutic doses of tamoxifen. This finding was unexpected given our previous observation that tamoxifen protects against acute ischemic stroke in a manner similar to that of 17β-E_2. Based on that finding, it was hypothesized many genes would be similarly regulated in a reproducible manner. However, only 15 genes were similarly up regulated 2-fold or greater by both treatments and 24 genes were down regulated 2-fold or greater by both treatment. Thus, only about 1.7% of regulated genes were influenced in a similar manner by 17β-E_2 and tamoxifen in the rat cerebral cortex. Given that tamoxifen is a selective estrogen receptor modulator, the finding that 2.8% of genes are differentially regulated is of great interest as it suggests that tamoxifen (and potentially other SERMs) may influence a small subset of 17β-E_2 regulated genes. Although it is not immediately clear as to why tamoxifen influences a largely different set of genes from 17β-E_2, it is possible tamoxifen differentially influences gene expression through the two known estrogen receptors, ER-α and ER-β. This is supported by reports that tamoxifen has a higher binding affinity for ER-α

as compared to ER-β. Tamoxifen may also recruit a different set of corepressors and coactivators to the DNA-receptor complex than estrogen as suggested by others (224).

Of the genes regulated by both 17β-E_2 and tamoxifen, several interesting classes of genes were identified, including genes implicated to have roles in synaptic plasticity, neurotransmission, growth factors, neuroprotection, signal transduction, transcriptional regulation, and neurogenesis (Table 6-7 – Table 6-13). Given the known role of 17β-E_2 in these functions, it is interesting to note that tamoxifen retains the ability to influence many of the genes involved in these important functions. For example, 17β-E_2 has long been known to influence neurotransmission by altering synaptic plasticity in the brain, especially the hippocampus. More recently, Silva et al. (221) demonstrated the ability of tamoxifen to similarly influence synaptic density. Together, these findings implicate estrogens in the regulation of synaptic plasticity. However, the molecular mechanisms underlying the plastic changes in the brain are poorly understood. In the present study, 17β-E_2 and tamoxifen regulate a wide array of genes related to synaptic function and neurotransmission. Of note, many components of glutamatergic signaling are influenced, suggesting a positive role for estrogens regulating excitatory neurotransmission in the brain. In particular, regulation of the expression of PSD-95, SAP102, and Chapsyn 110, which are well known to anchor and cluster glutamate receptors at the synapse, could provide a mechanism for facilitating efficient glutamate neurotransmission (225-226). As glutamate signaling is an important component of brain function, these findings may represent novel mechanisms of how 17β-E_2 exerts its purported beneficial effects on learning and memory. Furthermore, several members of the synaptic exocytotic mechanism were shown to be up-regulated by 17β-E_2 and/or tamoxifen. These include synaptotagmin and syntaxins 2, 7, and 8. Synaptotagmin is a component of the synaptic vesicle, which interacts with plasma membrane proteins (syntaxins, etc.) for docking and release of vesicle transmitter contents from the cell at the synapse. Thus, 17β-E_2 and tamoxifen regulation of these presynaptic proteins could influence the release of transmitter. Our gene chip results on 17β-E_2/tamoxifen regulation of syntaxins and synaptotagmin agrees with work by McEwen and coworkers who recently reported 17β-E_2 increases syntaxin protein in the hippocampus (227) and Yokomaku et al (228) who

showed 17β-E_2 increased syntaxin and synaptotagmin protein levels in the hippocampus, an effect correlated with increased glutamate release. Our work extends the regulatory effect to the cortex, and suggests the regulation may be exerted at the transcriptional level.

Aside from the regulation of many synapse-related genes, which may define how estrogens influence neurotransmission (and related processes such as learning and memory), numerous categories of novel genes were identified. Perhaps the most intriguing of these is genes implicated in neurogenesis. Neurogenesis is the process whereby neural stem cells (presumably in the subventricular zone of the cerebral cortex) differentiate from a multipotent cell into a terminally differentiated neuron. Recently, the possibility that 17β-E_2 may regulate neurogenesis was suggested in the dentate gyrus, a region of the hippocampus which also possesses neural stem cells (229). Although the present study did not directly address the issue of neurogenesis, the large number of genes related to this process that were regulated by estrogens implies this may occur in vivo. An increase in neurogenesis following 17β-E_2/SERM treatment may represent a mechanism for repair of the injured nervous system, as well as a mechanism to influence dynamic processes such as learning and memory. These findings likely will represent novel areas of research in the understanding of steroid hormone action in the CNS.

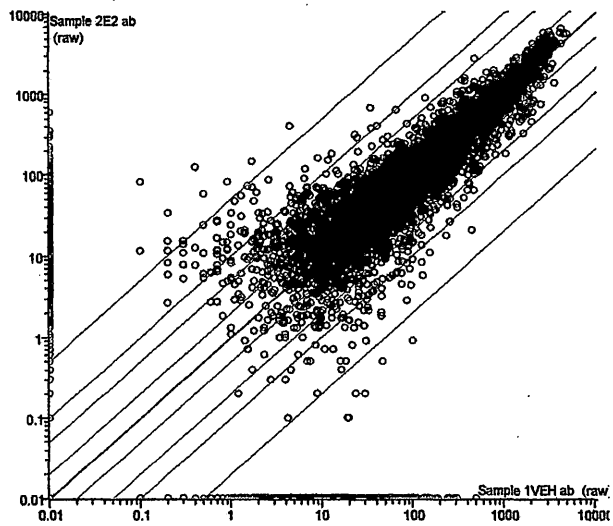
Although one of the aims of the present study was to identify estrogen regulated genes with neuroprotective potential, few anti-apoptotic genes were regulated by 17β-E_2 and tamoxifen. Given the ability of these compounds to protect the brain from injury, many pro-survival genes were expected to be regulated. However, many genes, such as growth factors, cytokines, metabolic factors, and growth factor receptors were increased following treatment. Given the cytoprotective ability of many of these factors, the neuroprotection observed following 17β-E_2 or tamoxifen treatment may involve one or more of these compounds. Additionally, both 17β-E_2 and tamoxifen led to a large increase in components of signaling pathways which have been implicated in neuronal survival. Interestingly, several pieces of the TGF-β – AP-1 (defined in Chapter 5) signaling cascade were induced by 17β-E_2/tamoxifen, including c-Fos, Fos related antigen, TGF-β type I receptor, and c-Jun interacting protein (Table 6-8, Table 6-9). This data may further implicate TGF-β and AP-1 in mediating the protective effect of estrogen/tamoxifen *in*

vivo. Additionally, other members of the TGF-β superfamily were up-regulated by 17β-E_2 and/or tamoxifen (BMP-3, BMP-4, GDNF, GDF-8), and significantly, decorin, a TGF-β binding proteoglycan that inhibits TGF-β action, was down-regulated by both 17β-E_2 and tamoxifen. IGF-1 and the glucose transporter GLUT4 were also up-regulated by 17β-E_2 and tamoxifen. This is interesting as Garcia-Segura and coworkers (230-231) have suggested that 17β-E_2 may regulate IGF-1 release from astrocytes to aid in its neuroprotection effects. Our gene chip results of 17β-E_2 and tamoxifen up-regulation of GLUT4 agrees with the recent work by Cheng et al (232) who showed similar up-regulation of GLUT4 in the primate cortex. The up-regulation of GLUT4 by 17β-E_2 and tamoxifen could mediate neuroprotection as up-regulation of GLUT4 in neurons has been shown to enhance survival of the neurons in the presence of cell death inducers.

As a whole, several interesting conclusions may be drawn from the present study. The well-documented neuroprotective effect of estrogens may involve a complex interplay of factors and potentially multiple cell types. This effect may involve growth factors, receptors, signaling pathways, metabolic regulation and other factors with yet unidentified functions (ie ESTs). Nonetheless, it is clear estrogens strongly regulate many genes related to synaptic plasticity and excitatory neurotransmission. These findings may further define the important role 17β-E_2 and tamoxifen have in the regulation of learning and memory. Finally, the large number of regulated genes related to neurogenesis, suggests the exciting possibility that estrogens may regulate the birth of new neurons in the CNS. This finding is especially intriguing as estrogens have recently been suggested to improve functional recovery of neurons following injury. Furthermore, neurogenesis may also be a mechanism whereby estrogens enhance learning and memory. Lastly, this study confirms the utility of high density microarrays to identify novel gene changes in whole animals. Future work will further characterize the present gene changes in the normal physiological functioning of the CNS.

Figure 6-1: Scatter plots of normalized intensity values from gene chip results. Starting from the center line, lines indicate a 2, 5, 10 and 50-fold up-regulation or down-regulation (left or right of center line, respectively). (A.) 17β-estradiol vs vehicle. (B.) Tamoxifen vs vehicle.

A.


Sample 2E2 ab (raw)
Sample 1VEH ab (raw
10000
1000
100
10
1
0.1
0.01
0.01
0.1
1
10
100
1000
10000

B.

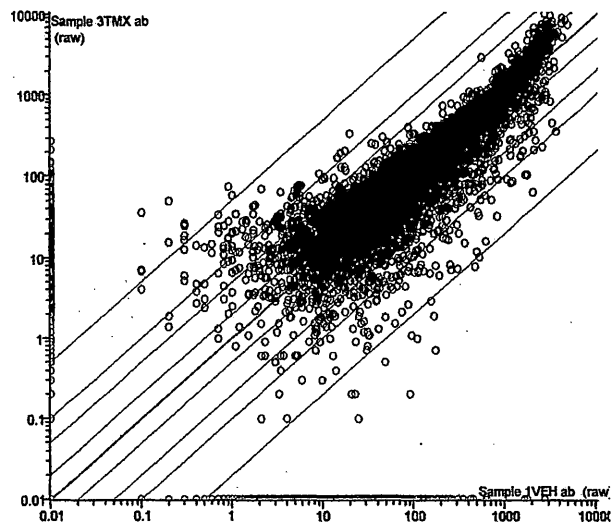
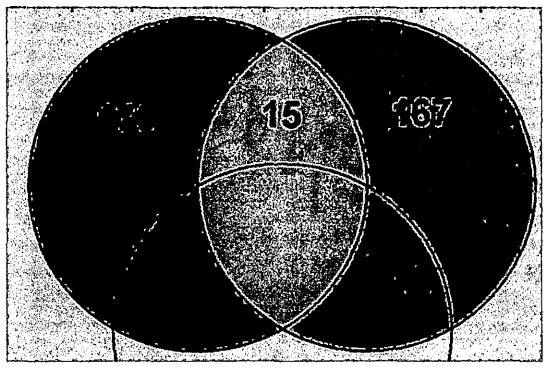

Sample 3TMX ab (raw)
Sample 1VEH ab (raw
10000
1000
100
10
1
0.1
0.01
0.01
0.1
1
10
100
1000
10000

Figure 6-2: Venn diagrams of gene chip data. (A.) Comparison of genes up-regulated two-fold and greater by 17β-E_2 and tamoxifen. (B.) Comparison of genes down-regulated two-fold and greater by 17β-E_2 and tamoxifen. (C.) Identification of genes differentially regulated by 17β-E_2 and tamoxifen.

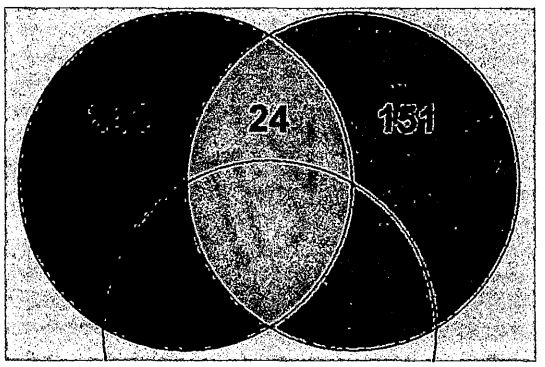
A.



Number of genes up-regulated two-fold and greater by:

- E_2: 194 total
- TMX: 182 total
- both E_2 and TMX: 15
- E_2 but not TMX: 179
- TMX but not E_2: 167

B.



Number of genes down-regulated two-fold and greater by:

- E_2: 268 total
- TMX: 175 total
- both E_2 and TMX: 24
- by E_2 but not TMX: 244
- by TMX but not E_2: 151

Figure 6-3: Cluster analysis of gene chip data. A 50K–Means cluster analysis was performed on the gene chip data utilizing GeneSpring software. Of these, 15 clusters were of genes which were not regulated two-fold or greater compared to vehicle and were discarded.

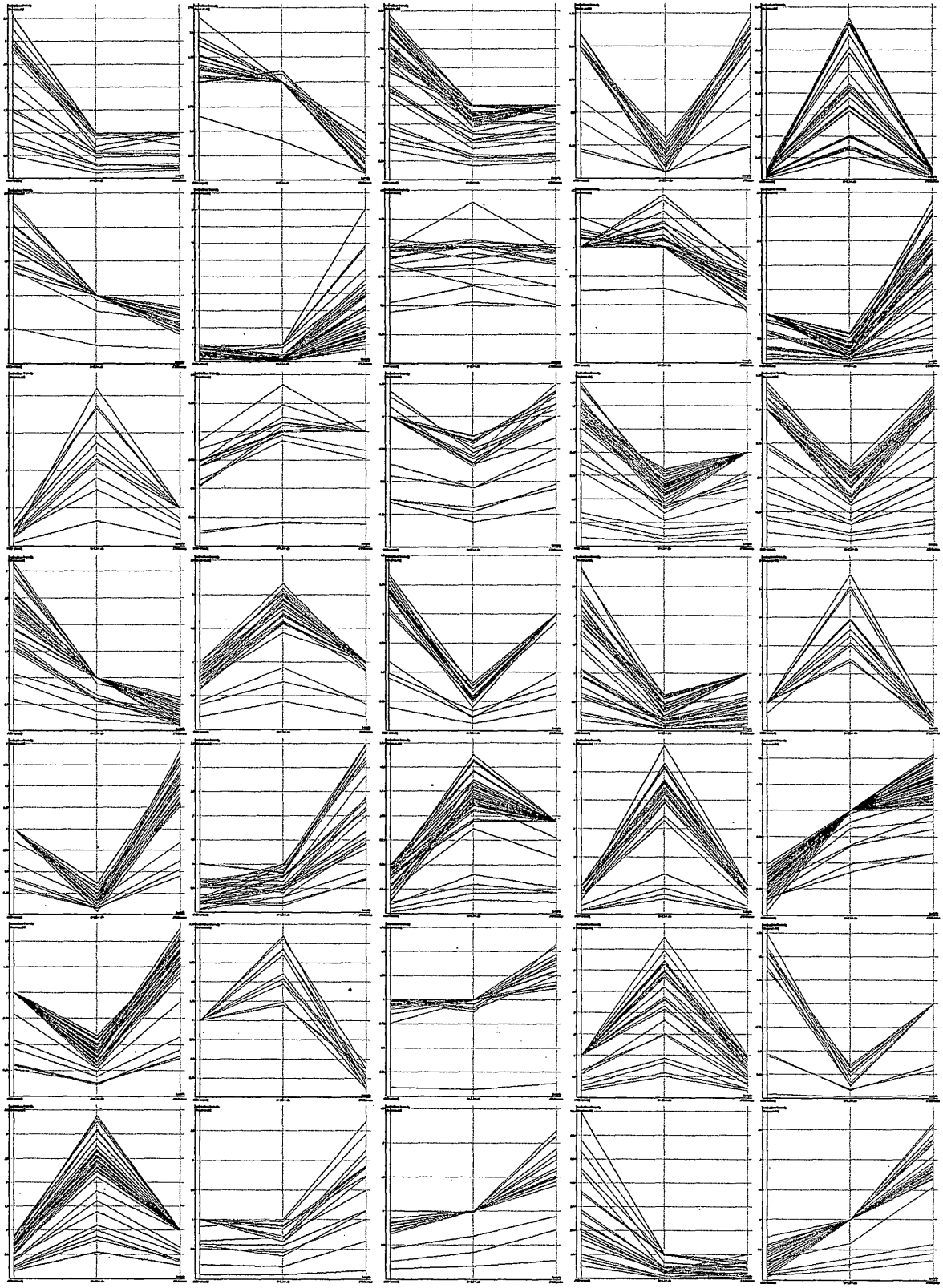


Figure 6-4: Hierarchal experiment gene tree.

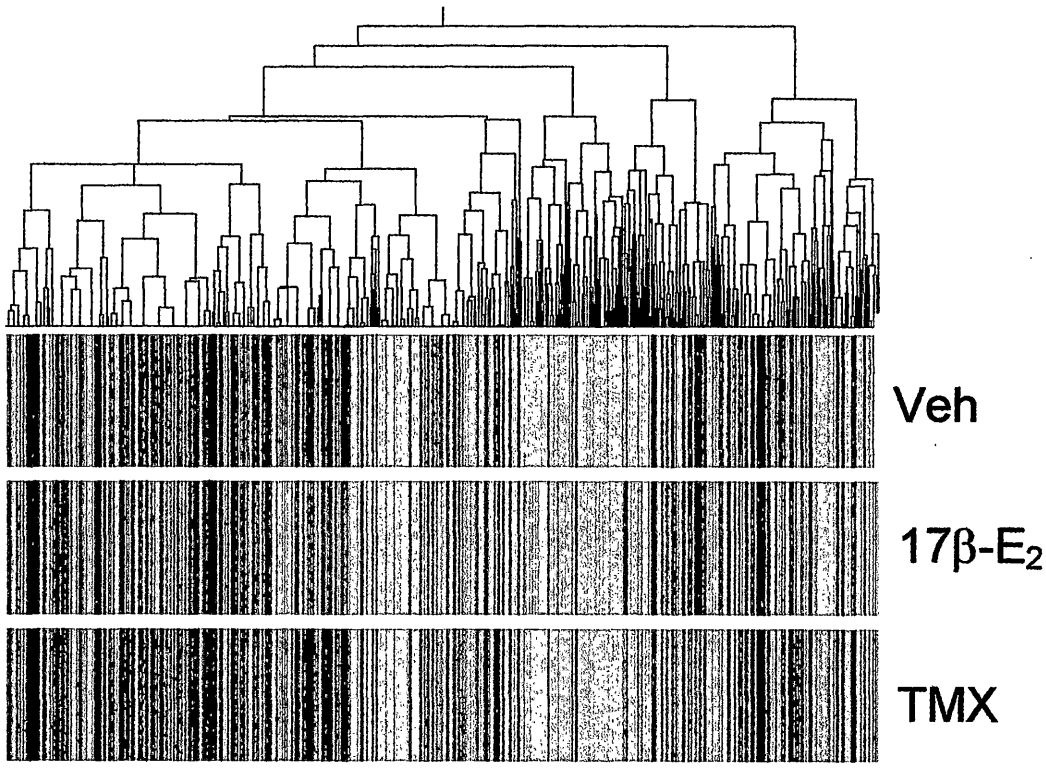

Veh
17β-E2
TMX

Figure 6-5: Real time RT-PCR confirmation of c-kit receptor. Microarray data (A.) was confirmed using real time RT-PCR (B.)

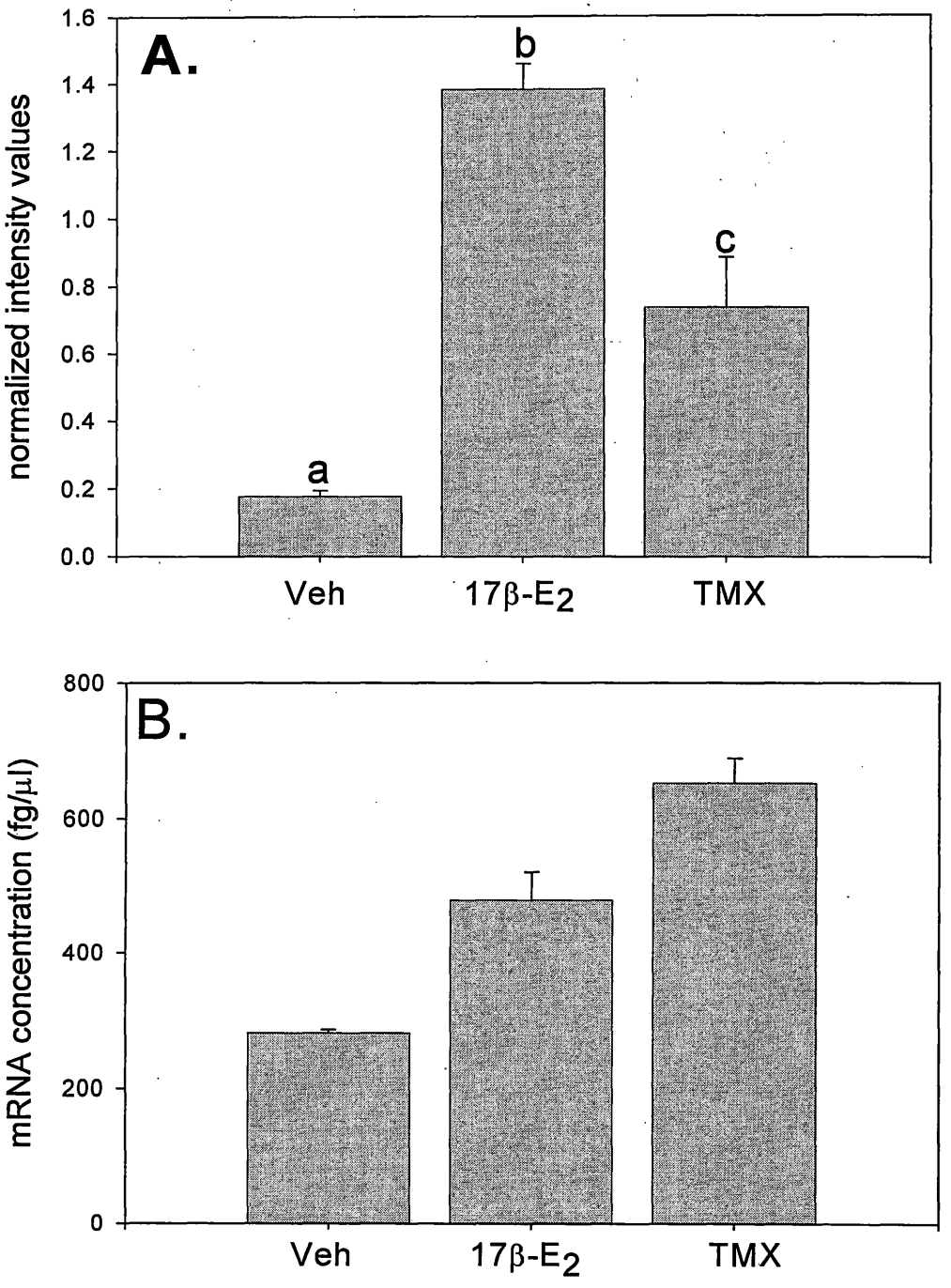
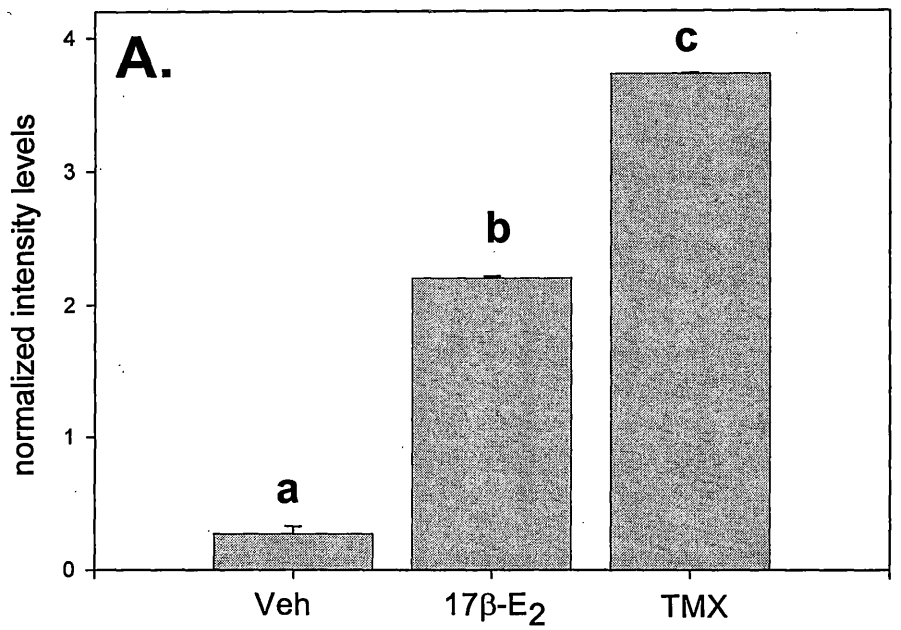

A.
normalized intensity values
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
Veh
17β-E2
TMX
B.
mRNA concentration (fg/μl)
800
600
400
200
0
Veh
17β-E2
TMX

Figure 6-6: Real time RT-PCR confirmation of stem cell factor. Microarray data (A.) was confirmed using real time RT-PCR (B.)


A.
normalized intensity levels
0
1
2
3
4
a
b
c
Veh
17β-E2
TMX

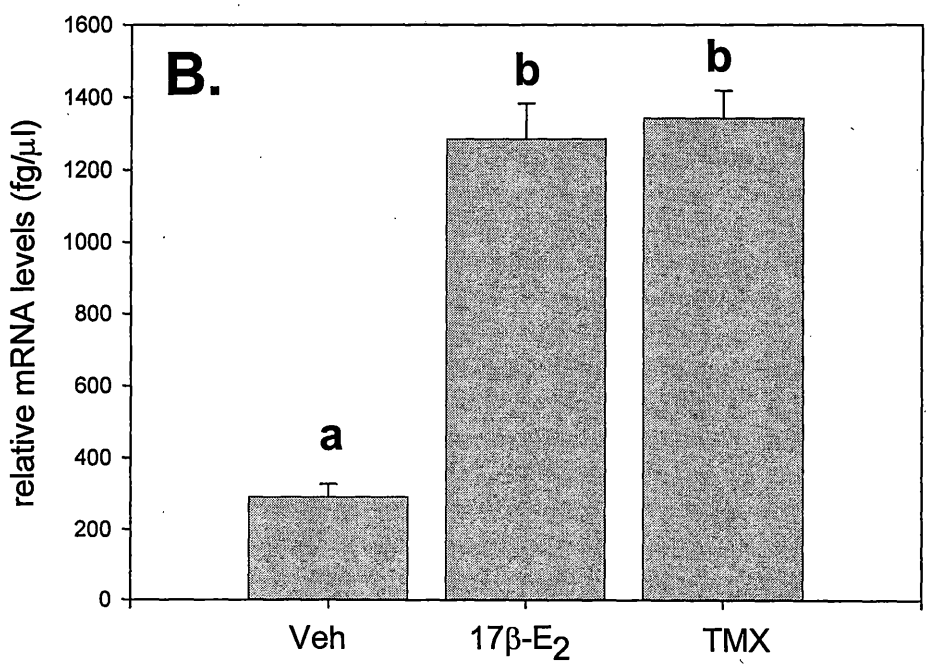
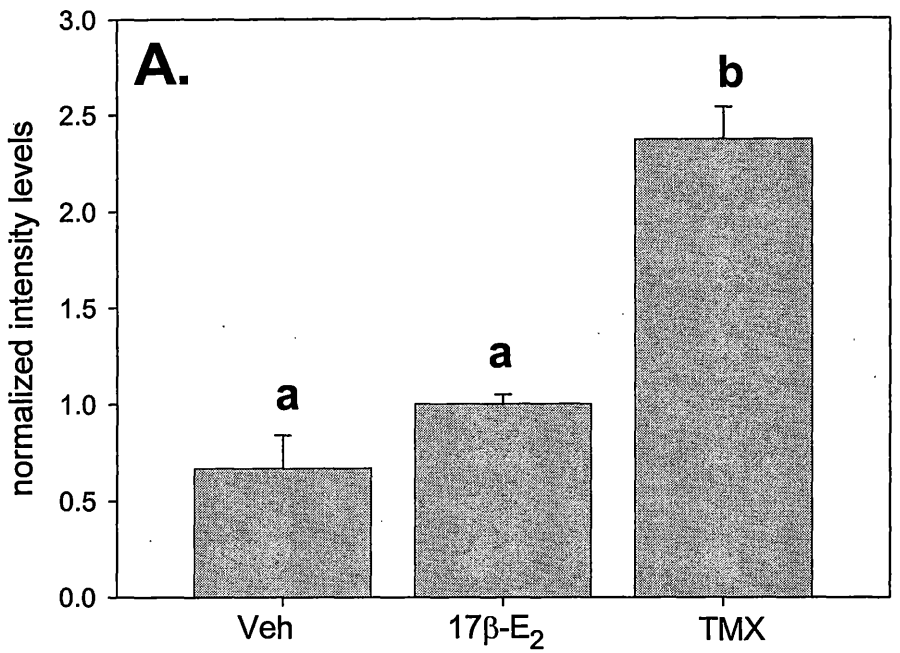

B.
relative mRNA levels (fg/μl)
0
200
400
600
800
1000
1200
1400
1600
a
b
b
Veh
17β-E2
TMX

Figure 6-7: Real time RT-PCR confirmation of stanniocalcin. Microarray data (A.) was confirmed using real time RT-PCR (B.)


A.
normalized intensity levels
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
Veh
17β-E2
TMX

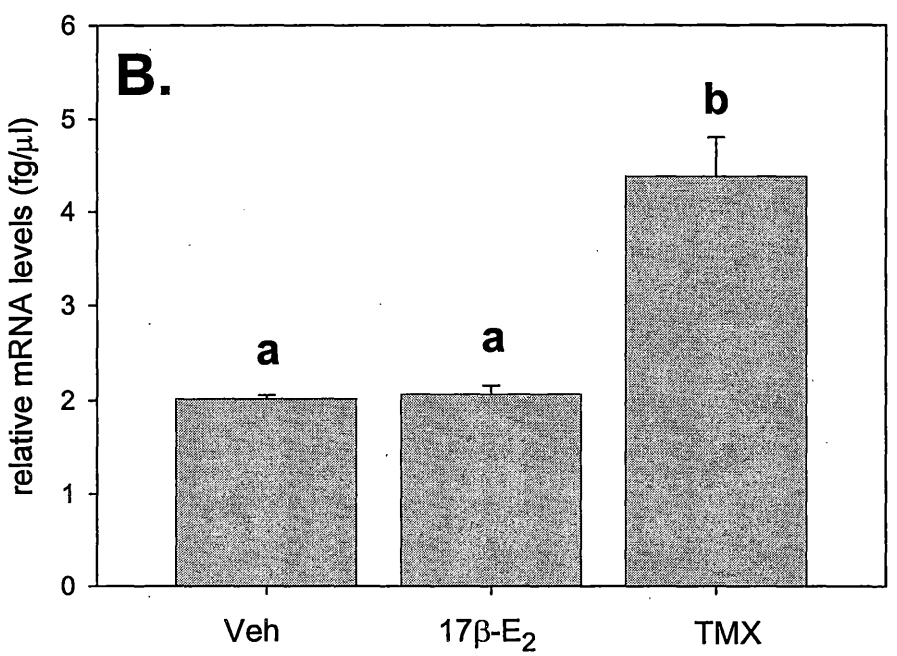
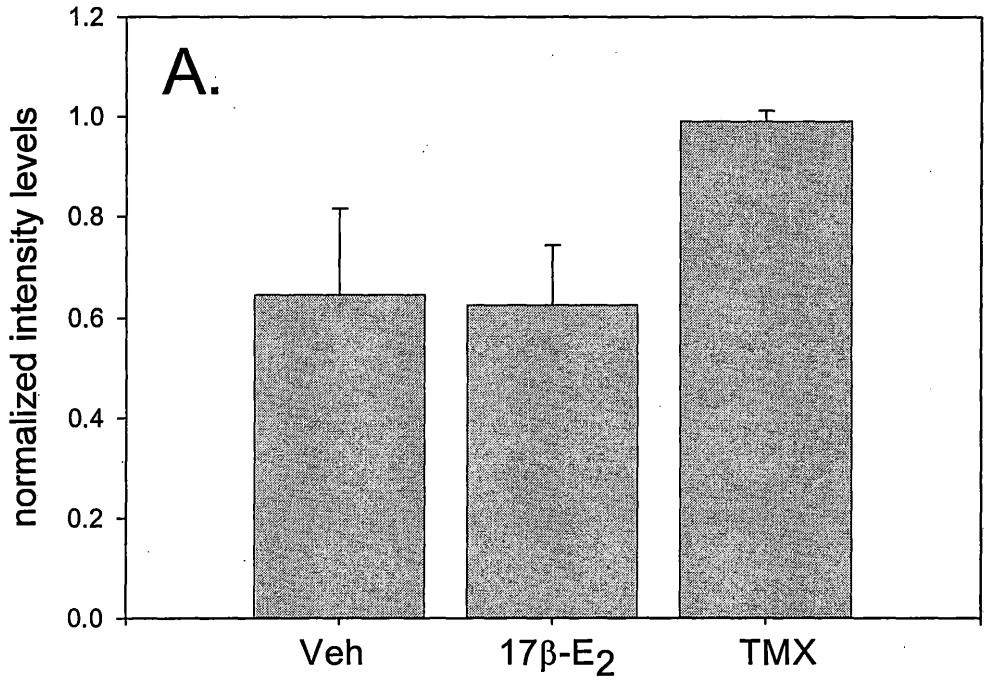

B.
relative mRNA levels (fg/μl)
6
5
4
3
2
1
0
a
a
b
Veh
17β-E2
TMX

Figure 6-8: Real time RT-PCR confirmation of DRM/gremlin. Microarray data (A.) was confirmed using real time RT-PCR (B.)


A.
normalized intensity levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Veh
17β-E2
TMX

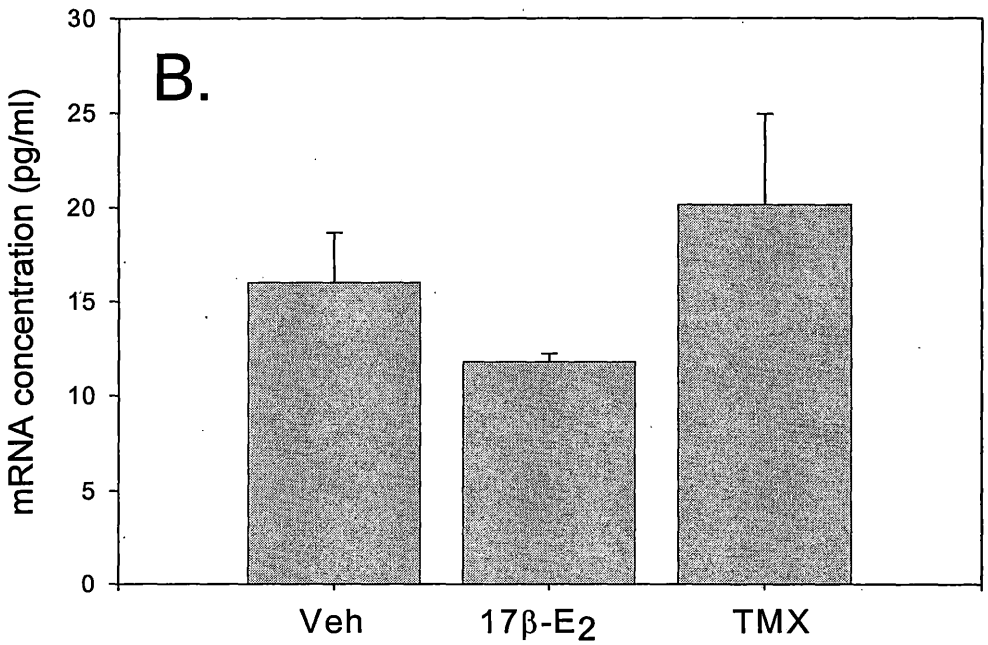
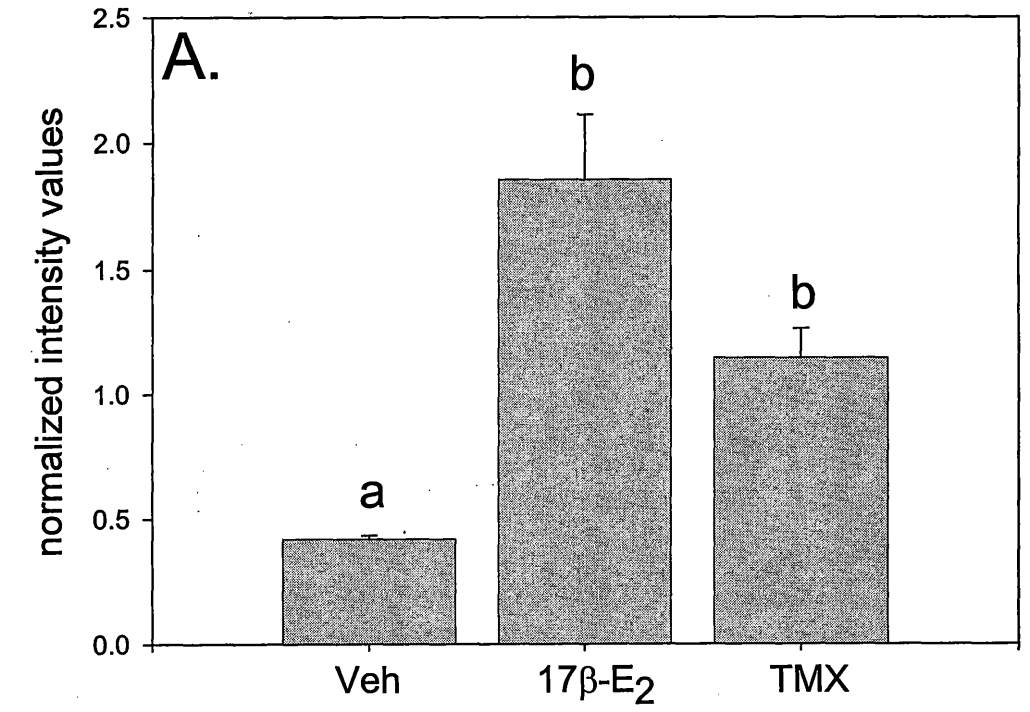

B.
mRNA concentration (pg/ml)
30
25
20
15
10
5
0
Veh
17β-E2
TMX

Figure 6-9: Real time RT-PCR confirmation of contactin 1. Microarray data (A.) was confirmed using real time RT-PCR (B.)


A.
normalized intensity values
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
Veh
17β-E2
TMX

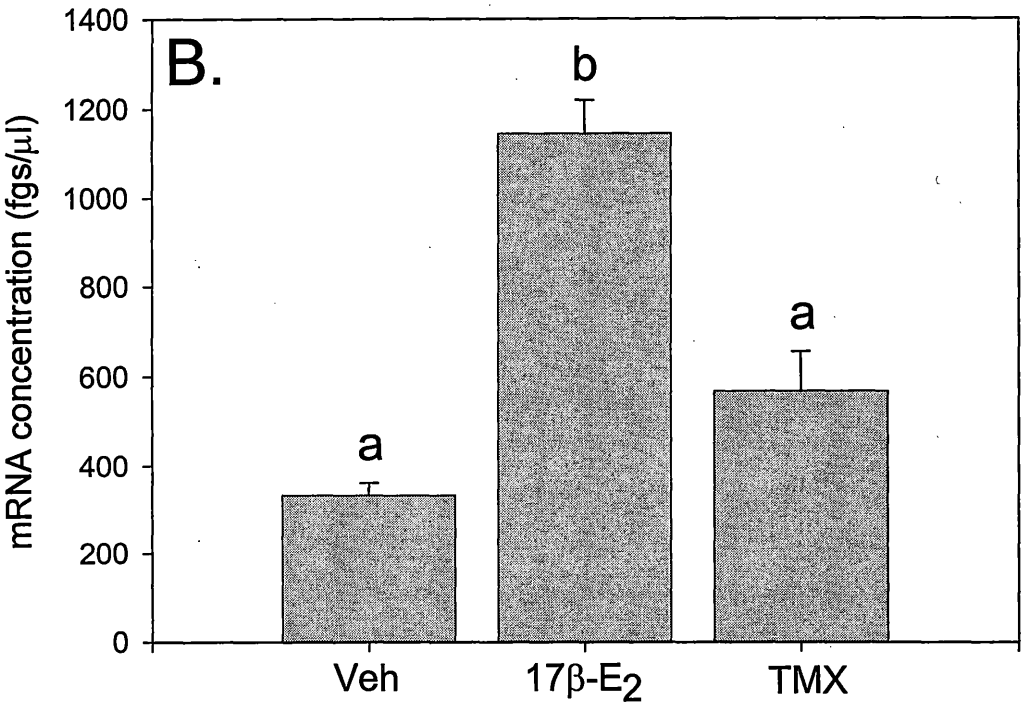
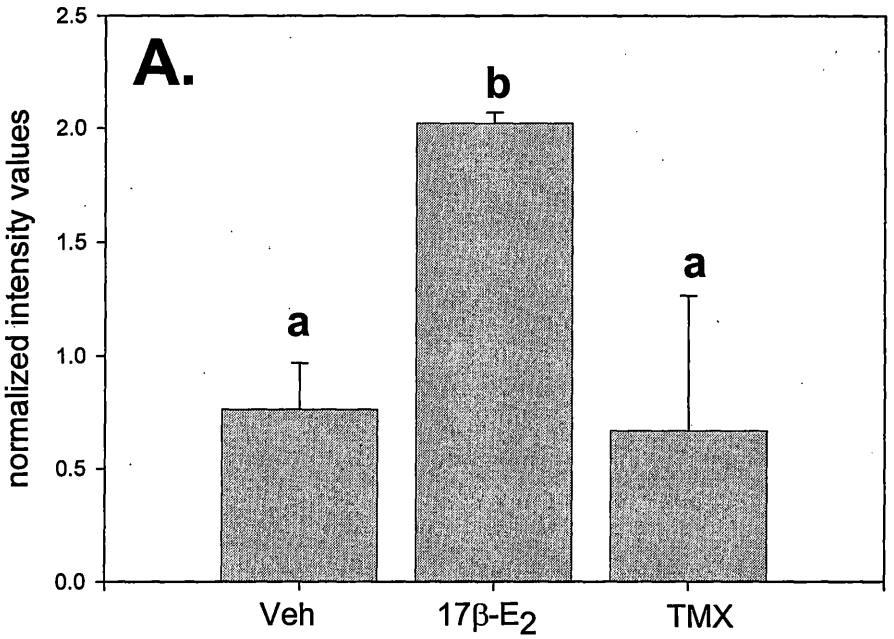

B.
mRNA concentration (fgs/μl)
1400
1200
1000
800
600
400
200
0
a
b
a
Veh
17β-E2
TMX

Figure 6-10: Real time RT-PCR confirmation of chapsyn-110. Microarray data (A.) was confirmed using real time RT-PCR (B.)


A.
normalized intensity values
2.5
2.0
1.5
1.0
0.5
0.0
a
b
a
Veh
17β-E2
TMX

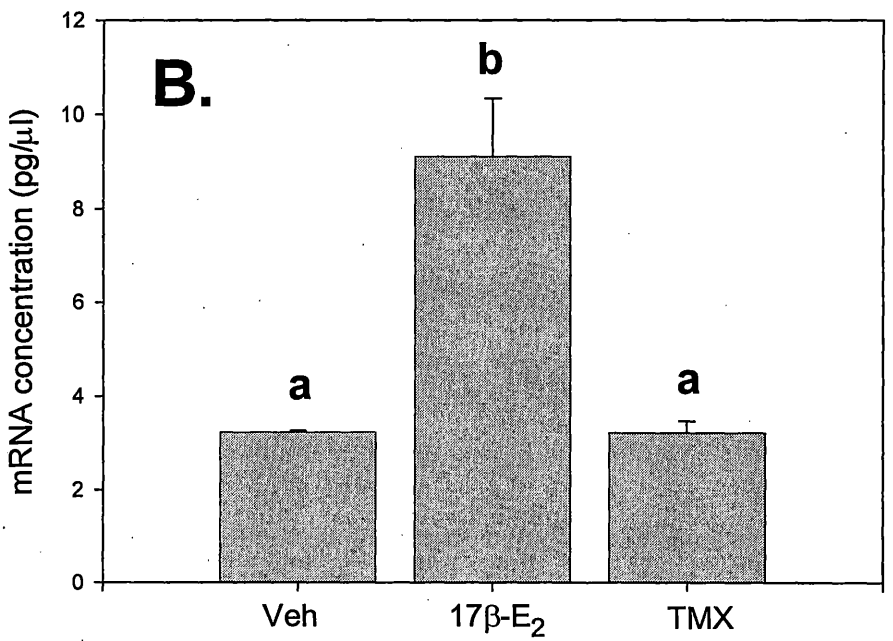

B.
mRNA concentration (pg/µl)
12
10
8
6
4
2
0
a
b
a
Veh
17β-E2
TMX

Figure 6-11: Real time RT-PCR confirmation of GnRH. Microarray data (A.) was confirmed using real time RT-PCR (B.)

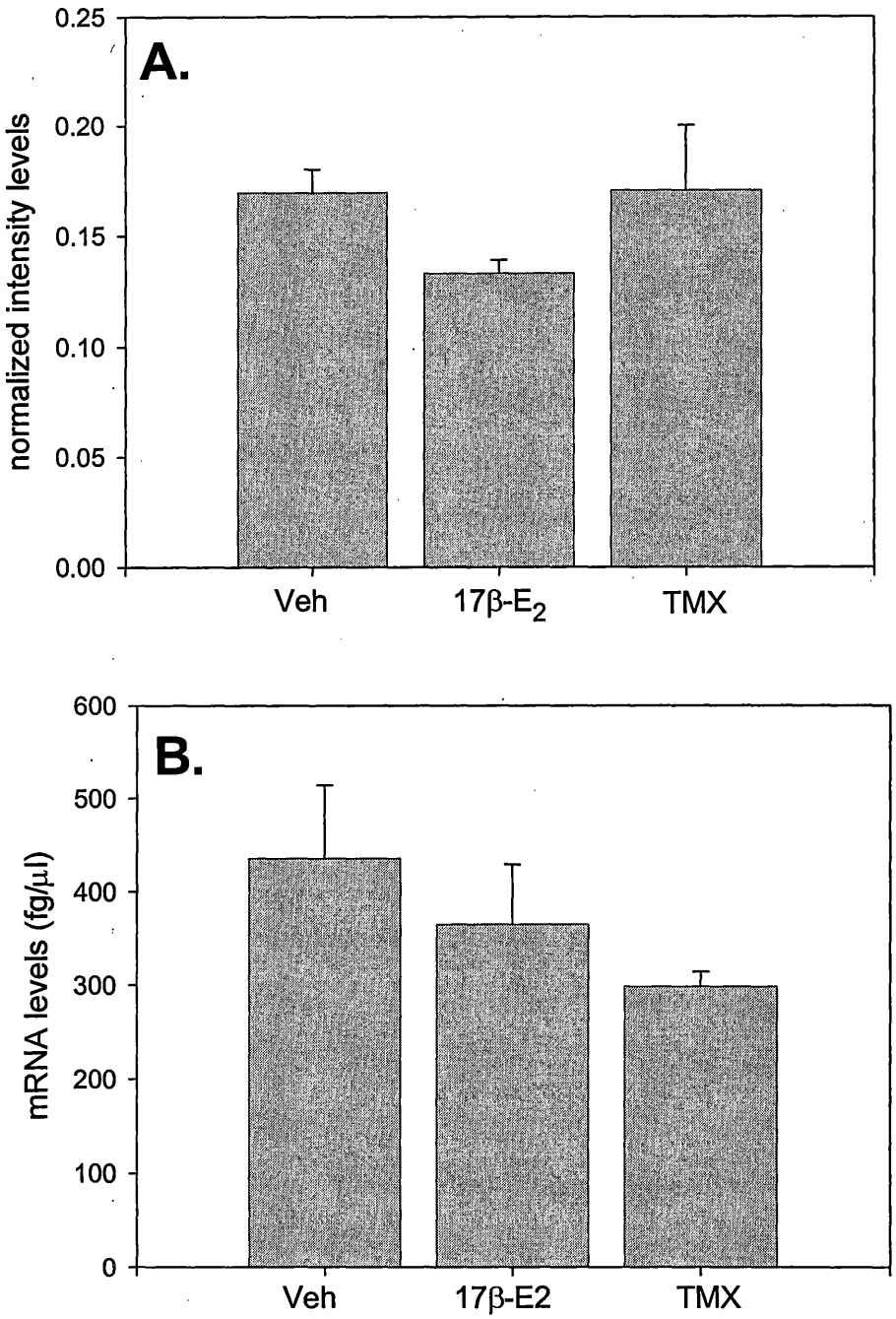
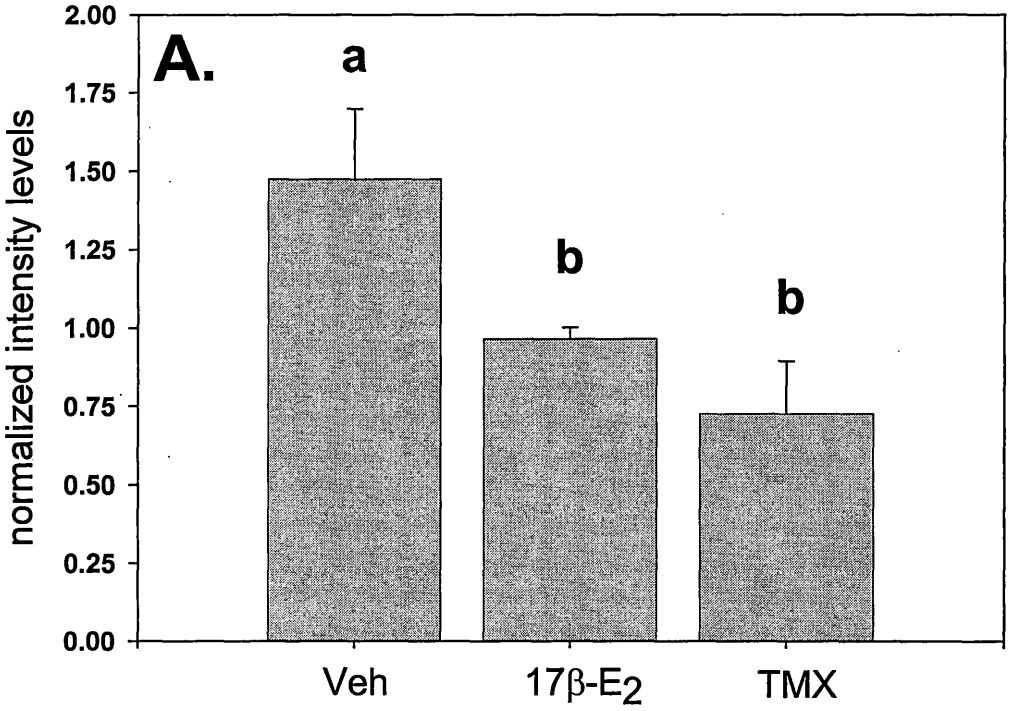

A.
normalized intensity levels
0.25
0.20
0.15
0.10
0.05
0.00
Veh
17β-E2
TMX
B.
mRNA levels (fg/μl)
600
500
400
300
200
100
0
Veh
17β-E2
TMX

Figure 6-12: Real time RT-PCR confirmation of Bax alpha. Microarray data (A.) was confirmed using real time RT-PCR (B.)


A.
a
b
b
normalized intensity levels
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Veh
17β-E2
TMX

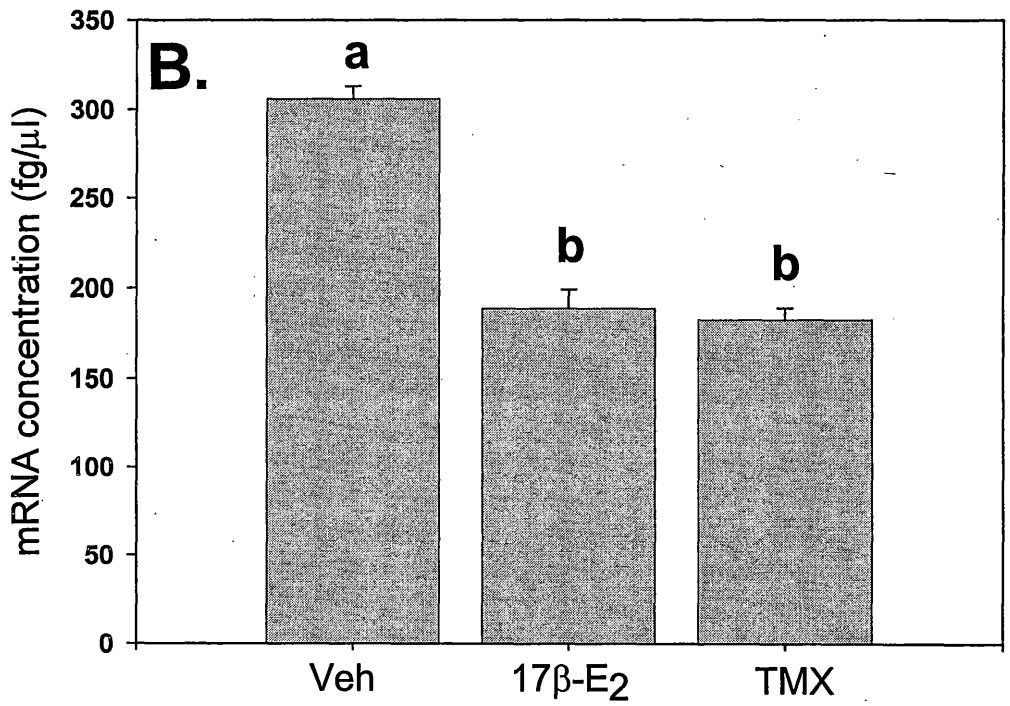

B.
a
b
b
mRNA concentration (fg/μl)
350
300
250
200
150
100
50
0
Veh
17β-E2
TMX

Table 6-1: List of primers used for real time RT-PCR confirmations. Sequence of primers used to confirm gene chip changes. Forward primer (FP) and reverse primer (RP) are listed for each of eight genes which were randomly selected from the regulated genes on the gene chip.

	Gene Name	Sequence (5'-3')
1.	Stanniocalcin-1	AGC CAA CAA CTT AGC GGA AA (FP)
		CCA ACC TGT AGG GCA CTG TT (RP)
2.	c-Kit receptor	GGC CGA GCC AGA GAC ATC AG (FP)
		CAT TCG GAA ACC TTC CTT GA (RP)
3.	Stem Cell Factor	CAA AAC TGG TGG CGA ATC TT (FP)
		GCC ACG AGG TCA TCC ACT AT (RP)
4.	Contactin-1	GAC GAA CGA TCG ATA CAG CA (FP)
		CCT TCC CTT CCT TCA CCT TC (RP)
5.	Chapsyn-110	GTC GGA GGT TTC CCA CAG TA (FP)
		CTG TGC AGC TCC ACC ATC TA (RP)
6.	GnRH	CAG GAC CAA AAT CAC TGC TCA GCC A (FP)
		GGG TCG AAG CAC GGG TTT AGA AAA G (RP)
7.	DRM/Gremlin	AGA GGA GGT GCT GCT TGA GTC CA (FP)
		TGA AAG GAC CCT TCC TCC TT (RP)
8.	Bax-alpha	CGA GCT GAT CAG AAC CAT CA (FP)
		CTC AGC CCA TCT TCT TCC AG (RP)

Table 6-2: List of genes upregulated by both 17β-estradiol and tamoxifen in the rat cerebral cortex. Listing of genes that were upregulated 2-fold or greater by 17β-estradiol and tamoxifen in two separate experiments using microarray analysis. Accession numbers (including Affymetrix tags) are listed prior to gene name or EST homology.

M86758_at	Rat estrogen sulfotransferase
S81289_i_at	Rat IgM kappa chain variable region {CDR1 to CDR3 region}, partial
rc_AA891559_at	No significant homology
rc_AA892069_s_at	98% homology (461/498 nt) to Rat Dipeptidyl peptidase 4
rc_AI234939_at	94% homology (197/460 nt) to Mouse NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4
D28561_s_at	Rat glucose transporter, GLUT4
D12524_at	Rat c-kit receptor tyrosine kinase
AF056034_s_at	Rat F-actin binding protein b-Nexilin
D90404_at	Rat cathepsin C
rc_AI177943_s_at	98% homology (463/463 nt) to Rat golgi SNAP receptor complex member 1
rc_AI639253_at	83% homology (271/285 nt) to Mouse OPPO 1
AF071204_g_at	Rat stem cell factor KL-1 precursor
AF055292mRNA_at	Rat signal transducer and activator of transcription 6 (stat6), partial
D38492_at	Rat neural adhesion molecule F3
AJ011607_at	Rat DNA polymerase alpha subunit III (primase), partial

Table 6-3: List of genes upregulated by 17β-estradiol, but not tamoxifen in the rat cerebral cortex. Listing of genes that were upregulated 2-fold or greater by 17β-estradiol and tamoxifen in two separate experiments using microarray analysis. Accession numbers (including Affymetrix tags) are listed prior to gene name or EST homology.

D00512_at	Rat mitochondrial acetoacetyl-CoA thiolase precursor
AF022081_at	Rat small nuclear RING finger protein (SNURF)
X95272_s_at	Rat mRNA with open reading frame
M58495mRNA_at	Rat NAD(P)H: quinone reductase
U64451_at	Rat short-branched chain acyl-CoA dehydrogenase precursor
U81518_at	Rat urea transporter (UT3)
AF036761_g_at	Rat stearoyl-CoA desaturase 2, partial
D14448_s_at	Rat Max
rc_AI145444_at	97% homology (192/526 nt) to Rat actin-binding protein neurabin
AF012282_at	Rat peptide HP (resection induced s14)
D88534_s_at	Rat pancreatic lipase, partial
Z22607_at	Rat bone morphogenetic protein 4
M92919_at	Rat phosphorylase kinase alpha-subunit (PhK-alpha-subunit), partial
AF008439_at	Rat natural resistance-associated macrophage protein 2 (Nramp2)
Z46614cds_at	Rat caveolin
D84667_g_at	Rat phosphatidylinositol 4-kinase
X89702cds_at	Rat TPCR18 protein
L26110_at	Rat transforming growth factor beta type I receptor
U84410_s_at	Rat interleukin-1beta-converting enzyme-related protease CPP32
rc_AI058393_s_at	99% homology (438/454 nt) to Rat Arg/Abl-interacting protein ArgBP2
D00698_s_at	Rat insulin-like growth factor I
U36773_g_at	Rat glycerol-3-phosphate acyltransferase, partial
AF056208_at	Rat PAM COOH-terminal interactor protein 1 (PCIP1)
rc_AI639007_at	No significant homology
L20823_at	Rat syntaxin 2
E05551cds_s_at	Vasoactive intestinal polypeptide receptor
M20722_s_at	Rat proline-rich protein (PRP-2), partial
rc_AA818499_at	Rat cytochrome P-450-LA-omega (lauric acid omega-hydroxylase)
rc_AA875244_at	91% homology (243/437 nt) to Mouse oxidative-stress responsive 1
AF065432_s_at	Rat Bcl-2 related ovarian death gene product BOD-M
rc_AA800908_at	No significant homology
AJ007488_at	Rat mitochondrion 16S ribosomal RNA, partial
rc_AI639198_at	No significant homology
U59801_at	Rat integrin alpha-M (Itgam), partial
M64699_s_at	Rat inositol 1,4,5-trisphosphate receptor (IP-3-R), partial
L23862_s_at	Rat Skn-1a
AI007530_f_at	82% homology (213/217 nt) to Human NADH:ubiquinone oxidoreductase MLRQ subunit homolog
U67995_s_at	Rat stearyl-CoA desaturase 2, partial
D82074_at	Rat BHF-1
AB008521_s_at	Rat dynein light intermediate chain 53/55, partial
D00569_at	Rat 2,4-dienoyl-CoA reductase precursor
rc_AA900380_at	87% homology (449/466 nt) to Mouse tumor necrosis factor I receptor (TNFR-1)
U92469mRNA_s_at	Rat gonadotropin-releasing hormone receptor gene, exon 1
AB002558_at	Rat glycerol 3-phosphate dehydrogenase
U14007_at	Rat aquaporin-4 water channel (AQP4)
M98826mRNA_g_at	Rat phosphorylase kinase gene, exons 2 and 3
D84336_at	Rat ZOG
AF036761_at	Rat stearoyl-CoA desaturase 2, partial
U35244_at	Rat vacuolar protein sorting homolog r-vps33a
AB002801_at	Rat cyclic nucleotide-gated channel
AF090135_at	Rat lin-7-Bb
AB015724_at	Rat nuclear receptor binding factor-1
AF107727_at	Rat sertolin

AF029690_at	Rat 8-oxoguanine-DNA-glycosylase (rogg1)
X53455cds_s_at	Rat microtubule-associated protein 2
U76714_at	Rat cell adhesion regulator (CAR1)
L07399_at	Rat immunoglobulin rearranged gamma-chain mRNA variable (V) region, partial
AF033109_at	Rat syntaxin 8
X60328_g_at	Rat cytosolic epoxide hydrolase
D28110_g_at	Rat MOBP (myelin-associated oligodendrocytic basic protein)
rc_AI639519_at	92% homology (125/517 nt) to Rat MIC2 like 1
rc_AA859700_at	90% homology (424/483 nt) to Mouse protoporphyrinogen oxidase
rc_AA892112_g_at	93% homology (307/433 nt) to Mouse proline oxidase 1
rc_AI639105_at	No significant homology
rc_AI044517_at	99% homology (352/417 nt) to Rat sodium-dependent high affinity glutamate transporter GLT-1A
X66693_f_at	Rat granzyme-like protein I
X62660mRNA_at	Rat glutathione transferase subunit 8
U63840_at	Rat nucleoporin p54
rc_AA818982_at	99% homology (406/519 nt) to Rat Thymopoietin (lamina associated polypeptide 2)
AF053100_g_at	Rat Pax4a
rc_AI008131_s_at	99% homology (489/496 nt) to Rat S-Adenosylmethionine decarboxylase 1A
rc_AI231164_s_at	99% homology (475/491 nt) to Rat splicing factor, arginine/serine-rich 10
D28562_s_at	Rat sugar transporter, GLUT5
AB011666_s_at	Rat utrophin, partial
AF019624_at	Rat myostatin
rc_AI639522_at	No significant homology
rc_AI639443_at	83% homology (342/458 nt) to Human roundabout, axon guidance receptor, homolog 2
X05111_at	Rat T-cell marker CD2 antigen
S53987_at	Rat nicotinic receptor alpha 7 subunit
U13895_s_at	Rat MSS1 protein, partial
U38653_s_at	Rat olfactory inositol 1,4,5-trisphosphate receptor, alternatively spliced variant, partial
D10757_at	Rat proteasome subunit R-RING12
D16308_at	Rat cyclin D2
X65747_at	Rat gustducin
S81353_s_at	Rat prosaposin; sulfated glycoprotein-1, partial
M18842_i_at	Rat T-cell receptor active beta-chain V-region (V-beta14-J-beta2.5), partial
rc_AA799683_at	No significant homology
U38812_s_at	Rat olfactory inositol 1,4,5-trisphosphate receptor, partial
rc_AI639309_at	No significant homology
AF031430_at	Rat syntaxin 7
S74265_s_at	Rat HMW MAP2=high molecular weight microtubule-associated protein, partial
AF059678_s_at	Rat VIP-receptor-gene repressor protein
rc_AA946368_at	98% homology (338/658 nt) to Rat cd36 antigen
M22340cds#1_s_at	Rat link protein, exon 5.
Z11558_at	Rat glia maturation factor beta
M63662_s_at	Rat peptidylglycine alpha-amidating monooxygenase
AF024712cds_at	Rat MHC class Ib M4 (RT1.M4) pseudogene
U14950_at	Rat synapse-associated protein 97
X06769cds_at	Rat c-fos
AF053987_at	Rat tissue-type vomeronasal neurons putative pheromone receptor V2R1, partial
rc_AI029183_s_at	99% homology (288/317 nt) to Rat connexin 43
M15481_at	Rat insulin-like growth factor I (IGF-I)
M84009_at	Rat dopamine receptor D4
AJ223184_at	Rat DORA protein
D13125_at	Rat neural visinin-like Ca2+-binding protein type 2 (NVP-2)
AF051155_g_at	Rat G beta-like protein GBL

J04563_at	Rat cAMP phosphodiesterase
U27558_at	Rat brain-specific inwardly rectifying K+ channel 1
AF035954_at	Rat kinesin-related protein KRP5 (KRP5), partial
AF065149_s_at	Rat rANK3.C-ter ankyrinG (Ank3), partial
rc_AA799786_s_at	99% homology (563/563 nt) to Rat sulfonylurea receptor
X90651_at	Rat P2X3 receptor
AF008912_at	Rat neuronal nitric oxide synthase, alternative untranslated exon 1a
L13207_at	Rat HNF-3/fork-head homolog-7 (HFH-7)
AF020211_s_at	Rat DLP1 splice variant 1, partial
rc_AI639392_at	No significant homology
rc_AI639530_at	85% homology (440/540 nt) to Mouse retrovirus readthrough RNA sequence
M16409_at	Rat m4 muscarinic acetylcholine receptor, partial
rc_AI639143_at	No significant homology
U18942_at	Rat double-stranded RNA-specific adenosine deaminase
AF020212_s_at	Rat DLP1 splice variant 2, partial
rc_AI008074_s_at	100% homology (397/493 nt) to Rat heat shock protein 90
rc_AI029920_s_at	96% homology (281/375 nt) to Rat Insulin-like growth factor-binding protein 5
U40790_at	Rat vascular protein tyrosine phosphatase-1 rDEP1
rc_AI639283_at	No significant homology
S75687_s_at	Rat GLAST; glutamate/aspartate transporter, partial
X56327cds_s_at	Rat epsilon 2 globin
X81449cds_g_at	Rat keratin 19.
S74898_s_at	Rat prostaglandin F2 alpha receptor, partial
X74565cds_g_at	Rat TBFII polypyrimidine tract binding protein
M24104_g_at	Rat vesicle associated membrane protein (VAMP-1)
S94371_g_at	Rat glutamate receptor subunit 4c, alternatively spliced
rc_AI169372_at	98% homology (205/651 nt) to Rat cytochrome P450 arachidonic acid epoxygenase
J02720_at	Rat liver arginase
U73586_at	Rat Fanconi anemia group C
H32189_s_at	98% homology (184/341 nt) to Rat Glutathione-S-transferase, mu type 2
U50842_at	Rat ubiquitin ligase (Nedd4) protein, partial
D28111_at	Rat MOBP (myelin-associated oligodendrocytic basic protein)
M24104_at	Rat vesicle associated membrane protein (VAMP-1)
rc_AA892680_at	91% homology (246/451 nt) to Human peptidylprolyl isomerase (cyclophilin)-like 3
D30781_at	Rat phospholipase A2 receptor, partial
L21995_s_at	Rat myelin/oligodendrocyte glycoprotein (MOG), partial
rc_AA859299_at	Rat nucleolar phosphoprotein of 140kD, Nopp140
M64795_i_at	Rat MHC class I antigen gene (RT1-u haplotype)
AJ011116_at	Rat endothelial nitric oxide synthase, 3' region, partial
J02942_at	Rat calcium/calmodulin-dependent protein kinase type II alpha-subunit
rc_AA852004_s_at	97% homology (368/368 nt) to Rat Glutamine synthetase (glutamate-ammonia ligase)
M84149_at	Rat IgH chain VJ region
rc_AA956332_at	Rat rabaptin
AF091247_s_at	Rat potassium channel (KCNQ3)
rc_AI639182_at	No significant homology
E01415cds_s_at	Rat glutathione S transferase.
D26495_at	Rat dynein-like protein 4, partial
rc_AI228548_at	97% homology (411/574 nt) to Rat S100 alpha
D14819_at	Rat calcium-binding protein P23k beta, partial
L08814_at	Rat CIIDBP
AF097593_g_at	Rat testicular N-cadherin
X68191_at	Rat sodium-calcium exchanger.
D16479_at	Rat mitochondrial long-chain 3-ketoacyl-CoA thiolase beta-subunit of mitochondrial trifunctional protein
U51584_s_at	Rat zinc finger homeodomain enhancer-binding protein-2 Zfhep-2), partial
D10770_s_at	Rat beta isoform of catalytic subunit of cAMP-dependent protein kinase

S81433_at	Rat heme oxygenase-2, 5' region, alternative splicing, partial
X04229cds_s_at	Rat glutathione S-transferase (GST) Y(b) subunit (EC 2.5.1.18)
E12275cds_s_at	Rat oxidosqualene lanosterol-cyclase
X60351cds_s_at	Rat alpha B-crystallin
L81137exon_s_at	Rat Rps2r2
AF004661_at	Rat Ra-reactive factor serine protease p100, partial
AF037071_at	Rat carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase (CAPON)
AF041066_at	Rat ribonuclease 4
D26154cds_at	Rat RB109 (brain specific protein)
U27767_at	Rat RGP4
Y15068_at	Rat Hsp70/Hsp90 organizing protein
U50147_at	Rat synapse-associated protein 102
U17254_at	Rat immediate early gene transcription factor NGFI-B
rc_AI228850_s_at	99% homology (414/414 nt) to Rat Microtubule-associated protein 2
U48247_at	Rat protein kinase C-binding protein Enigma
AB005743_at	Rat fatty acid transporter, partial
rc_AI639178_at	No significant homology
D28111_g_at	Rat MOBP (myelin-associated oligodendrocytic basic protein)
rc_AI180288_s_at	94% homology (412/417 nt) to Rat Caldesmon 1
U14398_at	Rat synaptotagmin IV homolog
rc_AA946532_at	98% homology (493/535 nt) to Rat ATP-binding cassette, sub-family D (ALD), member 3
X06769cds_g_at	Rat c-fos
X03518cds#3_s_at	Rat gamma-glutamyltranspeptidase (GGT)
U31866_g_at	Rat Nclone10
L13407_i_at	Rat calcium/calmodulin-dependent protein kinase II delta subunit, partial
AF062741_at	Rat pyruvate dehydrogenase phosphatase isoenzyme 2
X83231_at	Rat pre-alpha-inhibitor, heavy chain 3
rc_AA875327_at	95% homology (230/394 nt) to Mouse LIM-kinase1
U36773_at	Rat glycerol-3-phosphate acyltransferase mRNA, nuclear gene encoding mitochondrial protein, partial
rc_AI171090_at	99% homology (421/551 nt) to Rat 3-hydroxy-3-methylglutaryl CoA lyase
rc_AA893180_at	No significant homology
U39476_at	Rat p95 Vav proto-oncogene
U49049_at	Rat chapsyn-110

Table 6-4: List of genes upregulated by tamoxifen, but not 17β-estradiol in the rat cerebral cortex. Listing of genes that were upregulated 2-fold or greater by 17β-estradiol and tamoxifen in two separate experiments using microarray analysis. Accession numbers (including Affymetrix tags) are listed prior to gene name or EST homology.

rc_AI639463_at	No significant homology
M64381_at	Rat olfactory protein
rc_AI639386_at	No significant homology
rc_AI639397_s_at	88% homology (264/431 nt) to Mouse CEA-related cell adhesion molecule 11
rc_H31753_at	No significant homology
M34842_at	Rat thyroid stimulating hormone receptor
rc_AI639228_at	88% homology (537/568 nt) to Mouse squamous cell carcinoma antigen 2
rc_AI639224_at	96% homology (86/228 nt) to Rat ribosomal protein S13
rc_AI639248_r_at	No significant homology
rc_AI639313_at	98% homology (321/573 nt) to Rat Kalirin-9a
M99221_s_at	Rat pore-forming calcium channel alpha-1 subunit, partial
U11071_i_at	Rat polyadenylate-binding protein-related protein
L28818cds_at	Rat involucrin
U08986_s_at	Rat aryl hydrocarbon receptor nuclear translocator (ARNT), partial
M25490_at	Rat osteocalcin
M27902_at	Rat cardiac specific sodium channel alpha-subunit
M26199_s_at	Rat lutropin-choriogonadotropic hormone receptor
rc_AA892331_at	No significant homology
rc_AI071435_at	96% homology (394/446 nt) to Rat Sacm21/RT1-A intergenic region, haplotype RT1n and partial RT1-A gene for MHC Class I antigen
rc_AA875390_at	97% homology (210/427 nt) to Rat thioredoxin-like
rc_AA875405_at	82% homology (264/577 nt) to Human forkhead-like 18
rc_AA891032_at	92% homology (109/216 nt) to Mouse T10
rc_AA891758_at	No significant homology
rc_AA893781_at	98% homology (161/336 nt) to Mouse Centaurin beta 1
rc_AA945569_at	100% homology (583/664 nt) to Rat alpha-1-macroglobulin
rc_AA963682_at	90% homology (348/386 nt) to Rat 190 kDa ankyrin isoform
rc_AA956507_at	100% homology (440/440 nt) to Rat P450 (cytochrome) oxidoreductase
rc_AA900516_s_at	98% homology (295/403 nt) to Rat peptidyl arginine deiminase, type 2
rc_AA893148_at	87% homology (117/384 nt) to Mouse organic cationic transporter-like 3
rc_AI639091_at	No significant homology
rc_AA818198_at	84% homology (175/431 nt) to Rat cytochrome P450, 2c39
rc_AA818427_at	100% homology (586/586 nt) to Rat kinesin light chain C
rc_AA858977_at	99% homology (542/542 nt) to Rat Ras-related GTP-binding protein Rab29
rc_AI639047_at	No significant homology
rc_AA800172_at	No significant homology
rc_AA817854_s_at	99% homology (429/456 nt) to Rat GPI-anchored ceruloplasmin
rc_AA800665_at	86% homology (685/721 nt) to Mouse secretory carrier membrane protein 2
rc_AA874877_r_at	No significant homology
rc_AI013987_s_at	99% homology (463/463 nt) to Rat Protein kinase, interferon-inducible double stranded RNA dependent
rc_AI232284_i_at	99% homology (648/648 nt) to Rat MHC class I protein
rc_AI638973_at	No significant homology
rc_AA875090_at	No significant homology
U95368_at	Rat GABA-A receptor pi subunit
U94856_g_at	Rat paraoxonase, partial
AJ005046_g_at	Rat muscle fructose-1,6-bisphosphatase
X87884mRNA_s_at	Rat mitochondrial capsule selenoprotein
X89705cds_at	Rat TPCR38 protein
X99470_at	Rat DAX-1 protein
D26499_at	Rat dynein-like protein 8, partial
D12800cds_at	Rat histamine H1-receptor

X95096_at	Rat macrophage stimulating protein
D10693_at	Rat histamine N-methyltransferase
X59736mRNA_g_at	Rat sarcomeric mitochondrial creatine kinase
AF090692_g_at	Rat cystatin-related epididymal spermatogenic protein (CRES)
X70706cds_at	Rat T-plastin
AF053990_at	Rat tissue-type vomeronasal neurons putative pheromone receptor V2R2B, partial
AF055291mRNA_at	Rat signal transducer and activator of transcription 4 (stat4), partial
X63574_at	Rat putative G-protein coupled receptor
X62325cds_i_at	Rat TcRValphaT48a2 T cell receptor V-alpha J-alpha
X74833cds_at	Rat acetylcholine receptor beta-subunit
X17184_at	Rat glutamate receptor, AMPA subtype, GluR1
AB001452_at	Rat Sck, partial
Z17223_at	Rat Gax
U50949cds_at	Rat taste bud receptor protein TB 641
J03959_g_at	Rat uricase, 3' end
K01231_f_at	Rat alpha1-fetoprotein (AFP) 3' end
J05509Complete_seq_i_at	Rat cytochrome P450 cholesterol 7-alpha-hydroxylase (P450 VII)
U42719_at	Rat C4 complement protein, partial
AB004278_at	Rat protocadherin 2, partial
D63860_s_at	Rat prepro bone morphogenetic protein-3
L07315_at	Rat dipeptidase (dpep1)
D42019cds_at	Rat major histocompatibility complex class II H-beta, putative cytoplasmic domain, exon 5
Y07903cds_at	Rat transmembrane protein tMDC I
L05489_at	Rat heparin-binding EGF-like growth factor
U69278_at	Rat eph-related receptor tyrosine kinase homolog (Rek4)
Y00396mRNA_at	Rat c-myc oncogene and flanking regions
D44591_s_at	Rat inducible nitric oxide synthase
U65486_at	Rat beta-microseminoprotein precursor (PSP94)
U62667_at	Rat stanniocalcin
Z21780_at	Rat nup155
D67071exon_at	Rat regucalcin, exon1
U68491_at	Rat 5-hydroxytryptamine7 receptor, partial
L02926_s_at	Rat interleukin 10 (IL-10)
U04733_s_at	Rat cytochrome P450 arachidonic acid epoxygenase (cyp 2C23)
rc_AI638965_at	86% homology (210/450 nt) to Mouse AE binding protein 1
M81183Exon_UTR_g_at	Rat insulin-like growth factor I, 3' end of exon 6
L13235UTR#1_f_at	Rat polymeric immunoglobulin receptor, 3' untranslated sequence
U16655_at	Rat phospholipase C delta-4
AB013455_g_at	Rat NaPi-2 gamma
M64377_f_at	Rat olfactory protein
X99122mRNA_at	Rat T cell ecto-ADP-ribosyltransferase, exons 2,3,4,5, & 7
M99223_at	Rat calcium transporting ATPase
M22366_at	Rat MHC RT1.B-alpha precursor, exons 2-4
rc_AA799679_at	100% homology (435/659 nt) to Rat Solute carrier family 22, member 1
M88592_at	Rat peroxisome proliferator activated receptor (PPAR)
rc_AA945169_g_at	99% homology (552/552 nt) to Rat transthyretin
rc_AA894318_at	95% homology (366/569 nt) to Mouse hypothetical protein FLJ11560
rc_AA875646_at	No significant homology
rc_AA875072_at	No significant homology
U09583_at	Rat src related tyrosine kinase
rc_AI176462_at	93% homology (612/612 nt) to Mouse programmed cell death 2
AF034753UTR#1_s_at	Rat DC16, 3' UTR
rc_AI170380_g_at	99% homology (607/620 nt) to Rat surfactant, pulmonary-associated protein B

M20183_at	Rat cytochrome c oxidase subunit VIc (COX-VIc-1)
rc_AI144873_at	96% homology (346/346 nt) to Rat early growth response 4
rc_AA894030_at	87% homology (173/444 nt) to Human tyrosyl-DNA phosphodiesterase 1
rc_AI101320_at	99% homology (616/616 nt) to Rat jagged2 precursor
J04636_at	Rat nicotinic acetylcholine receptor beta-3 subunit precursor
L27651_g_at	Rat liver-specific transport protein
M29014_at	Rat insulin receptor
D87922exon_at	Rat 3'UTR of skeletal muscle sodium channel, partial
rc_AA892851_at	No significant homology
rc_AI639356_at	No significant homology
AF081557_at	Rat glial cells missing protein homolog (Gcm1)
rc_AI639207_at	No significant homology
AA859934_at	87% homology (197/438 nt) to Human DnaJ (Hsp40) homolog
M95578_g_at	Rat interleukin-1 receptor type I
rc_AA858626_at	90% homology (73/476 nt) to Rat synaptic vesicle glycoprotein 2 b
X57405_at	Rat notch
AF093569mRNA#1_s_at	Rat XLas protein (XLas), partial
U34932_at	Rat Fos-related antigen
AB002561_at	Rat FGF-16
AF052540_s_at	Rat calpain isoform Lp85
AF039584_at	Rat decay accelerating factor soluble-form precursor (DAF)
rc_AI639168_at	No significant homology
S40803cds#3_s_at	Rat luteinizing hormone/chorionic gonadotropin receptor, alternatively spliced
U53505_s_at	Rat type II iodothyronine deiodinase
rc_AI639376_at	92% homology (138/426 nt) to Human golgi autoantigen, golgin subfamily a, 1
rc_AA943872_g_at	99% homology (474/547 nt) to Rat adducin 1, alpha
M11901_f_at	Rat proline-rich salivary protein, partial
M37568cds_at	Rat homeobox protein R4
rc_AA998882_s_at	100% homology (262/477 nt) to Rat nucleolar phosphoprotein p130
rc_AI639036_r_at	82% homology (203/370 nt) to Rat spermine binding protein
U03699complete_seq_at	Rat inducible nitric oxide synthase (iNOS)
rc_AA859967_at	98% homology (381/529 nt) to Rat Rgc32 protein
rc_AA893074_at	No significant homology
AF084576_at	Rat Delta 3
U92897_s_at	Rat Kv4.3, partial
J00716mRNA_f_at	Rat gamma-E-crystallin (gamma 3-1)
U20110cds_at	Rat synaptotagmin VIII, partial
AB002584_at	Rat beta-alanine-pyruvate aminotransferase
X57523_at	Rat mtp1
L21672_at	Rat mRNA sequence
L26913_at	Rat interleukin-13 (IL-13)
rc_AA819793_at	98% homology (521/539 nt) to Rat Flap structure-specific endonuclease 1
J00710_at	Rat casein-alpha
rc_AI234828_at	97% homology (402/448 nt) to Rat Ig germline alpha H-chain C-region, exon 2
D42145_at	Rat ATP-sensitive potasium channel uKATP-1
rc_AA875097_at	Rat alpha-fibrinogen
L15305_s_at	Rat glial-derived neurotrophic factor
rc_AA800962_at	91% homology (495/495 nt) to Mouse talin
rc_AA799865_at	88% homology (378/381 nt) to Mouse low density lipoprotein receptor-related protein 4
AF063302mRNA#3_s_at	Rat carnitine palmitoyltransferase Ibeta 1-3, alternatively spliced products, partial

rc_AA866358_f_at	No significant homology
U85513_at	Rat synaptotagmin X, partial
rc_AI638946_at	No significant homology
D13912_s_at	Rat cytochrome P-450
D86345_at	Rat leukemia inhibitor factor receptor alpha-chain
rc_AA818403_at	Rat cytochrome P450 4F5 (CYP4F5)
AF016247_at	Rat RTK40 homolog (tyro10)
J02675_at	Rat prostatic spermine-binding protein (SBP)
AB003753cds#2_at	Rat high sulfur protein B2E and high sulfur protein B2F
L08492cds_s_at	Rat GABA-A receptor alpha-3 subunit
rc_AA866391_at	No significant homology
M18045_at	Rat testis-specific histone TH2B
AF021935_at	Rat mytonic dystrophy kinase-related Cdc42-binding kinase (MRCK)
rc_AI639009_at	85% homology (124/329 nt) to Mouse cell cycle p34 CDC2 kinase protein
rc_AA800120_at	98% homology (456/463 nt) to Rat carnitine/acylcarnitine carrier
L47281_at	Rat alpha-3 type IV collagen (COL4A3), partial
rc_AA892635_g_at	97% homology (314/478 nt) to Rat ras-like protein (Tc10)
rc_AA875126_at	No significant homology
AB003042_at	Rat C5a receptor
U71293_at	Rat hairless protein
M98049_s_at	Rat pancreatitis-associated protein precursor
U41183_at	Rat placental pre-progrowth hormone-releasing hormone (GHRH)
U03388_s_at	Rat cyclooxygenase 1
D14988_f_at	Rat hydroxysteroid sulfotransferase subunit
rc_AA799494_at	89% homology (453/453 nt) to Mouse tensin
U67915_at	Rat mast cell protease 1 precursor (RMCP-1)
rc_AI639418_at	99% homology (313/313 nt) to Rat deiodinase, iodothyronine, type 1
S78744_at	Rat activated protein C cofactor
rc_AI639285_at	No significant homology

Table 6-5: List of genes similarly downregulated by both 17β-estradiol and tamoxifen in the rat cerebral cortex. Listing of genes that were upregulated 2-fold or greater by 17β-estradiol and tamoxifen in two separate experiments using microarray analysis. Accession numbers (including Affymetrix tags) are listed prior to gene name or EST homology.

rc_AA892920_at	99% homology (274/549 nt) to Rat Na+/Pi cotransporter-1 isoform-a
rc_AI012183_at	99% homology (413/480 nt) to Rat nuclear receptor subfamily 2, group F, member2
M65149_at	Rat CELF
rc_AA799495_at	No significant homology
M37942exon#2-3_s_at	Rat adenosine monophosphate deaminase 1 (AMPD1), exons 14-16
M62930_at	Rat pancreatic stone protein
M60737_at	Rat S-antigen
rc_AA799861_g_at	92% homology (328/499 nt) to Mouse interferon regulatory factor 7
rc_AI639500_r_at	No significant homology
rc_AI639439_at	No significant homology
rc_AA800344_at	No significant homology
U35315_at	Rat serotonin 5-HT2C receptor, alternatively spliced truncated variant
U09022_at	Rat 15 kDa perforatorial protein PERF 15, partial
L38483_at	Rat jagged
rc_AA891045_at	99% homology (408/531 nt) to Rat nucleoporin p58
X59859_r_at	Rat decorin
rc_AA858570_at	No significant homology
D00833_g_at	Rat glycine receptor alpha 1
X92069_at	Rat P2X5 protein
rc_AI175900_g_at	EST 100% homology (355/458 nt) to Rat proto-oncogene (Ets-1)
X73371_at	Rat Fc gamma receptor
M81183Exon_UTR_at	Rat insulin-like growth factor I, 3' end of exon 6
X70900_at	Rat hepsin
M31603_at	Rat parathyroid-like peptide

Table 6-6: List of genes downregulated by 17β-estradiol, but not tamoxifen in the rat cerebral cortex. Listing of genes that were upregulated 2-fold or greater by 17β-estradiol and tamoxifen in two separate experiments using microarray analysis. Accession numbers (including Affymetrix tags) are listed prior to gene name or EST homology.

AF035673_at	Rat retinal pigment epithelium-specific protein (Rpe65)
rc_AA893870_at	98% homology (329/417 nt) to Rat 18S, 5.8S, and 28S ribosomal RNAs
rc_AA892296_at	95% homology (255/304 nt) to Mouse homeo box B7 (Hoxb7)
rc_AI638957_at	No significant homology
rc_AI639068_at	No significant homology
rc_AA859612_r_at	96% homology (122/314 nt) to Rat mitochondrial cytochrome oxidase subunits I,II, III
rc_AA859495_at	96% homology (382/450 nt) to Human TLS-associated SR proteins (TASR)
rc_AA875152_at	No significant homology
M31032cds#1_s_at	Rat contiguous repeat polypeptides (CRP)
M80633_at	Rat adenylyl cyclase type (IV)
D10261_at	Rat 59-kDa bone sialic acid-containing protein
rc_AI639197_at	No significant homology
rc_AI639361_at	No significant homology
rc_AI639258_at	No significant homology
rc_AI638986_s_at	No significant homology
rc_AI639254_at	98% homology (506/506 nt) to Rat sperm adhesion molecule (Spam)
S50879_at	Rat acetylcholinesterase T subunit, partial
X15143cds_g_at	Rat beta A3/A1 crystallin
rc_AI639183_at	88% homology (125/315 nt) to Mouse repetin
U19485_at	Rat spp-24 precursor, partial
U61772_at	Rat merlin (NF2), partial
K01878cds_s_at	Rat proopiomelanocortin (POMC), exon 2
rc_AI639210_at	94% homology (153/424 nt) to Mouse doublesex-Mab related 99B
U90260UTR#1_i_at	Rat developmentally-regulated cardiac factor (DRCF-1), 3' UTR sequence
U09957_at	Rat plasma membrane urea transporter
D29769_at	Rat bone morphogenic protein-7, partial
M15528mRNA_at	Rat SH-1 with gonadotropin-releasing hormone GnRH complete coding region encoded on opposite strand
AF016179_at	Rat putative pheromone receptor (Go-VN2)
rc_AA859921_at	99% homology (189/331 nt) to Human MINT28 colon cancer differentially methylated CpG island
X55246_at	Rat inhibitory glycine receptor alpha-1 subunit
rc_AA799489_at	97% homology (523/646 nt) to Rat acyl-coA oxidase
rc_AI639091_at	No significant homology
X05023_at	Rat mannan-binding protein (MBP) precursor
M83210_at	Rat neonatal submandibular gland proacinar cell protein precursor (SMGB1/SMGB2)
rc_AI008741_at	97% homology (276/501 nt) to Rat Hyaluronan synthase 2
rc_AA874849_at	Rat 18S, 5.8S, and 28S ribosomal RNAs
AB006137_at	Rat FTA mRNA for alpha 1,2-fucosyltransferase
AF027188UTR#1_at	Rat SA mRNA, partial 3'UTR
rc_AI639065_at	No significant homology
rc_AA892006_at	100% homology (191/443 nt) to Rat deoxyribonuclease I
AF013248_at	Rat beta-A3 crystallin (beta-A3), partial
D84480_s_at	Rat PMSG-induced ovarian mRNA, 3'sequence, N2
X92747_at	Rat PTP-S
AF058714_at	Rat sodium-dicarboxylate cotransporter SDCT1
rc_AA859928_at	No significant homology
L13192_i_at	Rat brain factor-2 (HFH-BF-2)
X62323_at	Rat Pan-1
rc_AI639062_at	No significant homology
rc_AI236945_s_at	99% homology (407/449 nt) to Rat Luteinizing hormone/ choriogonadotropin receptor

rc_AA800770_at	No significant homology
rc_AI639083_at	100% homology (78/483 nt) to Mouse Lysosomal-associated transmembrane protein 4A
rc_H31342_at	No significant homology
X83671cds_r_at	Rat CRYBB2
rc_AA892066_at	No significant homology
M20724_s_at	Rat proline-rich protein (PRP-4), partial
rc_AI071399_at	99% homology (588/595 nt) to Rat vesicle transport-related (RA410),
rc_AA858603_g_at	No significant homology
rc_AA893165_at	No significant homology
rc_AI044488_at	100% homology (527/527 nt) to Rat ferredoxin 1
S46131mRNA_r_at	Rat dopamine D1 receptor
AF016247_g_at	Rat RTK40 homolog (tyro10)
X80535cds_at	Rat thyrotropin-releasing hormone degrading enzyme
rc_AI639363_r_at	No significant homology
U67911_s_at	Rat mast cell protease 8 precursor (RMCP-8)
J02852_at	Rat cytochrome P450 IIA3, 3' end
U51919_at	Rat preprocortistatin
rc_AA900601_at	Rat vacuolar protein sorting homolog r-vps33a
U78889_at	Rat Delta1
rc_AA859612_f_at	Rat mitochondrial genome
L00981mRNA#1_at	Rat lymphotoxin (TNF-beta), tumor necrosis factor (TNF-alpha)
X67805_at	Rat SCP1
rc_AA894148_s_at	100% homology (251/447 nt) to Rat apolipoprotein A-IV
S62516_s_at	Rat SA, partial
L02529_at	Rat frizzled homologue
rc_AA799538_at	94% homology (512/512 nt) to Mouse splicing factor Sc35
AB009463_at	Rat LRp105
J04963_at	Rat ecto-ATPase
M64795_f_at	Rat MHC class I antigen gene (RT1-u haplotype)
rc_AI639248_i_at	No significant homology
rc_AA859532_at	No significant homology
S87544_g_at	Rat polyprotein 1-microglobulin/bikunin
X01454_at	Rat thyrotropin-beta (TSH)
X77117exon#1-3_at	Rat NADH-cytochrome b5 reductase
AJ002259_at	Rat Prx3A'
rc_AA859776_at	97% homology (443/461 nt) to Mouse ras homolog gene family, member E
rc_AI639437_at	No significant homology
rc_AA891962_at	No significant homology
rc_AA858573_s_at	99% homology (203/454 nt) to Rat spp-24 precursor
rc_AI639525_at	84% homology (421/528 nt) to Mouse adiponutrin
rc_AI639175_at	No significant homology
X63143_s_at	Rat neuroglycan, partial
M27151_at	Rat muscle regulatory factor MRF4
M33986mRNA_at	Rat aromatase cytochrome P450
rc_AI639462_at	No significant homology
rc_AA924591_at	Rat Cyp4a locus, encoding cytochrome P450 (IVA3)
rc_AI639069_at	No significant homology
M95780_at	Rat G protein gamma-5 subunit
rc_AI237884_s_at	98% homology (405/477 nt) to Rat Alpha-fetoprotein
X81449cds_at	Rat keratin 19
rc_AI639251_at	No significant homology
U73174_at	Rat glutathione reductase
U76551_at	Rat mucin Muc3, partial
rc_AA799729_at	100% homology (379/588 nt) to Rat phosphodiesterase

rc_H33120_at	85% homology (258/377 nt) to Mouse testis specific gene A2
rc_AA893351_at	92% homology (430/435 nt) to Mouse gliacolin
AF012891_at	Rat frizzled related protein frpAP
L07402_f_at	Rat immunoglobulin rearranged gamma-chain mRNA variable (V) region, partial
AF058787_at	Rat heme oxygenase-3 (HO-3)
M26686_at	Rat carboxyl methyltransferase
M36317_s_at	Rat thyrotropin-releasing hormone (TRH) precursor
L22339_g_at	Rat N-hydroxy-2-acetylaminofluorene (ST1C1)
AB000216_at	Rat CCA3
S61865_s_at	Rat HSPG core protein syndecan;heparan sulfate proteoglycan core protein
X06107_i_at	Rat insulin-like growth factor I
rc_H31554_at	No significant homology
rc_AA891288_g_at	No significant homology
rc_AA891633_f_at	95% homology (214/214 nt) to Rat Lysophospholipase 1
rc_AI102814_at	99% homology (585/585 nt) to Rat lysyl oxidase
rc_AI639330_at	No significant homology
rc_AI014135_at	99% homology (403/410 nt) to Rat CDK103
K00994mRNA_s_at	Rat intestinal calcium-binding protein (icabp) gene 2, 3' end and flank
rc_AA818403_at	Rat cytochrome P450 4F5 (CYP4F5)
AF000144_at	Rat fibroblast growth factor receptor 1 gene, alternatively spliced products and partial
D28557_s_at	Rat RYB-a
U75923cds_at	Rat isoleucyl tRNA synthetase mRNA, partial, 3' untranslated sequence
AF029109_at	Rat mint 3
rc_AI639455_at	92% homology (225/467 nt) to Mouse CDV-3B
M23995_g_at	Rat aldehyde dehydrogenase
U39571_at	Rat phosphatidylinositol 4-kinase
rc_AA875088_at	No significant homology
AF034899_i_at	Rat olfactory receptor-like protein (SCR D-9)
S62933_i_at	Rat receptor tyrosine kinase (TrkC(ki14), alternatively spliced
rc_AI013389_at	100% homology (382/387 nt) to Rat Calcium-binding protein, intestinal, vitamin D-dependent
M11597_at	Rat alpha-type calcitonin gene-related peptide
rc_AA859565_at	No significant homology
rc_AA859694_g_at	94% homology (176/452 nt) to Mouse ssDNA binding protein (SEB4D)
X74917gene_at	Rat T-cell receptor beta chain
rc_AI014135_g_at	99% homology (402/410 nt) to Rat CDK103
rc_AI638941_at	No significant homology
U38373_s_at	Rat huntingtin associated protein (rHAP1-A)
X03914mergedCDS_UTR_at	Rat IL-3 gene, exons 3-5 and 3' flanking region
AF014827_at	Rat vascular endothelial growth factor D (VEGF-D)
X06150cds_at	Rat glycine methyltransferase
rc_AA799773_at	No significant homology
rc_AA849035_at	98% homology (499/499 nt) to Rat Bmyc
U60096_at	Rat sciatic nerve integrin beta 4 subunit
rc_AA893219_at	100% homology (682/682 nt) to Rat Glycine methyltransferase
U53450cds_at	Rat Jun dimerization protein 1 (jdp-1)
M28671_at	Rat rearranged IgG-2b gene, last 4 exons
U72632_at	Rat membrane amine oxidase, partial
L05596cds_at	Rat serotonin receptor
rc_AI171962_s_at	99% homology (626/626 nt) to Rat Annexin 1 (p35) (Lipocortin 1)
rc_AA892677_at	No significant homology
L22655_i_at	Rat anti-acetylcholine receptor antibody, kappa-chain, VJC region
M75153_g_at	Rat ras p21-like small GTP-binding protein

rc_AA799636_at	No significant homology
rc_AI639533_g_at	No significant homology
rc_AI044110_s_at	100% homology (503/524 nt) to Rat Thymopoietin (lamina associated polypeptide 2)
AF021349_at	Rat natural killer cell protein group 2-C
rc_AA899552_at	93% homology (279/449 nt) to Mouse aggrecan 1
D16840_s_at	Rat angiotensin II type 2 receptor
J02643_at	Rat androgen-repressible liver protein SMP-2
M67465_at	Rat 3-beta-hydroxysteroid dehydrogenase/delta-5-delta-4-ene-isomerase
rc_AA799691_at	88% homology (628/628 nt) to Mouse solute carrier family 12, member 7
rc_AA965132_s_at	99% homology (241/258 nt) to Rat thiazide-sensitive sodium-chloride cotransporter
J05231_at	Rat neuronal nicotinic acetylcholine receptor-related protein
rc_AA800303_at	91% homology (336/569 nt) to Mouse phospholipid scramblase 3
rc_AA998164_s_at	99% homology (389/482 nt) to Rat cyclin B
rc_AA800059_at	95% homology (535/612 nt) to Rat syndecan 4
U75917_at	Rat clathrin-associated protein 17 (AP17)
J05592_at	Rat protein phosphatase inhibitor-1 protein
rc_AA858588_at	95% homology (236/472 nt) to Mouse CGI-45
rc_H31914_at	90% homology (169/397 nt) to Mouse ecotropic viral integration site 5
rc_AA892294_at	No significant homology
AB010275_s_at	Rat leukemia inhibitory factor
Z17319_at	Rat phosphoglyceromutase
rc_AI639349_i_at	99% homology (252/443 nt) to Rat Neonatal submandibular gland protein B
rc_AA799498_at	97% homology (441/683 nt) to Rat natriuretic peptide precursor type B
X69903_at	Rat interleukin 4 receptor
M80367_at	Rat isoprenylated 67 kDa protein
Z50052_at	Rat C4BP beta chain protein
L00603_at	Rat vesicular monoamine transporter
rc_AA799681_at	No significant homology
U08259_r_at	Rat N-methyl-D-aspartate receptor NMDAR2C subunit
AF091563_r_at	Rat isolate QIL-LD1 olfactory receptor, partial
X16002cds_s_at	Rat putative potassium channel subunit protein (RCK4).
U69550_at	Rat phospholipase D
rc_AI639315_f_at	88% homology (276/381 nt) to Mouse interferon-inducible protein 10 (IP-10) receptor
rc_AA945321_at	97% homology (503/536 nt) to Rat Albumin
rc_AI176191_at	No significant homology
AA850219_at	100% homology (184/464 nt) to Rat Annexin III (Lipocortin III)
L27421_at	Rat neuronal calcium sensor (NCS-1)
J01879_at	Rat brain-specific identifier sequence RNA
rc_AA945082_at	99% homology (362/561 nt) to Rat glutathione S-transferase Yc2 subunit
X89999cds_at	Rat 2B1 antigen protein
rc_AA955167_r_at	90% homology (434/442 nt) to Mouse myristoylated alanine rich protein kinase C substrate
rc_AA891753_at	No significant homology
M31178_g_at	Rat calbindin D28
rc_AI045794_at	98% homology (118/516 nt) to Rat H3 histone
U11418_s_at	Rat NMDAR1 glutamate receptor subunit
rc_AA866302_at	99% homology (271/405 nt) to Rat F alloantigen
rc_AI009682_s_at	99% homology (493/558 nt) to Rat aspartyl-tRNA synthetase
rc_AA875450_at	No significant homology
X59267_at	Rat drebrin A
U49099_at	Rat cis-Golgi p28
rc_AA894009_g_at	No significant homology
rc_AI639498_i_at	No significant homology

rc_AA945737_at	95% homology (150/620 nt) to Rat chemokine receptor LCR1
rc_AA891920_at	94% homology (561/561 nt) to Mouse NTF2-related export protein 1
U46034_at	Rat stromelysin 3
X70062_at	Rat gamma subunit of sodium potassium ATPase
rc_AA799575_f_at	93% homology (465/588 nt) to Rat peptidylglycine alpha-amidating monooxygenase precursor (PAM)
rc_AA859805_at	93% homology (433/450 nt) to Mouse lysyl oxidase-like
rc_AA892305_at	83% homology (347/534 nt) to Mouse brain protein 16
M21622_g_at	Rat high-affinity IgE receptor (Fc-epsilon-R-I)
rc_H31287_g_at	96% homology (352/403 nt) to Rat kinase (LOC246273),
rc_AI639529_s_at	No significant homology
D64062_at	Rat annexin V-binding protein (ABP-10), partial
AF059258_at	Rat monocarboxylate transporter MCT3
X56729mRNA_g_at	Rat calpastatin
rc_AI639347_at	No significant homology
rc_AI639448_at	100% homology (376/411 nt) to Rat A5D3
rc_AA817887_at	95% homology (476/574 nt) to Rat profilin
rc_AA893230_at	92% homology (401/646 nt) to Mouse calmodulin-like 4
AF078778exon#1_at	Rat microtubule-associated protein 1B mRNA, alternatively spliced non-coding exon 3U
M25890_at	Rat somatostatin
L17138_at	Rat 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase (3beta-HSD)
J04206_s_at	Rat cystatin S, 3' end.
U06273_i_at	Rat UDP-glucuronosyltransferase (UGT2B12)
rc_AI178024_at	99% homology (585/631 nt) to Rat Protein-L-isoaspartate (D-aspartate) O-methyltransferase
rc_AA894292_at	No significant homology
AB002561_at	Rat FGF-16
D43623_g_at	Rat carnitine palmitoyltransferase I like protein (CPTI like protein)
AA685376_f_at	88% homology (238/269 nt) to Human NADH:ubiquinone oxidoreductase MLRQ subunit homolog
X53858_at	Rat TTF-1 thyroid nuclear factor 1
U40999_at	Rat retinal protein (RRG4)
M21208mRNA_s_at	Rat 17-alpha-hydoxylase cytochrome P-450, 3' end
X77235_at	Rat ARL gene 4
D86641_at	Rat FK506-binding protein 12
rc_AA800015_at	98% homology (451/460 nt) to Mouse TAF10 RNA polymerase II, TATA box binding protein-associated factor
U94708_at	Rat EP2 prostanoid receptor
D49980_at	Rat 8-oxo-dGTPase, exon 3
S82649_r_at	Rat neuronal activity-regulated pentraxin (Narp)
rc_AA858621_at	100% homology (141/549 nt) to Rat CaM-kinase II inhibitor alpha
rc_AA892897_at	88% homology (441/629 nt) to Mouse procollagen lysine, 2-oxoglutarate 5-dioxygenase 2
U67309_at	Rat neuronal nitric oxide synthase
AF012714_at	Rat hepatic multiple inositol polyphosphate phosphatase (MIPP1), partial
S68736_at	Rat myosin heavy chain, partial
rc_AI059540_at	100% homology (423/458 nt) to Rat heparin-binding EGF-like growth factor
J04486_at	Rat insulin growth factor-binding protein
rc_AI639465_r_at	98% homology (251/315 nt) to Rat ring finger protein 28
rc_AA800260_at	No significant homology
rc_AA893634_at	89% homology (474/474 nt) to Mouse coatomer protein complex, subunit zeta 2
L14462_at	Rat R-esp1
rc_AA893314_at	89% homology (255/255 nt) to Mouse ankyrin repeat and SOCS box-containing protein 9
M58169_i_at	Rat prostatic 22-kD glycoprotein
X79860_at	Rat H1SHR

X61106cds_at	Rat P-glycoprotein ORF
M27467_at	Rat heart cytochrome oxidase subunit VIc (COX-VIc)
rc_AA998338_s_at	92% homology (169/186 nt) to Rat phospholipase D
rc_AA874848_s_at	94% homology (360/377 nt) to Rat thy-1 cell-surface glycoprotein
rc_AA818593_at	99% homology (155/492 nt) to Rat phosphatidate phosphohydrolase type 2a
D84479_at	Rat PMSG-induced ovarian mRNA, 3'sequence, N1.
rc_AA684631_at	No significant homology
rc_AA894087_at	100% homology (96/572 nt) to Rat potassium channel (erg2)
rc_AA799529_at	92% homology (193/566 nt) to Human MTG8-like protein (MTGR1)
AF033027_at	Rat prenylated SNARE protein Ykt6p
U75917_g_at	Rat clathrin-associated protein 17 (AP17)

Table 6-7: List of genes downregulated by tamoxifen, but not 17β-estradiol in the rat cerebral cortex. Listing of genes that were differentially regulated 2-fold or greater in two separate experiments using microarray analysis. Accession numbers (including Affymetrix tags) are listed prior to gene name or EST homology.

rc_AA891822_at	95% homology (364/534 nt) to Mouse solute carrier family 35 (CMP-sialic acid transporter)
rc_AA892888_g_at	No significant homology
rc_AA875165_at	No significant homology
rc_AA875215_at	92% homology (206/377 nt) to Mouse striatin, calmodulin binding protein 3
rc_AA874984_at	No significant homology
rc_AA891693_at	No significant homology
rc_AA891166_at	No significant homology
rc_AA999168_at	88% homology (427/484 nt) to Human NADH dehydrogenase (ubiquinone) 1 beta subcomplex
rc_AA997367_s_at	100% homology (191/398 nt) to Rat p21 (CDKN1A)-activated kinase 3 (Pak3),
rc_AA900850_g_at	98% homology (348/371 nt) to Rat T-complex 1 (Tcp1)
rc_AA799301_at	96% homology (527/545 nt) to Mouse ligatin
M94454_at	Rat serine/threonine protein kinase (Tpl-2), exons 1-7
M94043_at	Rat rab-related GTP-binding protein
M57705_g_at	Rat truncated thyroid peroxidase, 3' end.
M63574_at	Rat selenoprotein P
M60654_at	Rat alpha-1A-adrenergic receptor
rc_AA859661_at	No significant homology
rc_AA859474_at	89% homology (272/505 nt) to Mouse T-box18 (Tbx18)
rc_AA866455_g_at	95% homology (443/443 nt) to Mouse homeodomain interacting protein kinase 3 (Hipk3)
rc_AA874912_at	No significant homology
rc_AA800235_at	No significant homology
rc_AA858632_at	No significant homology
rc_AA858571_at	No significant homology
U71294_at	Rat Srg1 (Sytr1)
U60145_at	Rat fragile X protein (FMR1), partial
U94856_at	Rat paraoxonase, partial
U38481_at	Rat ROK-alpha
U57499_g_at	Rat protein tyrosine phosphatase SH-PTP2, partial
U87960exon_s_at	Rat leukocyte common antigen receptor, trans-spliced alternative untranslated exon
Z22607_at	Rat bone morphogenetic protein 4
Z27513exon_i_at	Rat carbamoylphosphate synthase I, exon 38
X51992_at	Rat GABA-A receptor alpha-5 subunit
X15958_at	Rat mitochondrial enoyl-CoA hydratase
X63253cds_s_at	Rat serotonin transporter
X62295cds_s_at	Rat vascular type-1 angiotensin II receptor
rc_AI639014_at	No significant homology
rc_AI638943_at	94% homology (286/519 nt) to Mouse carbonic anhydrase 6
S94371_g_at	Rat GluR-4c; glutamate receptor subunit 4c, alternatively spliced
U00964_at	Rat common salivary protein 1
U19516_at	Rat initiation factor eIF-2Be
U12184_at	Rat putative G protein-coupled receptor
S66618_at	Rat multidrug-resistance transporter P-glycoprotein, partial
S69161_s_at	Rat thyrotropin-releasing hormone receptor (TRH-R)
D16817_g_at	Rat metabotropic glutamate receptor mGluR7
D26073_at	Rat phosphoribosylpyrophosphate synthetase-associated protein (39 kDa)
L08227_at	Rat nicotinic acetylcholine receptor alpha 6 subunit
L25527_at	Rat E-selectin (ELAM-1)
L19180_at	Rat receptor-linked protein tyrosine phosphatase (PTP-P1)

AB010428_s_at	Rat acyl-CoA hydrolase
L20820_at	Rat syntaxin 3
L31621_s_at	Rat nicotinic acetylcholine receptor alpha 3 subunit
D86642_at	Rat FK506-binding protein 12.6
J04731_at	Rat potassium channel protein (BK2)
AF021137_s_at	Rat inward rectifier potassium channel (IRK1)
D38448_at	Rat 88kDa-diacylglycerol kinase (DGK-III)
AB001982_at	Rat growth hormone secretagogue receptor type 1a, partial
M18330_at	Rat protein kinase C delta
M15481_at	Rat insulin-like growth factor I
M26534_i_at	Rat kallikrein-like serine protease
AF073892_s_at	Rat potassium channel (elk2), partial
AJ000557cds_s_at	Rat Janus protein tyrosine kinase 2, JAK2
AF077338_at	Rat myosin binding protein H
AA686031_at	95% homology (260/265 nt) to Mouse NADH-ubiquinone oxidoreductase 75 kDa subunit
rc_AA893189_at	95% homology (318/413 nt) to Mouse glutathione reductase 1
rc_AA893939_at	91% homology (296/416 nt) to Mouse Deleted in split-hand /split-foot 1 region
X78461_at	Rat RB-IRK2
M21759mRNA_at	Rat profilaggrin
rc_AI639528_at	93% homology (137/400 nt) to Mouse oxysterol-binding protein-like 2
rc_AA799657_at	88% homology (425/502 nt) to Mouse ERCC2
S66545_at	Rat putative alkaline phosphatase
X64403_at	Rat c/ebp gamma
M33746mRNA#2_f_at	Rat UDP glucuronosyltransferase-5
rc_AI231213_at	99% homology (579/582 nt) to Rat kangai 1
rc_AI029279_at	97% homology (259/377 nt) to Rat neuroligin 1
M91599mRNA_at	Rat fibroblast growth factor receptor subtype 4 (FGFR4)
Z83757mRNA_g_at	Rat growth hormone receptor, 3' UTR
rc_AA893857_at	No significant homology
U79417_at	Rat 71 kDa component of rsec6/8 secretory complex p71
rc_AA946542_g_at	96% homology (266/655 nt) to Rat prolactin-like protein D
D14014_g_at	Rat cyclin D1
rc_AA799611_at	92% homology (591/611 nt) to Mouse notch4
AB000217mRNA_at	Rat GATA-3
D89730_g_at	Rat T16
U23438_at	Rat MAP kinase phosphatase (MKP-2)
D00568_s_at	Rat cytochrome P-450 11beta, variant precursor
rc_AI639485_s_at	No significant homology
X75785_at	Rat SCP3
U10071_at	Rat CART protein
D16339_at	Rat alpha-tocopherol transfer protein
rc_AA894298_s_at	99% homology (530/531 nt) to Rat membrane metalloendopeptidase
X16262_s_at	Rat smooth muscle myosin heavy chain, alternatively spliced
rc_AA799513_at	No significant homology
rc_AI069982_s_at	100% homology (311/382 nt) to Rat Ser-Thr protein kinase related to the myotonic dystrophy protein kinase
M64785_g_at	Rat vasopressin
L10073_at	Rat 5-hydroxytryptamine receptor (5HT5b)
U42755_at	Rat Bet1 homolog
rc_AA875587_at	No significant homology
X74293_s_at	Rat alpha 7A integrin
rc_AA875665_at	No significant homology
AF016387_at	Rat retinoid X receptor gamma, partial
rc_AI639289_at	No significant homology

U64689_at	Rat synaptotagmin interacting protein zyginII, partial
J00711_at	Rat beta-casein
rc_AA859299_at	Rat nucleolar phosphoprotein of 140kD, Nopp140
rc_AA965119_at	99% homology (321/399 nt) to Rat Wilms tumor 1
AB011528_at	Rat MEGF2
U18314_at	Rat lamina associated polypeptide 2 (LAP2)
U02320_s_at	Rat clone ndf40 neu differentiation factor, partial
rc_AI639184_at	No significant homology
S74572_g_at	Rat MPP beta; Mg2+ dependent protein phosphatase beta isoform, alternatively spliced
Y17162UTR#1_f_at	Rat G11 alpha subunit, 3'UTR
D29646_at	Rat ADP-ribosyl cyclase / cyclic ADP-ribose hydrolase (CD38)
AF081366_s_at	Rat ATP-regulated K+ channel ROMK2.1 isoform
rc_AA800786_at	90% homology (580/609 nt) to Mouse GATA binding protein 6 (Gata6)
S75280_s_at	Rat pre-mtHSP70
rc_AI104389_at	89% homology (488/488 nt) to Mouse ankyrin repeat, family A (RFXANK-like), 2 (Ankra2)
rc_AA817987_f_at	Rat hydroxysteroid sulfotransferase
M36151cds_s_at	Rat MHC class II A-beta RT1.B-b-beta, partial
M32062_g_at	Rat Fc-gamma receptor
rc_AI639170_at	No significant homology
AJ000556cds_at	Rat Janus protein tyrosine kinase 1, JAK1
M27223_at	Rat Na+ channel
AF062594_at	Rat nucleosome assembly protein
L13040_s_at	Rat calcitonin receptor C1b
J05519_at	Rat C1-tetrahydrofolate synthase
AF030088UTR#1_at	Rat activity and neurotransmitter-induced early gene 3 (ania-3), 3'UTR.
AB014722_at	Rat SALT-1(806)
E00775cds_s_at	Rat cardionatrin precursor
U19614_g_at	Rat lamina-associated polypeptide 1C (LAP1C)
AF022952_at	Rat vascular endothelial growth factor B, partial
L14936_at	Rat MAP kinase kinase (MKK2)
AF091580_s_at	Rat isolate EGL-TP1 olfactory receptor, partial
X06890cds_at	Rat rab4
U51584_s_at	Rat zinc finger homeodomain enhancer-binding protein-2 Zfhep-2), partial
D89730_at	Rat T16
L20678_at	Rat bone morphogenetic protein 2 related
D30647_at	Rat very-long-chain Acyl-CoA dehydrogenase
rc_AA892560_at	88% homology (217/624 nt) to Mouse neural-salient serine/arginine-rich
M21476_s_at	Rat iodothyronine 5'-monodeiodinase, partial
M58364_at	Rat GTP cyclohydrolase I
AF091572_s_at	Rat isolate HGL-SL3 olfactory receptor, partial
L02915_at	Rat mRNA fragment
A44407cds_at	unnamed protein product; Sequence 4 from Patent EP0655442.
D86711_at	87% homology (495/994 nt) to Mouse CGI-89 protein
U93197_at	Rat RN protein
rc_AI180145_s_at	91% homology (361/383 nt) to Rat protein tyrosine phosphatase, non-receptor type 1
rc_AA894340_at	No significant homology
U24652_at	Rat Lnk1
AF035955_at	Rat kinesin-related protein KRP6, partial
M29317_at	Rat gamma-interferon (IFN-gamma)
rc_AA858578_at	96% homology (359/416 nt) to Rat IkBL, vacuolar ATPase NG38, Bat1, and MHC class I antigen
AF041107_at	Rat tulip 2

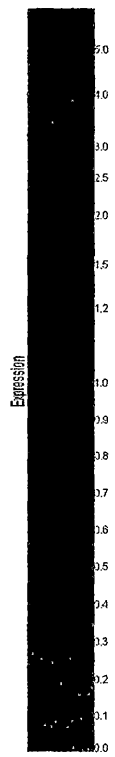
rc_AA892550_g_at	No significant homology
U49729_at	Rat Bax alpha
rc_H31625_at	No significant homology
rc_AI234730_at	99% homology (405/405 nt) to Mouse platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit
rc_AA893443_at	94% homology (548/548 nt) Mouse Ras-related protein RAP-1b
D63834_at	Rat MCT1 mRNA for monocarboxylate transporter
U56815_at	Rat syntaxin 6
U35775_g_at	Rat gamma-adducin
AF053988_at	Rat tissue-type vomeronasal neurons putative pheromone receptor V2R1-1, partial
AA108308_s_at	86% homology (73/186 nt) to Mouse Mdm2
rc_AA875307_at	93% homology (320/337 nt) to Mouse keratin complex 1, acidic
M23572_at	Rat 33, exon 4 and 3' end.
U73142_at	Rat p38 mitogen activated protein kinase
S55933_i_at	Rat GABAA receptor alpha 4 subunit
rc_AA892945_at	No significant homology
AJ006295_at	Rat AF-9
D12516_at	Rat HES-5
rc_AA891571_at	No significant homology
rc_AA799575_r_at	93% homology (465/588 nt) to Rat peptidylglycine alpha-amidating monooxygenase precursor (PAM)
M31174_g_at	Rat c-erbA-alpha-2-related protein
M95791cds_f_at	Rat transcription factor EF1(A)
D26178_at	Rat heart serine/threonine protein kinase

Table 6-8. Composite of selected genes of interest. Genes of interest were compiled according to gene expression levels and patterns, as indicated by the color bar at the bottom of the table.

Synapse-associated Proteins and Neurotransmission	Accession #	VEH	E_2	TMX
Genes regulated by E_2 (two-fold and greater):				
Syntaxin 2	L20823			
Syntaxin 7	AF031430			
Syntaxin 8	AF033109			
Synapse-associated protein (SAP)-97	U14950			
Synapse-associated protein (SAP)-102	U50147			
Chapsyn-110	U49049			
Synaptotagmin IV	U14398			
Synaptotagmin VIII	U20110			
Neuronal nitric oxide synthase (nNOS)	AF008912			
Carboxyl terminal PDZ ligand of nNOS (CAPON)	AF037071			
Vesicle-associated membrane protein (VAMP)-1	M24104			
Endothelial nitric oxide synthase (eNOS)	AJ011116			
Glutamate receptor (GluR1), AMPA subtype	S94371			
GLAST glutamate/aspartate transporter	S75687			
Testicular N-cadherin	AF097593			
Connexin 43 (EST, 99% homology)	AI029183			
nArgBP2 (EST, 99% homology)	AI058393			
Glutamate transporter, GLT-1A (EST, 99% homology)	AI044517			
Roundabout, (EST, 83% homology to human)	AI639443			
Inhibitory glycine receptor alpha-1 subunit	X55246			
Genes regulated by TMX (two-fold and greater)				
Syntaxin 6	U56815			
Synaptotagmin X	U85513			
Acetylcholinesterase T subunit, AChE	S50879			
Transcription Factors	Accession #	VEH	E_2	TMX
Genes regulated by E2 (two-fold and greater):				
c-Fos	X06769			
Max	D14448			
ZOG / Preadipocyte factor (PREF)-1	D84336			
Skn-1a (POU domain, class 2, transcription factor 3)	L23862			
Thyroid transcription factor (TTF)-1	X53858			
Genes regulated by TMX (two-fold and greater):				
Hairless protein	U71293			
c-Myc oncogene	Y00396			
Peroxisome proliferator-activated receptor (PPAR)-α	M88592			
CCAAT enhancer binding protein-gamma (C/EBP-γ)	X64403			
Fos-related antigen (FRA)-1	U34932			
Genes regulated by both (two-fold and greater):				
DNA polymerase alpha subunit III	AJ011607			
Nucleolar phosphoprotein p140; Nopp140(EST,100%)	AA859299			
CCAAT enhancer binding protein-delta (C/EBP-δ)	M65149			
Neurogenesis, Proliferation and Differentiation	Accession #	VEH	E_2	TMX
Genes regulated by E_2 (two-fold and greater):				
HNF-3/forkhead homology-7	L13207			
Cyclin D2	D16308			
Delta 1	U78889			

Genes regulated by TMX (two-fold and greater):				
Notch protein	X57405			
LIF receptor alpha chain	D86345			
Delta 3	AF084576			
NDF40 Neu differentiation factor	U02320			
Hairy and enhancer of split (HES)-5	D12516			
Cyclin D1	D14014			
Genes regulated by both (two fold and greater):				
c-Kit receptor	D12524			
Stem cell factor- kit ligand (SCF-KL)	AF071204			
Cell Signaling / Cell Adhesion	Accession #	VEH	E_2	TMX
Genes regulated by E_2 (two-fold and greater):				
Integrin-alpha M	U59801			
Cell adhesion regulator (CAR) -1	U76714			
cAMP-dependent protein kinase catalytic subunit β	D10770			
cAMP phosphodiesterase	J04563			
Inwardly-rectifying K+ channel-1	U27558			
Calcium/calmodulin-dependent PKIIα subunit	J02942			
Cystolic epoxide hydrolase	X60328			
Glutathione S-transferase mu type 2	H32189			
Caveolin	Z46614			
Protein kinase C (PKC)-binding protein Enigma	U48247			
Phospholipase A2 receptor	D30781			
Utrophin	AB011666			
Oxidative stress responsive 1 (EST; 87%, human)	AA875244			
Urea transporter (UT3)	U81518			
Fatty acid transporter	AB005743			
GLUT 5, glucose transporter	D28562			
NEDD 4 / ubiquitin ligase	U50842			
NAD(P)H quinone reductase	M58495			
Connexin 43 (EST; 99%)	AI029183			
Calpastatin	X56729			
Aromatase cytochrome P450	M33986			
c-jun interacting protein (JDP-1)	U53450			
Prepro-opiomelanocortin (POMC)	K01878			
Genes regulated by TMX (two-fold and greater)				
ATP-sensitive potassium channel, UKATP-1	D42145			
Osteocalcin	M25490			
Regucalcin	D67071			
Stanniocalcin	U62667			
Inducible nitric oxide synthase (iNOS)	U03699			
STAT 4	AF055291			
Ras-like protein (TC10), EST, 97% homology	AA892635			
SRC-related tyrosine kinase	U09583			
Cyclooxygenase I	U03388			
Pancreatitis-associated protein precursor (PAP)	M98049			
Protein kinase C (PKC)-delta subspecies	M18330			
P38 MAPK	U73142			
Heat shock protein-70	S75280			
Genes regulated by both (two-fold and greater)				
b-Nexilin	AF056034			
Estrogen sulfotransferase	M86758			

GLUT 4, glucose transporter	D28561			
Decorin	X59859			
Pancreatic stone protein (lithostathine)	M62930			
Growth Factors and Receptors	Accession #	VEH	E_2	TMX
Genes regulated by E2 (two-fold and greater)				
Bone morphogenetic protein (BMP)-4	Z22607			
Transforming growth factor type 1 receptor	L26110			
Growth/Differentiation factor (GDF)-8 (Myostatin)	AF019624			
Glia maturation factor-beta (GMF-β)	Z11558			
Inositol 1,4,5 triphosphate receptor (IP-3-R)	M64699			
Nicotinic receptor alpha-7 subunit	S53987			
Dopamine receptor D4	M84009			
Prostaglandin F2 (PGF2)-alpha receptor	S74898			
M4 muscarinic acetylcholine receptor	M16409			
Insulin growth factor (IGF)-1	M15481			
Insulin receptor	M29014			
Insulin growth factor (IGF)- binding protein-2	J04486			
Prostaglandin E receptor EP2 subtype	U94708			
Genes regulated by TMX (two-fold and greater)				
Heparin-binding EGF-like growth factor	L05489			
Bone morphogenetic protein (BMP)-3	D63860			
Glial-derived neurotrophic factor (GDNF)	L15305			
GABA-A receptor pi subunit	U95368			
GABA-A alpha 3 subunit	L08492			
Acetylcholine receptor beta subunit	X74833			
Interleukin 10	L02926			
Nicotinic acetylcholine receptor β3 subunit	J04636			
Interleukin-1 receptor type 1	M95578			
Genes regulated by both (two-fold and greater)				
Fibroblast growth factor (FGF)-16	AB002561			



7

SUMMARY OF RESULTS AND CONCLUSION

Recent evidence suggest that the ovarian steroid, 17β-E_2, may play a neuroprotective role in the brain following brain injuries such as acute ischemic stroke. However, to date, the precise mechanism whereby 17β-E_2 protects the brain is unclear. Further, the ability of selective estrogen receptor modulators (SERMs) to act as agonist in the brain is understudied. The present studies suggest there may be multiple components responsible for the beneficial effects of estrogen and SERMs on the brain. Potential mediation by astrocytes, regulation of neuroprotective growth factors, induction of neurogenesis, and regulation of energy balance are suggested to be several of the components that compromise the neuroprotective and neurotrophic effects of estrogen and SERMs.

The cerebral cortex, an area of the brain which is primarily affected by ischemic stroke, is dramatically protected from ischemic stroke damage in rats following administration of 17β-E_2 or tamoxifen. This region of the brain contains estrogen receptors, although localization studies do not identify a high number of neurons as estrogen receptor positive. Therefore, it is unclear how 17β-E_2 protects a large portion of the brain, without directly affecting all neurons via stimulation of estrogen receptors. We hypothesized astrocytes, which express estrogen receptors in vivo, may act as an intermediary cell type, allowing for the protection exhibited by 17β-E_2. In support of this hypothesis, no neuronal protection was observed in response to a wide variety of cell death paradigms (excitotoxicity, camptothecin induced apoptosis, hypoxia-ischemia, serum deprivation) following treatment with either 17β-E_2 or SERMs, at doses that approximate those which protect *in vivo*. Interestingly, the neurons used in these studies were positive for both known estrogen receptors, ER-α and ER-β. Two possibilities emerged from these observations. (1) A novel, yet unidentified estrogen receptor, such as the putative membrane ER mediates the neuroprotective effect of

17β-E_2 *in vivo* and is not present in the neuronal models utilized in the present study. Alternatively, (2) another cell type which is present *in vivo*, but not in our neuronal culture model (i.e. astrocytes) mediates the neuroprotective effect. Accordingly, astrocytes were estrogen receptor positive for both ER-α and ER-β and secreted a factor which protected neurons from cell death, making this an attractive candidate to mediate the effect of steroid hormones. TGF-β isoforms are astrocyte-derived factors which protect neurons from a wide array of cell death paradigms. TGF-β appears to protect neurons from cell death, at least in part, by stimulating AP-1 mediated gene transcription, via activation of the JNK-c-Jun signaling pathway. Additionally, the release of TGF-β1 and TGF-β2 from cultured cortical astrocytes was enhanced by the addition of 17β-E_2 or by the addition of SERMs, suggesting estrogens stimulate the release of an astrocyte-derived neuroprotective factor. If these data are extrapolated *in vivo*, we propose the presence of a novel estrogen-astrocyte-TGF-β pathway, which mediates the neuroprotection observed *in vivo*. This pathway is especially attractive as most neurons studied to date are positive for the TGF-β receptor. This would allow a widespread protection of neurons from cell death, including those which are estrogen receptor negative.

To further understand the mechanism of 17β-E_2 and SERM mediated protection *in vivo*, microarray analysis was utilized. Following treatment of ovariectomized female rats with physiological doses of 17β-E_2 or therapeutic concentrations of tamoxifen, both which protect in a model of ischemic stroke, a wide variety of genes were identified as differentially regulated. Rather unexpectedly, it was observed that 17β-E_2 and tamoxifen regulate a divergent set of genes, with few genes regulated in a similar pattern, suggesting these compounds may exhibit differential regulation of gene transcription in the brain. Nonetheless, numerous genes were regulated in a similar manner between the two groups, including genes implicated in synaptic plasticity, neurogenesis, growth factors and receptors, energy transport/balance and signal transduction/transcriptional regulation. The ability of estrogen and tamoxifen to regulate numerous genes related to synaptic function and

neurotransmission may explain, at least in part, the ability of estrogen to enhance processes such as learning and memory, which are known to involve excitatory neurotransmission. Numerous genes, such as growth factors and cytokines, are widely known to influence neuronal survival both during development and following brain injury and may thus play a role in mediating neuroprotection by estrogen. Along these lines, current work in the lab has implicated two genes regulated by estrogen in the gene chip, stem cell factor and its receptor, c-kit, in the protection of cultured neuronal cells. This exciting observation will be a focus of the laboratory in the future.

In addition to protection of neurons from cell death, several regulated genes are implicated in proliferation and differentiation, processes associated with stem cell differentiation. Given the recent suggestion that estrogen can induce neurogenesis in the dentate gyrus, the present observations may indicate a role for estrogen in cerebral cortical neurogenesis as well. Additionally, the possibility that tamoxifen, a SERM, may induce neurogenesis is an exciting possibility that is currently under exploration in the laboratory. Thus, in addition to a neuroprotective role for both estrogen and tamoxifen, the present gene chip findings suggest the possibility that neurogenesis may also be a regulated process, which would allow for neural repair following an injury, such as acute ischemic stroke.

In summary, the present studies suggest the possibility of a novel 17β-E_2 -astrocyte-TGF-β neuroprotective pathway. These findings may therefore provide a conceptual framework to identify potent SERMs which are better able to protect the brain from neurodegenerative diseases, such as ischemic stroke. They also identify astrocytes as an important target for steroid hormone actions in the cerebral cortex, a non-traditional endocrine target tissue. These studies demonstrate that astrocytes have important neuroprotective capabilities beyond simply serving a structural role in the brain. Additionally, through the use of high density gene chips, novel functions for estrogen and SERMs in the brain are suggested, such as the regulation of neurogenesis, which may influence functional recovery following injury, as well as normal physiological processes, such as learning and

memory. Together, the present findings suggest estrogen and SERMs may exert multiple mechanisms of action with regards to neuroprotection and brain function.

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