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SEX DIFFERENCES IN CELL DEATH AND STEROID HORMONE RECEPTORS IN CORTICAL EXPLANTS

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SEX DIFFERENCES IN CELL DEATH AND STEROID HORMONE RECEPTORS IN CORTICAL EXPLANTS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Amanda L. Trout
Lexington, Kentucky
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ABSTRACT OF THE DISSERATION

SEX DIFFERENCES IN CELL DEATH AND NUCLEAR HORMONE RECEPTORS IN CORTICAL EXPLANTS

Estrogens, such as the biologically active 17-β estradiol (E2) have many actions in the male and female brain. Not only does E2 regulate reproductive behavior in adults, it organizes and activates the brains of younger animals in a sex-specific manner. In addition, many human studies have shown E2 to provide protection against a variety of neurological disorders, including stoke. These studies have been controversial and depend largely on the type and timing of hormone replacement. Animal studies are much less controversial and clearly demonstrate a neuroprotective role for E2 following ischemic brain injury. Because much of E2 neuroprotection requires sex steroid hormone receptors, it is essential to understand expression patterns of these receptors. For the current studies, I evaluated estrogen receptor alpha (ER α), estrogen receptor beta (ER β) and androgen receptor (AR) expression in the cortex. It is known that these receptors change in expression at several times in an animal's life span including during early postnatal development and following ischemic brain injury. Here I used an *in vitro* cortical explant model to further examine how these receptors change both during development and following injury. This *in vitro* model is important because it provides a way to investigate changes in receptor expression pattern in the cortex without input from other brain regions. In addition to characterizing this model, I also evaluated the contribution of E2 to changes in receptor expression and on cell death following injury in the explants. To begin to decipher mechanisms for E2 mediated neuroprotection, I added antagonist for each of the receptors before and after injury. In each these experiments, I also examined potential sex differences by separating the female and male brains before I cultured the explants. Overall, these experiments showed that cortical explants are a good *in vitro* model. Here we found that E2 was protective in female, but not male cortical explants following injury. However, the exact mechanisms of E2-mediated neuroprotection are still to be deciphered.

KEYWORD: Sex Differences, Estrogen, Neuroprotection, Sex Steroid Hormones, Ischemia

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CHAPTER 1: GENERAL INTRODUCTION

Overview

Estrogens influence sexual differentiation and exert a multitude of effects throughout life in both females and males (McCarthy 2009; Wright, Schwarz et al. 2010; Eliot 2011). During perinatal development, estrogens organize neuronal circuits in the brain that regulate gonadal secretions and sexual receptivity. Estrogens also have non-reproductive roles in the brain during development, such as influencing spine density and synaptogenesis, which plays a role in learning and memory (McEwen, Coirini et al. 1991; McEwen and Woolley 1994; McEwen, Gould et al. 1995; Woolley 1998). Following development, estrogens can be neuroprotective and neurotrophic in animal models of disease (Dubal, Wilson et al. 1999; Dubal and Wise 2001; Garcia-Segura, Azcoitia et al. 2001; Wise 2003). The level of circulating estrogens in the brain influences the amount of brain damage following an experimental stroke, middle cerebral artery occlusion (MCAO). For example, in animals with lower levels of circulating estrogens such as gonadectomized females (Simpkins, Rajakumar et al. 1997; Dubal, Kashon et al. 1998; Toung, Traystman et al. 1998; Rusa, Alkayed et al. 1999) and males (Toung, Traystman et al. 1998), there is a larger amount of cell death following MCAO. Pretreatment with even low doses of the biologically active, 17β-estradiol (E2) is sufficient to exert dramatic protection from brain injury in both female (Dubal, Kashon et al. 1998; Dubal and Wise 2001) and male rats (Toung, Traystman et al. 1998).

In humans, the role of hormone replacement is not as clear. In fact, some clinical studies found that estrogens were not beneficial at all (Wilson, Garrison et al. 1985; Grodstein, Stampfer et al. 1996; Petitti, Sidney et al. 1998; Anderson, Limacher et al. 2004), while other studies did report benefits following stroke (reviewed in (Behl 2002; McCullough and Hurn 2003)). The discrepancies between different studies may be explained by differences in the timing of estrogen replacement and the age of women included in the studies. Animal studies are much less controversial and provide a simpler model to study the mechanisms of neuroprotection by E2 following ischemic brain injury.

A common model of focal ischemic stoke is MCAO which causes initial necrotic cell death in the striatum followed by apoptotic cell death in the cerebral cortex. In female rodents, treatment with E2 attenuates this cell death in the cortex via an estrogen receptor alpha (ERα)-dependent mechanism. In males, treatment with E2 can be protective, but the mechanism for E2 action is less clear. Another model of ischemic injury is treatment with a combination of 2-deoxyglucose and potassium cyanide (2DG/KCN) in cortical explants. 2DG/KCN blocks oxidative phosphorylation and glycolysis, which causes cell death that in some circumstances, can be attenuated by E2 treatment (Wilson, Dubal et al. 2000; Wise, Dubal et al. 2000; Wilson, Liu et al. 2002). There are several advantages of using this cortical explant model over using the whole animal (MCAO) or isolated neuron cultures. Explant cultures are a simplified environment in which pharmacological manipulations that are difficult or impossible in whole animal are possible. In explants, the cortex is isolated from

other regions of the brain, which allows us to examine innate cell responses that may indicate a sex difference in the way the cortex is originally organized during development. Additionally, this model is superior to isolated neurons because explant cultures maintain cytoarchitectural organization and neuronal/glial relationships. The following studies will employ this cortical explant model to investigate innate sex differences in the way steroid hormone receptors respond during development and following injury.

The studies described in this dissertation were designed to test the overall hypothesis that steroid hormone receptor expression, in response to injury, is different in the female and male brain. Specifically first I predicted that changes in sex steroid hormone receptor expression that occur during early postnatal development in the rodent cortex would also be maintained in the cortical explant model. If sex steroid hormone receptors do increase and/or decrease across development in culture in a similar pattern as we see in the cortex, it would suggest that regulation of these receptors is preprogrammed in the cortex and does not require input from other brain regions. Secondly, I predicted that 2DG/KCN treatment would increase cell death in cortical explants and that E2 treatment would attenuate this cell death in sex-specific manner. If E2-mediated neuroprotection following injury is sex-specific in explants, it would suggest that the mechanisms for E2-mediated neuroprotection are sex-specific and most likely innate to the cortex. Thirdly, I predicted that 2DG/KCN-induced injury would change expression of some or all of these steroid hormone receptors in a sex-specific manner. If there is a sex difference in the response of some or all of

these receptors, it may account for the sex-specific protection by E2 in females. Lastly, I predicted that blocking some or all of these receptors would increase ischemia-induced cell death and remove the E2 neuroprotection seen in female explants. If disruption of one of the receptors does change the amount of cell death or disrupts the E2-mediated neuroprotection, it would suggest that E2 is working via that receptor in our model.

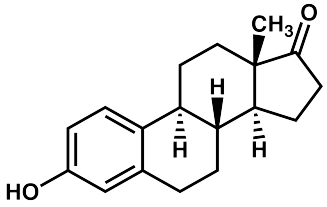
Below I have included a general literature review to describe some of the studies that provide that basic groundwork for the experiments included in these studies.

General Literature Review

Estrogens

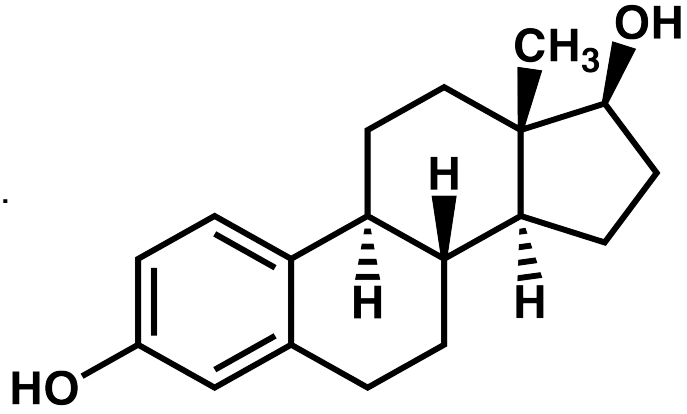
Estrogens are the rudimentary female sex hormone named for their significance in the estrous cycle. Estrogen was first discovered by Allen Edgar in 1923. The structure was independently isolated in 1929 by both Adolf Butenandt and Edward Adelbert. Estrogen has three isoforms: estrone (E1), estradiol (E2) and estriol (E3) (Figure 1.1) (Watson, Jeng et al. 2008). E1 is primarily made from adipose tissue, E2 from gonadal tissue and E3 in pregnancy (Berne RM 2004). E2, 17-β estradiol, is the most abundant and biologically active form of estrogen. Estrogen is a steroid hormone and a cholesterol derivative. Cholesterol or acetyl-coenzyme A (acetyl-CoA) is taken into the cell by endocytosis and transported to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR), where cholesterol is converted to pregnenolone

A.



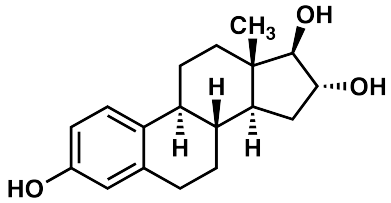
Estrone (E1)

B.



Estradiol (E2)

C.



Estriol (E3)

Figure 1.1. Chemical structure of estrogens. Modified from (Watson, Jeng et al. 2008) (A). Estrone also known as E1 has one hydroxyl group (OH). (B). Estradiol (E2), the most biologically active form of estrogen, has two hydroxyl groups and (C) Estriol has three hydroxyl groups and is also known as E3.

(Berne RM 2004; Kim, Kang et al. 2004). In the ovaries in females and the testis in males, cholesterol is converted into estrogens through a conglomerate of 6 or 7 enzymatic reactions (Berne RM 2004; McCarthy 2009). Estrogens can also be made extra-gonadally in adipose tissue (Simpson, Zhao et al. 1997), bone (Bruch, Wolf et al. 1992; Simpson, Rubin et al. 1999), heart (Taves, Gomez-Sanchez et al. 2011) and the brain (Simpson, Rubin et al. 1999; Zwain and Yen 1999; Ibanez, Guennoun et al. 2003; Hojo, Hattori et al. 2004; Tsutsui 2008; Mirzatoni, Spence et al. 2010; Fester, Prange-Kiel et al. 2011). In rodents, estrogen is synthesized in the brain during development (Ibanez, Guennoun et al. 2003; Tsutsui 2008; Nagarajan, Tsai et al. 2011) and also after injury (Mirzatoni, Spence et al. 2010). Astrocytes (Garcia-Segura, Wozniak et al. 1999) and neurons (Beyer 1999; King, Manna et al. 2002; Schaeffer, Meyer et al. 2010) contain the genes necessary to synthesize E2. While it is in debate if all areas of the brain are capable of local E2 synthesis from cholesterol (de novo neurosteroidogenesis), the cortex and hippocampus do have important enzymes such as StAR and aromatase, which suggests neurosteroidogenesis does occur (Zwain and Yen 1999; King, Manna et al. 2002; Fester, Ribeiro-Gouveia et al. 2006; Fester, Prange-Kiel et al. 2011). The fact that steroidogenesis occurs locally in certain brain regions highlights the evolutionary role of steroids in the brain even though not all areas are considered to be involved reproduction.

Estrogen is important for the organization and activation of sex specific characteristics during early development in humans and animals. Estrogens influence the growth, differentiation and functioning in many target tissues,

including the mammary gland, uterus, ovary, testis, and prostate (Kuiper, Carlsson et al. 1997). Estrogens also play an important role in bone maintenance and in the cardiovascular system (Kuiper, Carlsson et al. 1997; Simpson, Rubin et al. 1999; Baker, Meldrum et al. 2003), along with being neuroprotective and neurotrophic in the brain (Dubal, Wilson et al. 1999; Wise, Dubal et al. 2000; Dubal and Wise 2001; Garcia-Segura, Azcoitia et al. 2001; Wise, Dubal et al. 2001; Wise, Dubal et al. 2001; Wise 2003; Marin, Guerra et al. 2005). In the rodent hippocampus, estrogen treatment increases neurite length, spine density, and synaptogenesis (Gould, Woolley et al. 1990; Woolley 1998; McEwen and Alves 1999; McEwen, Tanapat et al. 1999; Gould, Tanapat et al. 2000).

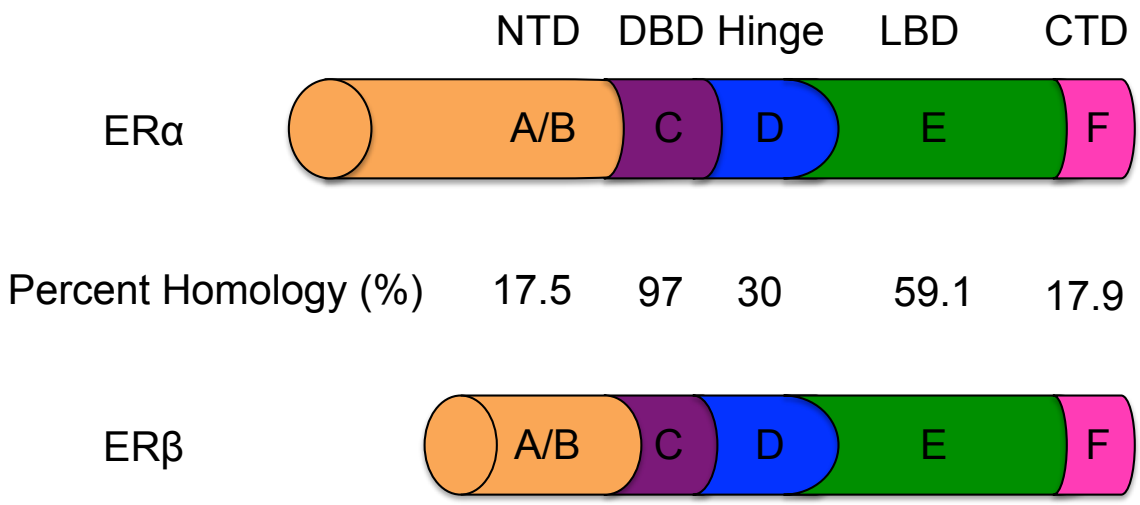
Initially, the actions of gonadal hormones were thought to be neuronal because of the high level of receptor expression in neurons (Simerly, Chang et al. 1990; Brown, Sharma et al. 1995; Shughrue, Lane et al. 1997). However, astrocytes and microglia also respond to gonadal hormones (Garcia-Estrada, Del Rio et al. 1993; Jones, Kinderman et al. 1997; Garcia-Estrada, Luquin et al. 1999; Bruce-Keller, Keeling et al. 2000). Specifically, following injury astrocytes were shown to express estrogen receptor alpha (ERα) and microglia to express androgen receptor (AR) (Garcia-Ovejero, Veiga et al. 2002). This indicates that part of the response to injury in cells of the brain is to up-regulate expression of sex steroid hormone receptors.

Sex Steroid Hormone Receptors

Estrogens can have both genomic and non-genomic actions on target tissues by binding to the well-characterized steroid hormone receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Green, Kumar et al. 1986; Koike, Sakai et al. 1987; Kuiper, Enmark et al. 1996; Berne RM 2004). Androgens have important actions by binding to Androgen receptor (AR). ERα and ERβ are not splice variants, but are completely separate genes. ERα, also known as ERS1, is located on chromosome 6 in humans, 10 in mice and 1 in rats. ERβ, also known as ERS2, is located on chromosome 14 in humans, 12 in mice and 6 in rats (Figure 1.2) (Saunders 1998; Bain, Heneghan et al. 2007). Although both receptors share a similar DNA binding domain, their overall sequence homology is low and they are generally expressed in different tissues (Katzenellenbogen, O'Malley et al. 1996; Shughrue, Lane et al. 1997; Saunders 1998; Denger, Reid et al. 2001; Berne RM 2004; Bain, Heneghan et al. 2007).

Another important sex steroid hormone receptor associated with sex differences is the androgen receptor (AR). Androgen receptor is also known as NR3C4 because it is a member of the 3rd nuclear receptor subfamily group C member 4 (Lu, Wardell et al. 2006). AR primarily mediates is actions with androgens (Roy, Lavrovsky et al. 1999) and is located on the X chromosome (Chang, Kokontis et al. 1988; Trapman, Klaassen et al. 1988; Chang, Kokontis et al. 1990). ERα, ERβ and AR are steroid hormone receptors located not only in the nucleus, but also in the plasma membrane, cytosol and mitochondria (Speroff 2000; Gonzalez, Cabrera-Socorro et al. 2007). AR expression is localized with

A.



B.

	Chromosome		
	Human	Mouse	Rat
ERα	6	10	1
ERβ	14	12	6

Figure 1.2. Structure of estrogen receptors. (A). Estrogen receptor alpha (ERα), top schematic, and estrogen receptor beta (ERβ), bottom schematic. Steroid hormone receptors are all composed of domains A-F, diagramed above. A/B is the N-terminal domain (NTD), C is the highly conserved DNA Binding Domain (DBD), D a flexible hinge region, E is the C-terminal Ligand Binding Domain (LBD) and F is an additional C terminal domain of unknown function. Percent homology between human ERα and human ERβ located between the two protein domain schematics. (B). Location of the ERα and ERβ genes in human, mouse and rat.

ERα and ERβ expression in many areas of the brain, specifically the cortex and hypothalamus (Patchev, Schroeder et al. 2004).

Sex steroid hormones exert genomic actions on target tissues by first binding to their receptors and homo or heterodimerizing (Murdoch and Gorski 1991; Berne RM 2004). Specifically, estrogen binds to ERα, ERβ or both (homo or heterdimerizing) and initiates transcription by attaching to an estrogen response element (ERE) (5'GGTCAXXXTGACC3') as a transcription factor (Murdoch and Gorski 1991; Berne RM 2004) (Green, Kumar et al. 1986; Koike, Sakai et al. 1987; Kuiper, Enmark et al. 1996; Berne RM 2004), Figure 1.3. Many genes utilize ER as a transcription factor to regulate their expression (Murdoch and Gorski 1991; McEwen and Alves 1999). The dimerized ERs can also bind to transcription factors such as activator protein (AP)-1 and NF-kB and initiate transcription factor cross talk through enhancer sequences, non-ERE signaling (Porter, Saville et al. 1997; Teyssier, Belguise et al. 2001), Figure 1.3.

E2 can also act through non-genomic pathways. The non-genomic actions include membrane initiated signaling pathways that are activated not only by ER α and ER β, but by G-protein-coupled receptors and ligand-gated ion channels (Watson, Jeng et al. 2008; Roman-Blas, Castaneda et al. 2009; Liu, Zhang et al. 2012). Estradiol has been shown to induce phosphorylation of extracellular signal-regulated kinase (ERK1/2) (Singh, Setalo et al. 1999; Setalo, Singh et al. 2002; Liu, Zhang et al. 2012). ERK is a part of the mitrogen-activated protein (MAP) kinase pathway (Singer, Figueroa-Masot et al. 1999; Watson, Jeng et al. 2008). These kinases regulate a number of cellular responses one of which is

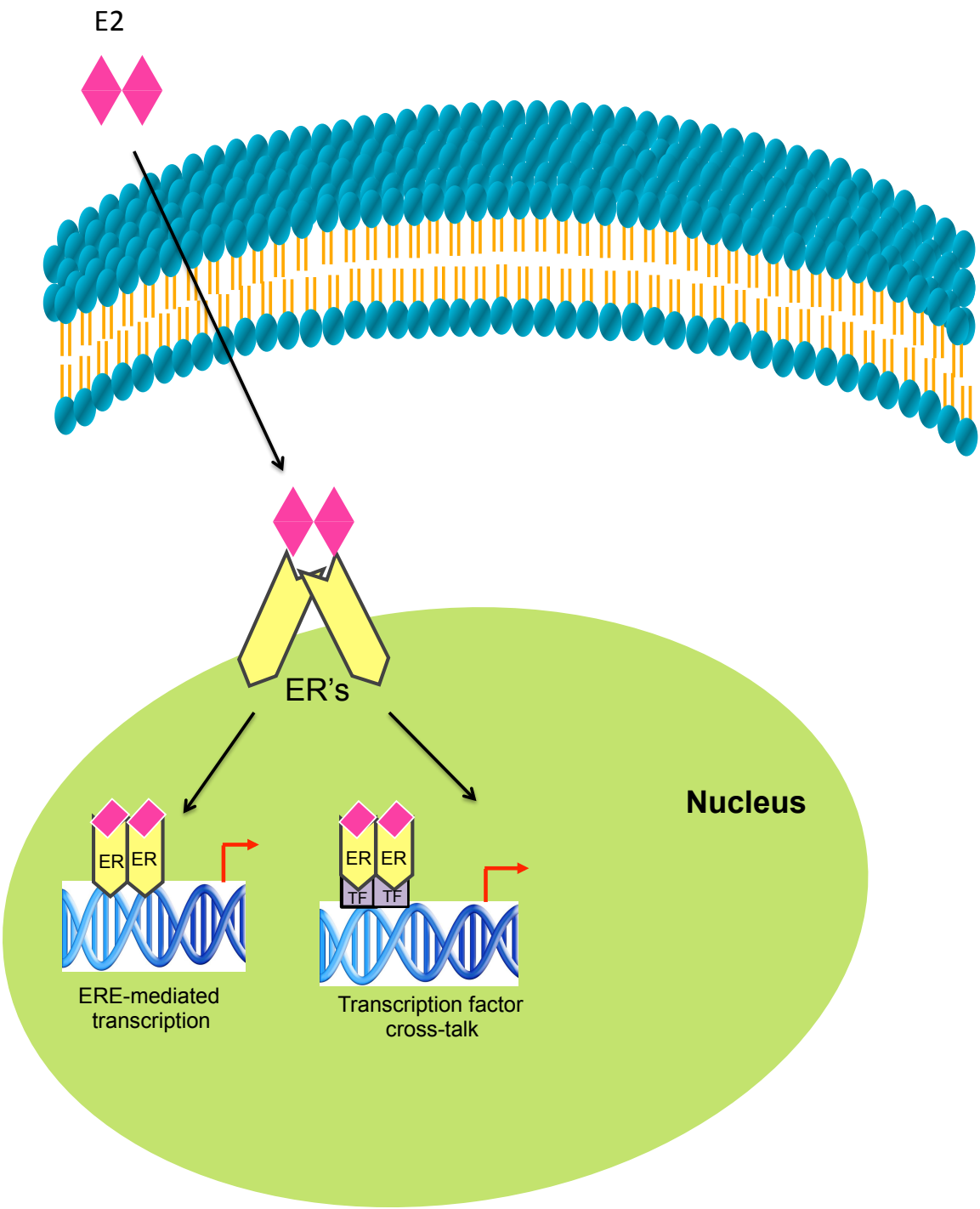


Figure 1.3. Intracellular estradiol signaling. Modified from (Roman-Blas, Castaneda et al. 2009) 17-β estradiol, E2, (pink rectangles) can cross the cell membrane and bind to estrogen receptors, ER (yellow receptors). These ERs diamerize and initiate transcription by binding to estrogen response elements (ERE's) or binding to transcription factors such as activator protein (AP)-1 and NF-kB and initiate transcription factor cross talk, ERE signaling.

cell death (Lee and McEwen 2001; Murphy and Blenis 2006).

Another second messenger signaling pathway that can be activated by E2 is the serine/threonine protein kinase, AKT, through the phosphatidylinositide 3'OH kinase (PI3 K) (Datta, Brunet et al. 1999; Wilson, Liu et al. 2002). ERs can be activated through phosphorylation in the absence of E2 by growth factors such as insulin-like grown factor (IGF)-1 or epidermal growth factor (EGF) or through PI3K/Akt or PKC/MAPK. Activation of one or more of these signaling cascades by E2 can promote cell survival.

Regulation of steroid hormone receptors

Steroid hormone receptor expression is dynamic throughout an animal's lifespan. In rodents, ERα mRNA expression changes across development in the cortex (Prewitt and Wilson 2007). ERα mRNA is high during early postnatal development when organization and sexual differentiation occurs in the brain (Zhang, Chang et al. 2000). Its expression is significantly decreased after PND10 and virtually absent in the uninjured adult cortex (Prewitt and Wilson 2007). ERβ mRNA is concentrated in the cerebral cortex and hippocampus and other brain regions that indicate non-reproductive actions of E2 (Shughrue, Lane et al. 1997; Mitra, Hoskin et al. 2003). It is important to note that while both ERα and ERβ are expressed in the hippocampus, a region of the brain involved in cognitive function (Bliss and Collingridge 1993), only ERβ is expressed in the adult rodent cortex (Shughrue, Lane et al. 1997). ERα and ERβ mRNAs are expressed throughout the human brain as well, but with distinctive patterns

(Osterlund, Grandien et al. 2000; Osterlund, Keller et al. 2000; Gonzalez, Cabrera-Socorro et al. 2007). It is not fully known which ER is responsible for mediating the effects of estrogen on cognition. However, accumulating evidence from knock out (KO) mice supports an important role for ERβ. ERβ KO mice-treated with E2 show impairments in acquisition of a spatial reference memory, implicating a role for ERβ in hippocampus dependent cognition (Rissman, Heck et al. 2002). Interestingly, AR expression also changes by increasing across early postnatal development in the cortex (Nunez, Huppenbauer et al. 2003) and in the adult cortex depending on the different phases of the estrus cycle (Feng, Weijdegard et al. 2010).

Women's Health Initiative

E2 has been shown to be beneficial for memory and cognition, and also reduces the incidence and severity of neurodegenerative diseases including Alzheimer's Disease and stroke (Paganini-Hill and Henderson 1994; Sherwin 1994; Paganini-Hill 1995; Henderson, Watt et al. 1996; Paganini-Hill and Henderson 1996; Sherwin 1996; Henderson 1997; Kawas, Resnick et al. 1997; Paganini-Hill 1997; Sherwin 1997; Green, Perez et al. 2000; Wise, Dubal et al. 2001; Wise, Dubal et al. 2001; Wise, Dubal et al. 2001; Suzuki, Brown et al. 2006; Chen, Cammarata et al. 2009). For example, pre-menopausal women have a lower incidence of stroke than men, but this discrepancy between men and women dissipates following menopause, presumably because of a decrease in protective E2. This population of postmenopausal women is increasing

because the average age of menopause has remained fixed at 51, while the average life expectancy in the US has increased to over 80 years (Suzuki, Brown et al. 2006). Thus, many women will spend a large portion of their lives in a hypo-estrogenic state. Many studies have been conducted to explore different hormone replacement regimes to counteract the negative effects of the loss of estrogen following menopause. Hormones and estrogen replacement therapies have received a great deal of attention in the popular media due to the early termination of the Women's Health Initiative (WHI). The WHI found an increased risk of cardiovascular complications, stroke and breast cancer in women using hormone replacement therapy (Rossouw 2002); however, part of this discrepancy can be explained by the timing of estrogen replacement and the age of women included in the studies (Sherwin 2009; Wise, Suzuki et al. 2009). Animal studies are much less controversial and clearly demonstrate a neuroprotective role for E2 following ischemic brain injury. Taken together these data indicate complex actions of estrogen and hormone replacement therapies that warrant further investigations. An important aspect of this investigation will be to determine how E2 acts through its estrogen receptors and how these receptors are regulated after injury.

Sex Differences in Neurodegenerative Diseases

Neurodegenerative diseases appear to be more robustly present in individuals later in life, when sex steroids have started to decline. Estrogen declines dramatically in women during menopause, while testosterone does also

decline with age but not as significantly. Many diseases that affect the brain are more prominent in one sex verses the other. The prefrontal cortex (PFC) is believed to play a role in these diseases. The PFC is rich in steroid hormones receptors (Bixo, Backstrom et al. 1995) and associated with sex specific responses to stress (Shansky, Glavis-Bloom et al. 2004; Bland, Schmid et al. 2005). Neuropsychatiric disorders including depression and anxiety are more prominent in females while alcohol induced dependencies, schizophrenia, autism and attention deficit disorders have a higher incidence in males (Davies and Wilkinson 2006). While Parkinson's Disease (Shulman and Bhat 2006) appears to be more prominent in the male population, Alzheimer's Disease (Pinkerton and Henderson 2005) and stroke have a higher incidence in females. The mechanisms that cause the sex differences in these diseases may be confounding. Women have fewer cortical neurons (de Courten-Myers 1999; Rabinowicz, Petetot et al. 2002) and experience a severe decrease in hormones with menopause. Since cell death occurs in a large number of neurodegenerative diseases, women may be more at risk of neuronal damage because of their lower number of cortical neurons to start with. Women also have a longer life expectancy than men (U.S Census 2012), which may increase their risk of developing diseases. The hormonal milieu in women and men is different along with their time frame of hormonal production. For example, estrogen may elicit protective effects in women and not men due to differences in hormone concentration and action (Vina, Gambini et al. 2011).

Neurodegenerative diseases have many parallel cellular responses in their histology and mechanisms of action. By definition a neurodegenerative disease is a result of loss of neuronal function resulting in ataxia or memory loss (dementia) (Ross and Poirier 2004). Themes that are common among these diseases include protein aggregation, calcium deregulation, oxidative stress, neural inflammation, excitotoxicity, neurotransmitter loss, and altered steroid genesis, and interestingly enough they seem to be interwoven. One theme leads to the initiation of another until a complete cascade that amplifies the original theme has begun. For example, protein aggregation causes oxidative stress and inflammation, which leads to excitotoxicity and calcium deregulation. The accumulation of these cascades leads to cell dysfunction by altering the signaling pathways, which then leads to additional protein aggregation. The integration of these common themes leads to apoptosis or necrosis of the neurons in various brain regions in disease states.

Estrogens can affect the many themes involved in neurodegenerative diseases. Estrogen not only has genomic and non-genomic effects by decreasing pro-apoptotic and increasing anti-apoptotic genes and pathways, respectively, but it can decrease neuroinflammation and free radicals (Scott, Zhang et al. 2012). It is also known that estrogen increases cognitive abilities which is demonstrated by estrogen's ability to affect many systems in the brain such as: dopaminergic, cholinergic, serotonergic, and noradrenergic (Pinkerton and Henderson 2005). This is important in neurodegeneration as neurotransmitters are a way of communication in the brain. The hippocampus

uses neurotransmitters to signal long-term potentiation, LTP, needed for memory. If there is a decrease in estrogen, causing a decrease in neurotransmitter the hippocampus will have fewer LTP, which affects memory. Interestingly, the Cache County study demonstrates that post menopausal women have a decreased memory, which strongly correlates with the decrease in estrogen leading to fewer LTP (Zandi, Carlson et al. 2002). Specifically in AD, there is also a sex specific irregular phosphorylation of Tau in the hypothalamus in 90% of men that occurs in less than 10% of women (Barnes, Wilson et al. 2005). Tau is a microtubule-associated protein that binds microtubules and initiates anteretrograde transport in the axon by promoting microtubule assembly that aids in transport of neurotransmitters and receptors from the cell body to the synapse in neurons (Lashuel, Hartley et al. 2002; Bossy-Wetzel, Schwarzenbacher et al. 2004). The hyperphosphorylation of Tau leads to the destabilization of microtubules and the disruption of axonal transport. The synapse will not receive adequate nutrients and the neuron will die. The damage in AD occurs mostly in the hippocampus and cortex, which are involved in memory and higher cognitive processes (Bossy-Wetzel, Schwarzenbacher et al. 2004; Ross and Poirier 2004).

In rodent models of disease, the concentration of estrogen administered plays a role in the mechanism of how estrogen is protective. Physiological concentrations of E2 are believed to work through typical steroid hormone receptor signaling, while pharmacological concentrations have more of an oxidative effect. Physiological concentrations of E2 mimic blood concentrations

seen during the reproductive cycle or during pregnancy in women and early life in men. Pharmacological concentrations are generally 10-10,000 fold higher than blood concentration levels (Wise, Dubal et al. 2001). In females, physiological concentrations of E2 vary depending on the phase of the reproductive cycle. In adult premenopausal humans, circulating levels of estrogen range from 15 - 350 pg/mL, while post-menopausal women have a decrease in E2 to less than 10 pg/ml. Interestingly adult men only have an average circulating level of 10 - 40 pg/ml. In rats, females range from 100 pg/ml in estrous to 400 pg/ml in proestrous (Shors 2005). Silastic capsules can be used in ovariectomized female rats to maintain a constant level of E2 and avoid changes in circulating levels. It is difficult to compare circulating levels of E2 to concentrations applied in vitro because of the lack of a linear relationship with specific brain region E2 concentrations and the concentration of circulating E2 (Konkle and McCarthy 2011). In vitro studies typically use 0.1 to 100 nM concentrations of E2 in media (Wise, Dubal et al. 2001). If you start out at 100 pg/ml (~estrous phase concentrations in rats), you end up 0.3671 nM.

Estrogen and Stroke

Stroke is the third leading cause of death in the U.S. (Lloyd-Jones, Adams et al. 2009). Ischemic stroke occurs when the tissue is deprived blood flow resulting in an inadequate supply of oxygen and glucose. This type of stroke occurs 87% of the time, while the other 13% of strokes are hemorrhagic caused by a weak blood vessel that ruptures and causes excess bleeding (Roger, Go et

al. 2012). Stroke can be classified as focal, which is confined to a specific region of the brain, or global, which involves wide areas of brain tissue.

Rodent models have been developed to mimic the most common type of ischemic type of stroke in humans. Permanent MCAO is a well-established model of focal ischemic stroke in rodents. In this model, there is a significant reduction in cerebral blood flow to the striatum and overlaying cortex (Dubal, Kashon et al. 1998). This decrease in blood supply leads to necrotic cell death in the striatum followed by apoptotic cell death in the overlaying cortex (Liu, Smith et al. 1999). Following MCAO, gonadectomized females (Simpkins, Rajakumar et al. 1997; Dubal, Kashon et al. 1998; Toung, Traystman et al. 1998; Rusa, Alkayed et al. 1999; Dubal and Wise 2001) and males (Toung, Traystman et al. 1998; Uchida, Palmateer et al. 2009) have a much larger MCAO-induced injury than animals with higher circulating estrogen concentrations. Pre-treatment with even low doses of E2 is sufficient to exert dramatic protection in the brains of both female (Dubal, Kashon et al. 1998; Dubal and Wise 2001) and male rats (Toung, Traystman et al. 1998). Estrogen receptors (ER) have been shown to be important for neuroprotection by estrogen. Generalized pharmacologic blockade of ER exacerbates ischemic injury in mice (Sawada, Alkayed et al. 2000) and blocks estrogen-induced neuroprotection in cultured neurons (Singer, Figueroa-Masot et al. 1999; Wilson, Dubal et al. 2000) and in cortical explant cultures (Wilson, Dubal et al. 2000). Studies using ERα knockout females demonstrate that neuroprotection by E2 following ischemia is dependent on the presence of ERα in the cortex (Dubal, Zhu et al. 2001), and that ERβ alone is not sufficient for

neuroprotection in females. In male ERα knockout mice, the absence of ERα did not increase the ischemia-induced damage, but these males were not given estradiol (Sampei, Goto et al. 2000). In both male and female rodents, ERα is only transiently expressed in the cortex during neonatal development and then is virtually absent in the adult (Miranda and Toran-Allerand 1992; Prewitt and Wilson 2007). 24 hours after MCAO, however, ERα mRNA and protein are increased in the cortex of female rats and mice (Dubal, Shughrue et al. 1999; Dubal, Rau et al. 2006). In OVX females, the increase in ERα mRNA occurs in both oil and E2-treated groups, but is seen earlier after injury with E2 (Dubal, Rau et al. 2006). These data suggest that in females, the ischemia-induced increase in ERα in the cortex is necessary for the neuroprotective effects of E2. In males, the injury-induced regulation of ERα and the mechanisms of E2 action are still largely unknown.

Injury Models and Cortical Explants

E2 has been shown to be protective in other models of injury. For example, E2 reduces cell death in cortical and hippocampal neurons along with cell line exposed to multiple types of injuries reviewed in (McCullough, Koerner et al. 2009; Herson and Hurn 2010). (Herson, Koerner et al. 2009; McCullough, Koerner et al. 2009; Liu, Kelley et al. 2010). These studies are predominately done in cell lines or primary cultures where only one cell type is present. We are specifically interested in the cortex, because it is a region of the brain that can be protected with estrogen treatment following stroke. Slices of the cortex can be

grown in culture where a heterogeneous population of neurons and glia cells can continue to cross talk as they did in the intact brain. In this model, E2 has also shown to be protective by reducing cell death in a chemical induced ischemic injury (Wilson, Dubal et al. 2000; Wilson, Liu et al. 2002). 2DG/KCN blocks glycolysis and oxidative phosphorylation, which are the two main areas of disruption when the brain experiences ischemia. By maintaining the synaptic connections of the whole brain slice, the heterogeneous population of neurons and glia can add insight to how E2 mediates protection in the cortex.

Cortical explants are taken from PND 3 or 4 rat pups because it is essential to isolate the explant while this part of the brain is still developing. Donor pups must be younger than PND7 do ensure viability (Staal, Alexander et al. 2011). If the explants were taken from a donor animal older than PND 7 they are not as likely to thrive outside input from other regions of the brain possible due to the tissue requiring more aerobic-base synthesis, such as oxygen and ATP from outside sources, for metabolism (Fuller and Dailey 2007). Healthy explants are transparent with smooth edges while overfed explants become opaque and underfed explants thin to the point that they are undetectable (Fuller and Dailey 2007).

Summary

To investigate the multi-faceted cellular pathways that are involved in cell death and neuroprotection, a simplified model is needed to isolate specific mechanisms on how sex differences occur in ischemia. My dissertation will

investigate sex differences at two time-periods. First, I will examine changes in sex steroid hormone receptor mRNA expression across time in culture of female and male cortical explants separately. Then, I will not only examine changes in sex steroid hormone receptor mRNA expression following injury but also sex differences in cell death and E2 mediated protection.

CHAPTER 2

STEROID HORMONE RECEPTOR EXPRESSION DURING EARLY POSTNATAL DEVELOPMENT IN THE RAT CORTEX *IN VIVO* AND *IN VITRO*

Introduction

Sex steroid hormones elicit numerous actions in the brain during development. These hormones can act through specific steroid hormone receptors, such as estrogen receptor alpha (ERα), estrogen receptor beta (ERβ) and androgen receptor (AR). To understand the many actions of steroid hormones it is important to examine the expression and distribution of their receptors. Expression of these receptors in the brain changes across an animal's lifetime. Specifically, during early postnatal development in the mouse, ERα mRNA expression is high in the cortex then decreases where it remains low in the uninjured adult cortex (Prewitt and Wilson 2007). Interestingly, ERα expression increases following injury in the adult cortex (Wilson, Westberry et al. 2008). Another example of the dynamic nature of steroid hormone receptor expression is ERβ, which increases across early postnatal development and decreases with aging (Wilson, Rosewell et al. 2002; Westberry, Trout et al. 2011). AR expression also changes by increasing across early postnatal development in the cortex (Nunez, Huppenbauer et al. 2003) and in the adult cortex depending on the different phases of the estrus cycle (Feng, Weijdegard et al. 2010). Little is known about changes in AR with age or following injury. By

understanding the regulation and expression patterns of these steroid hormone receptors during early postnatal development we can potentially understand how they are regulated at other times during an animal's lifetime, i.e. following a brain injury. In fact, one theory is that the brain reverts back to its developmental state following injury to aid in repair (Emery, Royo et al. 2003).

The experiments described in this chapter were designed to accomplish two goals: First, to test the hypothesis that sex steroid hormone receptor expression was regulated similarly during postnatal development in the rat cortex that was seen in previously published mouse studies. Specifically, we predicted that ERα mRNA expression would decrease and ERβ and AR mRNA expression would increase across early postnatal development. The second goal was to test the hypothesis that sex steroid hormone receptor mRNA expression also changed across time in rat cortical explants. I also wanted to test the hypothesis that E2 pretreatment would alter steroid hormone receptor expression in rat cortical explants. Specifically, since E2 alters steroid hormone receptor expression *in vivo* by positive and negative feedback, E2 would alter expression of steroid hormone receptors across time in culture, *in vitro*.

The cortex is a complex brain region with many changes occurring throughout development and following injury. One way to simplify how the cortex is studied and focus on its changes is to isolate it away from other regions of the brain and maintain it in culture. In fact, previous studies from our lab have used this model to study injury. These initial studies were done in rat cortical explants and showed that 17β-estradiol (E2) was protective following a chemically induced

ischemic injury (Wilson, Dubal et al. 2000; Wilson, Liu et al. 2002). One limitation from those studies was that the cortical explants had combined sections from both male and female rat pups. With our current knowledge of sex differences in cell death and in response to injury, we felt it was essential to repeat those studies separating cortical explants from female and male pups. Another limitation of those studies was that although they did explore E2 and neuroprotection using a variety of concentrations of E2 (0.1-100nM), they did not evaluate how E2 altered expression of steroid hormone receptors from the time that the cortical explants were plated. Here, we addressed these limitations by separating the pups based on sex before cortical explants were taken and by measuring sex steroid hormone receptor mRNA expression in rat cortical explants at several time-points after plating (1, 7, 15 and 22 days in culture, DIC) with and without the presence of E2.

More recently our lab was interested in the expression of steroid hormone receptors in the mouse cortex and in explants cultures taken from mice (Prewitt and Wilson 2007; Westberry, Prewitt et al. 2008). Our interest in mice was due to the possibility of utilizing knockout strains to learn E2 mediated neuroprotection mechanisms. A recent study from our lab compared expression patterns of ERα and ERβ across development *in vivo* and *in vitro* in mice (Prewitt and Wilson 2007). ERα mRNA and protein expression decreased and ERβ mRNA and protein expression increased across early postnatal development in the cortex and across time in mice cortical explants (Prewitt and Wilson 2007). Interestingly, no sex differences in mRNA or protein expression were reported in

the cortex or in cortical explants taken from mice (Prewitt and Wilson 2007). Although this study separated the mice pups based on sex before explant dissections, the influence of E2 on the expression of steroid hormone receptors across time in culture was not evaluated. It is important to know if E2 pretreatment caused any changes in steroid hormone expression even before the injury was induced. These studies provided a solid rationale to look at both mice and rat cortical explants. Unfortunately due to some initial technical difficulties with mice, including: small litter size and female to male ratios in litters, I decided to exclusively use rat cortical explants.

In the following studies, I tested the hypothesis that sex steroid hormone receptor (ERα, ERβ and AR) expression was regulated similarly during postnatal development in the rat cortex as the mouse cortex. I also wanted to test the hypothesis that ERα, ERβ and AR mRNA expression also changed across time in culture in rat cortical explants. To accomplish my goals for this chapter, I isolated mRNA from the cortex and from cortical explants of female and male rats and measured ERα, ERβ and AR mRNA expression. By understanding the regulation and expression patterns of these steroid hormone receptors during early postnatal development, we can potentially understand how they are regulated at other times during an animal's life, i.e. following brain injury.

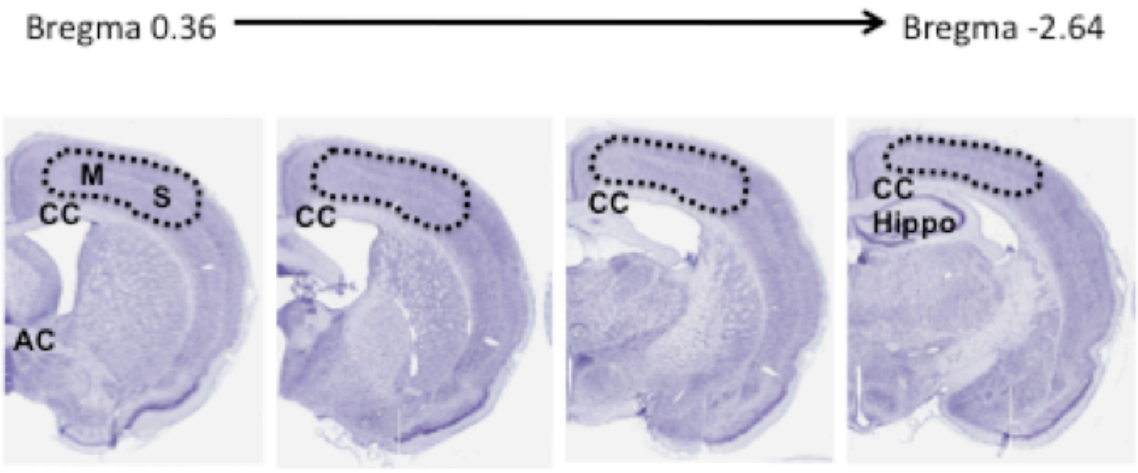
Methods

Animal Care and Housing

All animals used in these experiments were Sprague Dawley rats. Pregnant dams were purchased from Harlan Laboratories (Indianapolis, IN) and maintained in constant temperature conditions on a 12-hour light/dark cycle. Dams were provided food and water ad libitum. Pups were sexed and taken from their mother at postnatal day (PND) 3, 4, 10, and 18, with PND 0 as the day of birth. For the PND 25 time-point, pups were weaned from their mother at PND 22, sexed and placed in separate cages until PND 25. The Animal Care and Use Committee of the University of Kentucky approved all experimental procedures.

Collection of brain tissue

Animals were killed by rapid decapitation and the brains removed. On ice, the cortex was dissected from the corpus callosum with care not to include the piriform cortex, striatum and the hippocampus. The cortex was collected from Bregma -.36 to -2.64 mm (C 2007). See Figure 2.1. Tissue was collected on ice and frozen at -80 ^{o}C until processing.



AC- anterior commissure
CC- corpus callosum
M- motor cortex
S- somatosensory cortex
Hippo- hippocampus

Modified from Brainmaps.org and Paxinson and Watson, 6th edition

Figure 2.1. Diagram of the cortical region used in experiments. The cortex was collected from Bregma -0.36 to -2.64 mm. Regions of cortex that we collected included primary and secondary motor cortex along with some somatosensory cortex. The motor cortex controls voluntary movements by planning, control and execution of the movements. Neuronal axons from the motor cortex synapse onto motorneurons in the spinal cord, which have axons that synapse to a muscle.

Collection of cortical explants

Pups were taken at postnatal day 3 (PND3) with PND 0 considered day of birth. Pups were sexed and rapidly decapitated. As previously described (Wilson, Rosewell et al. 2002), brains were isolated and sectioned at 300 μm on a vibratome. Cortical pieces were dissected form Bregma -.36 to -2.64 mm (C 2007). Regions of cortex included primary and secondary motor cortex along with somatosensory cortex (see Figure 2.1 for representative brain sections and Figure 2.2 for diagram of explant procedure). The cortex was dissected away from the corpus callosum with care not to include the piriform cortex, striatum and the hippocampus. Approximately 8-10 slices were harvested per brain. The explants were sectioned in dissection media containing Gey's balanced salt solution (G9779, Sigma-aldrich, Saint Louis, MO), 0.2 M MgCl2 and 37.5% glucose in Geys BSS and kept in dissection media plus ketamine HCl (Ketaset, NLS Animal Heath, Pittsburg PA) on ice until the cortex was isolated and separated into individual hemispheres. Four individual cortices were plated on Millicell-CM membranes (PICMO3050, Fisher, Hampton, NH) in wells containing media of 1X Basal Medium Eagle (BME) (B9638, Sigma-aldrich, Saint Louis, MO), Hanks' Balanced Salt Solution (HBSS) (14025, Invitrogen), heat-inactivated horse serum (3H30074.03, Fisher, Hampton, NH), 37.5% glucose in Geys BSS, glutamax (35050, Invitrogen, Carlsbad, CA), and penicillin/streptomycin (15140, Invitrogen, Carlsbad, CA). The media also contained either 17 β-estradiol (E2) (1 nM in 0.01% EtOH) (1,3,5(10)-Estratrien-3,17-β-Diol, 122323, Steraloids, Inc, Newport, RI) or EtOH (0.01%) vehicle. Media containing E2 or vehicle was

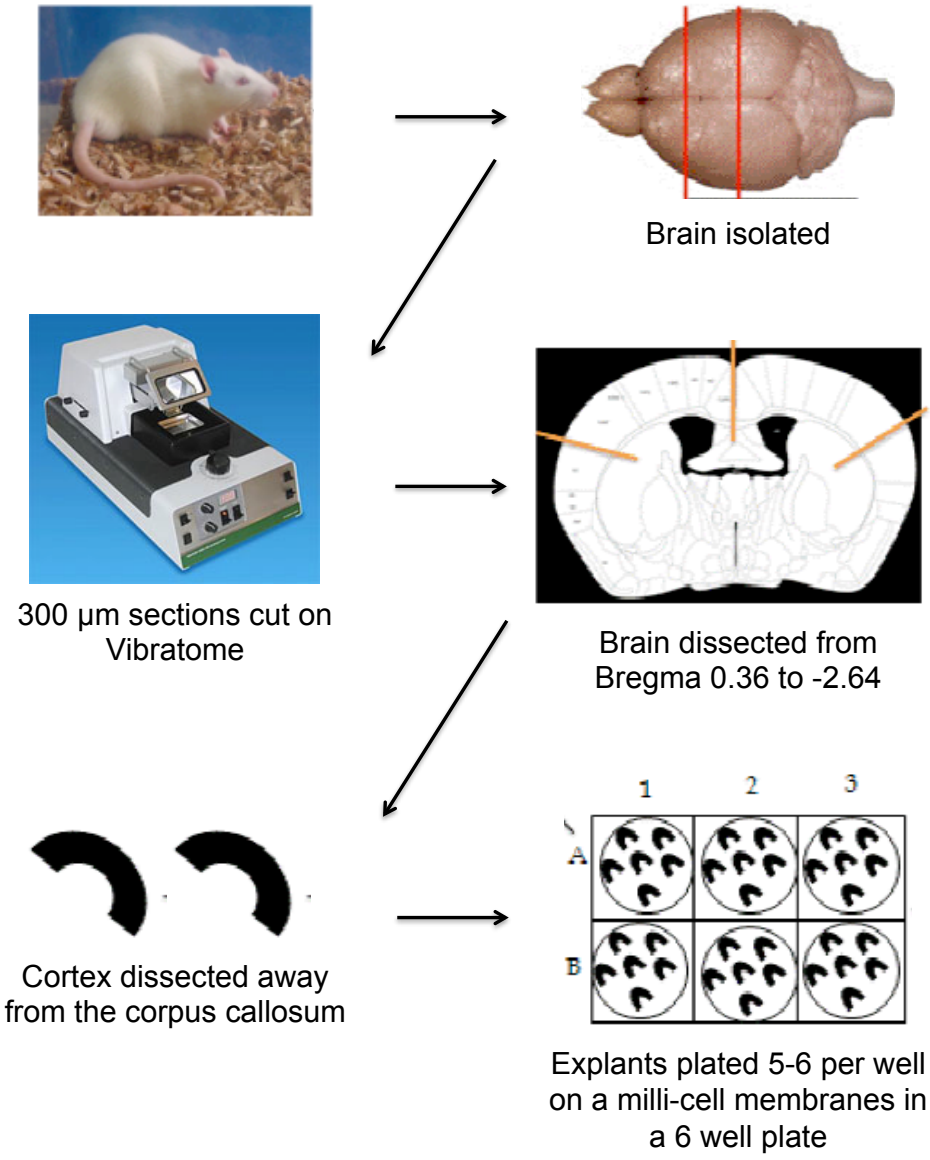


Figure 2.2. Summary of rat cortical explants protocol. Female and male PND3 rat pups were decapitated, the brain isolated and sectioned on a vibratome to 300 um. The cortex was isolated and plated on a milli-cell membrane inserted into a 6 well plate.

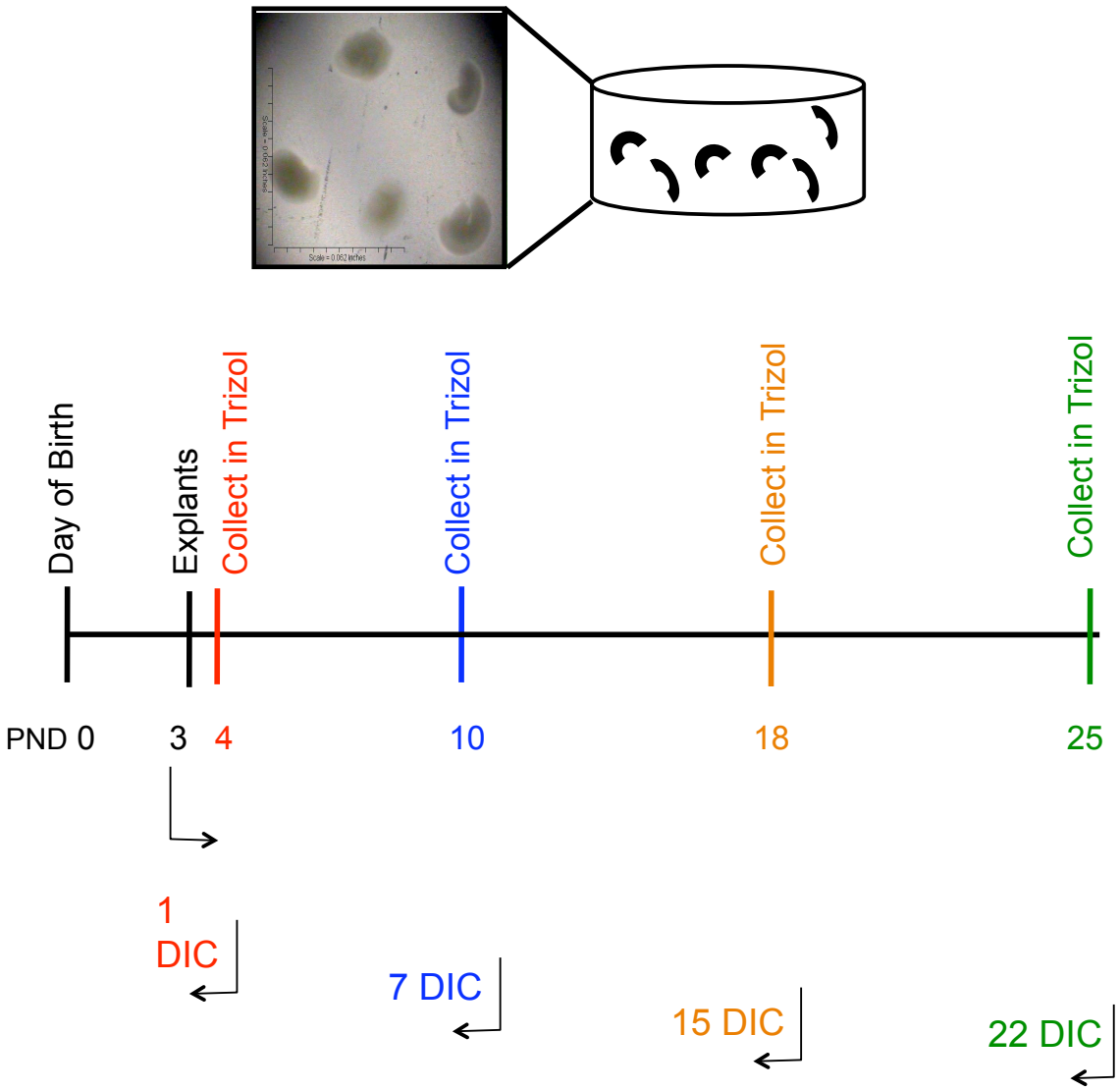


Figure 2.3. Time-line for the collection of cortical tissue and cortical explants. The above diagram shows a timeline of cortical tissue collection from day of birth (PND 0) to PND 25. Also diagramed is the timeline of cortical explants collection from 1 DIC to 22 DIC.

changed every three days. Explants remained in culture at 34°C with 5% CO_2 for 1 to 22 days.

RNA Isolation

Cortical tissue and explants were collected at PND 4 (1 DIC), PND 10 (7 DIC), PND 18 (15 DIC) and PND 25 (22 DIC) (see Figure 2.3 for time-line) to determine mRNA expression changes across time in culture. To collect RNA, cortical tissue or at least 3 explants were combined and homogenized in TriZol (Invitrogen, Carlsbad, CA). RNA was isolated, the resulting pellet was air-dried and resuspended in RNase-free water (DEPC) (BP561, Fisher). The RNA was then be incubated at 56°C for 10 minutes and stored at -80 °C until reverse transcription.

Reverse Transcription

One μg of total RNA was reverse transcribed to produce cDNA. DEPC H_20 was added to bring 1 μg of total RNA for each sample to a final volume of 20μl. 1 μl of Random Primers (58875, Invitrogen, Carlsbad, CA) and 1 μl of 10 mM dNTP's (U1515, Promega, Madison, WI) were added to each reaction. The samples were incubated at 65 °C for 5 minutes. 8 μl of Master Mix containing 4 μl of 5x first strand buffer (Y02321, Invitrogen, Carlsbad, CA), 2 μl of 0.1 M DTT (Y00147, Invitrogen, Carlsbad, CA), 1 μl of RNasin (N211B, Promega, Madison, WI) and 1 μl of Superscript II RT (100004925, Invitrogen, Carlsbad, CA) were added to each reaction. The samples were incubated at room temperature for 10

minutes, 42°C for 50 minutes, then 70 °C for 15 minutes. The cDNA was stored at -80 °C until quantitative real time PCR.

Quantitative Real Time PCR

For real time PCR, each reaction contained 10.125 μl DEPC H_20, 12.5 μl of Brilliant II SYBR@Green QPCR Master Mix (Agilent Technologies, catalog #600828, Santa Clara, CA), 0.375 μl of Reference Dye (diluted 1:500) (Agilent Technologies, Santa Clara, CA), 0.5 μl of forward primer, 0.5 μl of reverse primer and 1μg of appropriate cDNA. Primer specific concentrations were previously optimized for each gene and result in a single PCR product with no primer-dimer formation. Each 96 well plate contained a non-template control and each sample was run in triplicate. The cycling parameters were as follows: 1 cycle at 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds, annealing temperature for 1 minute, 72°C for 30 seconds, and 1 cycle of 95°C for 1 minute and 55°C for 30 seconds. The change in threshold cycle (ΔCt) for each sample was normalized to the constitutively expressed housekeeping control gene Histone 3.1 (Wilson and Handa 1997). The primers used for each gene are shown in Table 1. RNA was collected from at least 3 wells per condition at each time-point. At least 3 explants per time-point from each condition were visualized and each experiment was independently repeated 3 times.

For each gene of interest the change in threshold cycle (ΔCt) for each sample was normalized to the constitutively expressed housekeeping control gene Histone 3.1 (Wilson and Handa 1997). ΔΔCt were then calculated by

comparing the data to a earlier reference point, PND 4 or 1 DIC (Livak and Schmittgen 2001). For example, housekeeping Ct values are subtracted from ERα Ct values at PND 25 in females. This change in Ct values, ΔCt, at PND 25 was compared back to an early time-points PND4 ΔCt value creating a $\Delta\Delta$Ct. Real time PCR data was analyzed by logarithmically raising the changes in Ct at the two time-points, $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001) and graphed as a ratio of gene of interest to housekeeping gene. Error bars on each graph are standard error of the mean.

To look at relative levels of mRNA expression and not a fold change compared back to an earlier time-point, the average housekeeping Ct values for all corresponding samples of the same sex and time-point were divided by and individual sample housekeeping Ct value. The ratio of housekeeping Ct values (average housekeeping Ct value/ sample housekeeping Ct value) was multiplied by the Ct value for the gene of interest for the same sample.

Table 2.1. Primer Information. List of primers used for real time PCR.

Gene	GI #	Primer Location	Forward Primer (F) Reverse Primer (R)	Ref
His 3.1	318068040	208-425	F 5'-GCAAGAGTGCGCCCTCTACTG-3' R 5'GGCCTCACTTGCCTCCTGCAA-3'	(Wilson and Handa 1997)
ER alpha	6978814	1991-2055	F 5'-GGGCTTCCCCAACACCAT-3' R 5'-CGTTTCAGGGATTCGCAGAA-3'	(Takagi, Shibutani et al. 2005)
ER beta	6978816	454-715	F 5'-TTCCCGGCACGACCAGTAACCT-3' R 5'TCCCTCTTTGCGTTGGACTA-3'	(Kuiper, Carlsson et al. 1997)
AR	6978534	2739-2833	F 5'TGATTGCACCATTGATTGATAAATTTCG-3' R 5'GCTTACGAGCTCCCAGAGTCA-3'	(Bowman 2005)

Table 2.2 List of companies and locations of materials used in methods.

Company	**Home Office**
Agilent Technologies	Santa Clara, CA
Fisher	Hampton, NH
Invitrogen	Carlsbad, CA
NLS Animal Heath	Pittsburg PA
Promega	Madison, WI
Sigma-Aldrich	Saint Louis, MO
Steraloids, Inc	Newport, RI
Tocris	Bristol, United Kingdom

Statistics

In vivo

For mRNA studies, female and male cortical tissue was collected from at least 3 different litters at each of the time-points (PND 4, 10, 18 and 25). At least one female and one male were sampled from each of the 3 litters and at least 6 animals were taken at each time-point. For each gene of interest the change in threshold cycle (ΔCt) for each sample was normalized to the constitutively expressed housekeeping control gene Histone 3.1 (Wilson and Handa 1997). $\Delta\Delta$Ct was then calculated by comparing the ΔCt for the time-point (PND) to the ΔCt of PND 4 females. For example, housekeeping Ct values are subtracted from ERα Ct values at PND 25 in females. This change in Ct values, ΔCt, at PND 25 was compared back to an early time-point PND4 ΔCt value creating a $\Delta\Delta$Ct. Real time PCR data was analyzed by logarithmically raising the changes in Ct at the two time-points, $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). All data for the *in vivo* experiments were analyzed using a two-way Analysis of Variance (ANOVA) comparing the factors "sex" and "postnatal day (PND)." To determine what contributes to the main effect of PND or interactions between sex and PND a Student Neuman-Keuls post-hoc test was performed. Differences were considered significant at $p<0.05$.

In vitro

Statistical analyses of the cortical explant data were more elaborate. For these mRNA studies, 3 litters were used to dissect female and male cortical

explants. These explants were grown in media containing EtOH or E2 for various days in culture (1, 7, 15 and 22 DIC). Each time-point included the collection of at least 3 explants (N=1) per well that was repeated independently at least 3 times. For example, the 3 females explants grown in EtOH treated media for 1 DIC was collected for an N of 1. This was repeated at least 3 times with a new set of litters each time.

For each gene of interest the change in threshold cycle (ΔCt) for each sample was normalized to the constitutively expressed housekeeping control gene Histone 3.1 (Wilson and Handa 1997). $\Delta\Delta$Ct were then calculated by comparing the ΔCt for the time-point (PND) to the ΔCt of 1 DIC vehicle treated explants for the corresponding sex. For example, housekeeping Ct values are subtracted from ERα Ct values from the 22 DIC E2-treated females. This change in Ct values, ΔCt, at 22 DIC was compared back to an early time-point, 1 DIC vehicle-treated females cortical explants, ΔCt value creating a $\Delta\Delta$Ct. Real time PCR data was analyzed by logarithmically raising the changes in Ct values at the two time-points, $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

The effect of treatment across DIC was determined by a two-way ANOVA comparing the factors “treatment” (EtOH or E2 treated) and “DIC” for female explants and then for male explants. This allowed us to determine if treatment had and effect on the mRNA expression or if DIC had and affect on the mRNA expression in either female or male cortical explants. To determine what contributes to the DIC effect or interaction between treatment and DIC a Student

Neuman-Keuls post-hoc test was performed. Differences were considered significant at $p<0.05$.

Results

Part 1: Sex steroid hormone receptor expression in the female and male cortex across postnatal day (*in vivo)*.

Female and male rats were killed at postnatal day (PND) 4, 10, 18, and 25. Brains were removed and RNA was collected from the cortex. RNA was converted to cDNA and used for real time PCR to examine ER alpha (ERα), ER beta (ERβ) or androgen receptor (AR) mRNA expression. For each time point, at least 6 animals from different litters were included. Samples were run in triplicate and compared to the housekeeping gene Histone 3.1. All data were compared back to female PND 4.

Are there sex differences in sex steroid hormone receptor mRNA expression across development?

ERα

ERα mRNA significantly decreased across early postnatal development. Interestingly, there was not a sex difference in ERα mRNA expression (Figure 2.4). Sex differences across postnatal day were evaluated by a two-way analysis of variance (ANOVA) comparing the factors "sex" (female and male) and "postnatal day" (PND 4, 10, 18 and 25). Overall there was a significant main effect of postnatal day ($p= 0.0015$, $F= 5.693$, $df= 3, 72$), but no main effect of sex

and no interaction (Figure 2.4). To determine which days were different a Student Neuman-Keuls post-hoc test was performed and revealed that ERα mRNA expression was significantly decreased at both PND 18 and 25 compared to PND 10 and to PND 4 ($p < 0.05$). Overall, ERα mRNA expression decreased across postnatal development regardless of sex.

To look at relative levels of mRNA expression, not a change compared back to PND 4, the ratio of housekeeping Ct values (average specific PND housekeeping Ct value/ sample housekeeping Ct value) was multiplied by the Ct value for the gene of interest for the same sample, Table 2.3. I have included average Ct values for ERα and His 3.1 for female and males at each time-point, Table 2.4. This table highlights ERα Ct values normalized to average histones that can be compared across PND. The graphs, Figure 2.4, have Ct values logarithmically expressed relative to PND 4; however, the chart has raw ERα Ct values normalized to average histone Ct values that can be compared across PND. The chart Ct values show a substantial change in Ct values that are less than 30. For real time PCR, the lower the Ct values the more mRNA and changes that are less than 30 are considered "real." Ct values greater than 30 occur during the plateau phase of the logarithmic PCR cycle where there is an increase chance of error (reviewed in (Wong and Medrano 2005)). The chart Ct values correspond to the logarithmic change seen in the graphs. For the female and male cortex, ERα Ct values showed a decrease across PND.

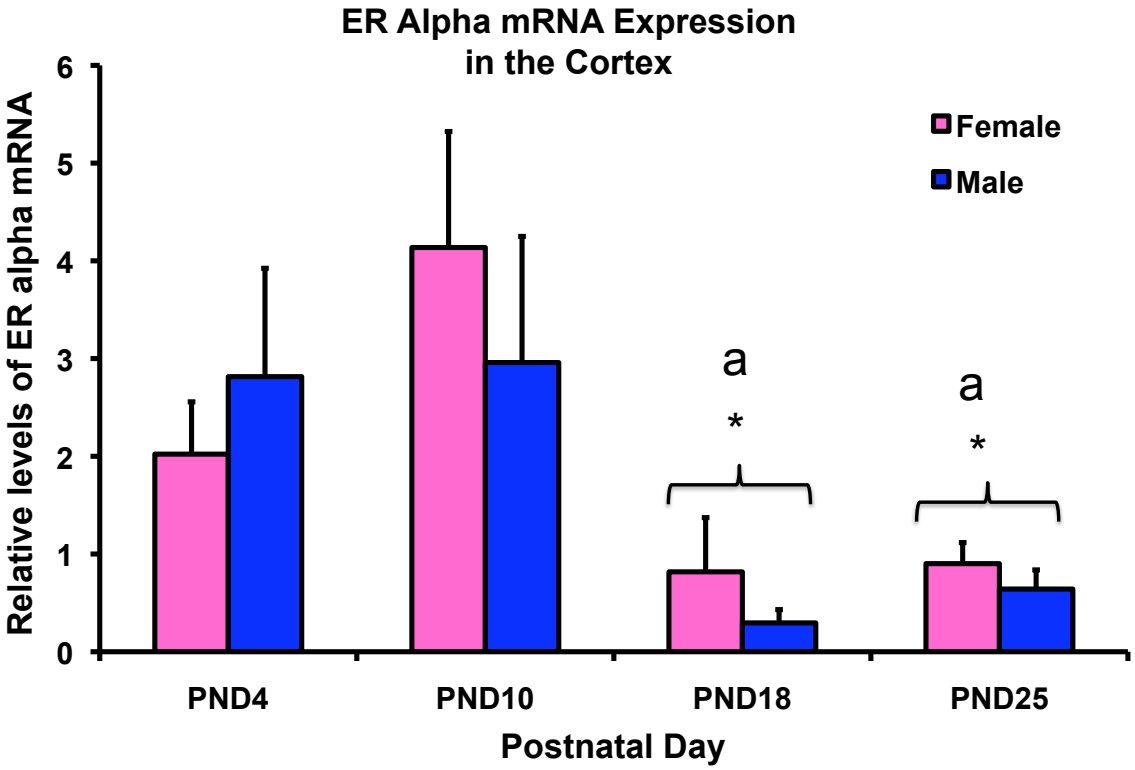


Figure 2.4. ERα mRNA expression in the rat cortex across early postnatal development. ERα mRNA decreased across early postnatal development in both female and male cortex. There were no sex differences in ERα mRNA expression at any of the postnatal days. The graph shows ERα relative to the housekeeping gene histone 3.1. Ct values from each postnatal day were compared to female PND 4. Asterisks on the graph indicate significant differences from PND 4, $p < 0.05$. The letter "a" on the graph indicates significant differences from PND 10, $p < 0.05$.

Table 2.3 Example calculations to look at relative levels of mRNA expression. The ratio of housekeeping Ct values (average housekeeping Ct value/ sample housekeeping Ct value) was multiplied by the Ct value for the gene of interest for the same sample.

PND	Gender	Histone Ct	ER alpha Ct	ER alpha * (avg histone/histone)
PND4	F	15.42	25.62	27.64
		15.41	25.71	27.75
PND4	F	14.46	25.19	28.98
		14.64	25.21	28.65
PND4	F	19.51	25.61	21.84
		17.69	25.59	24.06
		17.02	25.72	25.14
PND4	F	14.75	22.96	25.89
		14.24	22.93	26.79
		14.06	23.11	27.34
PND4	F	19.17	23.87	20.71
		19.16	23.95	20.79
		19.04	23.79	20.79
PND4	F	18.71	23.33	20.74
		18.56	23.46	21.03
		18.67	23.55	20.98
PND4	F	17.41	23.37	22.33
		16.07	23.1	23.91
		15.49	23.29	25.01
PND4	F	16.61	24.41	24.45
		16.84	24.11	23.82
		16.83	23.8	23.52
PND4	F	14.92	22.55	25.14
		15.07	23.04	25.43
		15.1	23.45	25.83
PND4	F	16.43	23.13	23.42
		16.34	25.19	25.64
		16.52	24.16	24.33
PND4	F	16.15	21.59	22.24
		16.27	21.45	21.93
		15.88	21.4	22.42
PND4	F	16.42	20.97	21.24
		16.27	21.06	21.53
		16.62	21.06	21.08
PND4	F	17.67	23.03	21.68
		18.02	23.01	21.24
		18.16	23.11	21.17
PND4	F	16.44	21.06	21.31
		16.64	21.25	21.24
		16.72	21.46	21.35
Average		16.64	23.34	23.51

Table 2.4 Average real time PCR Ct values from housekeeping gene histone 3.1 and ERα on postnatal day 4, 10, 18 and 25. Bolded box on Table A (females) and Table B (males) indicates ERα Ct values normalized to average histones for each postnatal day. These ERα and histone Ct values show a normalized value for ERα throughout postnatal day. These values allow us to look at the change in ERα across postnatal day. The normalized Ct value indicates the relative amount of mRNA in the sample taking into consideration amount of total mRNA. Table A (female) and Table B (male) correspond to the overall decrease in ERα mRNA throughout early postnatal development. Note: higher Ct values indicate a lower expression level of mRNA by representing a greater number of PCR cycles to reach a maximum.

A.

Sex	PND	Average Ct value		Ct value normalized to average histone
		Histone	ER alpha	
Female	4	16.64	23.34	23.51
	10	17.75	23.72	23.31
	18	17.71	26.37	26.65
	25	20.8	28.03	28.33

B.

Sex	PND	Average Ct value		Ct value normalized to average histone
		Histone	ER alpha	
Male	4	16.77	23.46	23.64
	10	17.28	23.8	23.95
	18	17.92	27.56	27.67
	25	20.2	27.96	28.1

ERβ

ERβ mRNA significantly increased across early postnatal development. Interestingly, there was not a sex difference in ERβ mRNA expression (Figure 2.5). Sex differences across postnatal day were evaluated by a two-way analysis of variance (ANOVA) comparing the factors "sex" (female and male) and "postnatal day" (PND 4, 10, 18 and 25). Overall there was a significant main effect of postnatal day ($p < 0.0001$, $F = 47.289$, $df = 3, 67$), but no main effect of sex and no interaction. To determine which days are different a Student Neuman-Keuls post-hoc test was performed and revealed that ERβ mRNA expression was significantly increased at PND 25 compared to PND 4, 10 and 18 ($p < 0.05$), Figure 2.5. Overall, ERβ mRNA expression increased across postnatal development regardless of sex.

To look at relative levels of mRNA expression, not a change compared back to PND 4, the ratio of housekeeping Ct values (average specific PND housekeeping Ct value/ sample housekeeping Ct value) was multiplied by the Ct value for the gene of interest for the same sample, Table 2.5. I have included average Ct values for ERβ and His 3.1 for female and males at each time-point, Table 2.5. This chart highlights ERβ Ct values normalized to average histones that can be compared across PND. For the female and male cortex, ERβ Ct values showed that there was an increase in mRNA expression across PND. The graphs, Figure 2.5, have Ct values logarithmically expressed relative to PND 4; however, the table has raw ERβ Ct values normalized to average histone Ct values that can be compared across PND. The chart Ct values show a

substantial change in Ct values that are less than 30. For real time PCR, the lower the Ct values the more mRNA and changes that are less than 30 are considered “real.” Ct values greater than 30 occur during the plateau phase of the logarithmic PCR cycle where there is an increase chance of error (reviewed in (Wong and Medrano 2005)). The chart Ct values correspond to the logarithmic change seen in the graphs. For the female and male cortex, ERβ Ct values showed an increase across PND.

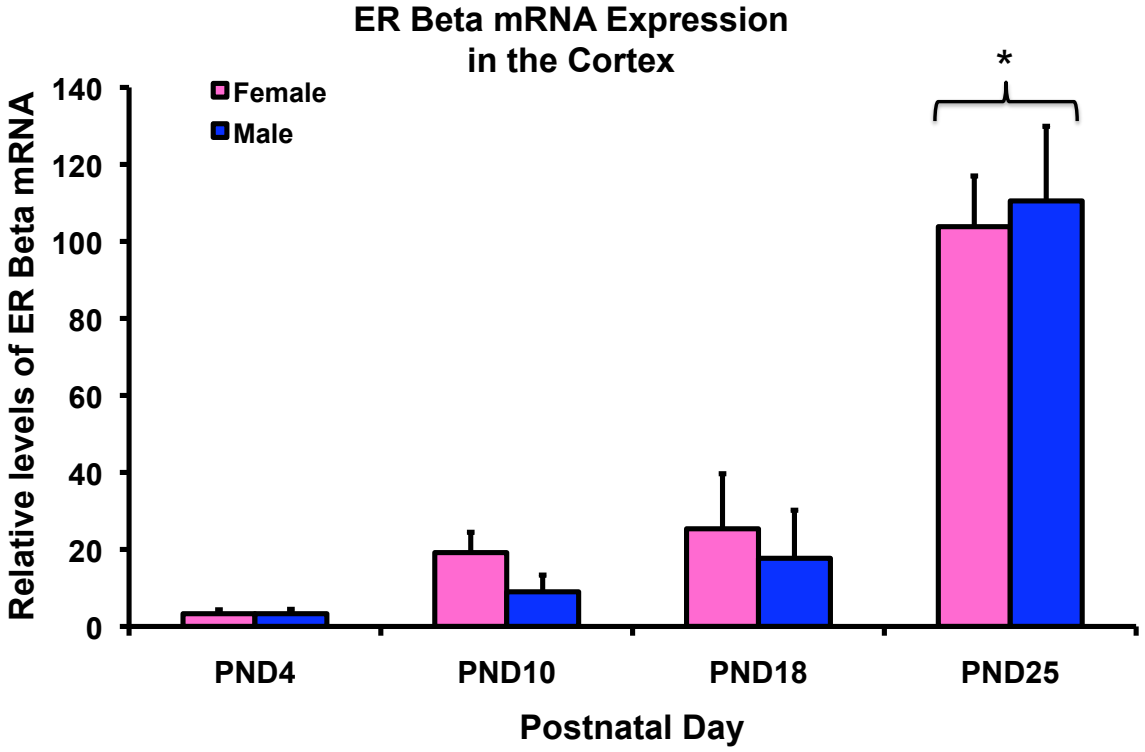


Figure 2.5. ERβ mRNA expression in the cortex across postnatal development. ERβ mRNA increased across early postnatal development in both female and male cortex. There was no sex difference in ERβ mRNA expression at any of the postnatal days. The graph shows ERβ relative to the housekeeping gene histone 3.1. CT values from each postnatal day were compared to female PND 4. Asterisks on the graph indicate significant increase in ERβ mRNA at PND 25 from PND 4,10 and 18, $p < 0.05$.

Table 2.5. Average real time PCR Ct values from housekeeping gene histone 3.1 and ERβ on postnatal day 4, 10, 18 and 25. Bolded box on Table A (females) and Table B (males) indicates ERβ Ct values normalized to average histones for each postnatal day. These raw numbers show a normalized value for ERβ throughout postnatal day. The normalized Ct value indicates the relative amount of mRNA in the sample taking into consideration amount of total mRNA. Table A (female) and Table B (male) correspond to the overall increase in ERβ mRNA throughout early postnatal development (Figure 2.5). Note: higher Ct values indicate a lower expression level of mRNA by representing a greater number of PCR cycles to reach a maximum.

A.

Sex	PND	Average Ct value		Ct value normalized to average histone
		Histone	ER beta	
Female	4	16.64	29.55	27.33
	10	17.58	28.76	28.44
	18	17.71	27.56	27.75
	25	19.89	26.27	26.27

B.

Sex	PND	Average Ct value		Ct value normalized to average histone
		Histone	ER beta	
Male	4	16.77	30.05	27.56
	10	17.28	27.95	28.1
	18	17.76	28.77	28.96
	25	19.46	25.8	25.8

AR

AR mRNA significantly increased across early postnatal development. Interestingly, there was no sex difference in AR mRNA expression (Figure 2.6). Sex differences across postnatal day were evaluated by a two-way analysis of variance (ANOVA) comparing the factors “sex” (female and male) and “postnatal day” (PND 4, 10, 18 and 25). Overall there was a significant main effect of postnatal day ($p< 0.0001$, $F= 16.764$, $df= 3, 66$), but no main effect of sex and no interaction. To determine which days were different a Student Neuman-Keuls post-hoc test was performed and revealed that AR mRNA expression was significantly increased at PND 18 and 25 compared to PND 4 and 10 and significantly increased at PND 25 compared to PND 18 ($p< 0.05$), Figure 2.6. Overall, AR mRNA expression increased across postnatal development regardless of sex.

To look at relative levels of mRNA expression, not a change compared back to PND 4, the ratio of housekeeping Ct values (average specific PND housekeeping Ct value/ sample housekeeping Ct value) was multiplied by the Ct value for the gene of interest for the same sample, Table 2.6. I have included average Ct values for AR and His 3.1 for female and males at each time-point, Table 2.6. This chart highlights AR Ct values normalized to average histones that can be compared across PND. For the female and male cortex, AR Ct values showed that there was an increase in mRNA expression across PND. The graphs, Figure 2.6, have Ct values logarithmically expressed relative to PND 4; however, the table has raw AR Ct values normalized to average histone Ct

values that can be compared across PND. The chart Ct values show a substantial change in Ct values that are less than 30. For real time PCR, the lower the Ct values the more mRNA and changes that are less than 30 are considered “real.” Ct values greater than 30 occur during the plateau phase of the logarithmic PCR cycle where there is an increase chance of error (reviewed in (Wong and Medrano 2005)). The chart Ct values correspond to the logarithmic change seen in the graphs. For the female and male cortex, AR Ct values showed an increase across PND.

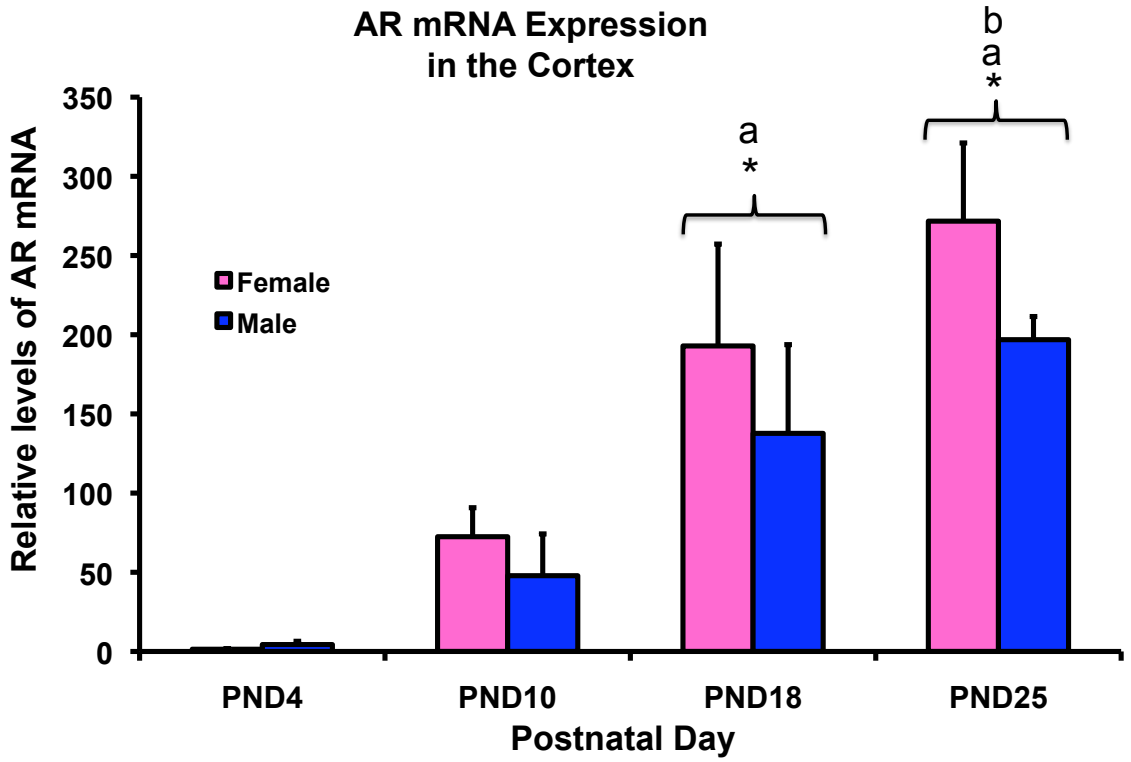


Figure 2.6. AR mRNA expressions in the cortex across postnatal development. AR mRNA increased across early postnatal development in female and male in the cortex. There was no sex difference in AR mRNA expression at any of the postnatal days. The graph shows AR expression relative to the housekeeping gene histone 3.1. Ct values from each postnatal day were compared to female PND 4. Asterisk on the graph indicate significant increases from PND 4, $p<0.05$. The letter "a" on the graph indicates significant increases from PND 10, $p< 0.05$. The letter "b" on the graph indicates significant increases from PND 18, $p< 0.05$.

Table 2.6 Average real time PCR Ct values from housekeeping gene histone 3.1 and AR on postnatal day 4, 10, 18 and 25. Bolded box on table A (females) and table B (males) indicates AR Ct values normalized to average histones for each postnatal day. These raw numbers allow you to evaluate a normalized value for AR throughout postnatal day. The normalized Ct value indicates the relative amount of mRNA in the sample taking into consideration amount of total mRNA. Table A (female) and Table B (male) correspond to the overall increase in AR mRNA throughout early postnatal development (Figure 2.6). Note: higher Ct values indicate a lower expression level of mRNA by representing a greater number of PCR cycles to reach a maximum.

A.

Sex	PND	Average Ct value		Ct value normalize to average histone
		Histone	AR	
Female	4	16.66	30.3	28.59
	10	17.58	25.55	25.58
	18	17.71	24.08	24.28
	25	19.89	26.27	26.27

B.

Sex	PND	Average Ct value		Ct value normalize to average histone
		Histone	AR	
Male	4	16.77	30.08	27.65
	10	17.15	26.92	25.85
	18	17.92	25.67	25.65
	25	19.46	25.36	25.37

We found that the expression patterns of sex steroid hormone receptors were the same in the rat cortex as in the mouse cortex across development. My hypothesis that ERα decreased and ERβ and AR increased in mRNA expression across early postnatal development in the rat cortex was confirmed. Additionally, there was not a sex difference in relative level or pattern of expression in ERα, ERβ or AR mRNA across development.

Part 2: Steroid hormone receptor mRNA expression in female and male rat cortical explants across time in culture (*in vitro).*

Cortical explants were isolated from postnatal day 3 (PND 3) rat pups and grown for 1 to 22 days in culture with media containing either 17β-estradiol (E2) (1 nM in 0.01% EtOH) or EtOH (0.01%) vehicle. Explants were collected for RNA at 1, 7, 15 and 22 days in culture (DIC). Each time-point included the collection of at least 3 explants (N=1) per well that was repeated independently at least 3 times. For example, 3 females explants were grown in EtOH treated media for 1 DIC were collected and combined as an N of 1. This was repeated at least 3 times with a new set of litters each time. RNA was isolated from the explants and converted to cDNA where it was used for real time PCR to examine ER alpha (ERα), ER beta (ERβ) or androgen receptor (AR) mRNA expression. Samples were run in triplicate and compared to the housekeeping gene Histone 3.1. All data were compared back to 1 DIC for each vehicle-treated sex.

ERα

Does E2 influence ERα mRNA expression in rat cortical explants across time in culture?

To answer this question, we analyzed mRNA expression by statistically comparing treatment (EtOH and E2) and days in culture (DIC) separately for female cortical explants and male cortical explants. ERα mRNA expression significantly decreased in female and male cortical explants that were EtOH and E2 treated.

In female cortical explants, ERα mRNA expression decreased in cortical explants across time in culture (1 to 22 DIC), but treatment in the feed media was not a factor (Figure 2.7). For the **female** explants grown in media containing vehicle and E2, a two-way ANOVA comparing the factors "treatment" and "DIC" revealed a significant main effect of DIC ($p= 0.002$, $F= 7.839$, $df= 3, 65$), but no main effect of treatment and no interaction. To determine which DIC were different a Student Neuman-Keuls post-hoc test was performed and revealed that ERα mRNA expression was significantly decreased at 7, 15 and 22 DIC compared to 1 DIC ($p< 0.05$). Overall, these data demonstrate that ERα mRNA expression decreased in female rat cortical explants regardless if E2 was present in the feed media.

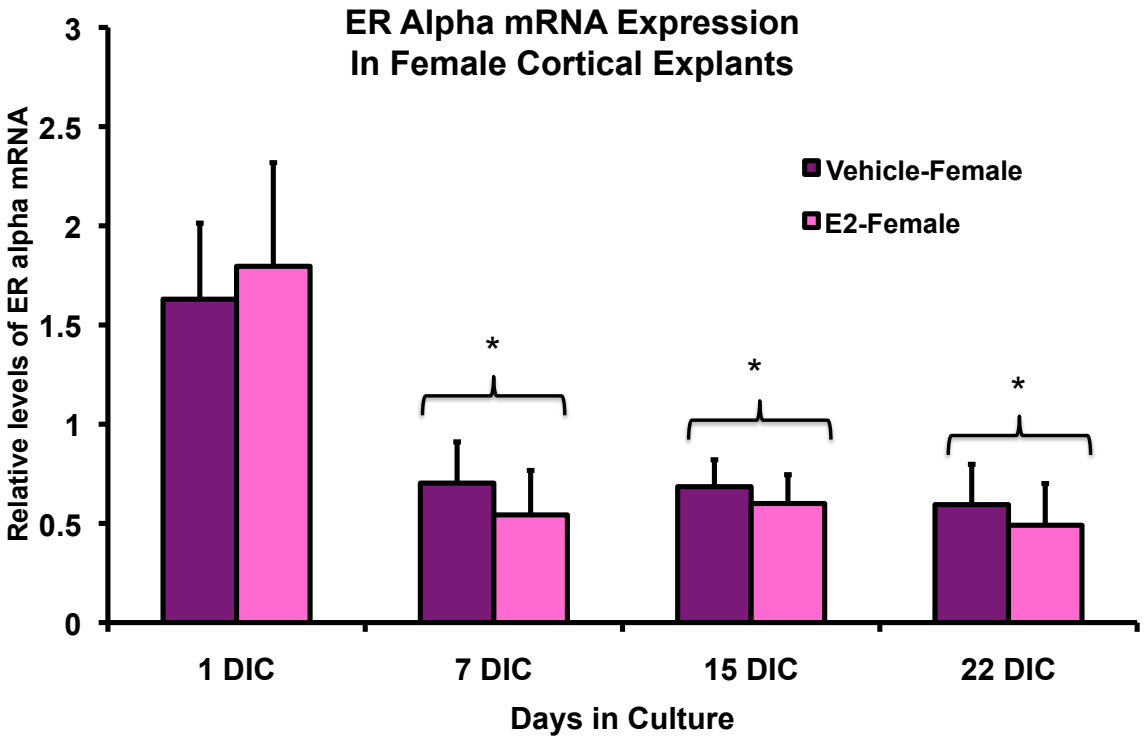


Figure 2.7. ERα mRNA expression in female cortical explants grown in culture. ERα mRNA decreased across time in culture in vehicle and E2-treated female cortical explants. There was no treatment difference in ERα mRNA expression at any of the postnatal days. The graph above shows ERα relative to the housekeeping gene histone 3.1. Ct values from each postnatal day are compared to 1DIC vehicle-treated females explants. Asterisks on the graph indicate significant decreases at 7, 15, and 22 DIC, $p < 0.05$.

In male cortical explants, ERα mRNA expression decreased across time in culture (1 to 22 DIC) and E2 influenced the expression. This is different from what we see in female cortical explants (Figure 2.7), because female cortical explants did not have an effect of treatment. For the **male** explants grown in media containing vehicle and E2, a two-way ANOVA comparing the factors "treatment" and "DIC" revealed a significant main effect of DIC ($p=0.0345$, $F=3.091$, $df= 3, 54$) and of treatment ($p=0.0190$, $F= 5.852$, $df= 1, 54$), but no interaction. Vehicle (EtOH)-treated male cortical explants had an increased ERα mRNA expression compared to E2-treated male cortical explants. To determine which DIC were different a Student Newman-Keuls post-hoc test was performed and revealed that 1 DIC was significantly greater than 22 DIC when both E2 and vehicle were collectively compared against 1 DIC ($p< 0.05$), indicated by an asterisk in Figure 2.8. Overall, male cortical explants had a decrease in ERα mRNA expression and E2-treatment significantly lowered this expression.

To look at relative levels of mRNA expression, not a change compared back to vehicle-treated explants grown for 1 DIC, the ratio of housekeeping Ct values (average specific PND housekeeping Ct value/ sample housekeeping Ct value) was multiplied by the Ct value for the gene of interest for the same sample. The average Ct values for ERα and His 3.1 are located in Table 2.7 for vehicle and E2 treated female and male cortical explants at each time-point. This table highlights ERα Ct values normalized to average histones that can be compared across PND. For the female and male cortical explants, ERα Ct values decreased across PND. ERα Ct value normalized to average histone in

both female and male were not significantly different from each other, but they did change significantly over time, represented in Figures 2.7-2.8.

To look at relative levels of mRNA expression, not a change compared back to 1 DIC, the ratio of housekeeping Ct values (average specific 1 DIC housekeeping Ct value/ sample housekeeping Ct value) was multiplied by the Ct value for the gene of interest for the same sample, Table 2.7. I have included average Ct values for ERα and His 3.1 for female and males at each time-point, Table 2.7. This chart highlights ERα Ct values normalized to average histones that can be compared across PND. For the female and male cortex, ERα Ct values showed that there was a decrease in mRNA expression across time in culture. The graphs, Figure 2.7-2.8, have Ct values logarithmically expressed relative to PND 4; however, the table has raw ERα Ct values normalized to average histone Ct values that can be compared across 1 DIC. The chart Ct values show a substantial change in Ct values that are less than 30. For real time PCR, the lower the Ct values the more mRNA and changes that are less than 30 are considered "real." Ct values greater than 30 occur during the plateau phase of the logarithmic PCR cycle where there is an increase chance of error (reviewed in (Wong and Medrano 2005)). The chart Ct values correspond to the logarithmic change seen in the graphs. For the female and male cortex, ERα Ct values showed a decrease across DIC.

As demonstrated in mice cortical explants, ERα mRNA expression decreased in the cortical explants across time in culture in both female and male rat cortical explants. These data suggest that the expression patterns of steroid

hormone receptors also change in rat cortical explants, which confirms our hypothesis that ERα mRNA expression will decrease across time in culture.

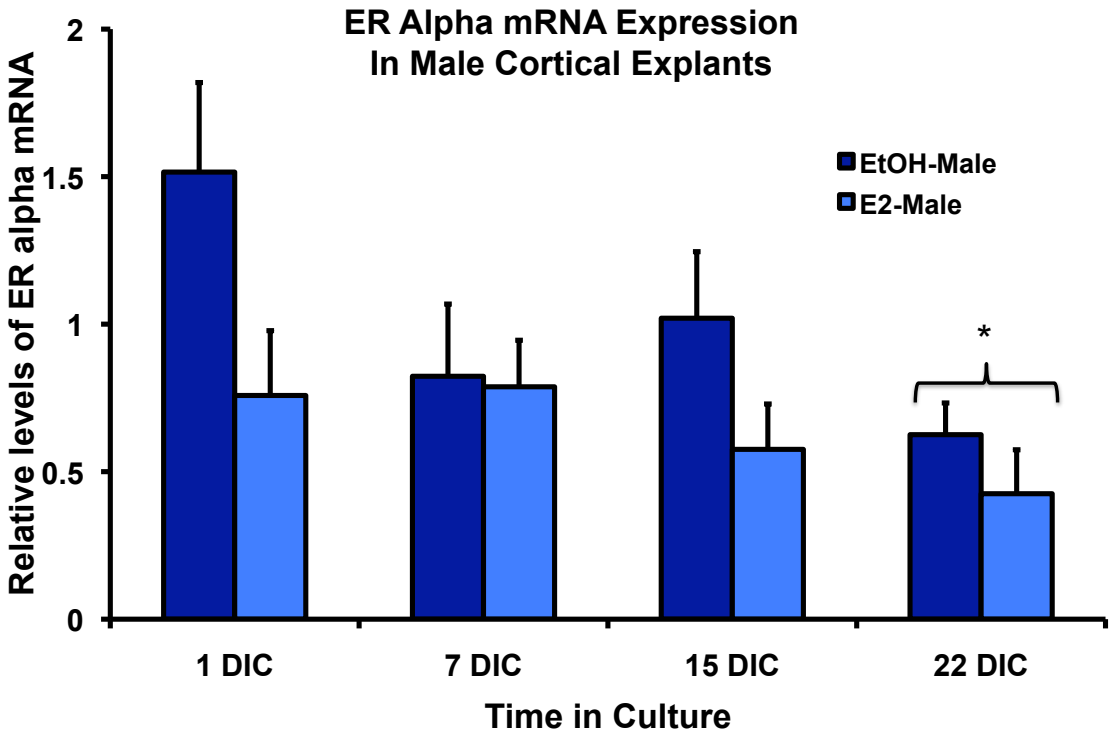


Figure 2.8. ERα mRNA expression in male cortical explants. ERα mRNA decreased across time in culture in vehicle and E2-treated male cortical explants. ERα mRNA expression at 1 DIC was significantly greater than at 22 DIC for both E2 and vehicle-treated explants, indicated by an asterisk, $p < 0.05$. E2 treatment significantly lowered ERα mRNA expression compared to vehicle, not indicated on the graph.

Table 2.7 Average real time PCR Ct values from housekeeping gene histone 3.1 and ERα in cortical explants grown for 1, 7, 15 and 22 days in culture (DIC). Bolded box on table A and B (females) and table C and D (males) indicates ERα Ct values normalized to average histones for each postnatal day. Note: higher Ct values indicate a lower expression level of mRNA by representing a greater number of PCR cycles to reach a maximum.

A

Sex	DIC	EtOH Treated Average Ct Value		Ct value normalize to average histone
		Histone	ER alpha	
Female	1	18.94	26.34	25.77
	7	18.54	27.33	26.37
	15	20.19	27.61	27.63
	22	19.69	28.84	28.58

B

Sex	DIC	E2 Treated Average Ct Value		Ct value normalize to average histone
		Histone	ER alpha	
Female	1	19.46	26.23	26.34
	7	18.45	28.16	26.83
	15	19.68	27.37	27.42
	22	19.45	29.85	29.87

C

Sex	DIC	EtOH Treated Average Ct Value		Ct value normalize to average histone
		Histone	ER alpha	
Male	1	19.85	26.21	26.43
	7	18.78	26.72	25.91
	15	19.77	26.58	26.19
	22	19.84	27.33	27.27

D

Sex	DIC	E2 Treated Average Ct Value		Ct value normalize to average histone
		Histone	ER alpha	
Male	1	19.04	26.44	26.59
	7	19.72	26.72	25.59
	15	19.53	26.72	26.88
	22	19.51	28.33	28.19

ERβ

Does E2 influence ERβ mRNA expression in rat cortical explants across time in culture?

To answer this question, we analyzed mRNA expression by statistically comparing treatment (EtOH and E2) and days in culture (DIC) separately for female cortical explants and male cortical explants. ERβ mRNA expression significantly increased in both vehicle and E2-treated female and vehicle and E2-treated male cortical explants across time in culture.

In **female** cortical explants, ERβ mRNA expression increased in cortical explants across time in culture (1 to 22 DIC), but treatment in the feed media is not a factor. For the female explants grown in media containing vehicle and E2, a two-way ANOVA comparing the factors "treatment" and "DIC" revealed a significant main effect of DIC ($p= 0.0043$, $F= 4.815$, $df= 3, 66$), but no main effect of treatment and no interaction. To determine which DIC were different a Student Neuman-Keuls post-hoc test was conducted and revealed that ERβ mRNA expression was significantly increased at 15 DIC compared to 1 DIC ($p< 0.05$) and significantly increased at 15 and 22 DIC compared to 7 DIC ($p< 0.05$) (Figure 2.9). These significant differences include data from both vehicle and E2 treated female cortical explants because there was not an effect of treatment. Overall, these data demonstrate that ERβ mRNA expression increased across time in culture in female rat cortical explants regardless if E2 was present in the feed media.

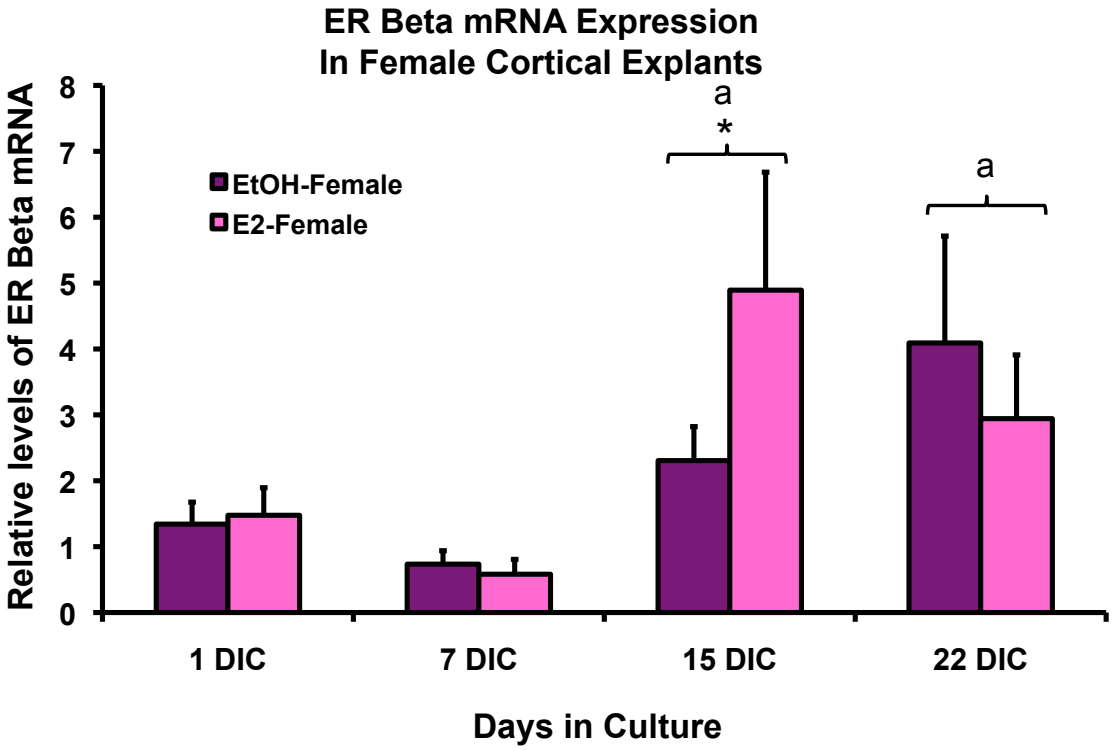


Figure 2.9. ERβ mRNA expression in female cortical explants. ERβ mRNA increased across time in culture in vehicle and estrogen-treated cortical explants. The graph above shows ERβ relative to the housekeeping gene histone 3.1. Ct values from each postnatal day are compared to 1 DIC for each media treatment. Significant increases at 15 DIC compared to 1 DIC are indicated by asterisks on the graph and significant increases at 15 and 22 DIC compared to 7 DIC are indicated by an "a", $p < 0.05$.

In **male** cortical explants, ERβ mRNA expression increased in cortical explants across time in culture (1 to 22 DIC), but treatment in the feed media is not a factor. For the male explants grown in media containing vehicle and E2, a two-way ANOVA comparing the factors "treatment" and "DIC" revealed a significant main effect of DIC ($p < 0.0001$, $F = 8.718$, $df = 3, 63$), but no main effect of treatment and no interaction. To determine which DIC were different a Student Newman-Keuls post-hoc test was conducted and revealed that ERβ mRNA expression significantly increased at 15 and 22 DIC compared to 1 and 7 DIC ($p < 0.05$) (Figure 2.10). These significant differences include data from both vehicle and E2 treated male cortical explants because there was not an effect of treatment. Overall, male cortical explants had an increase in ERβ mRNA expression across time in culture.

To look at relative levels of mRNA expression, not a change compared back to 1 DIC, the ratio of housekeeping Ct values (average specific 1 DIC housekeeping Ct value/ sample housekeeping Ct value) was multiplied by the Ct value for the gene of interest for the same sample, Table 2.8. I have included average Ct values for ERβ and His 3.1 for female and males at each time-point, Table 2.8. This chart highlights ERβ Ct values normalized to average histones that can be compared across PND. For the female and male cortex, ERβ Ct values showed that there was a decrease in mRNA expression across time in culture. The graphs, Figure 2.9-2.10, have Ct values logarithmically expressed relative to PND 4; however, the table has raw ERβ Ct values normalized to average histone Ct values that can be compared across 1 DIC. The chart Ct

values show a substantial change in Ct values that are less than 30. For real time PCR, the lower the Ct values the more mRNA and changes that are less than 30 are considered “real.” Ct values greater than 30 occur during the plateau phase of the logarithmic PCR cycle where there is an increase chance of error (reviewed in (Wong and Medrano 2005)). The chart Ct values correspond to the logarithmic change seen in the graphs. For the female and male cortex, ERβ Ct values showed an increase across DIC.

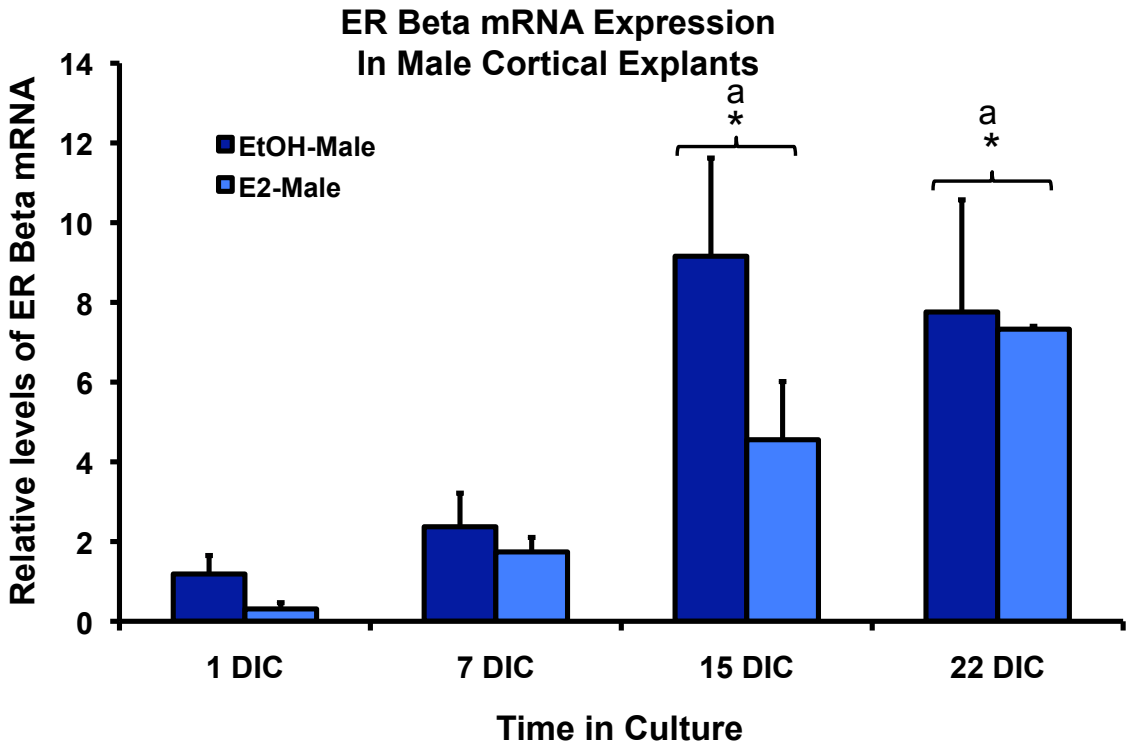


Figure 2.10. ERβ mRNA expression in male cortical explants. ERβ mRNA increased across time in culture in vehicle and E2-treated male cortical explants. The graph above shows ERβ relative to the housekeeping gene histone 3.1. Ct values from each postnatal day are compared to 1 DIC for each media treatment. Asterisk on the graph indicate significant increases from PND 4, $p<0.05$. The letter "a" on the graph indicates significant increases from PND 10, $p< 0.05$.

Table 2.8. Average real time PCR Ct values from housekeeping gene histone 3.1 and ERβ in cortical explants grown for 1, 7, 15 and 22 days in culture (DIC). Bolded box on table A and B (females) and table C and D (males) indicates ERβ Ct values normalized to average histones for each postnatal day. Note: higher Ct values indicate a lower expression level of mRNA by representing a greater number of PCR cycles to reach a maximum.

A.

Sex	PND	EtOH Treated Average Ct Value		Ct value normalized to average histone
		Histone	ER beta	
Female	4	18.94	30.16	30.83
	10	19.98	31.41	30.61
	18	20.27	29.57	29.59
	25	19.74	29.46	29.53

B.

Sex	PND	E2 Treated Average Ct Value		Ct value normalized to average histone
		Histone	ER beta	
Female	4	19.28	30.20	29.62
	10	18.44	32.99	30.60
	18	19.82	28.62	28.68
	25	19.45	29.03	29.10

C.

Sex	PND	EtOH Treated Average Ct Value		Ct value normalized to average histone
		Histone	ER beta	
Male	4	19.67	30.47	30.49
	10	19.97	30.64	30.16
	18	20.29	29.22	29.17
	25	20.14	29.19	29.36

D.

Sex	PND	E2 Treated Average Ct Value		Ct value normalized to average histone
		Histone	ER beta	
Male	4	19.04	31.9	31.93
	10	20.45	30.21	30.46
	18	19.51	29.81	30.01
	25	19.51	39.91	29.58

AR

Does E2 influence AR mRNA expression in rat cortical explants across time in culture?

We analyzed mRNA expression by statistically comparing treatment (EtOH and E2) and days in culture (DIC) separately for female cortical explants and male cortical explants. AR mRNA expression significantly increased in female and male cortical explants that were EtOH and E2 treated.

In female cortical explants, AR mRNA expression increased across time in culture (1 to 22 DIC), but treatment in the feed media is not a factor. For the **female** explants grown in media containing vehicle and E2, a two-way ANOVA comparing the factors “treatment” and “DIC” revealed a significant main effect of DIC ($p < 0.0001$, $F = 11.610$, $df = 3, 65$), but no main effect of treatment and no interaction. To determine which DIC were different a Student Neuman-Keuls post-hoc test was conducted and revealed that AR mRNA expression was significantly increased at 15 and 22 DIC compared to 1 and 7 DIC ($p < 0.05$), Figure 2.11.

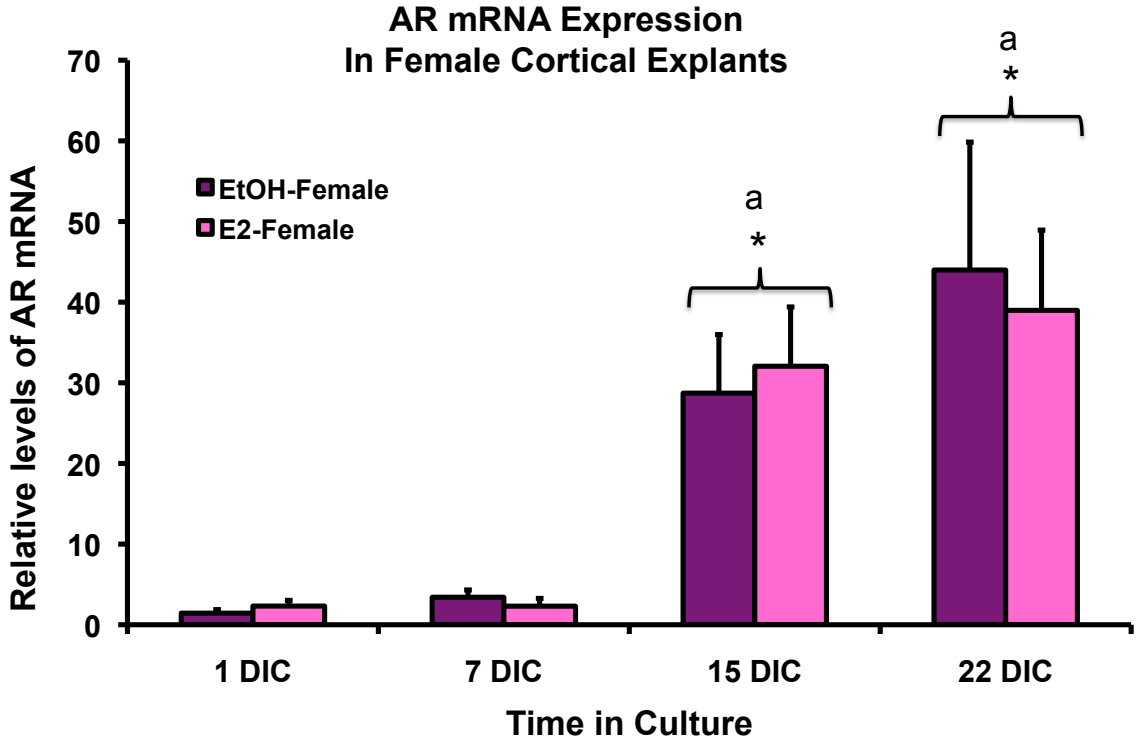


Figure 2.11. Androgen receptor mRNA expression in female cortical explants. AR mRNA increased across time in culture in vehicle and E2-treated cortical explants. The graph above shows AR relative to the housekeeping gene histone 3.1. Ct values from each postnatal day are compared to 1 DIC for each media treatment. Asterisk on the graph indicate significant increases from PND 4, $p<0.05$. The letter "a" on the graph indicates significant increases from PND 10, $p< 0.05$.

In male cortical explants, AR mRNA expression increased across time in culture (1 to 22 DIC) and E2 did influence the expression. For the **male** explants grown in media containing vehicle and E2, a two-way ANOVA comparing the factors “treatment” and “DIC” revealed a significant main effect of DIC ($p< 0.0001$, $F= 11.093$, $df= 3, 58$), but no main effect of treatment and no interaction. To determine which DIC were different a Student Newman-Keuls post-hoc test was conducted and revealed that AR mRNA expression significantly increased at 15 and 22 DIC compared to 1 and 7 DIC and 15 DIC to 22 DIC ($p< 0.05$), Figure 2.12. These significant differences include data from both vehicle and E2 treated male cortical explants because there was not an effect of treatment. Overall, male cortical explants had an increase in AR mRNA expression.

To look at relative levels of mRNA expression, not a change compared back to 1 DIC, the ratio of housekeeping Ct values (average specific 1 DIC housekeeping Ct value/ sample housekeeping Ct value) was multiplied by the Ct value for the gene of interest for the same sample, Table 2.9. I have included average Ct values for AR and His 3.1 for female and males at each time-point, Table 2.9. This chart highlights AR Ct values normalized to average histones that can be compared across PND. For the female and male cortex, AR Ct values showed that there was a decrease in mRNA expression across time in culture. The graphs, Figure 2.11-2.12, have Ct values logarithmically expressed relative to 1 DIC; however, the table has raw AR Ct values normalized to average histone Ct values that can be compared across 1 DIC. The chart Ct values show a substantial change in Ct values that are less than 30. For real time PCR, the

lower the Ct values the more mRNA and changes that are less than 30 are considered “real.” Ct values greater than 30 occur during the plateau phase of the logarithmic PCR cycle where there is an increase chance of error (reviewed in (Wong and Medrano 2005)). The chart Ct values correspond to the logarithmic change seen in the graphs. For the female and male cortex, AR Ct values showed an increase across DIC.

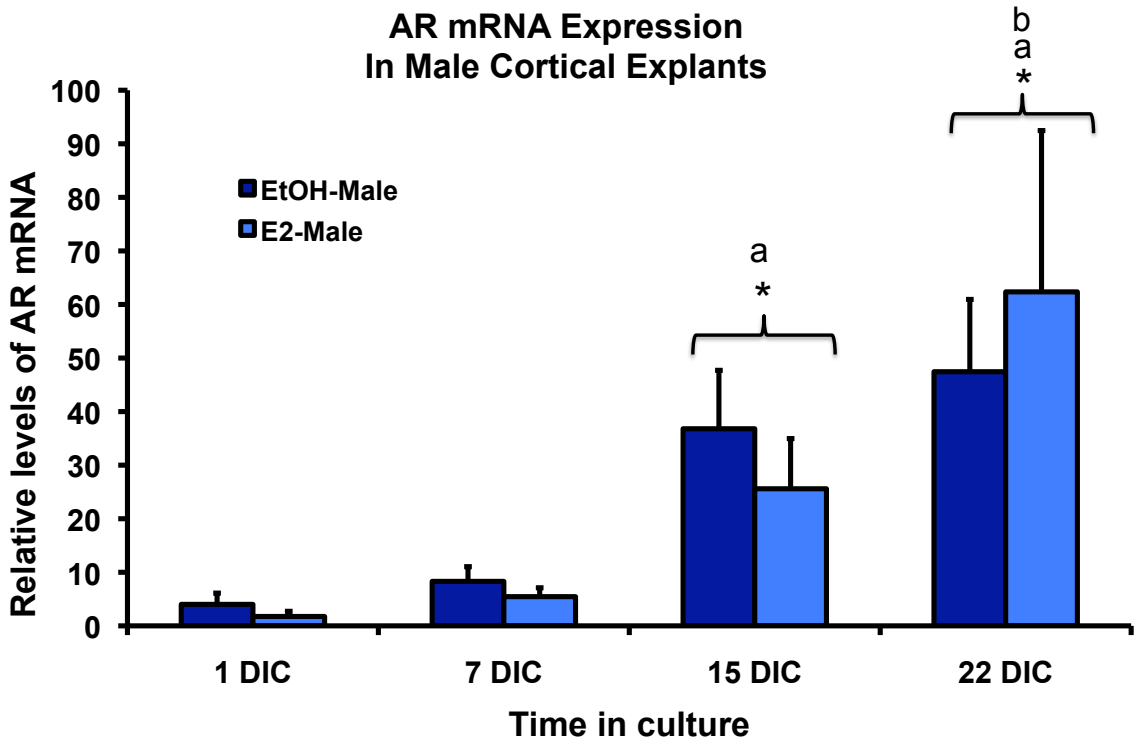


Figure 2.12. Androgen receptor mRNA expression in male cortical explants. AR mRNA increased across time in culture in vehicle and E2-treated male explants.The graph above shows AR relative to the housekeeping gene histone 3.1. Ct values from each postnatal day are compared to 1 DIC for each media treatment. Asterisk on the graph indicate significant increases from PND 4, $p<0.05$. The letter “a” on the graph indicates significant increases from PND 10, $p< 0.05$. The letter “b” on the graph indicates significant increases from PND 18, $p< 0.05$.

Table 2.9 Average real time PCR Ct values from housekeeping gene histone 3.1 and AR in cortical explants grown for 1, 7, 15 and 22 days in culture (DIC). Bolded box on table A and B (females) and table C and D (males) indicates AR Ct values normalized to average histones for each postnatal day. Note: higher Ct values indicate a lower expression level of mRNA by representing a greater number of PCR cycles to reach a maximum.

A.

Sex	DIC	EtOH Treated Average Ct Value		Ct value normalized to average histone
		Histone	AR	
Female	1	18.94	32.33	32.54
	7	18.71	30.83	30.37
	15	20.27	28.38	28.42
	22	19.9	29.33	29.58

B.

Sex	DIC	Estrogen Treated Average Ct Value		Ct value normalized to average histone
		Histone	AR	
Female	1	19.25	31.20	31.57
	7	18.45	31.82	30.47
	15	19.68	27.58	27.61
	22	19.45	28.98	29.10

C.

Sex	DIC	EtOH Treated Average Ct Value		Ct value normalized to average histone
		Histone	AR	
Male	1	19.77	32.18	33.15
	7	19.38	29.22	29.47
	15	19.77	28.29	27.03
	22	20.13	28.04	28.24

D.

Sex	DIC	Estrogen Treated Average Ct Value		Ct value normalized to average histone
		Histone	AR	
Male	1	19.04	33.34	33.60
	7	20.1	29.99	30.72
	15	19.57	28.69	28.89
	22	19.51	29.03	27.66

Discussion

In these studies, I compared expression of three sex steroid hormone receptors, ER alpha (ERα), ER beta (ERβ) and androgen receptor (AR) in the rat cortex and in cortical explants taken from male and female rats. My first goal was to test the hypothesis that these sex steroid hormone receptors were regulated similarly during postnatal development that was previously published in the mouse. My second goal was to test the hypothesis that these sex steroid hormone receptors change across time in culture in rat cortical explants when the cortex is isolated away from other regions of the brain. By understanding the expression patterns of steroid hormone receptors during postnatal development, we can potentially understand how they are regulated at other times during an animal's lifetime due to the theory that the brain reverts back to its developmental state to aid in repair following brain injury (Emery, Royo et al. 2003).

As expected in the female and male rat cortex, ERα mRNA expression significantly decreased, ERβ mRNA expression significantly increased and AR mRNA expression also significantly increased all compared to a housekeeping gene across early postnatal development, Table 2.10. This confirms our hypothesis that the mRNA for these sex steroid hormone receptors changes similarly during rat postnatal development that was previously published in the mouse. Specifically, ERα mRNA significantly decreased at PND 18, ERβ mRNA expression significantly increased at PND 25 and AR mRNA expression significantly increased at PND 18, Table 2.10. For these sex steroid hormone receptors there was no sex difference across postnatal development. This

Table 2.10. Summary Table of Sex Steroid Hormone Receptors. The expression pattern for ERα, ERβ and AR mRNA across early postnatal development in the female and male cortex and changes in expression patterns across time in culture in cortical explants take female and male rat pups were summarized.

	Female		Male	
	Cortex	Cortical Explants	Cortex	Cortical Explants
ERα mRNA expression	↓	↓	↓	↓
ERβ mRNA expression	↑	↑	↑	↑
AR mRNA expression	↑	↑	↑	↑

follows a similar pattern of what was previously published (Prewitt and Wilson 2007).

As expected in cortical explants taken from either female or male rats, ERα mRNA expression significantly decreased, ERβ mRNA expression significantly increased and AR mRNA expression also significantly increased across time in culture, Table 2.10. This confirms our second hypothesis that these sex steroid hormone receptors would change across time in culture. This validates the explant model by showing the cortex, when isolated away from other regions of the brain, can maintain its dynamic regulation of the sex steroid hormone receptors mRNA expression. Specifically, ERα mRNA significantly decreased in female cortical explants at 7 DIC, regardless of treatment, Figures 2.7. However, when vehicle and E2-treated males explants were compared there was a significant effect of treatment, where E2 treatment decreased ERα mRNA expression compared to vehicle treatment. There was also a significant decrease in ERα mRNA expression at 22 DIC in male cortical explants, Figure 2.8. Sex differences cannot be compared directly because female and male cortical explants were not analyzed directly. They were compared within sex looking at the effect of treatment. In female cortical explants, ERα mRNA expression significantly decreased at 7 DIC, where male cortical explants did not have a significant decrease until 22 DIC. E2 also decreased ERα mRNA expression in males, but did have an effect in female cortical explants.

ERβ mRNA significantly increased at 15 DIC when vehicle and E2 treated female or male explants are compared independently, Figures 2.9-2.10. AR

mRNA expression significantly increased at 15 DIC, regardless of sex or treatment.

The additional presence of E2 in the feed media only alters ERα mRNA expression in only male cortical explants. This could be due to a change in the hormonal environment that the male cortical explants experience from the time of plating that is different from in the intact brain, *in vivo.* Males do not have alpha-fetal protein to bind to E2 and prevent it from crossing the blood brain barrier. Therefore E2 masculinizes the brain in male rodents. The E2 concentration in the intact cortex may vary from the E2 concentration in the feed media, which causes ERα mRNA expression to be lower compared its vehicle control. Since ERα is high while ERβ and AR mRNA expression are low at the time of plating, ERα mRNA expression could be more susceptible to an alteration in hormonal environment and decrease in mRNA expression with the presence or change in concentration of E2.

Sex steroid hormone protein expression was not correlated to the mRNA expression. Protein, not mRNA, expression can directly correlate to function. Changes in ERα, ERβ and AR mRNA expression have been correlated to changes in protein expression. Ideally protein and mRNA expression would have been measured across early postnatal development in the intact cortex and across time in culture in cortical explants instead of just mRNA expression. However due to lack of specificity in antibodies for ERβ and AR western blots (Skliris, Parkes et al. 2002) (Snyder, Smejkalova et al. 2010), protein expression could not be accurately measured. Specifically, ERβ protein was detected in an

ERβ knockout where there was no ERβ expressed (Snyder, Smejkalova et al. 2010).

The cytoarchitecture of the cortex is known to change and mature during postnatal development. Steroid hormones and their receptors influence this cytoarchitecture by influencing synaptogenesis (Mong, Roberts et al. 2001), cell differentiation (De Vries, Rissman et al. 2002; Simerly 2002), apoptosis (Chung, Swaab et al. 2000; Forger 2006), neurite outgrowth (Toran-Allerand 1976; Toran-Allerand 1976), connectivity and migration (Parent, Naveau et al. 2011; Peper, Hulshoff Pol et al. 2011; Peper, van den Heuvel et al. 2011). Specifically the higher expression of ERβ and AR that occurs from postnatal day 18 to 25 correlates with steroidogenesis that is beginning in the sex organs during the second postnatal week (Carson and Smith 1986; Dufau, Khanum et al. 1987). The brain is sexual differentiated around PND 18 when an enzyme (aromatase) that converts testosterone, from steroidogenesis in males, to estrogen is present (Green and Simpkins 2000). Estrogen produced from steroidogenesis in females is bound to alpha-fetoprotein, which inhibits its access across the blood brain barrier (Keller, Pawluski et al. 2010). However, the male brain is masculinized by the presence of estrogen and the lack of alpha-fetoprotein (Keller, Pawluski et al. 2010). Although the male brain is believe to be masculinized by estrogen and the female brain by the lack there of, the amount of *de novo* estrogen production is variable. The enzymes for de novo estrogen production are present throughout development (Ibanez, Guennoun et al. 2003; Tsutsui 2008; Nagarajan, Tsai et al. 2011) and can be compensating for the presence of alpha-

fetoprotein by locally producing estrogen in the female brain. The first two weeks of postnatal development in the cortex are when cells are migrating and forming neuronal circuits within the cortex and with other regions of the brain. The increase in steroid hormones and their receptors correlated with the cellular organization of the cortex. Disruption of these hormones and receptors cause delays in cell death and disruption in cytoarchitecture (Nunez, Jurgens et al. 2000; Nunez, Lauschke et al. 2001; Nunez, Huppenbauer et al. 2003). The concentrations of hormones present across postnatal development vary depending on brain region. For example, the hypothalamus had higher E2 concentrations than the cortex, which had higher concentrations than the hippocampus. These changes in E2 concentration that occurred in the brain across the first 10 days of life were not sex specific in the hypothalamus or the hippocampus. However, there were sex differences in E2 concentration in the cortex. Specifically, males had a peak concentration of E2 in the cortex on PND 4, while females did not peak until PND 6. Following the initial sex difference in days when E2 concentrations peaked, both sexes had a similar pattern of decrease in E2 across postnatal development. Interestingly, females and males that were adrenalectomized and gonadectomized before they were 12 hours old, also maintained a similar hormonal pattern in the brain to animals with intact adrenal glands and gonads when they were 3 days old. These data demonstrated that the brain regulates hormone concentrations independently of the rest of the body. This may be a protective mechanism to ensure the brain receives a certain amount of hormones independently of the body to ensure

proper brain development. Cortical explants also appear to be maintaining homeostasis by regulating sex steroid hormone receptor expression when changes occur in the hormone environment, such as a difference in ERα mRNA expression seen in male cortical explants in the presence of E2.

ERα mRNA expression, in female cortical explants, significantly decreases earlier (7 DIC) than intact cortex. In male cortical explants, ERα mRNA decreased later (22 DIC) compared to the intact cortex (PND 18). Changes in ERβ mRNA expression were also seen earlier in cortical explants, 15 DIC, than across cortical development. AR has a similar increase in mRNA expression in cortical explants, 15 DIC, and *in vivo*, PND 18. In general, cortical explants had a similar change in expression in ERα, ERβ and AR that is seen in cortical development, *in vivo*. However, generally we see a significant change in mRNA expression earlier when cortical explants are grown in culture. This could be due to numerous possibilities. One possibility is a lack of inhibitory cues from other parts of the brain such as the striatum, a region of the brain that located directly next to the cortex (Figure 2.1). The axons from the cortex not only project into the striatum and back into the cortex, but also to the thalamus and brainstem (Rosell and Gimenez-Amaya 1999; Hooks, Hires et al. 2011; Mao, Kusefoglu et al. 2011).

Another possibility is the change of nutrients that the cortical explants receive compared to the cortex during development. In cortical development there is a vast blood supply with numerous growth factors. Cortical explants are isolated from this blood supply and grown on millicell membranes with access to

feed media. So it is important to note that while there are differences we can correlate the overall expression patterns. The changes that occur across time in culture will allow us to evaluate steroid hormones and how they affect steroid hormone receptors across development and following injury.

One limitation of the cortical explant model is determining the relative age of the cortical explant. The explants are taking out of a PND 3 rat pup and allowed to "age" in culture. When the organization of the neurons and glia cells in the cortex of mature animals are compared to explants taken from PND3 rat pups and "aged," the glia and neuronal cells due not exhibit the same pattern of organization (Staal, Alexander et al. 2011). Explants isolated from neonatal animals and grown in culture have increased glia expression and loss of cytoarchitectural neuronal organization of the different layers of the cortex compared to the cortex taken from a mature animal (Staal, Alexander et al. 2011). Aged cortical explants have a loss of glia limitans and display additional growth on the periphery of slice from possible progenitor like cells (Staal, Alexander et al. 2011). The have, however, been shown to be a reliable model for studying injury (Wise, Dubal et al. 2000; Wilson, Liu et al. 2002)

The cortical explant model allows us to look at a specific region of the brain, the cortex, with out input from other regions of the brain. This is important because the cortex is a region of the brain that is protected by estrogen following injury. This model allows us to isolate the cortex and look at changes across development and to simplify an injury and determine if the neuroprotection is endogenous to the cortex or if the cortex requires input from other regions of the

brain. The explant model is unique because it is a heterogeneous population of neurons and glial cells that other *in vitro* models do not incorporate. The neuronal glial cross talk is essential to take into consideration in any model of the brain because these cells work together in development and in injury.

The expression of 3 sex steroid hormone receptors ERα, ERβ and AR were described across postnatal development in the cortex and across time in culture in cortical explants. My first hypothesis was confirmed that these sex steroid hormone receptors were regulated similarly during postnatal development that was previously published in the mouse. My second hypothesis was also confirmed that these sex steroid hormone receptors also change across time in culture in rat cortical explants. By understanding the expression patterns of steroid hormone receptors during postnatal development, we can potentially understand how they are regulated at other times during an animal's lifetime, ie following brain injury. Specifically the cortical explant model will allow us to evaluate mechanism of neuroprotection inside the cortex and allow us to pharmacologically manipulate only the cortex to further outline the mechanisms involved in neuroprotection.

CHAPTER 3

SEX DIFFERENCES AND ESTROGEN-MEDIATED NEUROPROTECTION IN MODEL OF ISCHEMIA

Introduction

Brain ischemia occurs when the brain is deprived of blood flow, which results in an inadequate supply of oxygen and glucose. Tissues need oxygen and glucose to meet their metabolic cellular demands. Insufficient blood supply for seconds in the brain can result in cell death and tissue damage. This ischemia-induced cell death is only one type of stroke, but occurs the majority (87%) of the time, while the other 13% of strokes are hemorrhagic (Roger, Go et al. 2012). Ischemia can be classified as focal, which is confined to a specific region of the brain, or global, which involves wide areas of brain tissue. Our lab has used an animal model to study focal ischemia caused by middle cerebral artery occlusion (MCAO). As described in the General Introduction, MCAO causes a significant reduction in cerebral blood flow to the striatum and over laying cortex (Dubal, Kashon et al. 1998). This decrease in blood supply leads to necrotic cell death in the striatum followed by apoptotic cell death in the over laying cortex (Liu, Smith et al. 1999).

The effects of ischemic cell death are heavily influenced by an animal's steroid hormone background. Following MCAO, gonadectomized females (Simpkins, Rajakumar et al. 1997; Dubal, Kashon et al. 1998; Toung, Traystman

et al. 1998; Rusa, Alkayed et al. 1999; Dubal and Wise 2001) and males (Toung, Traystman et al. 1998; Uchida, Palmateer et al. 2009) have a much larger MCAO-induced injury than animals with higher circulating estrogen concentrations. Pretreatment with even low doses of E2 is sufficient to exert dramatic protection in the brains of both female (Dubal, Kashon et al. 1998; Dubal and Wise 2001) and male rats (Toung, Traystman et al. 1998). In humans, the role of hormone replacement is not as clear. In fact, some clinical studies found that estrogens were not beneficial at all (Wilson, Garrison et al. 1985; Grodstein, Stampfer et al. 1996; Petitti, Sidney et al. 1998; Anderson, Limacher et al. 2004), while other studies did report benefits following stroke (reviewed in (Behl 2002; McCullough and Hurn 2003)). The discrepancies between different studies may in part be explained by differences in the timing of estrogen replacement and the age of women included in the studies. As described above, animal studies are much less controversial and provide a simpler model to study the mechanisms of neuroprotection by E2 following ischemic brain injury.

An important factor in the extent of brain damage following ischemic injury in animal models is the level and expression pattern of estrogen receptors (ER). For example, generalized pharmacologic blockade of ER exacerbates ischemic injury in mice (Sawada, Alkayed et al. 2000) and blocks estrogen-induced neuroprotection in cultured neurons (Singer, Figueroa-Masot et al. 1999; Wilson, Dubal et al. 2000) and in cortical explant cultures (Wilson, Dubal et al. 2000). Specifically, ERα and not ERβ seem to be very important, while AR has not been

evaluated. Studies using ERα knockout females demonstrate that neuroprotection by E2 following ischemia is dependent on the presence of ERα in the cortex (Dubal, Zhu et al. 2001), and that ERβ alone is not sufficient for neuroprotection in females. As described in chapter 2, ERα is only transiently expressed in the cortex during early postnatal development and then is virtually absent in the uninjured adult cortex (Miranda and Toran-Allerand 1992; Prewitt and Wilson 2007). 24 hours after MCAO, however, ERα mRNA and protein are increased in the cortex of female rats and mice (Dubal, Shughrue et al. 1999; Dubal, Rau et al. 2006). In OVX females, the increase in ERα mRNA occurs in both oil and E2-treated groups, but is seen earlier after injury with E2 (Dubal, Rau et al. 2006). These data suggest that in females, the ischemia-induced increase in ERα in the cortex is necessary for the neuroprotective effects of E2. Interestingly, although male rodents can be protected by E2 (depending on the concentration and time frame of treatment), ERα expression did not change following injury in gonadally intact males (Westberry, Prewitt et al. 2008). The previous animal studies show a clear role for E2 and estrogen receptors in neuroprotection following MCAO. This model is quite complicated and does not allow us to focus on understanding mechanisms specific to the cortex.

For the studies included in this chapter, I will use the *in vitro* cortical explant model described in chapter 2 as a simplified way to discern sex-specific mechanisms of neuroprotection that are innate to the cortex. This model is better than using isolated neuronal cultures because it maintains the important neuronal/glial relationship. Here, cortical cultures were separated based on sex

and treated with 2-deoxyglucose and potassium cyanide (2DG-KCN) to block glycolysis and oxidative phosphorylation, which simulates ischemia. Previous studies from our lab have used this model to study injury by treating cortical explants grown for one week in culture with 2DG-KCN and showed that pretreatment of E2 attenuated this cell death (Wilson, Dubal et al. 2000; Wilson, Liu et al. 2002). Those studies did not look at sex-specific changes in cell death or response of receptors. With our current knowledge of sex differences in cell death and changes in receptors with response to injury, we felt it essential to repeat those studies separating cortical explants from female and male pups.

In the first part of this chapter, I wanted to test the hypothesis that 2DG/KCN treatment increased cell death and determine if increased cell death occurred in a sex-specific manner. I also wanted to measure the influence of E2 pre-treatment on 2DG/KCN induced cell death. In the second part of the chapter, the experiments were designed to determine how injury affects steroid hormone receptor expression and how E2 influenced changes in expression. To accomplish these goals, I evaluated cell death by measuring propidium iodide uptake and measured changes in sex steroid hormone receptors following treatment with 2DG/KCN in both female and male cortical explants separately. By understanding the mechanisms of E2 neuroprotection in cortical explants and the regulation and expression patterns of steroid hormone receptors following injury, I plan to pharmacologically manipulate this protection in the explants to outline pathways that are involved.

Methods

Injury

To induce an ischemic like injury, explants were treated with 1 mM 2-DG (D8375, Sigma-aldrich, Saint Louis, MO) and 0.5 mM KCN (207810, Sigma-aldrich, Saint Louis, MO) for 2 hours, 2 mM 2-DG and 1 mM KCN for 2 hours or 2 mM 2-DG and 1 mM KCN for 1 hour in 1X Basal Medium Eagle, BME, (injury) or BME (control). 2DG/KCN chemically mimics "ischemic" conditions by inhibiting glycolysis and oxidative phosphorylation (Wilson, Liu et al. 2002). Following injury, media was replaced. At several time-points after injury (4, 8, 12 and 24 hours), explants were processed for RNA isolation or treated with propidium iodide to determine the extent of cell death.

Cell Death

To measure cell death, explants were washed with 0.1 M PBS and incubated with 5 μg/ml of propidium iodide (PI) (P4170, Sigma-Aldrich, Saint Louis, MO) (stock concentration of 1mg/mL in H2O) in BME for 30 minutes at 4, 8, 12 and 24 hours following injury. The PI was washed off with 0.1M PBS. Explants were visualized using a fluorescent microscope. PI entered cells that had a porous cell membranes, indicating damage, and bound to DNA. PI uptake indicated cell death and fluoresced red (emission at 630 nm) under green light (excited at 495 nm). Pictures, 20X magnification of explants, were captured using an image capture program, SPOT Advanced. Red (dead) cells per frame

were then counted using a NIH program, Image J. Pictures were coded and analyzed blindly. Injured explants were compared back to the corresponding vehicle treated non-injury for each hour and sex.

Statistics

For the cell death (described above) and mRNA (described in chapter 2 methods) studies, 3 litters were used to dissect female and male cortical explants. These explants were grown in media containing 0.01% EtOH or 1 nM E2 in 0.01% EtOH for 1 week (7 days). At least 3 explants per condition at each time-point following injury were collected and repeated at least 3 times. For example, 3 female explants were analyzed that were EtOH treated and injured 4 hours from the time of collection, N=1. This was repeated at least 3 times with a new set of litters each time.

The effect of treatment and injury was determined by a two-way ANOVA comparing the factors “treatment” (EtOH or E2) and “injury” (non injured or 2DG/KCN treated) evaluated at various time-points following injury (4, 8, 12 and 24 hours). This allowed us to determine if treatment had an effect on the PI or mRNA expression or if injury had an effect on the PI or mRNA expression in either female or male cortical explants. If a significant interaction between treatment and injury was detected, Student Neuman-Keuls post-hoc tests were performed. Differences were considered significant at $p<0.05$.

Results

Initially, three different injury paradigms were tested to determine which concentration of 2DG/KCN to apply and how long to apply the injury. My preliminary data on the previously published injury (2 mM 2-DG and 1 mM KCN for 2 hours) (Wise, Dubal et al. 2000; Wilson, Liu et al. 2002) did not consistently produce a visible injury in the explants when female and males were separated. Since this concentration had previously injured the explants, we hypothesized that when explants were separated based on sex they were more susceptible to injury causing a faster and harsher cell death with 2DG/KCN. The explants did not appear healthy and most of the cell death appeared to have washed away when we changed media indicating an overpowering injury. To weaken the injury we cut the concentration in half and the time the injury was applied in half. Both injuries that were halved either in concentration or time resulted in consistent injury. We decided to continue with the injury, 1 mM 2-DG and 0.5 mM KCN for 2 hours, which halved the original concentration of 2 mM 2-DG and 1 mM KCN for 2 hours, because this was the lowest concentration that still consistently injured both the female and male cortical explants. The concentration of injury in the remaining studies is 1 mM 2-DG and 0.5 mM KCN for 2 hours.

Part 1: Cell Death in female or male cortical explants following injury.

Cortical explants were isolated from postnatal day 3 (PND 3) female or male rat pups and grown for 7 days in culture with media containing either 17 β-estradiol (E2) (1 nM in 0.01% EtOH) or EtOH (0.01%) vehicle. Explants were

treated with 1 mM 2-DG and 0.5 mM KCN for 2 hours in BME (injury) or BME (control). Following the 2 hour injury, media was replaced. At several time-points after injury (4, 8, 12 and 24 hours), explants were stained with propidium iodide to determine the extent of cell death.

Does E2 influence cell death at 4, 8, 12 or 24 hours following 2DG/KCN treatment in female or male cortical explants?

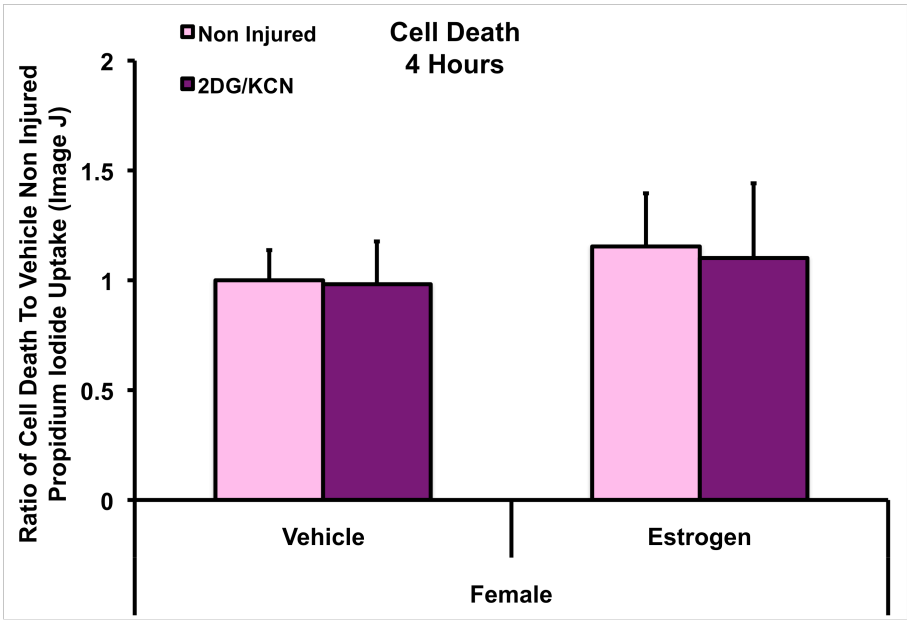
In females there was no effect of 2DG/KCN induced injury or E2 treatment on cell death 4, 8 or 12 hours following injury (determined by two-way ANOVA's at each time-point (4 hours, Figure 3.1A) (8 hours, Figure 3.2A) (12 hours, Figure 3.3A). However, 2DG/KCN produced a significant increase in cell death at 24 hours (main effect of injury; $p< 0.0001$, $F= 24.369$, $df= 1, 76$). A two-way ANOVA showed an interaction between injury and treatment ($p<0.05$, $F= 4.350$, $df= 1, 76$). Post-hoc t-tests revealed that cell death was significantly increased following 2DG/KCN injury in the EtOH group ($p<0.05$ compared to EtOH non-injured), and significantly increased following 2DG/KCN injury in the E2 group ($p<0.05$ compared to EtOH non-injured) (Figure 3.4A). Interestingly, this increase in cell death from 2DG/KCN was attenuated by the pretreatment of E2 ($p<0.05$, compared to EtOH 2DG/KCN injured), (Figure 3.4A).

In males there was no effect of injury or treatment on cell death 4 or 8 hours following 2DG/KCN injury (4 hours, Figure 3.1B) (8 hours, Figure 3.2B). However, 2DG/KCN produced a significant increase in cell death at 12 hours (main effect of injury; $p= 0.0322$, $F= 4.979$, $df= 1, 35$; Figure 3.3B) and at 24

hours (main effect of injury; $p < 0.0001$, $F = 24.958$, $df = 1, 98$; Figure 3.4B). Pretreatment with E2 did not alter the amount of cell death induced by 2DG/KCN at either time point.

Figure 3.5 depicts PI uptake (10X pictures) 24 hours following treatment with 2DG/KCN in vehicle (EtOH) and E2-treated female and male cortical explants. Overall, male cortical explants exhibited earlier increases in cell death in response to 2DG/KCN injury than females. E2 attenuated the 2DG/KCN induced cell death in female, but not male, cortical explants.

A.



B.

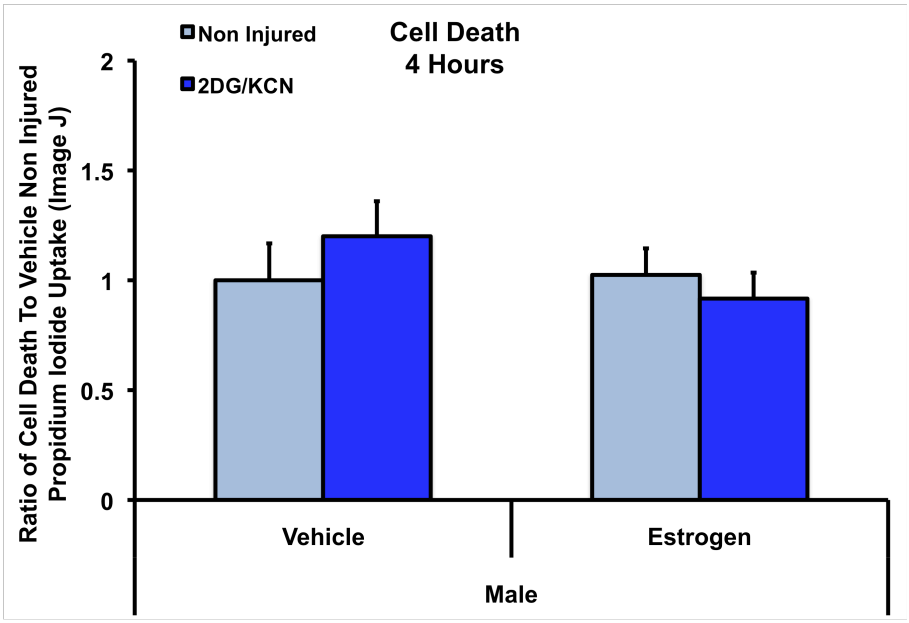


Figure 3.1. Cell death 4 hours following injury with 2DG/KCN in cortical explants. At 4 hours there was not a significant increase in cell death with 2DG/KCN in female (A) or male explants (B) or an effect of E2. The graphs above show PI uptake in non-injured (light pink (A) or light blue (B) bars) and 2DG/KCN-injured (pink (A) or blue (B) bars) cultures for vehicle or estrogen treatment. Error bars represent SEM.

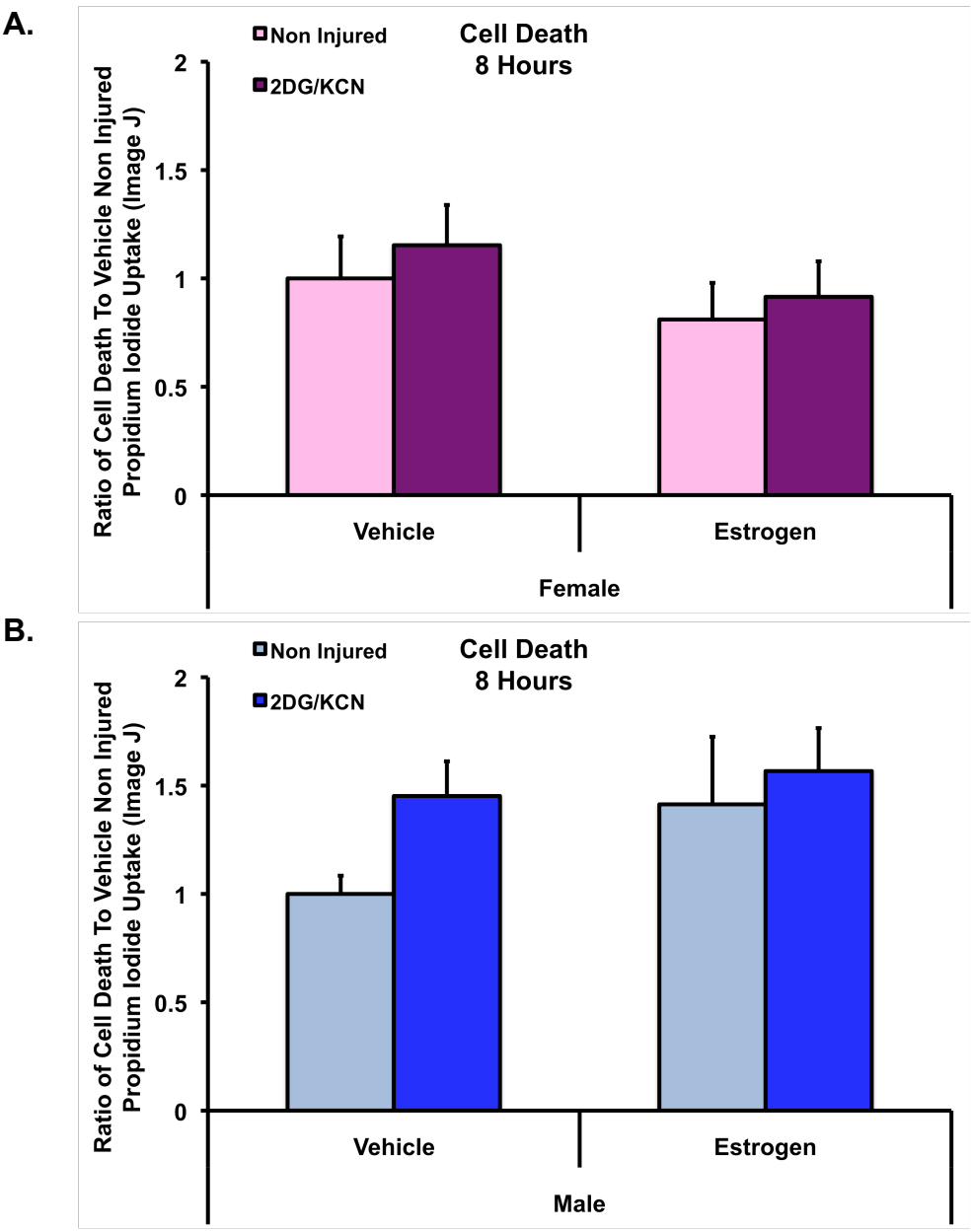
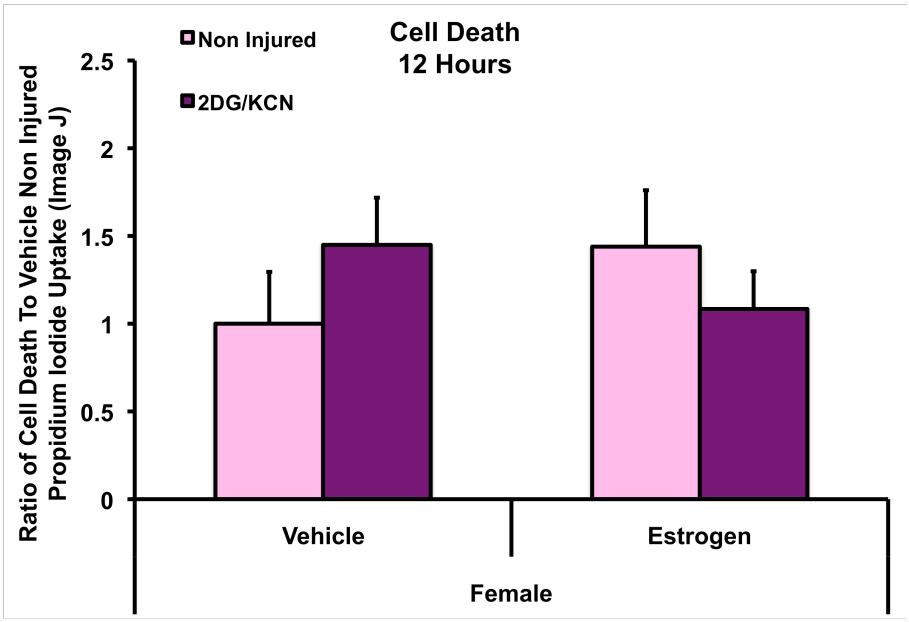


Figure 3.2. Cell death 8 hours following injury with 2DG/KCN in cortical explants. At 8 hours there was not a significant increase in cell death with 2DG/KCN in female (A) or male explants (B) or an effect of E2. The graphs above show PI uptake in non-injured (light pink (A) or light blue (B) bars) and 2DG/KCN-injured (pink (A) or blue (B) bars) cultures for vehicle or estrogen treatment. Error bars represent SEM.

A.



B.

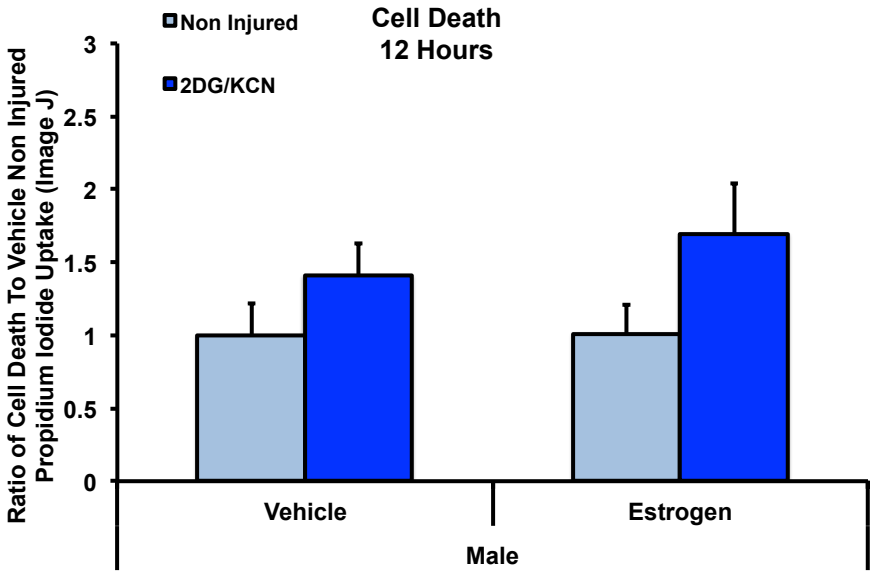
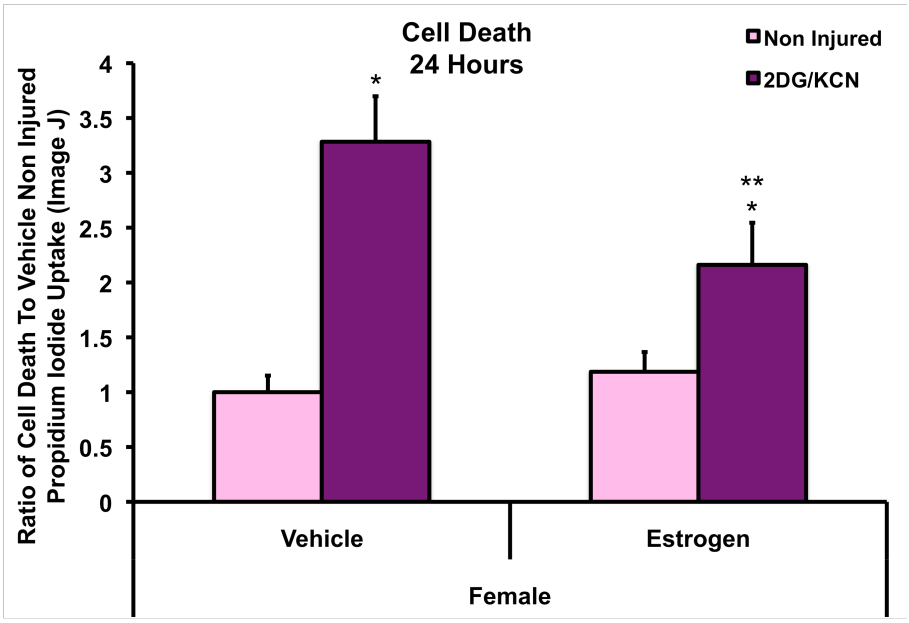


Figure 3.3. Cell death 12 hours following injury with 2DG/KCN in cortical explants. (A). **At 12 hours,** there was not an effect of 2DG/KCN injury or an effect of E2 in female cortical explants. (B). In male explants, at 12 hours there was a significant increase in cell death with 2DG/KCN injury, but no effect of E2. The graphs above show PI uptake in non-injured (light pink (A) or light blue (B) bars) and 2DG/KCN injured (pink (A) or blue (B) bars) cultures for vehicle or estrogen treatment. Error bars represent SEM.

A.



B.

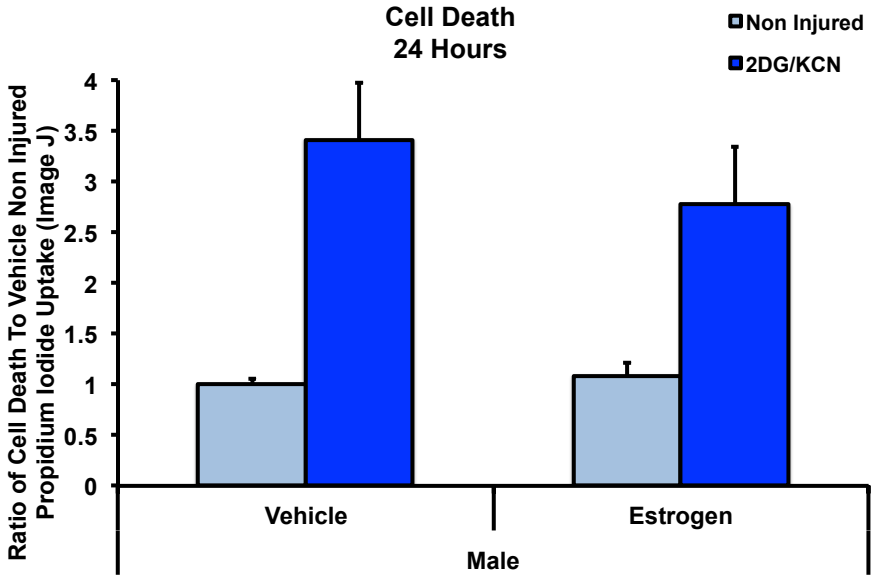
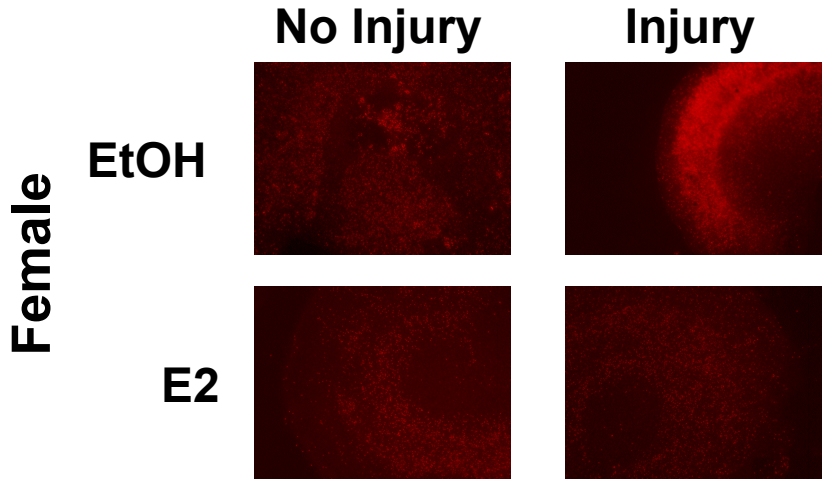


Figure 3.4. Cell death 24 hours following injury with 2DG/KCN in cortical explants. (A). In female explants, there was significant increase in cell death with 2DG/KCN injury. E2 significantly decreased this 2DG/KCN induced cell death. (B). In male explants, there was a significant increase in cell death with 2DG/KCN, but no effect of E2. The graphs above show PI uptake in non-injured (light pink (A) or light blue (B) bars) and 2DG/KCN-injured (pink (A) or blue (B) bars). Single asterisks indicate a significant increase in cell death from respective non-injured groups and double asterisks indicate a significant difference from vehicle-treated 2DG/KCN, $p < 0.05$. Error bars represent SEM.

A.



B.

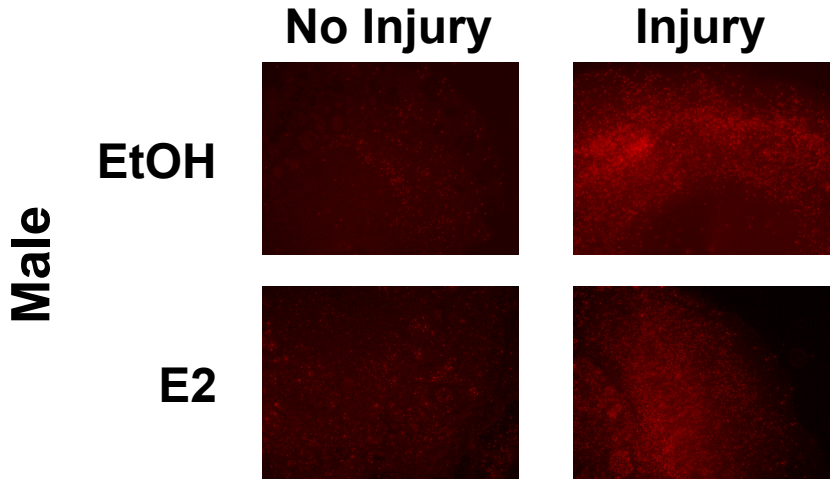


Figure 3.5 Representative photographs of PI uptake at 24 hours. (A). In female explants, there was increased cell death (red dots) with 2DG/KCN treatment. E2 significantly decreased this cell death. (B). In male explants, there was increased cell death with 2DG/KCN, but no effect of E2 on cell death.

Part 2: Sex steroid hormone receptor expression in female and male cortical explants following treatment with 2DG/KCN.

Cortical explants were isolated from postnatal day 3 (PND 3) female or male rat pups and grown for 7 days in culture with media containing either 17 β-estradiol (E2) (1 nM in 0.01% EtOH) or EtOH (0.01%) vehicle. Explants were treated with 1 mM 2-DG and 0.5 mM KCN for 2 hours in BME (injury) or BME (control). 2DG/KCN chemically mimics "ischemic" conditions by inhibiting glycolysis and oxidative phosphorylation (Wilson, Liu et al. 2002). Following the 2 hour injury, media was replaced. At several time-points after injury (4, 8, 12 and 24 hours), explants were collected and RNA extracted for real time PCR. Each time-point included the collection of at least 3 explants (N=1) per well that was repeated independently at least 3 times.

ERα

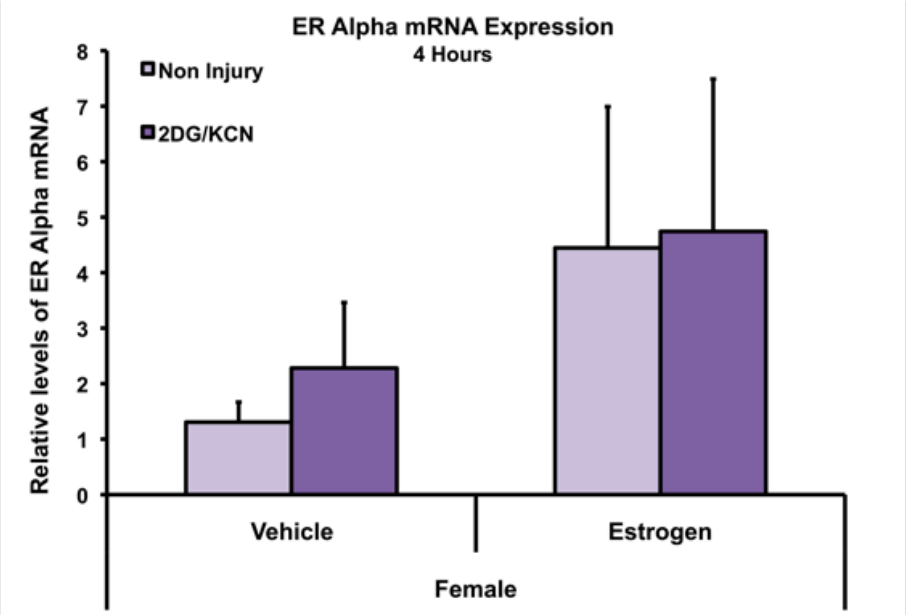
Does E2 influence ERα mRNA expression at 4, 8, 12 or 24 hours following 2DG/KCN treatment in female or male cortical explants?

In females, 2DG/KCN or E2 pretreatment did not affect ERα mRNA expression at 4 (Figure 3.6A) or 8 hours (Figure 3.7A). However, at 12 hours (Figure 3.8A) 2DG/KCN resulted in a significant increase in ERα mRNA expression (main effect of injury; p=0.0011, F=15.146, df=1,18). E2 pretreatment reduced ERα mRNA expression (main effect of treatment; p= 0.0060, F= 9.682, df= 1,18), preferentially in the 2DG/KCN injured cultures (interaction effect; p=0.0071, F=9.238, df=1,18). Post-hoc t-tests revealed that ERα mRNA was

significantly increased by 2DG/KCN injury in EtOH treated cultures, ($p<0.0001$ compared to EtOH non-injured cultures; $p<0.0001$ compared to the E2 non-injured cultures). E2 pretreatment significantly attenuated the increase in ERα mRNA expression due to 2DG/KCN ($p<0.001$ compared with EtOH 2DG/KCN injured) to a level equivalent to that in uninjured E2 treated cultures. Interestingly, by 24 hours ERα mRNA expression had returned to baseline (Figure 3.9A; no effect of injury, $p=0.9570$, $F=0.003$, $df=1,29$ and no effect of treatment; $p=0.6942$, $F=0.158$, $df= 1,29$).

Neither 2DG/KCN nor E2 pretreatment affected ERα mRNA expression at 4 (Figure 3.6B), 8 (Figure 3.7B), or 12 (Figure 3.8B) hours in male cortical explants. Interestingly, 2DG/KCN significantly reduced ERα mRNA expression in male cortical explants at 24 hours (main effect of injury, $p= 0.0290$ $F=5.830$, $df=1, 15$) (Figure 3.9B).

A.



B.

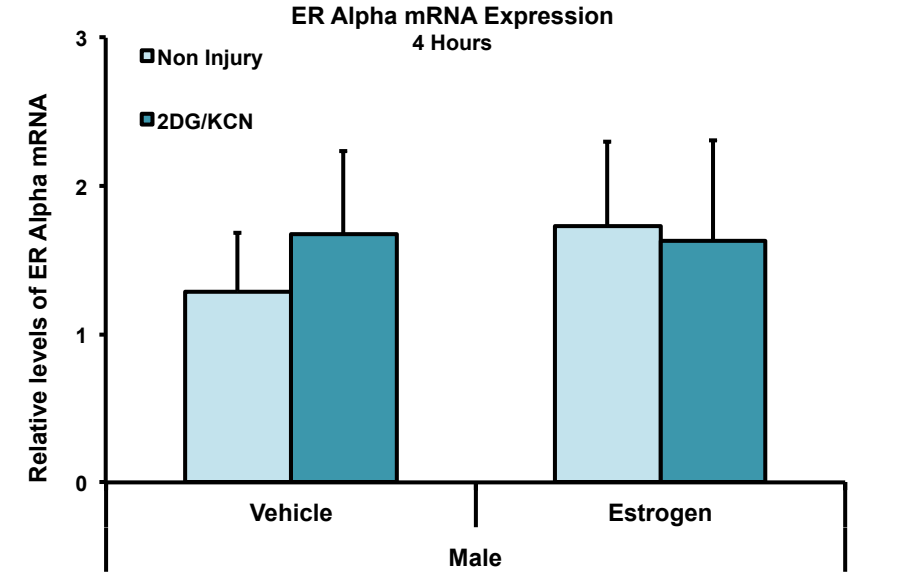
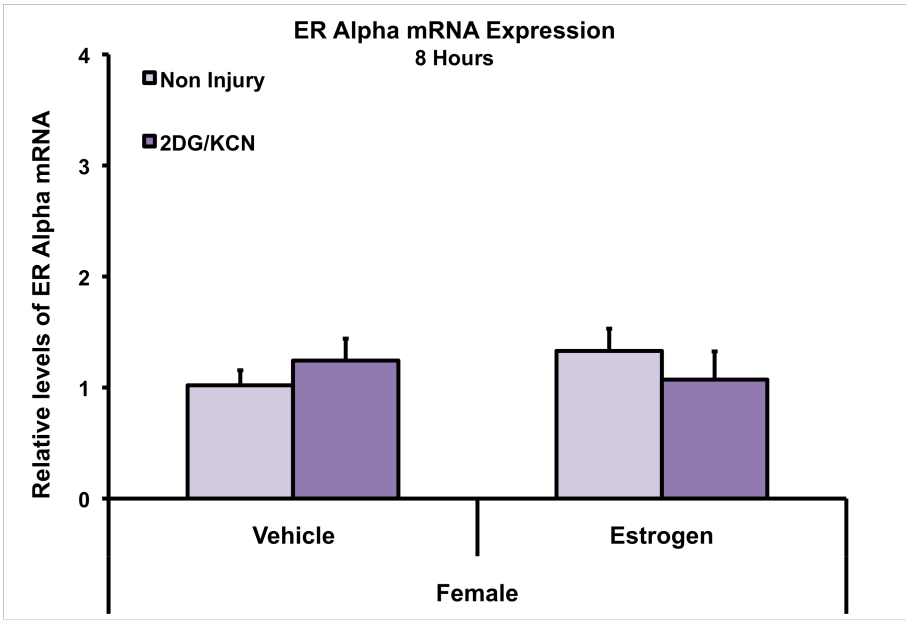


Figure 3.6. ERα mRNA expression 4 hours after injury. There was no significant increase in ERα mRNA expression with 2DG/KCN injury or E2 pretreatment in either the (A). female or (B). male explants. The graph above shows ERα relative to the housekeeping gene histone 3.1 in non-injured (light purple (A) and light teal (B) bars) and 2DG/KCN treated (purple (A) and teal (B) bars) cultures. Error bars represent SEM.

A.



B

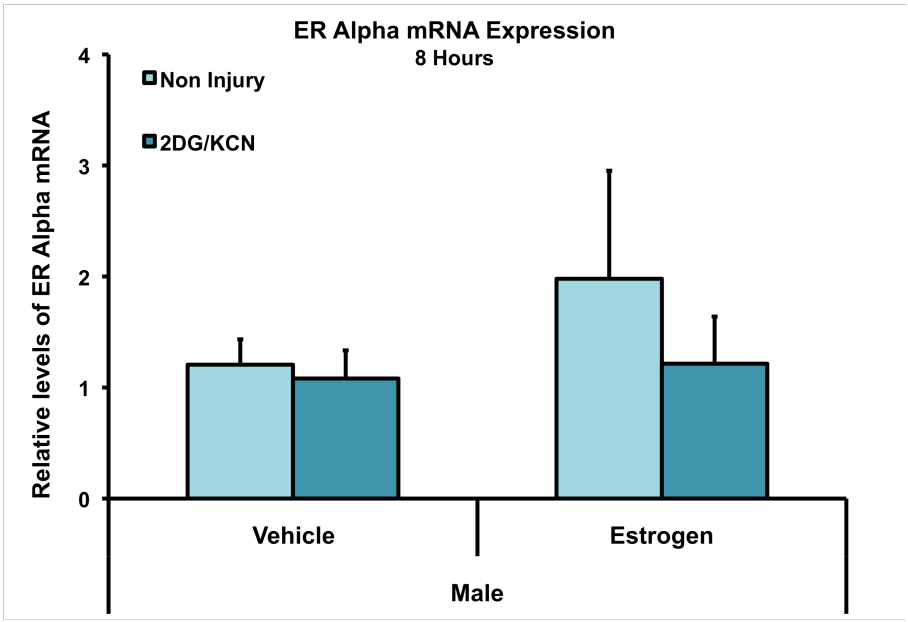
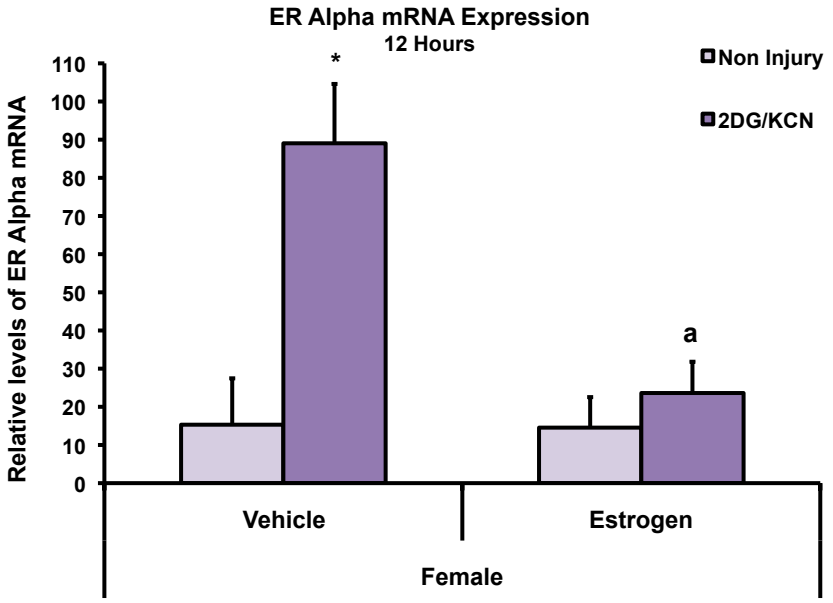


Figure 3.7. ERα mRNA expression 8 hours after injury. There was no significant increase in ERα mRNA with 2DG/KCN injury or E2 pretreatment in either the (A). female or (B). male explants. The graph above shows ERα relative to the housekeeping gene histone 3.1 in non-injured (light purple (A) and light teal (B) bars) and 2DG/KCN treated (purple (A) and teal (B) bars) cultures. Error bars represent SEM.

A.



B.

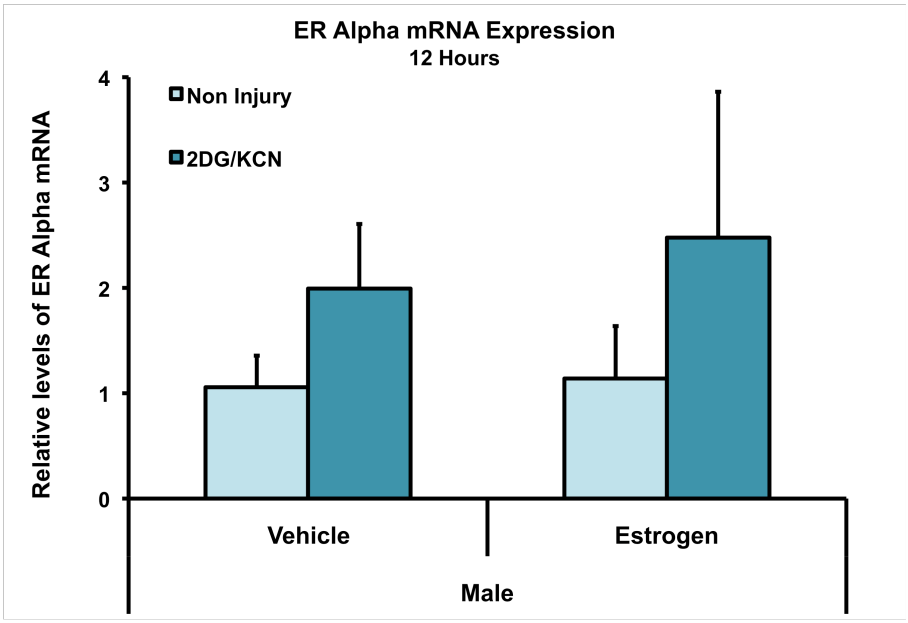
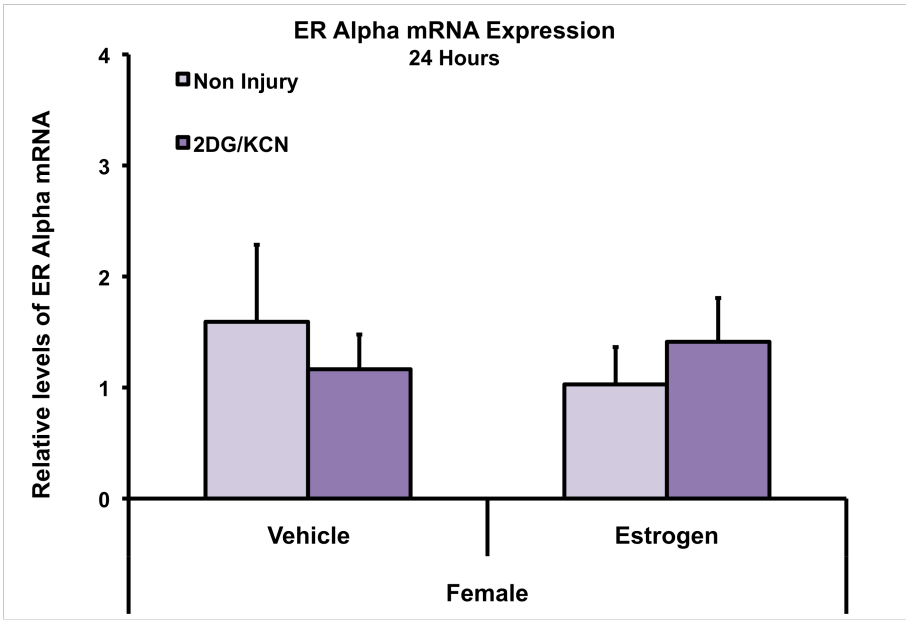


Figure 3.8. ERα mRNA expression 12 hours following injury. (A). In female explants ERα mRNA expression increased following 2DG/KCN injury in vehicle-treated cortical explants, indicated by an asterisks. E2 treatment prevented this increase, indicated by an "a" on the graph. (B). In males, there was no significant 2DG/KCN injury or E2 induced difference in ERα mRNA expression. The graph above shows ERα relative to the housekeeping gene in non-injured (light purple (A) and light teal (B) bars) and 2DG/KCN injured (purple (A) and teal (B) bars) cultures. Asterisks indicate a significant increase in ERα mRNA expression from vehicle non-injured group and the letter "a" indicates a significant decrease from vehicle 2DG/KCN injured cultures, $p < 0.05$. Error bars represent SEM.

A.



B.

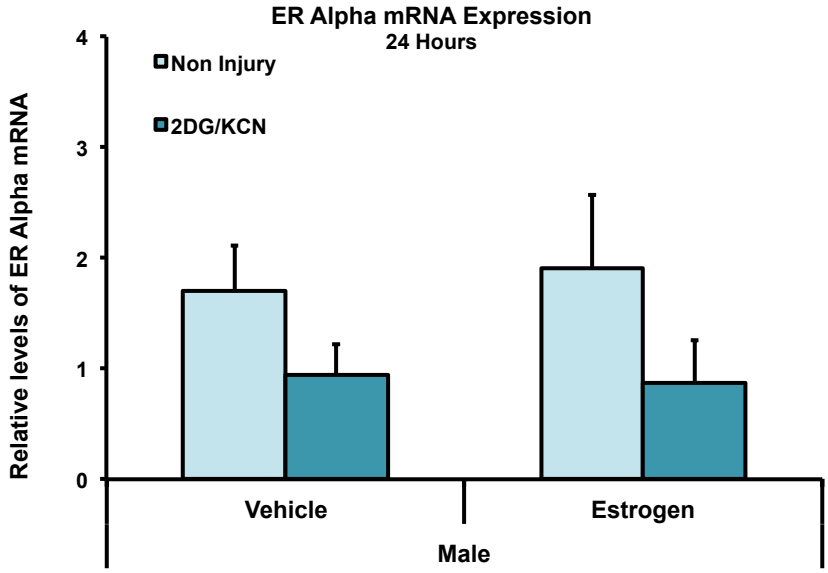


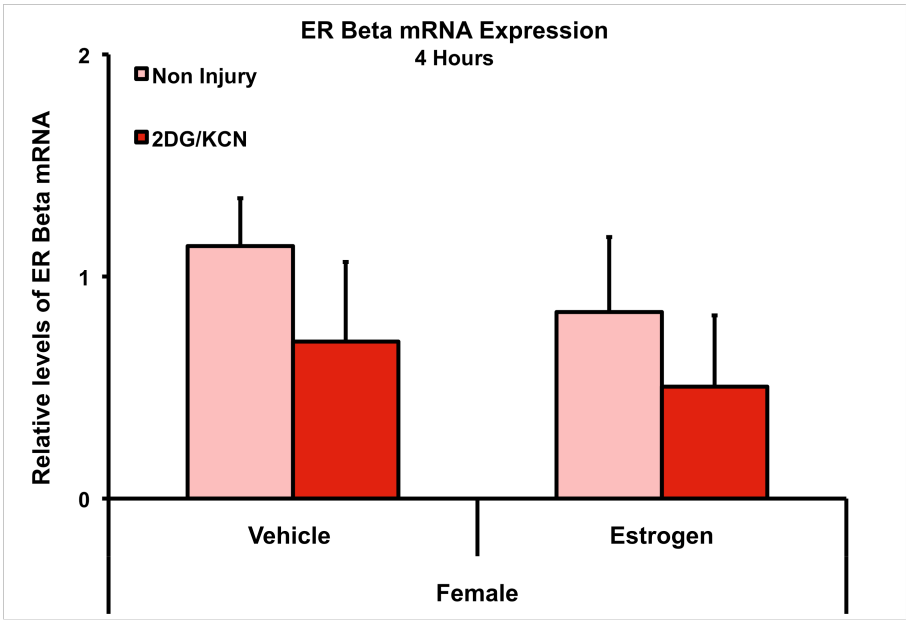
Figure 3.9. ERα mRNA expression 24 hours following injury. (A). In females, there was no significant change in ERα mRNA expression. (B). In males, ERα mRNA expression was significantly decreased 24 hours following injury with 2DG/KCN. The graph above shows ERα relative to the housekeeping gene in non-injured (light purple (A) and light teal (B) bars) and 2DG/KCN injured (purple (A) and teal (B) bars) cultures. Error bars represent SEM.

ERβ

Does E2 influence ERβ mRNA expression at 4, 8, 12 or 24 hours following 2DG/KCN treatment in female or male cortical explants?

Two-way ANOVAs comparing the factors injury and treatment were conducted at each time-point, separately for females and males. There was no effect of E2 treatment or 2DG/KCN injury on ERβ mRNA expression at 4 (Figure 3.10), 8 (Figure 3.11), 12 (Figure 3.12) or 24 (Figure 3.13) hours in female or male cortical explants.

A.



B.

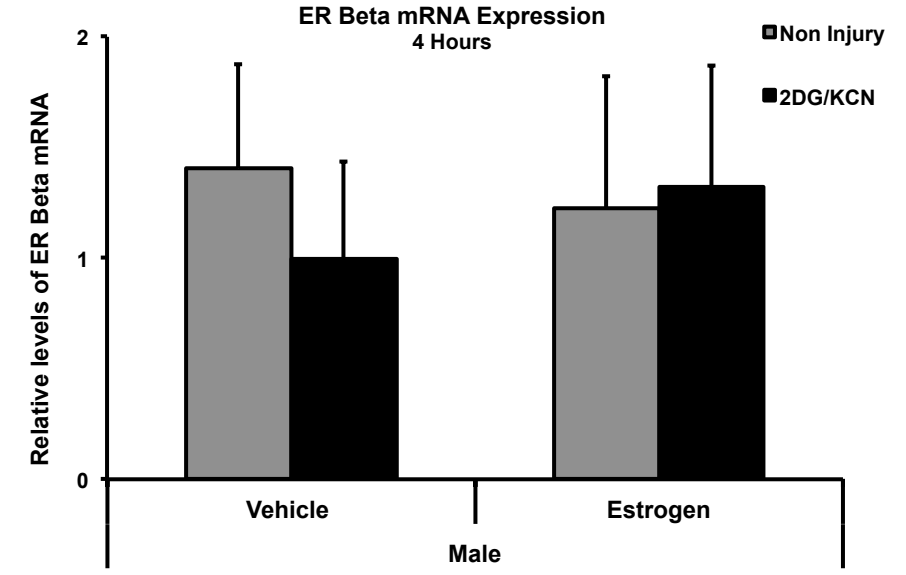
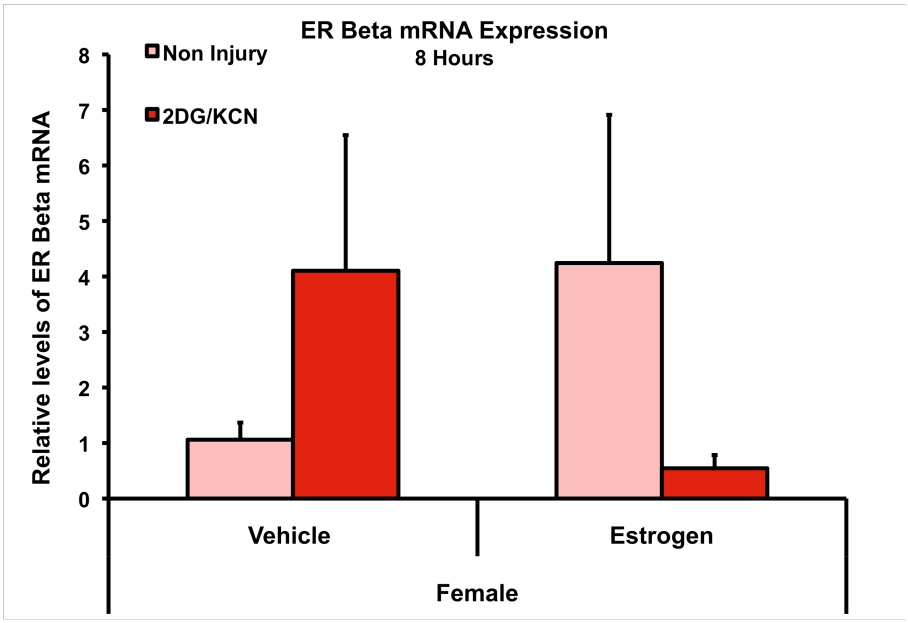


Figure 3.10. ER beta mRNA expression 4 hours after injury. There was no significant difference in ERβ mRNA expression at 4 hours after injury in (A). female or (B). male explants. The graph above shows ERβ relative to the housekeeping gene histone 3.1 in non-injured (pink (A) and grey (B) bars) and 2DG/KCN treated (orange (A) and black (B) bars). Error bars represent SEM.

A.



B.

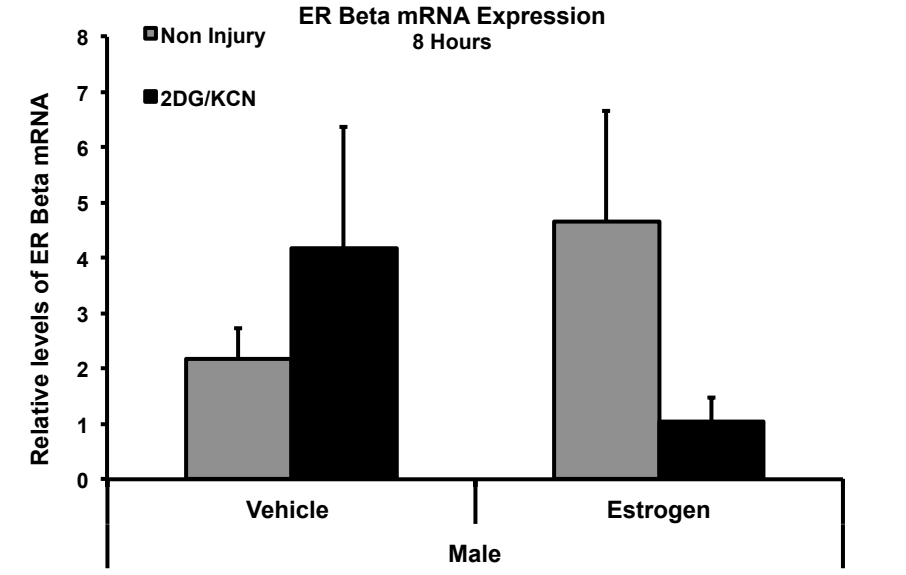
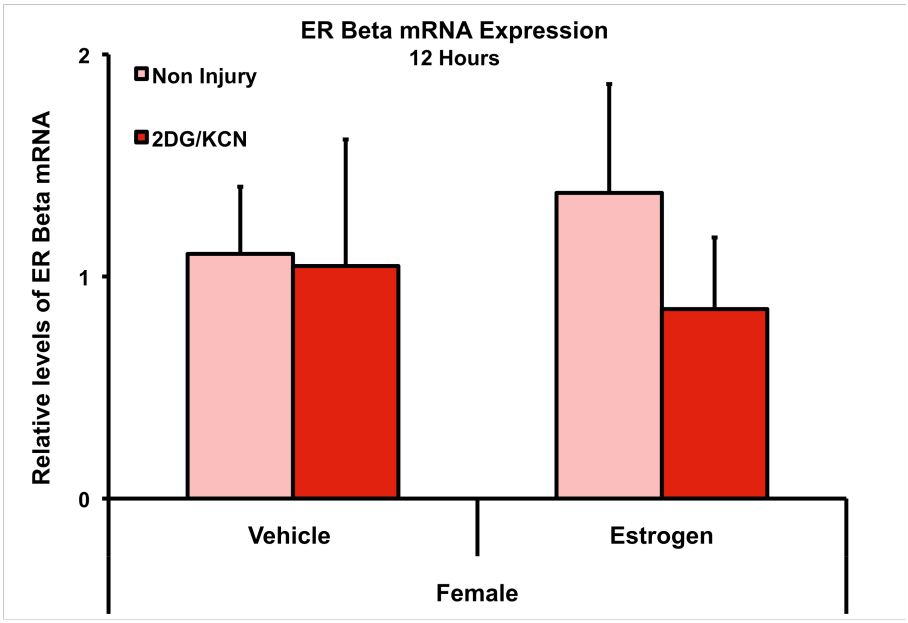


Figure 3.11. ER beta mRNA expression 8 hours after injury. There was no significant difference in ERβ mRNA expression at 8 hours after injury in (A). female or (B). male explants. The graph above shows ERβ relative to the housekeeping gene histone 3.1 in non-injured (pink (A) and grey (B) bars) and 2DG/KCN treated (orange (A) and black (B) bars). Error bars represent SEM.

A.



B.

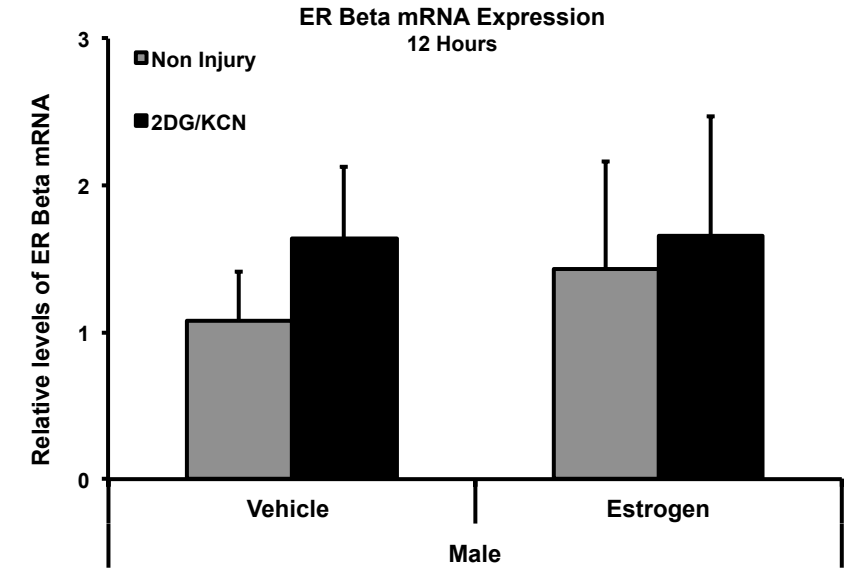
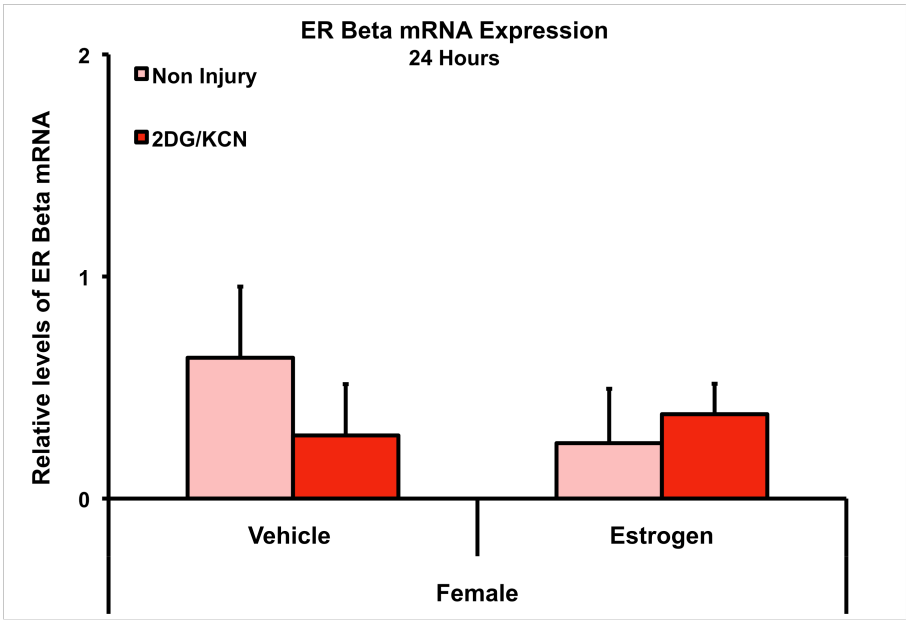


Figure 3.12. ER beta mRNA expression 12 hours after injury. There was no significant difference in ERβ mRNA expression at 12hours after injury in (A). female or (B). male explants. The graph above shows ERβ relative to the housekeeping gene histone 3.1 in non-injured (pink (A) and grey (B) bars) and 2DG/KCN treated (orange (A) and black (B) bars). Error bars represent SEM.

A.



B.

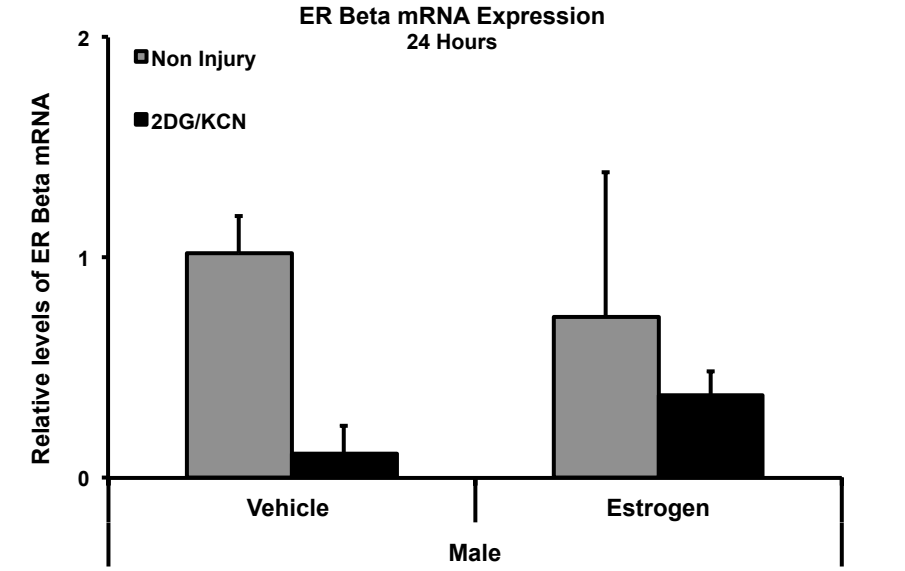


Figure 3.13. ER beta mRNA expression 24 hours after injury. There was no significant difference in ERβ mRNA expression at 24 hours after injury in (A). female or (B). male explants. The graph above shows ERβ relative to the housekeeping gene histone 3.1 in non-injured (pink (A) and grey (B) bars) and 2DG/KCN treated (orange (A) and black (B) bars). Error bars represent SEM.

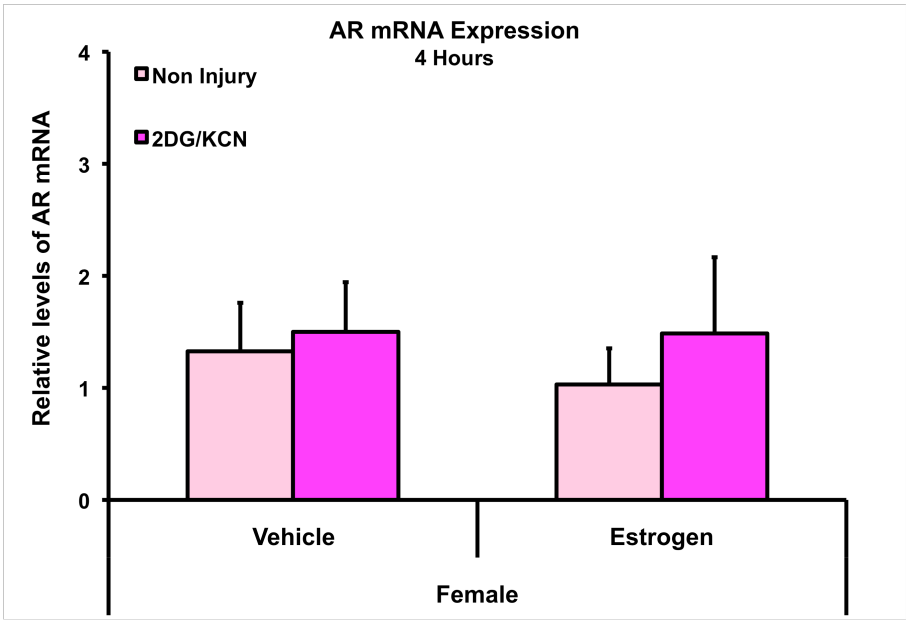
AR

Does E2 influence AR mRNA expression at 4, 8, 12 or 24 hours following injury?

In females, 2DG/KCN or E2 pretreatment did not affect AR mRNA expression at 4 hours (Figure 3.14A). However, at 8 hours (Figure 3.15A) 2DG/KCN transiently increased AR mRNA expression in female cortical explants (main effect of injury, p=0.0096, F=8.789, df=1,15), but this effect was not dependent on E2 pretreatment. Interestingly by 12 hours (Figure 3.16A) AR mRNA expression was no longer significantly increased following injury with 2DG/KCN (no effect of injury, p=0.0616, F=4.050, df= 1,16) and E2 pre-treatment still did not affect cell death. AR mRNA expression was back down to baseline by 24 hours (Figure 3.17A) following injury with 2DG/KCN (p=0.3880, F=791, df=1,15), and was not dependent on E2 treatment.

In contrast, neither 2DG/KCN nor E2 pre-treatment affected AR mRNA expression at 4 (Figure 3.14B), 8 (Figure 3.15B), 12 (Figure 3.16B) or 24 (Figure 3.17B) hours in male cortical explants.

A.



B.

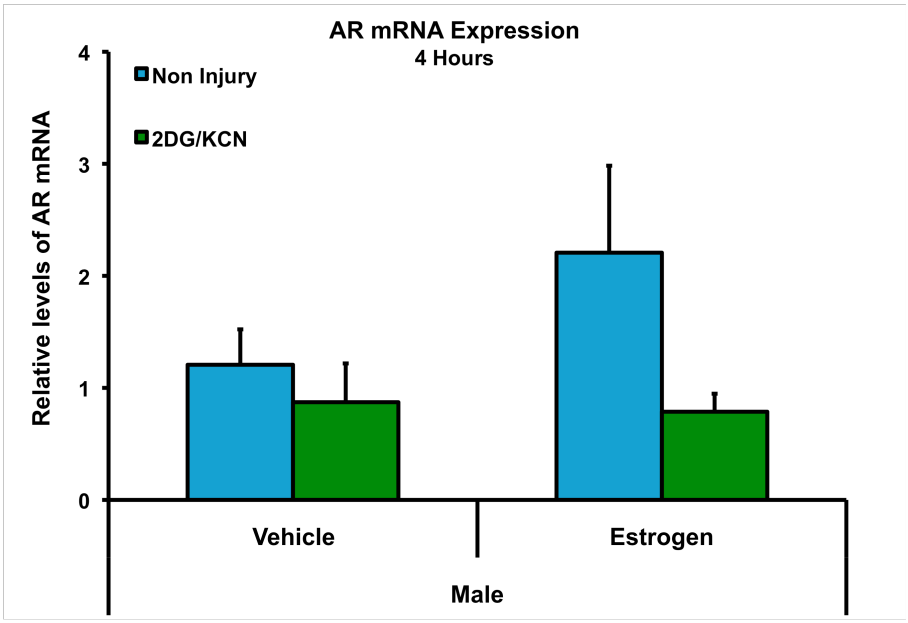
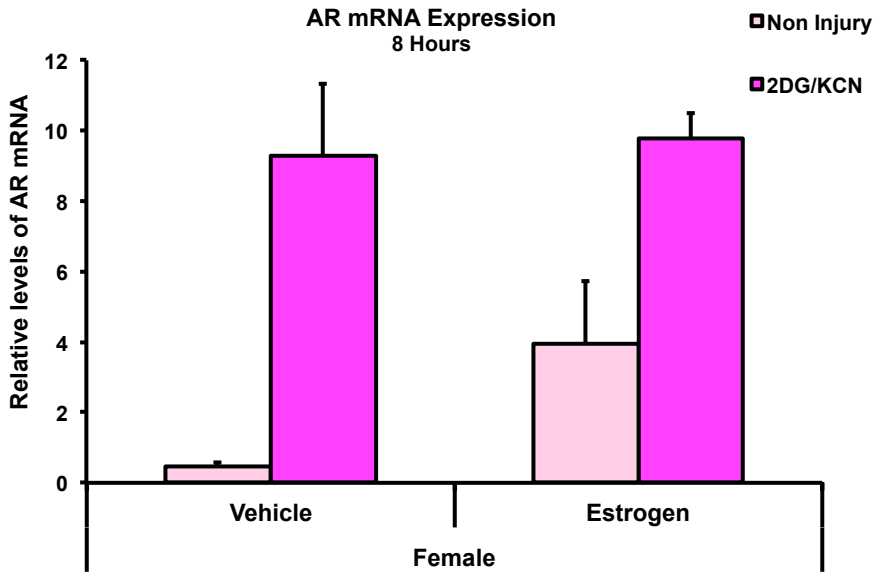


Figure 3.14. AR mRNA expression 4 hours after injury. There were no significant differences in AR mRNA expression at 4 hours after injury in (A). female or (B). male explants. The graph above shows AR relative to the housekeeping gene histone 3.1 in non-injured (light pink (A) and blue (B) bars) and 2DG/KCN treated (pink (A) and green (B) bars). Error bars represent SEM.

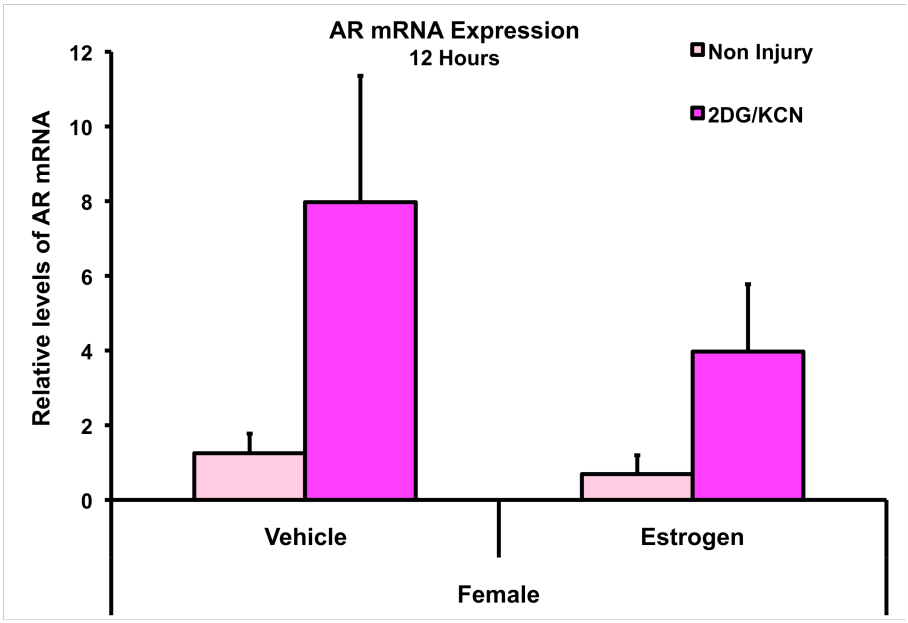
A.



B.

Figure 3.15. AR mRNA expression 8 hours after injury. (A). In females, there was a significant increase in AR mRNA expression following 2DG/KCN injury at 8 hours with no effect of E2 pre-treatment. (B). There was no significant effect of 2DG/KCN injury or E2 pre-treatment on AR mRNA expression in male explants. The graph above shows AR relative to the housekeeping gene histone 3.1 in non-injured (light pink (A) and blue (B) bars) and 2DG/KCN treated (pink (A) and green (B) bars). Error bars represent SEM.

A.



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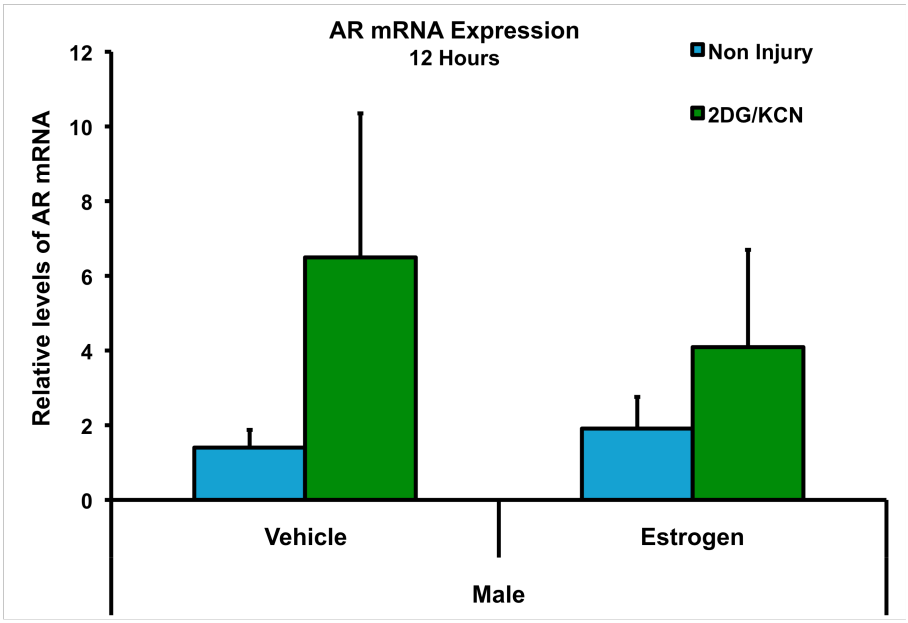
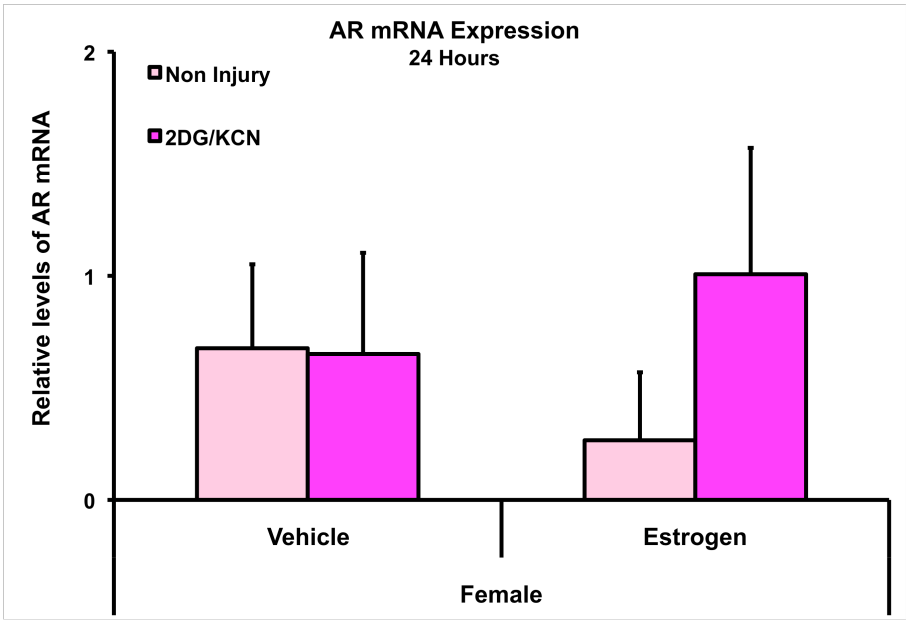


Figure 3.16. AR mRNA expression 12 hours after injury. There were no effects of 2DG/KCN injury or E2-pre-treatment on AR mRNA expression at 12 hours in (A). female or (B). male explants. The graph above shows AR relative to the housekeeping gene histone 3.1 in non-injured (light pink (A) and blue (B) bars) and 2DG/KCN treated (pink (A) and green (B) bars). Error bars represent SEM.

A.



B.

Figure 3.17. AR mRNA expression 24 hours after injury. There were no effects of 2DG/KCN injury or E2-pre-treatment on AR mRNA expression at 24 hours in (A). female or (B). male explants The graph above shows AR relative to the housekeeping gene histone 3.1 in non-injured (light pink (A) and blue (B) bars) and 2DG/KCN treated (pink (A) and green (B) bars). Error bars represent SEM.

Discussion

This chapter describes cell death and sex steroid hormone receptor expression in both male and female cortical explants following injury. In both male and female explants, treatment with 2DG/KCN significantly increased cell death. This increase occurred at 12 and 24 hours in males, but only at 24 hours in females. There was also a sex difference in response to E2 pretreatment such that in females, but not males, E2 attenuated cell death following 2DG/KCN induced injury.

Changes in steroid hormone receptor expression following 2DG/KCN injury were sex-specific and were influenced by E2 in the culture media, summarized in Table 3.1 (female cortical explants) and Table 3.2 (male cortical explants. In vehicle (EtOH) treated female cortical explants, 2DG/KCN injury increased ERα mRNA expression at 12 hours. Interestingly, E2 pre-treatment prevented this increase in ERα mRNA expression following the 2DG/KCN injury in females. ERα mRNA expression was transiently increased at 12 hours and had returned to the baseline expression by 24 hours. There was no similar increase in ERα mRNA expression in male explants at any time-point with 2DG/KCN or E2 treatment. ERβ mRNA expression did not change in response to 2DG/KCN induced injury or with E2 pretreatment in either female or male explants. AR mRNA expression was significantly increased with 2DG/KCN injury at 12 hours, but had no effect of E2 pre-treatment in female cortical explants. There was no change in AR mRNA expression due to ischemic injury induced by 2DG/KCN or E2 pre-treatment in male explants at any of the time-points.

Table 3.1. Summary of Changes in Cell Death and Sex Steroid Hormone Receptor mRNA Expression Following E2 Pre-treatment and injury with 2DG/KCN in Female Cortical Explants. Female cortical explants pre-treated with E2 were examined at 4, 8, 12 and 24 hours following an injury with 2DG/KCN to evaluate changes in cell death and along with changes in ERα, ERβ and AR mRNA expression.

	Female Cortical Explants			
	Non-injured		**2DG/KCN injured**	
	Vehicle (EtOH)	**E2**	**Vehicle (EtOH)**	**E2**
Cell Death (PI uptake)	No effect	No effect	↑ from EtOH non-injured	↑ from EtOH non-injured ↓ from EtOH 2DG/KCN
ERα mRNA expression	No effect	No effect	↑ from EtOH non-injured	↓ from EtOH 2DG/KCN
ERβ mRNA expression	No effect	No effect	No effect	No effect
AR mRNA expression	No effect	No effect	↑ from non-injured	↑ from non-injured

Table 3.2. Summary of Changes in Cell Death and Sex Steroid Hormone Receptor mRNA Expression Following E2 Pre-treatment and injury with 2DG/KCN in Male Cortical Explants. Male cortical explants pre-treated with E2 were examined at 4, 8, 12 and 24 hours following an injury with 2DG/KCN to evaluate changes in cell death and along with changes in ERα, ERβ and AR mRNA expression.

	Male Cortical Explants			
	Non-injured		**2DG/KCN injured**	
	Vehicle (EtOH)	**E2**	**Vehicle (EtOH)**	**E2**
Cell Death (PI uptake)	No effect	No effect	↑ from non-injured	↑ from non-injured
ERα mRNA expression	No effect	No effect	↓ from non-injured	↓ from non-injured
ERβ mRNA expression	No effect	No effect	No effect	No effect
AR mRNA expression	No effect	No effect	No effect	No effect

After MCAO in female rat cortex, ERα mRNA expression increases between 4 and 16 hours (Dubal, Rau et al. 2006). In female cortical explants treated with 2DG/KCN ERα mRNA expression significantly increased at 8 hours and had returned to a baseline expression by 24 hours. ERα mRNA does return to baseline following MCAO (unpublished observations), but it takes a longer period of time. These data suggest that in both cases the ERα mRNA response to injury is innate to the cortex, although the time course for expression is different in each model. ERα mRNA does not increase following MCAO in male rats (Westberry, Prewitt et al. 2008; Broughton, Brait et al. 2012) and did not increase here in male explants at any time point after injury. In both models, there is a sex-specific increase in ERα mRNA. It is interesting that in the cortical explants where there are no connections to other brain regions, this sex-specific response remains indicating that the cortex is preprogrammed as male or female in how it responds to injury. These results may have implications for how we treat other neurodegenerative diseases in males and females.

Following MCAO in female rat cortex, ERβ mRNA expression is initially elevated and then dramatically declines by 16 and 24 hours (Dubal, Rau et al. 2006). Pretreatment with E2 prevents the injury-induced decrease in ERβ at 16 and 24 hours after injury (Dubal, Rau et al. 2006). ERβ mRNA expression also does not increase following MCAO in male rats (Westberry, Prewitt et al. 2008; Broughton, Brait et al. 2012). Here, ERβ mRNA expression did not change in female or male cortical explants following treatment with 2DG/KCN. There is a difference in how ERβ responds to injury in the cortical explant model (*in vitro*)

verse the MCAO model (in vivo) in females. Since ERβ decreases in the female cortex following injury *in vivo* with input and connections from other brain regions and does not change when the cortex is isolated in culture could indicate that changes in ERβ mRNA expression in the cortex are influenced from other regions of the brain outside the cortex.

AR mRNA was significantly increased at 12 hours following treatment with 2DG/KCN in female cortical explants and was decreased by 24 hours. AR mRNA expression increases similarly to 2DG/KCN injury in female cortical explants regardless of E2 treatment. In male cortical explants, AR mRNA expression did not change following injury. These data are the first to demonstrate sex specific changes in AR following injury in the cortex.

E2 was protective against an ischemic injury in female, but not male cortical explants. ERα mRNA expression increased in response to injury and E2 treatment prevented this increase in ERα mRNA expression. Previously published injury models (MCAO) show that an increase in ERα mRNA is needed for E2-mediated protection (Suzuki, Brown et al. 2007). E2 was protective following injury. In response to 2DG/KCN injury, ERα mRNA expression increased and E2 treatment prevented this increase. AR mRNA expression also increased in response to injury, but E2 did not effect the increase as it did in ERα mRNA expression. ERβ mRNA expression did not change in response to injury or E2 treatment. Although there was not specific changes in sex steroid hormone receptor mRNA that were associated with the E2-mediated protection, these receptors may still play a role. E2 can also non-genomic actions.

Signaling pathways can be initiated by receptors on the cell membrane, including ERα, ERβ, AR, GPRs and ligand-gated ion channels (Watson, Jeng et al. 2008; Roman-Blas, Castaneda et al. 2009; Liu, Zhang et al. 2012). Specifically, AKT (serine/threonine protein kinase) becomes activated in the presence of E2 to promote cell survival via PI3 K (phosphatidylinositide 3'OH kinase) (Wilson, Liu et al. 2002). Pharmacological manipulations to block the activity of these receptors will decipher the role of these receptors in E2-mediated protection.

A potential pitfall of this cortical explant model includes cell death that occurred as a part of normal time in culture. We have investigated this cell death and have found that we are still able to see changes associated with 2DG/KCN damage and E2 protection. An additional limitation of this model that makes it harder to compare to the MCAO model is that the cortex has been completely separated from the striatum. In the MCAO model, most cortical damage is a result of secondary cell death caused by reduced blood flow to the striatum. Here data from our 2DG/KCN cell death must be interpreted differently, as the cell death is primary and not a result of secondary damage.

Previously published studies using 2DG/KCN to chemical induce an ischemic injury in cortical explants allowed the explants to maintain in culture for 7 days before adding the injury (Wise, Dubal et al. 2000; Wilson, Liu et al. 2002). When female explants are grown for 7 days in culture ERα mRNA expression has decreased, but males do not significantly decrease until 22 DIC. ERβ and AR mRNA expression are still low and have not increased, which occurs at 15 DIC in both female and male cortical explants. In females, all three sex steroid

hormone receptor mRNA's are low and in males ERα is still high. A more optimal design would have been to injure the cortical explants with 2DG/KCN at 22 DIC when ERα is low and ERβ and AR are high, which correlates to mRNA levels seen in the adult rodents cortex when MCAO's are performed. To maintain cortical explants for over three weeks in culture and then injure with 2DG/KCN would add additional factors to our model, such as the explants are now much thinner than at 7 DIC and the injury may affect the explants differently. The thinner explants may be more susceptible to damage. However, because E2 is still protective in the cortical explants grown for 7 DIC and ERα increases in female cortical explants following injury, correlating with the MCAO data, we feel that this model is still valuable.

Data from cortical explants will allow us to conduct more complicated manipulations at more time-points that will translate back to the MCAO model and allow us to use fewer animals. The cortical explant model can be furthered utilized to describe sex differences in injury by applying pharmacological manipulations to the cultures to block specific sex steroid hormones receptors to see how they are involved in E2 neuroprotection.

CHAPTER 4

ROLE OF SEX STEROID HORMONE RECEPTORS IN CELL DEATH FOLLOWING AN ISCHEMIC INJURY

Introduction

In the previous chapters, I investigated changes in sex steroid hormone receptor expression during development in the cortex and across time in culture and after injury in cortical explants. This chapter will focus on the role of endogenous sex hormone receptors following ischemic injury. Specifically, I will use pharmacological inhibitor to block estrogen receptor alpha (ERα), estrogen receptor beta (ERβ) or androgen receptor (AR) to determine the contribution of each receptor to cell death following injury.

The cortical explant model has several advantages to evaluate the effects of these inhibitors over adding these inhibitors directly in the brain using *in vivo* models. Inhibitors can be added directly to the feed media to see direct effects on cortex. In whole animal models, inhibitors are either added to the blood stream and may or may not cross the blood brain barrier or by direct cannulation into the brain. Direct injection in the brain requires surgery with general anesthetic and the drug can affect additional regions of the brain in close proximity. Additional of these inhibitors to the cortical explants allows us to see how these receptors are important by blocking them in a model that maintains the neuron/glial relationship without influence from other brain regions.

Previous studies have used sex steroid hormone receptor inhibitors in explants to evaluate their importance after injury. For example, addition of the nonspecific ER antagonist (ICI 182,780) blocked the neuroprotective effects of E2 following injury (Wise, Dubal et al. 2001). ICI blocks both ERα and ERβ, therefore these studies did not differentiate the roles of one receptor versus the other. In another study, addition of 17α-estradiol, an isomer of 17β-estradiol (E2) that does not bind well to the estrogen receptors, did not reduce cell death (Wise, Dubal et al. 2001). E2 was also shown to be protective when ERα, compared to vector DNA, was transfected into PC12 cells (Gollapudi and Oblinger 1999; Gollapudi and Oblinger 1999). Interestingly, these studies did not look specifically at each sex steroid receptor individually to decipher their role separately in E2 mediated protection. While these studies do indicate a role for sex steroid receptors, they did not evaluate each receptor independently to determine their role in E2 mediated protection and ischemic induced cell death.

The initial goal of these studies was to determine which receptor is necessary for the attenuation of cell death in female explants at 24 hours. Because there was no effect of E2 on cell death in the males or at any other time point in the females, the secondary goal was to determine if the presence of each receptor may actually inhibit the ability of E2 to be protective. To accomplish these goals, I evaluated cell death by measuring propidium iodide uptake and evaluating changes in sex steroid hormone receptors following treatment with 2DG/KCN in both female and male cortical explants separately. Sex steroid

hormone receptor antagonists were added either before or after treatment with 2DG/KCN to see how they influence cell death cause by ischemic injury.

Methods

Inhibitors

Cortical explants were isolated from postnatal day 3 (PND 3) female or male rat pups and grown in culture with media containing either 17 β-estradiol (E2) (1 nM in 0.01% EtOH) or EtOH (0.01%) vehicle. Antagonists were either added 24 hours prior to injury or 6 hours post injury. The inhibitors used are listed below:

1. ER alpha antagonist, **MPP dihydrochloride** (1,3-*Bis*(4-hydroxyphenyl)-4-methyl-5 -[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride) (1991, Tocris, Minneapolis, MN) was made up in EtOH to a stock concentration of 100 mM and added into feed media to a final concentration of 1 uM (previously published optimized *in vitro* concentration) (Harrington, Sheng et al. 2003; Ben-Jonathan, Chen et al. 2009). MPP Dihydrochloride is a selective silent antagonist at the ERα receptor with more than 200 fold selectivity for ERα than ERβ. A silent antagonist is a competitive receptor antagonist that doesn't intrinsically activate the receptor.
2. ER beta antagonist, **PHTPP** (4-[2-Phenyl-5,7-*bis* (trifluoromethyl) pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol) (2662 Tocris, Minneapolis, MN), was made up in EtOH to a stock concentration of 100 mM and added into feed media to a final concentration of 1 uM (previously published optimized *in*

vitro concentration) (Ben-Jonathan, Chen et al. 2009). PHTPP is a selective ERβ receptor antagonist with more than 36 fold selectivity for ERβ than ERα.

3. AR antagonist, **Flutamide** (2-Methyl-*N*- (4-nitro-3-[trifluoromet hyl]phenyl) propanamide) (4094 Tocris, Minneapolis, MN), was made up in EtOH to a stock concentration of 100 mM and added into feed media to a final concentration of 100 nM. Flutamide is a selective anti-androgenic antagonist for AR.

Pre-treatment studies

For pre-treatment, on the 6th day in culture (24 hours before injury), 1 uM antagonist was added into the feed media that contained vehicle (EtOH) or E2. 24 hours later the feed media with or without the antagonist was removed and explants were injured using 1 mM 2-DG and 0.5 mM KCN for 2 hours in BME (injury) or BME (control). Feed media with or without the antagonist and with or without E2 was replaced after the two hours and cell death was analyzed using propidium iodide (PI) 24 hours after the addition of 2DG/KCN.

Post-treatment studies

For post-treatment of the antagonist, on the 7th day in culture, explants maintained in the presence of EtOH or E2 were injured using 1 mM 2-DG and 0.5 mM KCN for 2 hours in BME (injury) or BME (control). 6 hours after injury, 1 nM

or 1 uM of the antagonist was added into the feed media that contained EtOH or E2. 24 hours following the injury cell death was analyzed using PI.

At each treatment (EtOH/E2), inhibitor (vehicle/drug) and injury (non injured/ 2DG/KCN treated) condition at least 3 explants were processed per well. For example, 1 well of at least 3 females explants that were grown in EtOH treated media and pretreated with an antagonist for 24 hours before injury were processed 24 hours after injury with propidium iodide to determine the extent of cell death. Each explant in the well was considered an N of 1. This was repeated at least 3 times with a new set of litters each time. Explants were visualized using a fluorescent microscope. Pictures of explants at 20X were captured using an image capture program, SPOT Advanced. Red (dead) cells were then counted using an NIH program, Image J. Pictures were coded and analyzed blindly. Injured explants were compared back to the corresponding vehicle treated non-injury for each hour and sex.

Statistics

For the cell death studies, 3 litters were used to dissect female and male cortical explants. Cortical explants were taken from female rat pups at postnatal day 3 (PND 3) and maintained in culture media containing vehicle or estrogen. Explants were either pre-treated or post-treated with inhibitors (described above) then each inhibitor scenario was split into non-injured and 2DG/KCN treated groups. At each treatment (EtOH/E2), drug (vehicle/antagonist) and injury (non-injured/ 2DG/KCN treated) condition at least 3 explants were processed per well.

For example, 1 well of at least 3 females explants that were grown in EtOH treated media and inhibited for 24 hours before injury were processed 24 hours after injury with PI to determine the extent of cell death. Each explant in the well was considered an N of 1. This was repeated at least 3 times with a new set of litters each time.

Since we were interested if antagonizing a specific sex steroid receptor had an effect on cell death, we evaluated the effect of each antagonist on cortical explants injured with 2DG/KCN and pre-treated with E2. A three-way ANOVA comparing the factors “drug” (vehicle/ antagonist), “treatment” (EtOH/ E2) and “injury” (non injured/ 2DG/KCN) were evaluated for each receptor antagonist scenario. If a significant interaction between drug, treatment or injury was detected, Student Newman-Keuls post hoc test were performed. All three inhibitors have a 24 hour pre-treatment at 1uM and a 6 hour post-treatment at 1 nM and 1 uM.

Results

Overview

Drugs were either added as a pre-treatment as described in previous studies or as a post-treatment. Because we did not see the level of cell death we anticipated, we were concerned that the drug in the culture media before injury may be influencing the ability of 2DG/KCN to induce cell death. To eliminate this problem, we included two additional paradigms where inhibitor drugs were added following injury. First, I added inhibitor 6 hours post-injury to determine if this would interfere with the cell death in response to 2DG/KCN treatment. We also added a lower concentration of the antagonists at 6 hours post-injury to determine if the inhibitor's effects were dose dependent.

Drugs were either added as a pre-treatment as described in previous studies or as a post-treatment. Because we did not see the level of cell death we anticipated, we were concerned that the drug in the culture media before injury may be influencing the ability of 2DG/KCN to induce cell death. To eliminate this problem, we included two additional paradigms where inhibitor drugs were actually added following injury. First, I added inhibitor 6 hours post injury to determine if this would interfere with the cell death in response to 2DG/KCN treatment. We also added a lower concentration of the antagonists at 6 hours post-injury to determine if the drug concentration alone was affecting cell death.

Part 1: Role of each sex steroid hormone receptor in FEMALE explants.

Does inhibiting ERα influence cell death in female cortical explants?

MPP is a high affinity specific ERα antagonist that inhibits at the receptor. We choose this inhibitor over previously published inhibitors because this particular antagonist has a selectivity of 200 fold more affinity for ERα over ERβ. ICI 182,780 was used in previous studies (Wise, Dubal et al. 2001), but this inhibitor affects both ERα and ERβ, which would not allow me to decipher which receptor is specifically involved.

MPP Pre-treatment

To determine the effects of the ERα antagonist, MPP, and the presence of E2 on cell death following injury with 2DG/KCN in female cortical explants, a three-way ANOVA was performed comparing the factors “drug” (vehicle and MPP), “injury” (non-injured and 2DG/KCN treated) and “treatment” (EtOH and E2). This test revealed no main effects of drug, injury or E2 treatment, but did reveal an interaction between drug and injury ($p<0.0001$, $F=16.642$, $df=1,104$), Figure 4.1. Post-hoc t-tests revealed three significant differences. First, 2DG/KCN produced a significant increase in cell death in the vehicle (no drug) ($p<0.0001$; compared to non-injured vehicle (no drug) group). Next, MPP pre-treatment increased cell death in the non-injured group ($p<0.05$; compared to the non-injured vehicle group). Lastly, MPP pre-treatment significantly decreased cell death in the 2DG/KCN injured explants ($p<0.005$; compared to the vehicle (no

drug) 2DG/KCN injured explants). This decrease in cell death indicates that when 2DG/KCN is applied in the presence of MPP that this drug significantly protects female cortical explants from the 2DG/KCN induced injury. Interestingly, when female cortical explants were pre-treated with MPP, 2DG/KCN did not produce a significant injury ($p>0.05$; compared to MPP non-injured). To summarize these results, 2DG/KCN caused a significant injury in the vehicle (no drug) group, and MPP pretreatment increased cell death in uninjured cultures but protected against cell death in cultures subjected to 2DG/KCN injury.

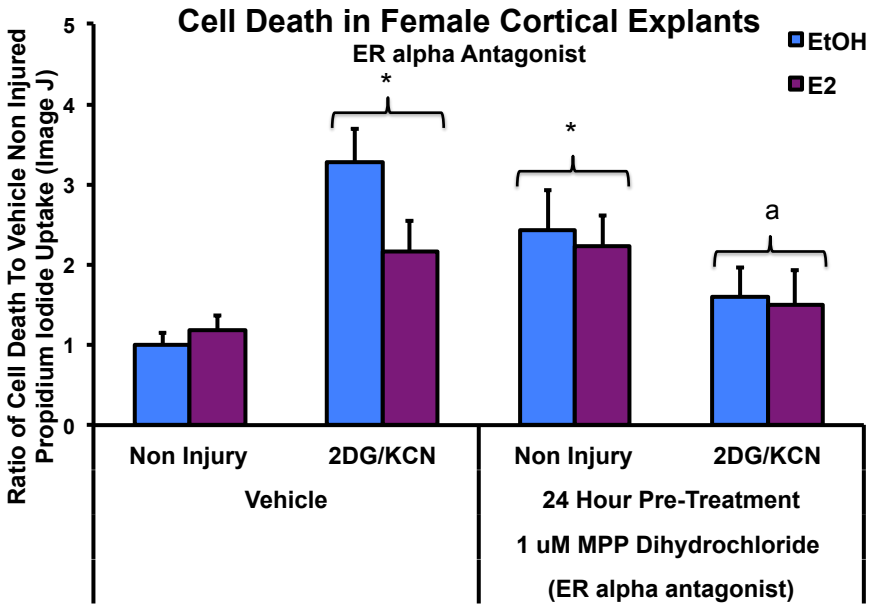


Figure 4.1. Cell death in female cortical explants after pre-treatment with MPP, an ERα antagonist. Overall, 2DG/KCN increased cell death in the vehicle (no drug) explants. MPP pre-treatment significantly increased cell death in the non-injured explants. Asterisks indicate a significant increase in cell death from the vehicle (no drug) non-injured group. Interestingly, in the 2DG/KCN injured explants, MPP pre-treatment decreased cell death compared to vehicle (no drug) explants. The letter "a" on graph indicates a decrease in cell death from vehicle 2DG/KCN injured explants. Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

MPP Post-treatment

2DG/KCN did not produce a significant injury when the female cortical explants were pre-treated with MPP. To determine if MPP, the ERα antagonist, was affecting 2DG/KCN's ability to block oxidative phosphorylation and glycolysis (ischemic injury), MPP was added after the 2DG/KCN injury at two different paradigms (described in methods).

6 hour post-treatment of 1 uM MPP Dihydrochloride

The same concentration of MPP (1 uM) used for the 24 hour pre-treatment was added 6 hours following the 2DG/KCN injury. A three-way ANOVA comparing the factors drug, injury and treatment revealed a significant main effect of injury ($p=0.0006$, $F=12.522$, $df=1,105$) and an interaction of drug and injury ($p=0.0328$, $F=4.680$, $df=1,105$). Post-hoc t-test revealed three significant differences, Figure 4.2. First, 2DG/KCN increased cell death in the vehicle (no drug) group ($p <0.001$; compared to the non-injured vehicle (no drug) group and $p<0.05$; compared to the non-injured MPP group). Lastly, MPP treatment significantly decreased cell death in the 2DG/KCN injured explants ($p<0.05$; compared to vehicle (no drug) 2DG/KCN injured explants). This decrease in cell death indicates that post-treatment with MPP protects female cortical explants from an increase in cell death due to 2DG/KCN. Interestingly, 2DG/KCN did not produce a significant injury within the MPP group ($p>0.05$; compared to MPP non-injured explants). To summarize these results, 2DG/KCN causes a significant injury in the vehicle (no drug) group compared to the vehicle no injury

and to the MPP no injury. A significant 2DG/KCN induced injury could not be reached with in the MPP group at 1 um. A lower concentration of MPP was also applied post-treatment to see if the MPP concentration was too high.

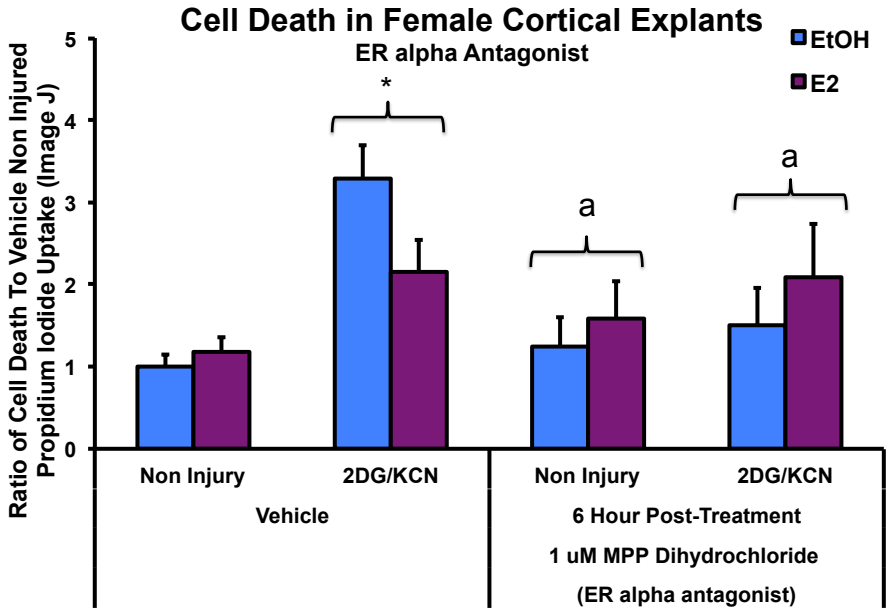


Figure 4.2. Cell death in female cortical explants after post-treatment with 1 uM MPP. Overall, 2DG/KCN increased cell death in the vehicle (no drug) explants. Asterisks indicate a significant increase in cell death from the vehicle (no drug) non-injured group. Interestingly, MPP post-treatment decreased cell death from the vehicle 2DG/KCN injured. The letter "a" indicates less cell death as compared to the 2DG/KCN vehicle. Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

6-hour post-treatment with 1 nM MPP

A 1000 fold weaker concentration (1 nM) of MPP than was previous used was added 6 hours after injury with 2DG/KCN to see if 2DG/KCN can induce significant injury in the presence of 1 nM MPP. A three-way ANOVA comparing the factors drug, injury and treatment was conducted and revealed a significant main effect of injury ($p<0.0001$, $F=16.369$, $df=1, 105$), but no main effect of drug or treatment and no interactions, Figure 4.3. These data indicate that 2DG/KCN significantly increased cell death regardless if the explants were vehicle (no drug) or 1 nM MPP treated 6 hours after injury.

Note: p value for an interaction between drug and treatment was 0.054, which is not significant, but extremely close. This indicates a trend that in the vehicle (no drug) group, E2 had less cell death than in the absence of E2. However, the MPP post-treatment group had a trend for an increase in cell death in the presence of E2.

These results did not confirm our initial hypothesis that blocking ERα activity, either with a 24 hour pretreatment or a 6 hour post-treatment relative to 2DG/KCN induced injury, would remove the E2-mediated protection seen in female cortical explants.

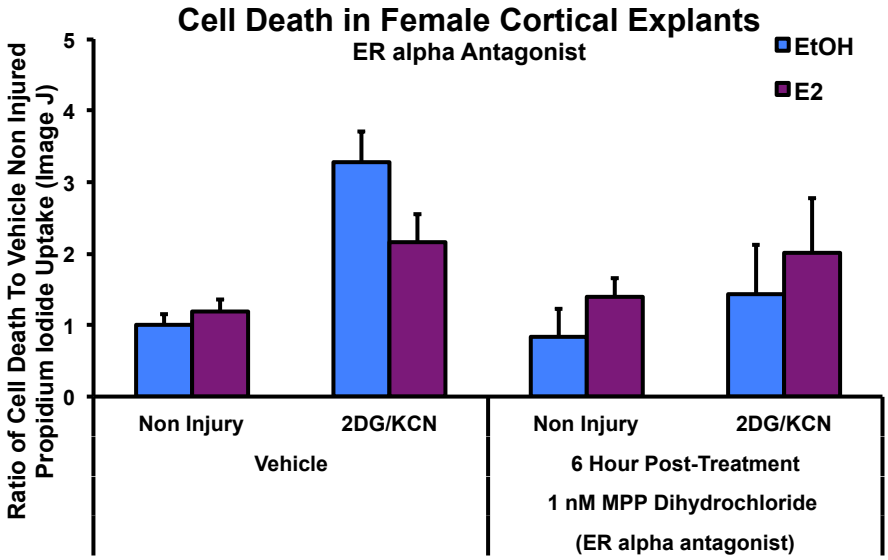


Figure 4.3. Cell death in female cortical explants after post-treatment with ERα inhibitor 1 nM MPP. Overall, 2DG/KCN increased cell death in both the vehicle and MPP treated groups. Error bars represent SEM.

Does inhibiting ERβ influence cell death in female cortical explants?

PHTPP is a selective ERβ receptor antagonist. We choose this inhibitor over previously published inhibitors because this particular antagonist has a 36 fold more affinity for ERβ than ERα. ICI was used in previous studies (Wise, Dubal et al. 2001), but this inhibitor affects both ERα and ERβ which would not allow me to decipher which receptor is specifically involved.

PHTPP Pre-treatment

To determine the effects of the ERβ antagonist, PHTPP, and the presence of E2 on cell death following injury with 2DG/KCN in female cortical explants, a three-way ANOVA comparing the factors "drug" (Vehicle and PHTPP), "injury" (non-injured and 2DG/KCN treated) and "treatment" (EtOH and E2) was performed. This test revealed a significant main effect of injury ($p=0.0148$, $F=6.127$, $df=1,110$) and an interaction of drug and injury ($p=0.0006$, $F=12.489$, $df=1,110$). Post-hoc t-tests revealed that 2DG/KCN significantly increased cell death in the vehicle (no drug) group ($p<0.05$; compared to vehicle (no drug) non-injured and $p<0.05$; compared to PHTPP non-injured). PHTPP decreased cell death in the 2DG/KCN group ($p<0.05$; compared to vehicle (no drug) 2DG/KCN injured). This indicates that pre-treatment with PHTPP protects female cortical explants from cell death induced by 2DG/KCN. Interestingly, 2DG/KCN did not induce a significant injury within the PHTPP group ($p<0.05$; compared to PHTPP non-injured). To summarize these results, 2DG/KCN caused a significant injury

in the vehicle (no drug) group PHTPP pre-treatment protected against cell death in the 2DG/KCN injured cultures.

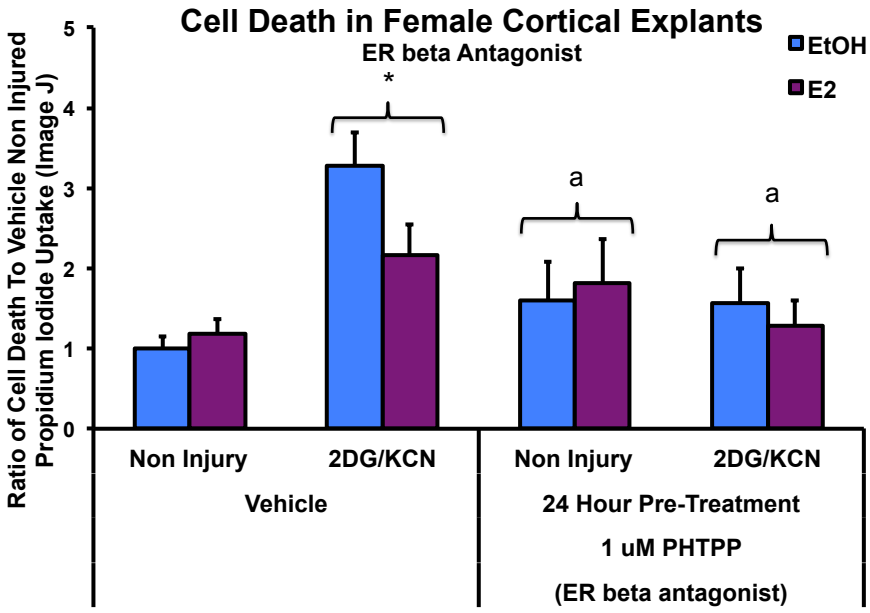


Figure 4.4. Cell death in female cortical explants after pre-treatment with PHTPP, an ERβ antagonist. Overall, 2DG/KCN increased cell death in the vehicle (no drug) explants. Asterisks indicate a significant increase in cell death from the vehicle (no drug) non-injured group. Interestingly, PHTPP pre-treatment decreased cell death from the vehicle 2DG/KCN injured. The letter "a" indicates less cell death as compared to the 2DG/KCN vehicle. Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

6 hour post-treatment of 1 uM PHTPP

The same concentration of PHTPP (1 uM) use for the 24 hour pre-treatment was added 6 hours following the 2DG/KCN injury. A three-way ANOVA comparing the factors “drug”, “injury” and “E2 treatment” was conducted and revealed a significant main effect of injury ($p=0.004$, $F=8.66$, $df=1,102$), an interaction of drug and injury ($p=0.0112$, $F=6.66$, $df=1,102$) and an interaction of injury and treatment ($p=0.050$, $F=5.17$, $df=1,102$).

Post-hoc tests between drug and injury revealed 3 interactions, Figure 4.5. First, 2DG/KCN significantly increased cell death in the vehicle (no drug) group ($p<0.001$; compared to vehicle (no drug) non-injured and $p<0.05$; compared to the PHTPP non-injured). PHTPP post-treatment significantly reduced cell death in the 2DG/KCN injured explants ($p<0.05$; compared to the vehicle (no drug) 2DG/KCN injured). Interestingly, 2DG/KCN did not induce a significant injury within the PHTPP. To summarize these results 2DG/KCN caused a significant injury in the vehicle (no drug) group and PHTPP significantly reduced cell death from the 2DG/KCN vehicle (no drug).

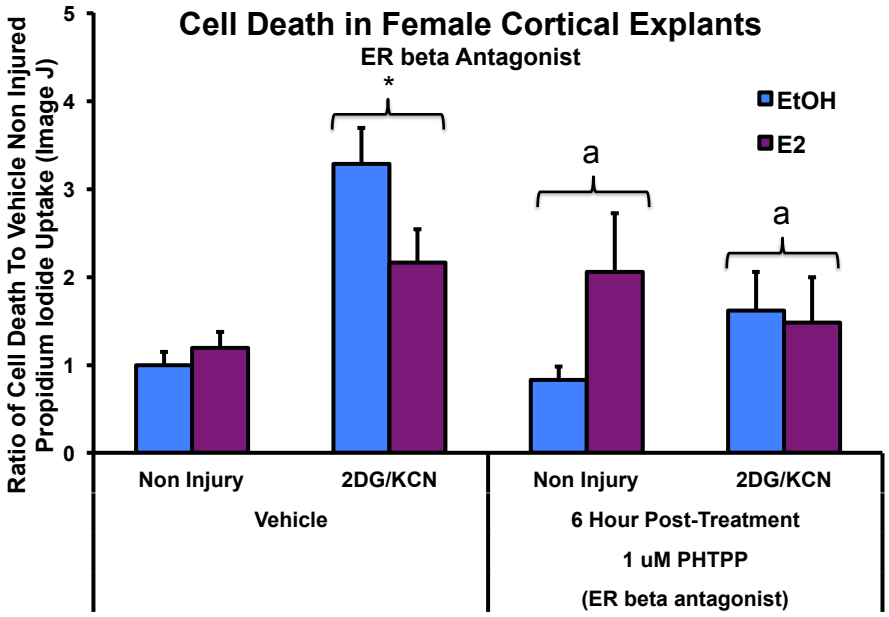


Figure 4.5. Cell death in female cortical explants after post-treatment with 1 uM PHTPP, an ERβ antagonist, illustrating injury and drug effects. Overall, 2DG/KCN increased cell death in the vehicle (no drug) explants. Asterisks indicate a significant increase in cell death from the vehicle (no drug) non-injured group. Interestingly, PHTPP post-treatment, regardless of injury with 2DG/KCN, decreased cell death from the vehicle 2DG/KCN injured. The letter "a" indicates less cell death as compared to the 2DG/KCN vehicle. Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

Post-hoc tests between injury and treatment revealed 4 interactions, Figure 4.6. First, 2DG/KCN significantly increased cell death in the EtOH group ($p<0.05$; compared to EtOH non-injured and $p<0.05$; compared to E2 non-injured). E2 pre-treatment significantly increased cell death in the non-injured explants ($p<0.05$; compared to the EtOH non-injured). 2DG/KCN significantly increased cell death in the E2 pre-treated group ($p<0.05$; compared to EtOH non-injured). Interestingly, there was no additional cell death with 2DG/KCN injury in the E2 pre-treated explants above E2 non-injured.

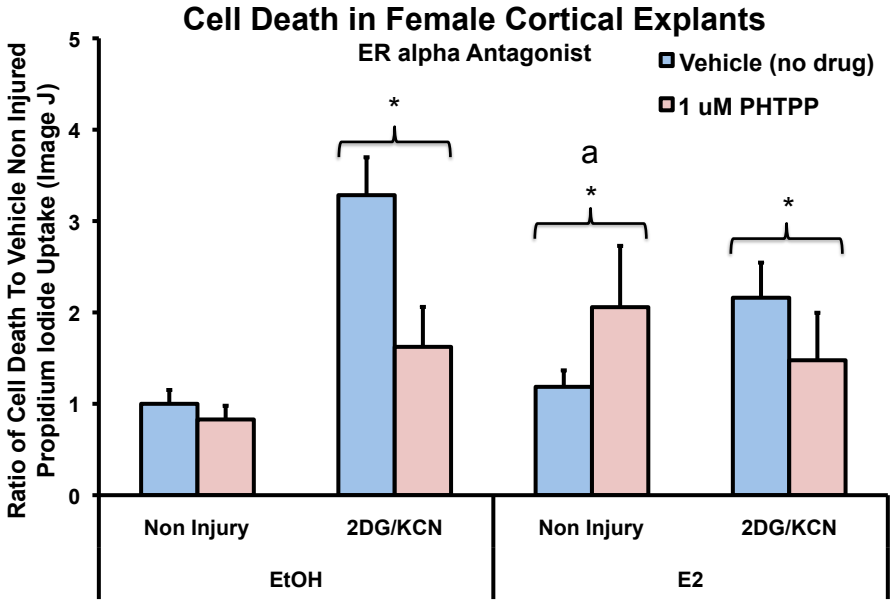


Figure 4.6. Cell death in female cortical explants after post-treatment with 1 uM PHTPP, an ERβ antagonist, illustrating injury and E2 treatment effects. Overall, 2DG/KCN increased cell death in the EtOH group. E2 pre-treatment increased cell death from the EtOH group. Asterisks indicated a significant increase in cell death from the EtOH (vehicle) non-injured group. E2 pre-treated non-injured explants had significantly lower cell death than the 2DG/KCN EtOH explants (indicated by the letter "a" on graph). Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

6 hour post-treatment of 1 nM PHTPP

A 1000 fold weaker concentration (1 nM) of PHTPP than was previous used in the post-treatment experiment was added 6 hours after 2DG/KCN injury to see if 2DG/KCN can induce significant injury. A three-way ANOVA comparing the factors “drug”, “injury” and “treatment” revealed a significant main effect of injury ($p<0.0005$, $F=12.77$, $df=1, 103$), but no main effect of drug or treatment and no interactions, Figure 4.7. These data indicate that 2DG/KCN significantly increased cell death regardless if the explants were vehicle (no drug) or 1 nM PHTPP treated 6 hours after injury and regardless of whether explants were pretreated with E2.

These results did not confirm our initial hypothesis that blocking ERβ activity, either with a 24 hour pre-treatment or a 6 hour post-treatment relative to 2DG/KCN injury, would remove the E2-mediated protection seen in female cortical explants.

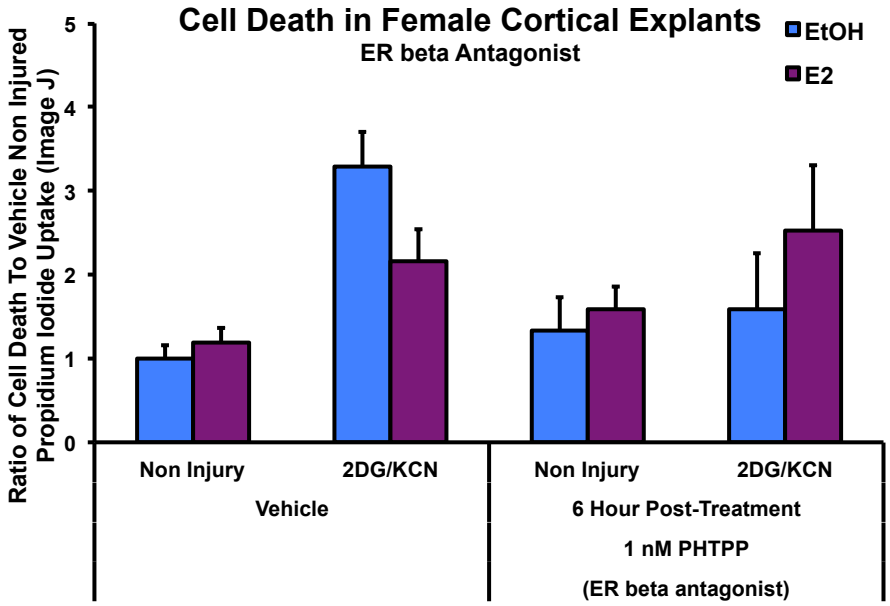


Figure 4.7. Cell death in female cortical explants after post-treatment with 1 nM ERβ antagonist PHTPP. Overall, 2DG/KCN increased cell death in both the vehicle (no injury) and the 1 nM PHTPP groups. Error bars represent SEM.

Does Flutamide influence cell death in female cortical explants?

Flutamide is a selective non-steroidal antiandrogen antagonist for the androgen receptor.

Flutamide Pre-treatment

To determine the effects of the AR antagonist, Flutamide, and the presence of E2 on 2DG/KCN induced cell death in female cortical explants, a three-way ANOVA comparing the factors “drug” (vehicle and Flutamide), “injury” (non-injured and 2DG/KCN injured) and “treatment” (EtOH and E2) was conducted. This test revealed a main effect of injury ($p=0.0012$, $F=10.904$, $df=1,134$), an interaction with drug and injury ($p<0.0001$, $F=23.107$, $df=1,134$) and an interaction with injury and treatment ($p=0.0430$, $F=4.176$, $df=1,134$).

Post-hoc t-tests looking at the interaction between drug and injury (Figure 4.8) revealed 2DG/KCN significant increased cell death in the vehicle group ($p<0.001$; compared to the vehicle non-injured and $p<0.001$; compared to PHTPP non-injured). Flutamide pretreatment caused an increase in cell death among the non-injured ($p<0.05$; compared to EtOH non-injured). Pre-treatment with Flutamide decrease cell death among the 2DG/KCN injured ($p<0.001$; compared to vehicle 2DG/KCN injured). Interestingly, 2DG/KCN did not cause a significant injury when Flutamide was present ($p>0.05$; compared to Flutamide non-injured).

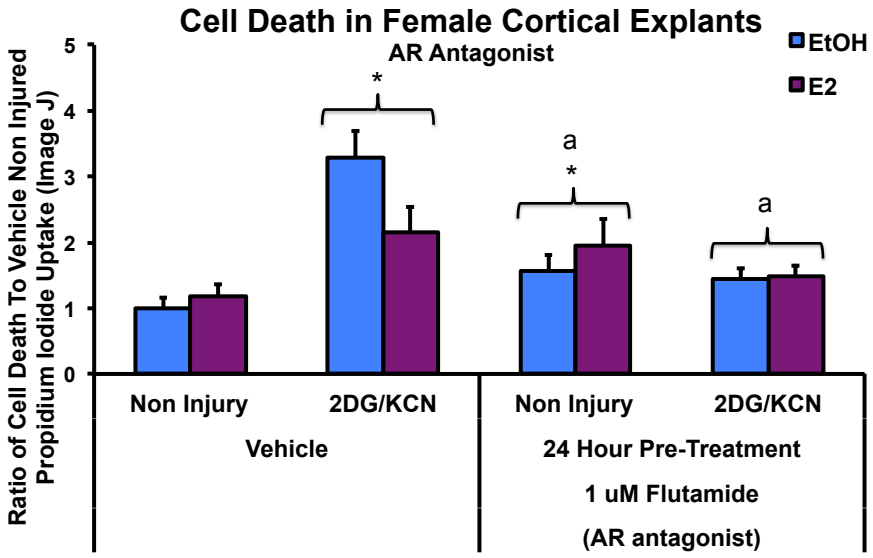


Figure 4.8. Cell death in female cortical explants after pre-treatment with 1 uM AR antagonist, Flutamide, illustrating injury and drug effects. Overall, 2DG/KCN increased cell death in the vehicle group. Pre-treatment with Flutamide increased cell death in the non-injured group. Asterisks indicate a significant increase in cell death from the vehicle (no drug) non-injured group. Pre-treatment with Flutamide significantly decreased cell death from the vehicle (no drug) 2DG/KCN injured. The letter "a" indicates less cell death as compared to the 2DG/KCN vehicle. Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

Post hoc t-tests comparing injury and treatment revealed 3 significant effects, Figure 4.9. 2DG/KCN significantly increased cell death in the EtOH group ($p<0.001$, compared to EtOH non-injured and $p<0.05$, compared to E2 non-injured). E2 pre-treatment significantly decreased cell death ($p<0.05$; compared to EtOH 2DG/KCN injured). Interestingly, significantly injury could not be reached with 2DG/KCN within the pre-treatment Flutamide group.

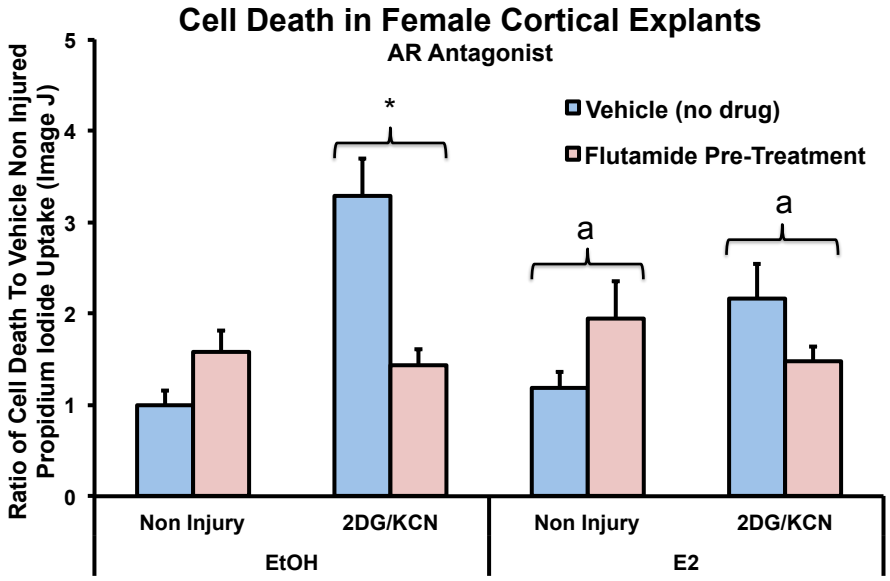


Figure 4.9. Cell death in female cortical explants after pre-treatment with 1 uM AR antagonist, Flutamide, illustrating injury and treatment effects. Overall, 2DG/KCN significant increased cell death in the EtOH group. Asterisks on the graph indicate significant increases from EtOH non-injured. Pre-treatment with E2 regardless of injury with 2DG/KCN significantly reduced cell death from the EtOH 2DG/KCN induced injury (indicated by the letter “a” on graph). Asterisks (*) and the letter “a” on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

Flutamide Post-treatment

To determine if the antagonist was affecting 2DG/KCN's ability to block oxidative phosphorylation and glycolysis (ischemic injury), we added the inhibitor after the injury in two different paradigms (described in methods sections)

6 hour post-treatment of 1 uM Flutamide

A three-way ANOVA comparing the factors "drug", "injury" and "treatment" revealed an interaction of drug and injury ($p=0.0007$, $F=12.218$, $df=1,108$). The post-hoc t-test revealed 3 interactions, Figure 4.10. First, 2DG/KCN significantly increased cell death in the vehicle group ($p<0.05$; compared to vehicle non-injured). Post-treatment with Flutamide increased cell death in the non-injured explants ($p<0.05$; compared to vehicle non-injured). However, post-treatment with Flutamide in the presence of 2DG/KCN decreased cell death ($p<0.05$; compared to vehicle 2DG/KCN). Interestingly, 2DG/KCN did not produce a significant injury within the Flutamide post-treated group ($p>0.05$; compared to Flutamide non-injured).

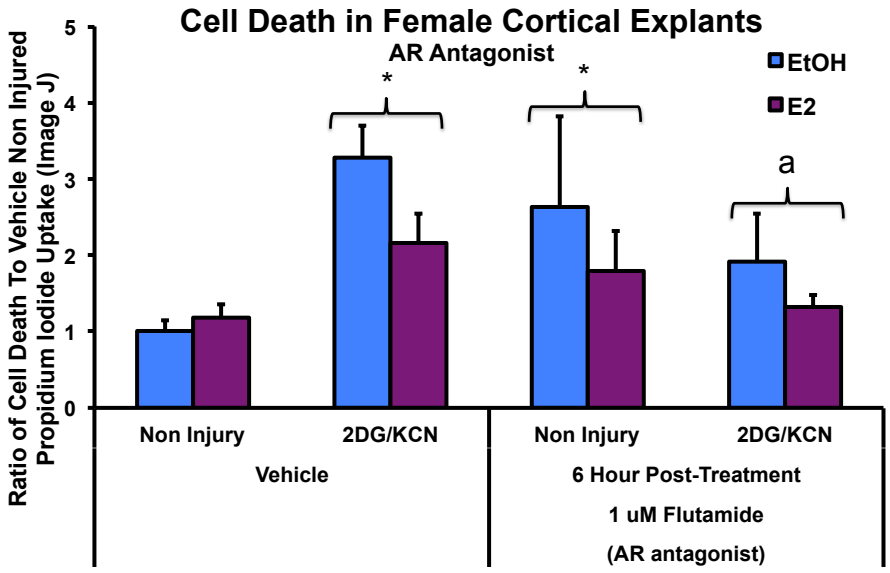


Figure 4.10. Cell death in female cortical explants after post-treatment with 1 uM AR antagonist, Flutamide. Overall, 2DG/KCN increased cell death in the vehicle group. Post-treatment with Flutamide increased cell death in the non-injured explants. Asterisks indicate a significant increase in cell death from the vehicle (no drug) non-injured group. Interestingly, there was a decrease in cell death with the post-treatment of Flutamide in the 2DG/KCN-injured explants. The letter "a" on the graph indicates decreases in cell death from vehicle 2DG/KCN injured. Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

6-hour post-treatment with 1 nM Flutamide

Because I wanted to see not only how adding a lower concentration, 1 nM, of Flutamide after injury affected cell death, but also how E2 pre-treatment and 2DG/KCN induced injury affected cell death a three-way ANOVA comparing the factors “drug”, “injury” and “treatment” was conducted. This test revealed a significant main effect of injury ($p=0.0047$, $F=8.323$, $df=1, 115$), an interaction with drug and injury ($p=0.0003$, $F=14.039$, $df=1,115$) and an interaction with all three factors (drug, treatment and injury).

The post hoc t-tests evaluating the interaction between injury and drug (Figure 4.11) revealed 2DG/KCN significant increased cell death in the vehicle group ($p<0.001$; compared to vehicle non-injured and $p<0.05$; compared to Flutamide non-injured). Flutamide post-treatment decreased cell death in the 2DG/KCN injured explants ($p<0.001$; compared to vehicle 2DG/KCN injured).

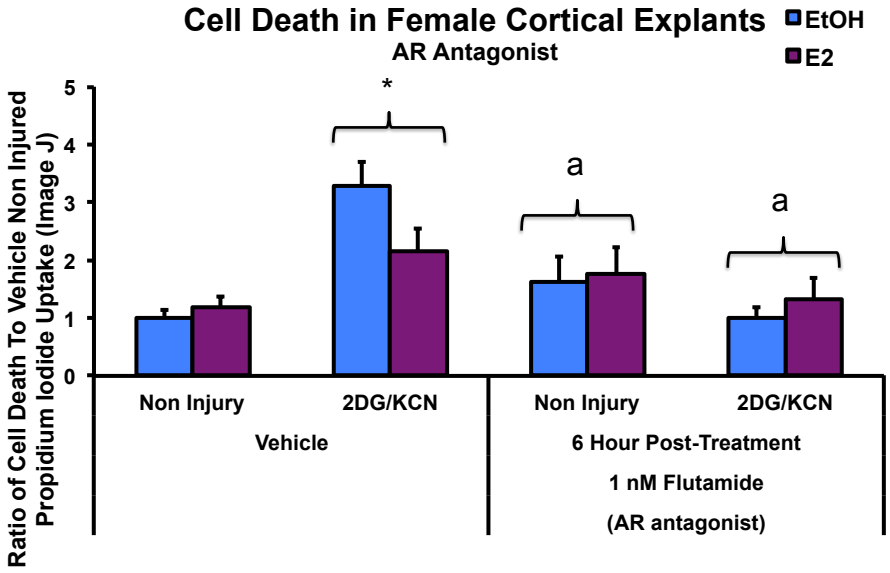


Figure 4.11. Cell death in female cortical explants after post-treatment with 1 nM AR antagonist, Flutamide, illustrating injury and drug effects. Overall, 2DG/KCN increased cell death in the vehicle group. Asterisks indicate a significant increase in cell death from the vehicle (no drug) non-injured group. Interestingly, there was a decrease in cell death with the post-treatment of Flutamide in both the non-injured and the 2DG/KCN injured explants. The letter "a" indicates less cell death as compared to the 2DG/KCN vehicle. Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

The post hoc to evaluate the interaction between drug, treatment and injury revealed 7 interactions (Figure 4.12). 2DG/KCN increased cell death in the EtOH vehicle (no drug) group compared to all non-injured groups ($p<0.001$; compared to non-injured EtOH vehicle (no drug), $p<0.001$; compared to non-injured E2 vehicle (no drug), $p<0.05$; compared to Flutamide EtOH non-injured, and $p<0.05$; compared to Flutamide E2 non-injured). E2 pre-treatment and Flutamide post-treatment decreased cell death from the vehicle 2DG/KCN injured explants ($p<0.05$, compared to the EtOH vehicle (no drug) 2DG/KCN injured). In summary, 2DG/KCN induced significant cell death in vehicle/EtOH (control) cultures. This cell death was reduced by E2 pre-treatment, but protection was not affect by Flutamide. EtOH vehicle (no drug) 2DG/KCN injured explants had significantly more cell death than all of the other experimental groups. In the presence of Flutamide, 2DG/KCN does not cause significant injury.

These results did not confirm our initial hypothesis that blocking AR activity, either with a 24 hour pre-treatment or a 6 hour post-treatment relative to 2DG/KCN injury, would remove the E2-mediated protection seen in female cortical explants. An AR antagonist was included because AR mRNA expression increased in the cortex and in cortical explants across development. AR along with ERβ mRNA expression was high at 22 DIC suggesting a role for these receptors in the adult cortex.

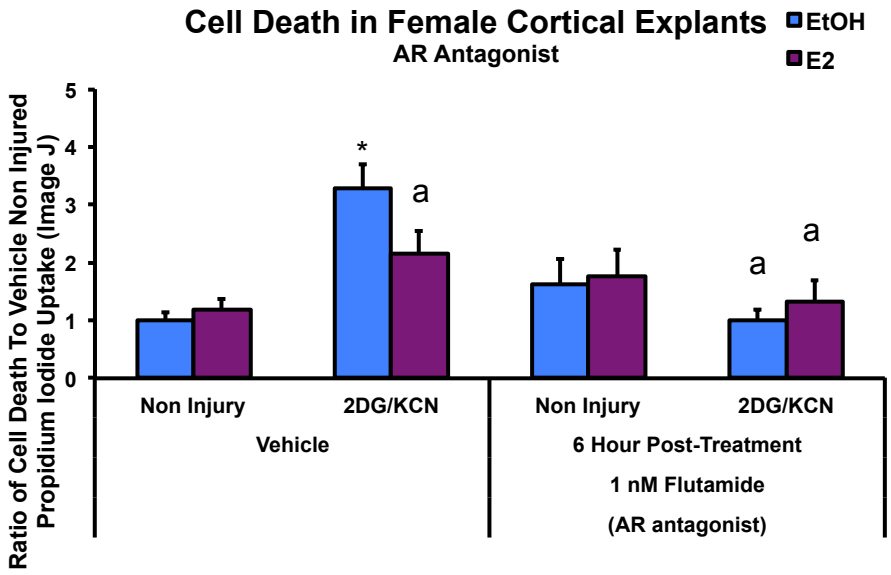


Figure 4.12. Cell death in female cortical explants after post-treatment with AR inhibitor Flutamide, illustrating drug, injury and treatment effects. Overall, 2DG/KCN increased cell death in the EtOH vehicle non-injured compared to both EtOH and E2 non-injured vehicle explants. Asterisks indicated a significant increase in cell death from all non-injured group. E2 pre-treatment or Flutamide post-treatment significantly reduced cell death from EtOH vehicle (no drug) 2DG/KCN injured explants. The letter “a” indicates a decrease from EtOH vehicle (no drug) 2DG/KCN injured. Asterisks (*) and the letter “a” on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

Part 2. How does antagonizing sex steroid hormone receptors affect cell death in MALE cortical explants following injury?

Male cortical explants were also evaluated with each inhibitor scenario. Initially we evaluated the sex steroid hormone antagonist in female cortical explants in hopes of removing the E2 mediated protection. However, in the female cortical explants, when we inhibited ERα, ERβ or AR there was a further reduction in cell death in both EtOH and E2-treated explants. This led us to believe that the sex steroid hormone levels were involved in the cell death induced by 2DG/KCN and by antagonizing ERα, ERβ or AR we could attenuate cell death following a 2DG/KCN induce injury in a E2 treatment independent pathway. In chapter one we evaluated mRNA levels of these receptors, ERα, ERβ or AR, across time in culture and did not see any sex difference in expression. However, in chapter two we evaluated these receptors following injury and discovered specific sex response following injury in ERα and AR mRNA expression. Even though only female explants had a significant increase in mRNA following injury does not remove the theory that these sex steroid hormones are having an effect in male explants also. The protein levels of these receptors directly correlate to the activity level of these receptors. Therefore, male cortical explants were also evaluated in each inhibitor scenario to determine what effect ERα, ERβ or AR had following injury.

Does inhibiting ERα influence cell death in MALE cortical explants?

MPP Dihydrochloride (MPP) is a high affinity specific ERα antagonist that inhibits at the receptor.

MPP Pre-treatment

To determine the effects of the ERα antagonist, MPP, on 2DG/KCN induced cell death in E2 treated male cortical explants, a three-way ANOVA comparing the factors “drug” (vehicle and MPP), “injury” (non-injured and 2DG/KCN treated) and “treatment” (EtOH and E2). This test revealed a main effect injury ($p=0.0023$, $F=9.676$, $df=1,125$), but no main effect of drug and no interactions, Figure 4.13. This indicated that 2DG/KCN caused significant injury, which was not dependent on pretreatment with MPP or E2. Interestingly, male, unlike female, cortical explants did not have an increase in cell death in the non-injured explants with the pretreatment of MPP.

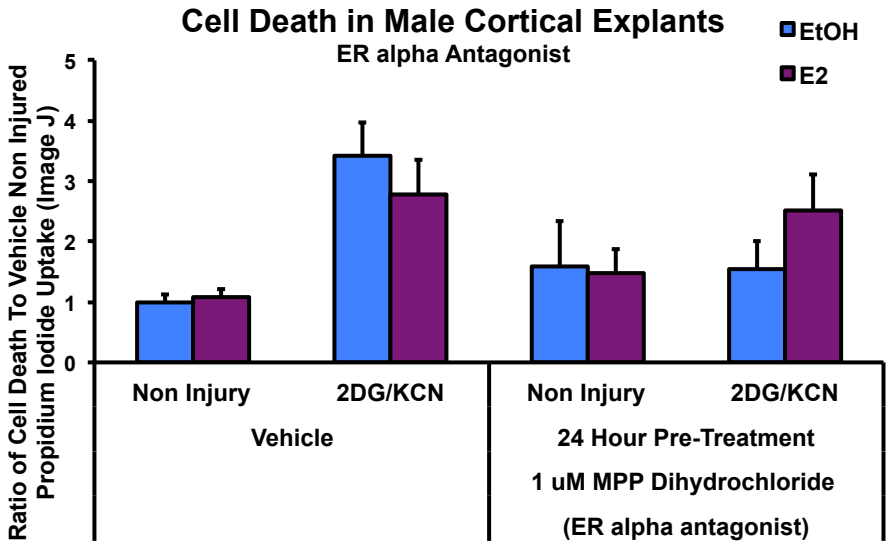


Figure 4.13. Cell death in male cortical explants after pre-treatment with MPP, ERα antagonist. Overall, 2DG/KCN caused significant injury, which was not dependent on pretreatment with MPP or E2. Error bars represent SEM.

6 hour post-treatment of 1 uM MPP Dihydrochloride

The same concentration of MPP (1 uM) that was used for the 24 hour pre-treatment was added 6 hours following injury with 2DG/KCN. A three-way ANOVA comparing the factors drug, injury and treatment was conducted and revealed a significant main effect of injury ($p=0.0220$, $F=5.375$, $df=1,126$) and an interaction of drug and injury ($p=0.0107$, $F=6.721$, $df=1,126$). The post hoc t-test revealed that 2DG/KCN caused significant injury in the vehicle (no drug) group ($p<0.05$; compared to vehicle non-injured). 2DG/KCN did not cause significant injury when the male cortical explants were post-treated with MPP.

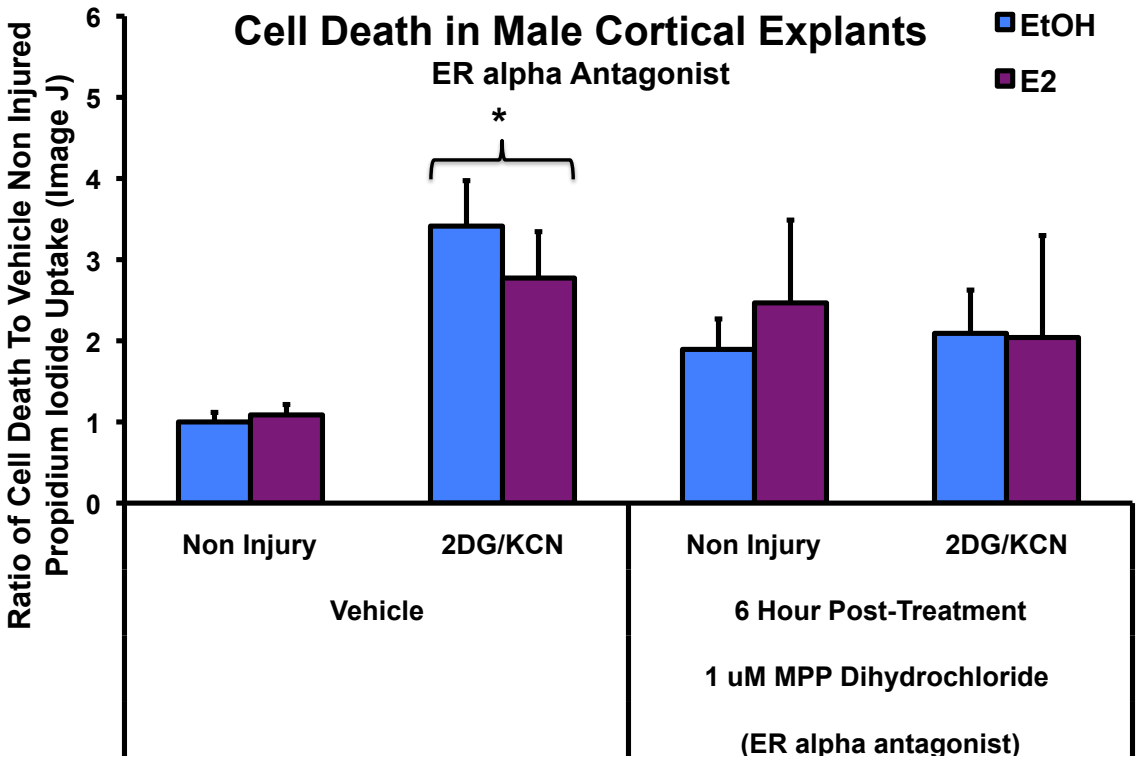


Figure 4.13. Cell death in male cortical explants after post-treatment with 1 uM MPP, ERα antagonist. Overall, 2DG/KCN increased cell death in the vehicle (no drug) group. Asterisks indicated a significant increase in cell death from the vehicle (no drug) non-injured group. Asterisks (*) on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

6-hour post-treatment with 1 nM MPP

1000 fold weaker concentration (1 nM) of MPP than was previous used (1 uM) in the post-treatment experiment of MPP was added 6 hours after 2DG/KCN injury to see if 2DG/KCN can induce significant cell death in the presence of MPP. A three-way ANOVA comparing the factors “drug”, “injury” and “treatment” were conducted. This test also revealed a main effect injury ($p=0.0008$, $F=11.675$, $df=1,128$), but no main effects of drug or treatment and no interactions. This indicated that 2DG/KCN induced significant injury regardless of E2 pretreatment or application of 1 nM MPP after injury. This is interesting because 2DG/KCN produced significant cell death when explants were pretreated with 1 uM MPP, but not when they were post-treated with 1 uM MPP. The weaker concentration (1 nM) of MPP that was applied post-treatment no longer blocked 2DG/KCN-induced injury.

In summary, 2DG/KCN induced significant injury, from vehicle (no drug) non-injured, when 1 um MPP was applied 24 hours before injury or 1 nM MPP was applied 6 hours following injury. However, in these scenario’s 2DG/KCN did not induce a significant injury within the MPP group, compared to MPP non-injured. This indicates that MPP increased cell death in the non-injured MPP group and removed 2DG/KCN’s ability to cause significant cell death above the MPP non-injured group, although the MPP 2DG/KCN injury was significantly different from the vehicle non-injured. Interestingly, when 1 uM MPP was added 6 hours following injury, 2DG/KCN did not induce a significant injury. These

results did confirm our hypothesis for male cortical explants that blocking ERα activity would alter the injury induced by 2DG/KCN.

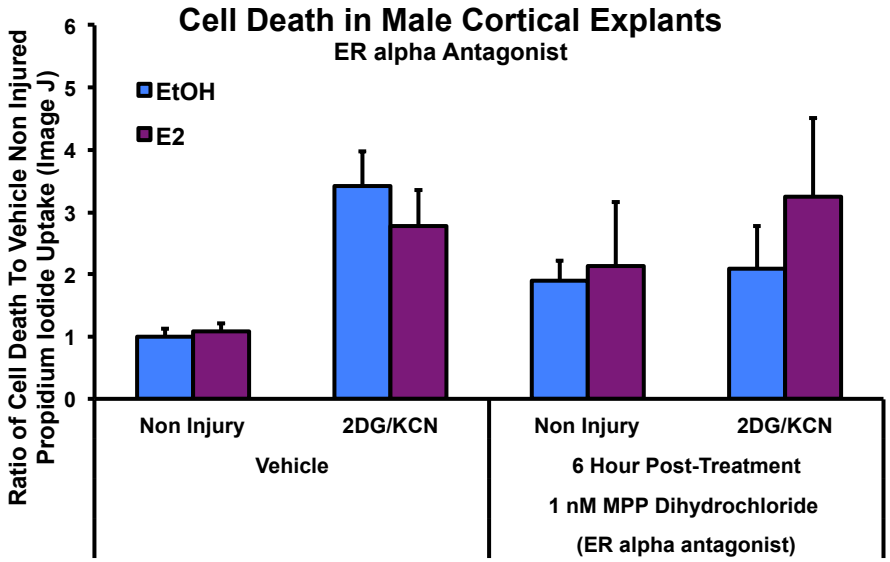


Figure 4.14. Cell death in male cortical explants after post-treatment with 1 nM MPP, ERα inhibitor. Overall, 2DG/KCN increased cell death in the vehicle (no drug) and in the MPP pre-treatment group. Error bars represent SEM.

24 hour pre-treatment of 1 uM PHTPP

To determine the effects of the ERβ antagonist, PHTPP, on cell death in male cortical explants, a three-way ANOVA comparing the factors “drug” (Vehicle and PHTPP), “injury” (non injured and 2DG/KCN treated) and “treatment” (EtOH and E2) was conducted. This test revealed a main effect of injury ($p=0.0101$, $F=6.765$, $df=1,127$) and an interaction between drug and injury ($p=0.0193$, $F=5.616$, $df=1,127$. Post-hoc t-tests revealed 2DG/KCN increased cell death in the vehicle group ($p<0.05$; compared to vehicle non-injured). Pre-treatment with PHTPP decreased cell death in the 2DG/KCN group ($p<0.05$; compared to vehicle 2DG/KCN injured). Pre-treatment with PHTPP also prevented 2DG/KCN from inducing significant injury ($p>0.05$; compared to PHTPP non-injured).

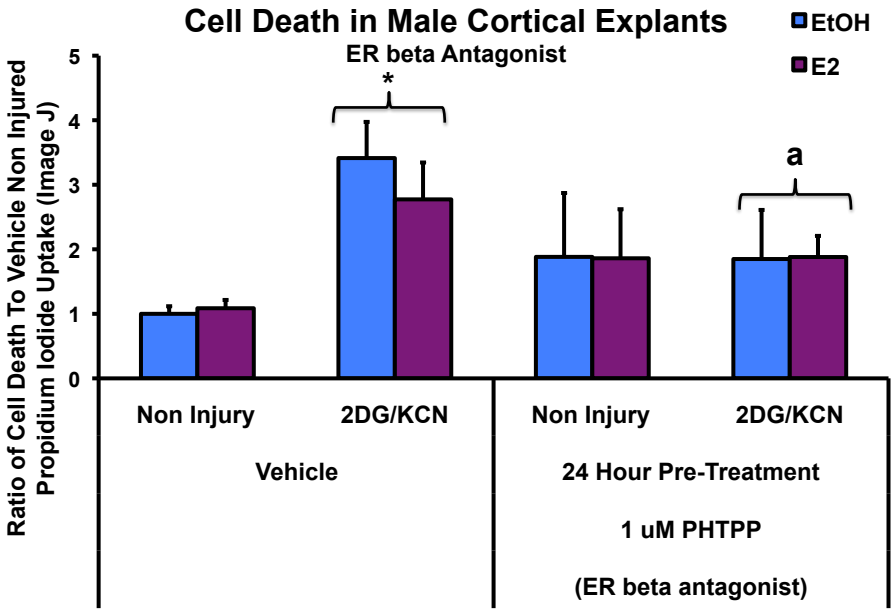


Figure 4.15. Cell death in male cortical explants after pre-treatment with PHTPP, an ERβ antagonist. Overall, 2DG/KCN increased cell death in the vehicle (no drug). Asterisks indicated a significant increase in cell death from the vehicle (no drug) non-injured group. Interestingly, there was a decrease in cell death with the pre-treatment of PHTPP in the 2DG/KCN injured explants. The letter "a" on graph indicates a significant decrease in cell death from the vehicle 2DG/KCN. Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

6 hour post-treatment of 1 uM PHTPP

The same concentration of PHTPP (1 uM) used for the 24 hour pre-treatment was added 6 hours following injury with 2DG/KCN. A three-way ANOVA comparing the factors “drug”, “injury” and “treatment” was conducted. This test revealed a significant main effect of injury ($p=0.020$, $F=5.549$, $df=1, 125$) and an interaction with drug and injury ($p=0.006$, $F=7.768$, $df=1,125$). Post-hoc t-tests revealed that 2DG/KCN induced significant injury in the vehicle group ($p<0.001$; compared to vehicle non-injured). PHTPP post-treatment increased cell death in the non-injured ($p<0.05$; compared to vehicle (no drug) non-injured) and in the 2DG/KCN injured ($p<0.05$; compared to vehicle (no drug) non-injured).

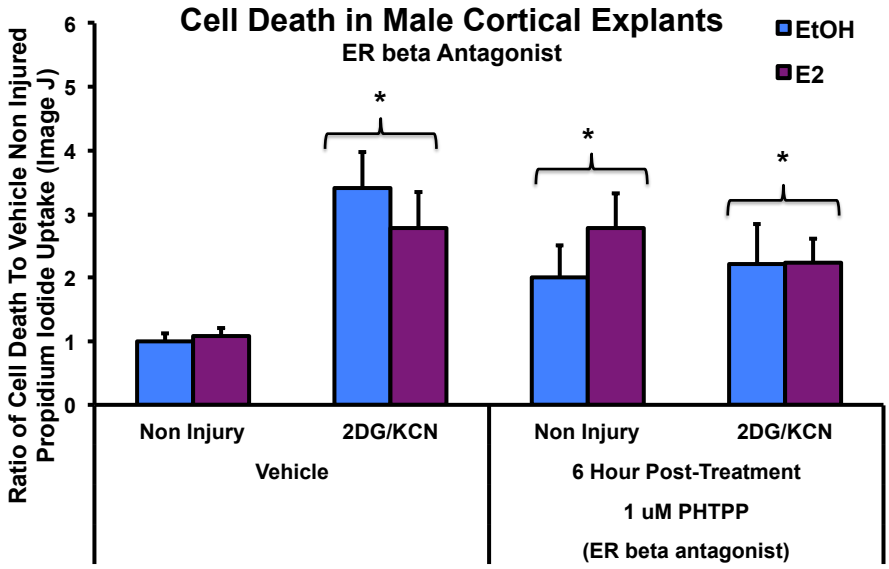


Figure 4.16. Cell death in male cortical explants after post-treatment with 1 uM PHTPP, an ERβ antagonist. Overall, 2DG/KCN increased cell death in the vehicle (no drug) and in PHTPP compared to vehicle non-injured. Post-treatment with PHTPP also increased cell death in the non-injured group. Asterisks indicated a significant increase in cell death from the vehicle (no drug) non-injured group, $p < 0.05$. Error bars represent SEM.

6 hour post-treatment of 1 nM PHTPP

A three-way ANOVA comparing the factors “drug”, “injury” and “treatment” was conducted and revealed a significant main effect of injury ($p=0.0135$, $F=6.277$, $df=1, 120$) and an interaction with drug and injury ($p=0.0061$, $F=7.769$, $df=1,125$). Post-hoc t-tests revealed that 2DG/KCN induced a significant injury in the vehicle and PHTPP group ($p<0.001$; compared to vehicle non-injured). PHTPP caused significant cell death in the non-injured ($p<0.05$; compared to vehicle non-injured). This is interesting because PHTPP post-treatment increased cell death in the non-injured, but PHTPP pre-treatment did not increase cell death in the non-injured. These results did confirm our hypothesis for male cortical explants that blocking ERβ activity 24 hours before 2DG/KN would alter the cell death induced by 2DG/KCN. However, post-treatment with PHTPP did not confirm our initial hypothesis because cell death was equivalent to the vehicle (no drug) groups, suggesting blocking ERβ post-treatment did not alter injury with 2DG/KCN.

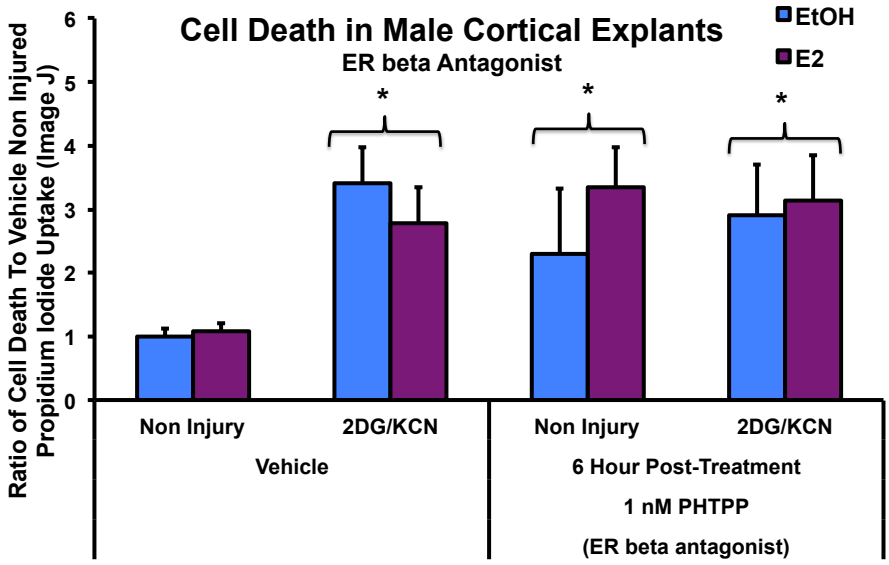


Figure 4.17. Cell death in male cortical explants after post-treatment with 1 nM PHTPP, an ERβ antagonist. Overall, 2DG/KCN increased cell death in the vehicle (no drug) and PHTPP groups compared to vehicle non-injured explants. Post-treatment with PHTPP also increased cell death in the non-injured group. Asterisks indicated a significant increase in cell death from the vehicle (no drug) non-injured group, $p < 0.05$. Error bars represent SEM.

Does Flutamide influence cell death in male cortical explants?

Flutamide is a selective non-steroidal anti-androgen antagonist for the androgen receptor.

Flutamide Pre-treatment

To determine the effects of the AR antagonist, Flutamide, and the presence of E2 on cell death following injury with 2DG/KCN in male cortical explants, a three-way ANOVA comparing the factors “drug” (Vehicle and Flutamide), “injury” (non-injured and 2DG/KCN treated) and “treatment” (EtOH and E2) revealed a main effect of injury ($p=0.0029$, $F=9.226$, $df=1,129$), main effect of drug ($p=0.0031$, $F=9.085$, $df=1,129$), and an interaction between drug and injury ($p=0.0100$, $F=6.834$, $df=1,129$). Post-hoc t-tests revealed that 2DG/KCN increased cell death in the vehicle group ($p<0.001$, compared to vehicle non-injured and $p<0.001$; compared to Flutamide non-injured). Flutamide pre-treatment decreased cell death in the 2DG/KCN group ($p<0.001$; compared to vehicle 2DG/KCN). In summary, 2DG/KCN induced injury in the vehicle and the presence of Flutamide significantly lowered this cell death.

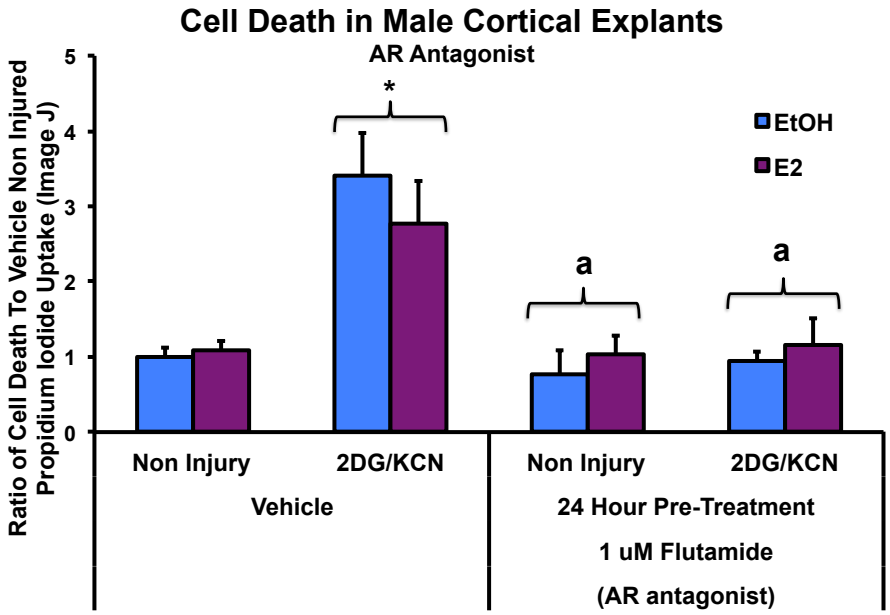


Figure 4.18. Cell death in male cortical explants after pre-treatment with Flutamide, an AR inhibitor Flutamide. Overall, 2DG/KCN increased cell death in the vehicle (no drug). Asterisks indicated a significant increase in cell death from the vehicle (no drug) non-injured group. Pre-treatment with Flutamide decreased cell death from the vehicle 2DG/KCN injured. The letter "a" indicates less cell death as compared to 2DG/KCN vehicle. Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

6 hour post-treatment of 1 uM Flutamide

The same concentration of Flutamide (1 uM) used for the 24 hour pre-treatment was added 6 hours following injury with 2DG/KCN. A three-way ANOVA comparing the factors drug, injury and treatment revealed an effect of injury ($p=0.0052$, $F=8.084$, $df=1,136$), an interaction with drug and injury ($p=0.0024$, $F=9.590$, $df=1,136$). Post-hoc t-tests revealed 2DG/KCN significantly increased cell death in the vehicle ($p<0.05$; compared to vehicle non-injured). Post-treatment with Flutamide significantly reduced cell death in the 2DG/KCN group ($p<0.05$; compared to vehicle 2DG/KCN injured). 2DG/KCN did not induce significant injury ($p>0.05$; compared to Flutamide non-injured) when the explants were treated 6 hours following injury with Flutamide.

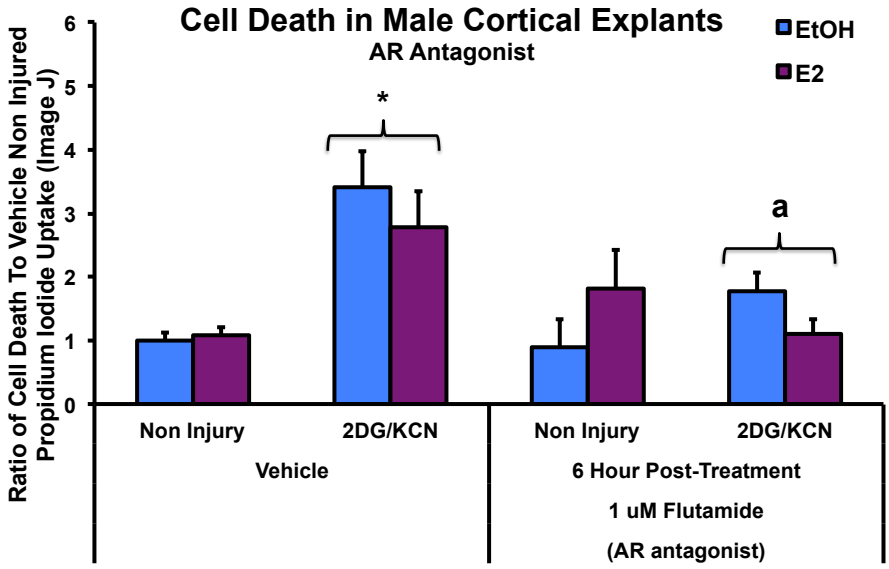


Figure 4.19. Cell death in male cortical explants after post-treatment with 1 uM Flutamide, an AR inhibitor Flutamide. Overall, 2DG/KCN increased cell death in the vehicle (no drug). Asterisks indicated a significant increase in cell death from the vehicle (no drug) non-injured group. Post-treatment with Flutamide reduced cell death in the 2DG/KCN group. The letter "a" indicates a decrease in cell death from the 2DG/KCN vehicle group. Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

6-hour post-treatment with 1 nM Flutamide

1000 fold weaker concentration (1 nM) of Flutamide than was previous used (1 uM) in the post-treatment experiment of Flutamide was added 6 hours after 2DG/KCN injury to see if 2DG/KCN can induce significant cell death in the presence of Flutamide. A three-way ANOVA comparing the factors “drug”, “injury” and “treatment” were conducted. This test also revealed a main effect of injury ($p=0.0018$, $F=10.109$, $df=1,134$), a main effect of drug ($p=0.0151$, $F=6.061$, $df=1, 134$) and an interaction with drug and injury ($p=0.0095$, $F=6.918$, $df=1,134$). Post-hoc t-tests revealed 2DG/KCN significantly increased cell death in the vehicle ($p<0.05$; compared to vehicle non-injured).

Post-treatment with Flutamide significantly reduced cell death ($p<0.05$; compared to vehicle 2DG/KCN injured). 2DG/KCN did not induce significant injury ($p>0.05$; compared to Flutamide non-injured) when the explants were treated 6 hours following injury with Flutamide. These results did confirm our hypothesis for male cortical explants that blocking AR activity would alter the injury induced by 2DG/KCN.

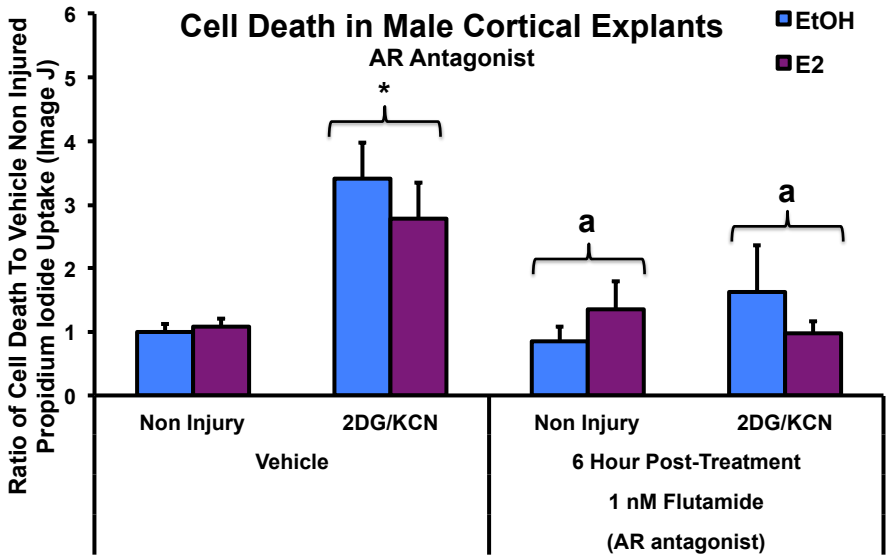


Figure 4.20. Cell death in male cortical explants after post-treatment with 1 nM Flutamide, an AR inhibitor Flutamide. Overall, 2DG/KCN increased cell death in the vehicle (no drug). Asterisks indicated a significant increase in cell death from the vehicle (no drug) non-injured group. Post-treatment with Flutamide decreased cell death from the vehicle 2DG/KCN injured. The letter "a" indicates less cell death as compared to the 2DG/KCN vehicle. Asterisks (*) and the letter "a" on the graph indicate significant differences, $p < 0.05$. Error bars represent SEM.

Discussion

Here, we describe the role of sex steroid hormone receptors in female and male cortical explants treated with 2DG/KCN. Sex steroid hormone antagonists were added either before or after injury. In female cortical explants, pretreatment or post-treatment with any of the sex steroid hormones did not remove the E2 mediated protection following 2DG/KCN treatment. Interestingly, treatment with the inhibitors alone provided additional protection for the female explants following injury with 2DG/KCN. This did not confirm our initial hypothesis that if E2 neuroprotection in female cortical explants is mediated by a particular receptor, blocking that receptor will increase cell death and remove the E2 mediated protection. In fact, most inhibitors further reduced cell death in the 2DG/KCN (injury) treated female explants, results summarized in Table 4.1. The hypothesis for the male cortical explants that these receptors are also aiding in the cell death caused by 2DG/KCN, was confirmed and summarized in Table 4.2. Interestingly, male cortical explants followed a similar pattern of protection when the sex steroid hormone receptors were inhibited.

In female explants treated with MPP, an ERα antagonist, the drug did affect cell death. However, this affect was not what we predicted. In the 2DG/KCN injured explants, addition of 1 uM MPP prior or 6 hours after the injury decreased cell death. We predicted that blocking ERα would actually increase cell death if E2 were acting via ERα. Interestingly, pre-treatment with MPP to the non-injured explants actually increased cell death, suggesting the drug is having

Table 4.1. Summary of the Role of Sex Steroid Hormone Receptor Activity.
24 hour pre-treatment and 6 hour post-treatment with ERα, ERβ and AR

antagonist in female cortical explants injured with 2DG/KCN. Note: ↑ indicates an increase in cell death, ↓ indicates a decrease in cell death and V indicates vehicle (no drug).

				Cell Death
Female Cortical Explants	**24 Hour Pre-Treatment**	**MPP**	**Non Injured**	↑ from V non-injured
			2DG/KCN	↓ from V 2DG/KCN
		PHTPP	**Non Injured**	↓ from V 2DG/KCN
			2DG/KCN	↓ from V 2DG/KCN
		Flutamide	**Non Injured**	↑ from V non-injured ↓ from V 2DG/KCN
			2DG/KCN	↓ from V 2DG/KCN
	1 uM 6 Hour Post-Treatment	**MPP**	**Non Injured**	↓ from V 2DG/KCN
			2DG/KCN	↓ from V 2DG/KCN
		PHTPP	**Non Injured**	↓ from V 2DG/KCN
			2DG/KCN	↓ from V 2DG/KCN
		Flutamide	**Non Injured**	↑ from V non-injured
			2DG/KCN	↓ from V 2DG/KCN
	1 nM 6 Hour Post-Treatment	**MPP**	**Non Injured**	No Effect
			2DG/KCN	↑ from V non-injured
		PHTPP	**Non Injured**	↓ from V 2DG/KCN
			2DG/KCN	↓ from V 2DG/KCN
		Flutamide	**Non Injured**	↓ from V 2DG/KCN
			2DG/KCN	↓ from V 2DG/KCN

Table 4.2. Summary of the Role of Sex Steroid Hormone Receptor Activity. 24 hour pre-treatment and 6 hour post-treatment with ERα, ERβ and AR antagonist in male cortical explants injured with 2DG/KCN. Note: ↑ indicates an

increase in cell death, ↓ indicates a decrease in cell death and V indicates vehicle (no drug).

				Cell Death
Male Cortical Explants	**24 Hour Pre-Treatment**	**MPP**	**Non Injured**	No Effect
			2DG/KCN	↑ from V non-injured
		PHTPP	**Non Injured**	No Effect
			2DG/KCN	↑ from V non-injured
		Flutamide	**Non Injured**	↓ from V 2DG/KCN
			2DG/KCN	↓ from V 2DG/KCN
	1 uM 6 Hour Post-Treatment	**MPP**	**Non Injured**	No Effect
			2DG/KCN	No Effect
		PHTPP	**Non Injured**	↑ from V non-injured
			2DG/KCN	↑ from V non-injured
		Flutamide	**Non Injured**	No Effect
			2DG/KCN	↓ from V 2DG/KCN
	1 nM 6 Hour Post-Treatment	**MPP**	**Non Injured**	No Effect
			2DG/KCN	↑ from V non-injured
		PHTPP	**Non Injured**	↑ from V 2DG/KCN
			2DG/KCN	↑ from V 2DG/KCN
		Flutamide	**Non Injured**	↓ from V 2DG/KCN
			2DG/KCN	↓ from V 2DG/KCN

an effect even when there is no injury. When cell death was evaluated in all 3 scenarios, pre or post treatment with MPP, 2DG/KCN did not induce significant

injury. This indicated that when MPP is present before or after 2DG/KCN treatment, a significantly injury cannot be induced.

Treatment with 1 uM PHTPP (ERβ antagonist) 24 hours before or 6 hours following injury also caused a significant decrease in cell death in the 2DG/KCN injured explants. Interestingly a lower concentration, 1 nM, of PHTPP increased cell death, regardless of a 2DG/KCN injury, when added 6 hours following injury.

Treatment with Flutamide, the AR antagonist, also significantly decreased cell death in the 2DG/KCN treated and an increase in cell death in the non-injured when the inhibitor was added 24 hours before injury. Post-treatment with the two different concentrations did result in significant differences. Post-treatment with Flutamide increased cell death in the non-injured at 1 um, but not 1 nM. There was a significant decrease in the 2DG/KCN injured groups when post-treated with Flutamide.

In male cortical explants, MPP either pre or post-treatment did not affect cell death in the non-injured. 2DG/KCN increased cell death in the pre-treatment and 1 nM post-treatment from the vehicle non-injured. Pre-treatment with PHTPP also did not influence cell death in the non-injured, but did decrease cell death in the 2DG/KCN injured. Post-treatment with PHTPP regardless of concentration and injury increased cell death from the vehicle non-injured. However, Flutamide pre and post treatment decreased cell death from the vehicle 2DG/KCN injured; except for the 1 uM post-treated non-injured, which did not have an effect.

The increase in cell death in the non-injured female explants could indicate that these receptors are needed at a certain concentration to maintain a healthy explant and by inhibiting these receptors the explant loses its cellular regulation. The exact roles of these sex steroid hormone receptors are not well defined. Since these receptors are located in the mitochondria, (Solakidi, Psarra et al. 2005; Arnold and Beyer 2009; Vasconsuelo, Pronsato et al. 2011) they may be necessary for transcription of mitochondrial genes. There are 37 genes contained in the mitochondrial DNA, some of which encode enzymes for the oxidative phosphorylation. Disruption of the mitochondrial sex steroid hormone receptors may disrupt the electron transport chain by down regulating the transcription of oxidative phosphorylation enzymes needed to maintain normal cellular respiration and homeostasis.

Another possible reason for the additional damage is that the inhibitor cocktail itself is damaging to the explant and somehow prevents additional injury when the explants are treated with 2DG/KCN. However, because the effects of each inhibitor were dependent on the concentration and timing of treatment, it is unlikely the inhibitor cocktail but rather a base-line concentration of receptors are needed.

In summary, female cortical explants had an effect of E2 with post-treatment of PHTPP at 1uM and pre-treatment with Flutamide. Post-treatment of 1 uM PHTPP inhibited cell death induced by 2DG/KCN in the E2 pre-treated female explants. However, EtOH (controls) did have a significant increase in cell death with 2DG/KCN. E2 pre-treatment increased cell death. Pre-treatment with

Flutamide, also had an increase with 2DG/KCN in the EtOH group and E2 protected this increase. Interestingly, E2 pre-treatment did not influence cell death in male cortical explants treated with antagonist or injured with 2DG/KCN. This correlates to the protective effect of E2 in female, but not male cortical explants. We initially hypothesized that if E2 neuroprotection in female cortical explants is mediated by a particular receptor, blocking that receptor will increase cell death and remove the E2 protection. We did not find any data to support that hypothesis. In fact, most inhibitors further reduced cell death when injured with 2DG/KCN.

Overall, the E2 mediated protection seen in female cortical explants was not mediated through ERα, ERβ or AR in a classical fashion. In fact, when these receptors were inhibited in female cortical explants there was a decrease in cell death in the 2DG/KCN injured. In female cortical explants, blocking ERα, ERβ or AR following injury with 2DG/KCN did not add additional cell death to the non-injured, depending on the antagonist and the concentration. In male cortical explants, 2DG/KCN caused significant cell death in the MPP scenarios from the vehicle non-injured. PHTPP post-treatment generally increased cell death from the vehicle non-injured. However, PHTPP pre-treatment and Flutamide pre and post-treatment decreased cell death from the vehicle 2DG/KCN injured. These results are extremely perplexing. Concentration and timing of the antagonist influences cell death, indicating different roles of the specific sex steroid hormone receptors throughout protection and injury in both female and male cortical explants.

These data indicate that sex steroid hormone receptors may play a role in the ability of 2DG/KCN to induce injury. Only treatment with 2DG/KCN induces a significant injury with in the vehicle group. Studies have shown that ERα, ERβ (Solakidi, Psarra et al. 2005; Pedram, Razandi et al. 2006; Arnold and Beyer 2009; Vasconsuelo, Pronsato et al. 2011) and AR (Solakidi, Psarra et al. 2005) are present in the mitochondria. KCN in our injury inhibits oxidative phosphorylation by inhibiting cytochrome c oxidase in complex IV of the electron transport chain (ETC). E2 mediated protection in another injury models, hemorrhagic shock, was mediated through ERβ in the mitochondria by upregulating the genes of complex IV in the ETC (Hsieh, Yu et al. 2006). Disruption of complex IV by cyanide (CN-) treatment inhibited the E2 mediated protection (Hsieh, Yu et al. 2006). E2 also increased expression of ERα and ERβ in the mitochondria (Chen, Delannoy et al. 2004; Chen, Eshete et al. 2004). Since E2 can increase expression of ER in the mitochondria where there is a balance of normal cellular respiration from the ETC, in our model the balance of hormone receptors could be such that when E2 is present and the explants are injured, E2 is able to compensate for the inhibition of cytochrome c oxidase and mediate protection. However, when we potentially disrupt the activity of these sex steroid hormone receptors in the mitochondria we can see an increase in cell death without the addition of 2DG/KCN that could be interfering with our injury or inducing secondary pathways to maintain a homeostatic environment. Our results are timing and concentration specific when we do not see additional cell death to the non injured and can further reduce injury in the 2DG/KCN treated.

Sex steroid hormones receptors may have additional roles in the mitochondria, which allow additional protection from injury or increase cell death in a basal (non injured) state regardless of E2 treatment when these receptors are inhibited.

Cell survival can be promoted through genomic and non-genomic actions of steroid hormone receptors. E2 can have both genomic and non-genomic actions on target tissues by binding to the well-characterized steroid hormone receptors, ERα and ERβ (Green, Kumar et al. 1986; Koike, Sakai et al. 1987; Kuiper, Enmark et al. 1996; Berne RM 2004). Androgens, testosterone and dihydrotestosterone, have important actions by binding to AR (Roy, Lavrovsky et al. 1999). ERα, ERβ and AR are steroid hormone receptors located not only in the nucleus, but also the plasma membrane, cytosol and mitochondria (Speroff 2000; Gonzalez, Cabrera-Socorro et al. 2007). AR expression is localized with ERα and ERβ expression in many areas of the brain, specifically the cortex and hypothalamus (Patchev, Schroeder et al. 2004).

Sex steroid hormones can have both genomic and non-genomic actions on target tissues (Murdoch and Gorski 1991; Berne RM 2004). Here we have eliminated the role of E2 working through the classical genomic pathways in our model of E2-mediated neuroprotection. The non-genomic actions of steroid hormones include membrane initiated signaling pathways that are activated not only by ERα, ERβ and GPR30 (Watson, Jeng et al. 2008; Roman-Blas, Castaneda et al. 2009; Liu, Zhang et al. 2012), but by AR (Foradori, Weiser et al. 2008) (Peterziel, Mink et al. 1999). E2 induces phosphorylation of extracellular signal-regulated kinase (ERK1/2) (Singh, Setalo et al. 1999; Setalo, Singh et al.

2002; Liu, Zhang et al. 2012). ERK is a part of the mitrogen-activated protein (MAP) kinase pathway (Singer, Figueroa-Masot et al. 1999; Watson, Jeng et al. 2008). Another second messenger signaling pathway that can be activated is the serine/threonine protein kinase, AKT, through the phosphatidylinositide 3'OH kinase (PI3 K) by E2 (Datta, Brunet et al. 1999; Wilson, Liu et al. 2002) and testosterone (Yu, Akishita et al. ; Yu, Akishita et al.). These kinases regulate a number of cellular responses including cell death (Yu, Akishita et al. ; Yu, Akishita et al. ; Lee and McEwen 2001; Murphy and Blenis 2006). Activation of one or more of these signaling cascades by E2 can promote cell survival. In our studies, these non-genomic actions of E2 may be responsible for our results.

These data are the first to show that E2 mediated protection is not mediated through ERα, ERβ or AR in the classical receptor mediated fashion. In fact, depending on concentration and timing of the antagonist, ERα, ERβ and AR antagonist could reduce cell death following treatment with 2DG/KCN with out affecting the non-injured explants. The specific roles of sex steroid hormone receptors to increase injury have not been described in the female or male cortex. These data demonstrate that cortical responses to injury may be innate and preprogrammed based on sex and hormonal background.

CHAPTER 5: GENERAL DISCUSSION

Summary

The studies included in this dissertation provide valuable insight into how sex steroid hormone receptors in the cortex respond during early postnatal development and following injury. The isocortex is a nonconventional area of the brain to study sex steroid hormone receptor dynamics. These receptors have been studied more extensively in other regions of the brain, such as the hypothalamus, that are responsible for the reproductive action of steroids. Estrogen and androgens are two main sex steroid hormones that are associated with sex differences. ERα, ERβ and AR are the typical sex steroid hormone receptors associated with estrogens and androgens. It is important to understand expression of these receptors in the cortex at the time of injury to see how these receptors respond to injury.

During cortical development, the expression of several sex steroid hormones was dynamic. ERα mRNA expression was elevated then decreased to a very low level across early postnatal development. ERβ and AR mRNA expression were low and then increased across early postnatal development in the cortex. Interestingly, around the same time the changes in sex steroid hormone receptors occur in the cortex, steroidogenesis is beginning in the female and male sex organs, ovaries and testes. Reproductive regions of the brain respond to steroidogenesis by up or down regulating receptors and signaling cues to establish a positive and negative feedback loop to regulate the

reproductive axis. Although the cortex is normally not thought of as being reproductively dynamic, it may also respond to changes in the hormone milieu. Interestingly, although females and males have different sex organs and different primary sex hormones, estrogen for females and testosterone for males, there was no sex difference in how ERα, ERβ or AR mRNA expression changed across early postnatal development. The fact that ERα mRNA expression is high then decreases and ERβ and AR mRNA expression are low then increase in both the female and male cortex across early postnatal development suggest a role for ERβ and AR in the adult cortex.

Cortical explants were used to evaluate the innate changes that occur as the cortex "ages" across time in culture because this *in vitro* model maintains the neuronal/glial cross talk and cellular cytoarchitectural organization of the cortex. During early postnatal development ERα, ERβ and AR expression are dynamic in the intact cortex. To evaluate whether these changes are innate to the cortex during development or require input from other brain regions we measured sex steroid hormone receptor mRNA expression in cortical explants across time in culture. Female and male cortical explants were plated in either EtOH (vehicle) or E2 to evaluate the innate changes of these sex steroid hormone receptors that occur within the cortex and also to determine the affects of E2 on these receptors across time in culture. ERα mRNA expression was high then decreased while ERβ and AR mRNA expression were low then increased across time in culture in both female and male cortical explants. Interestingly, treatment with E2 only had an affect on ERα mRNA expression in male cortical explants across time in

culture. Here the changes in sex steroid hormone receptor mRNA expression that occurred in cortical explants were similar to the pattern of changes that we see in the intact cortex. These data are the first to show that regulation of steroid hormone receptors in the rat cortex is innate and still occurs when it is isolated from other brain regions.

Changes in sex steroid hormone receptors following injury

Another time-point where sex steroid receptors are known to change is in the cortex following an injury. 24 hours after MCAO, ERα mRNA and protein are increased in the cortex of female rats and mice (Dubal, Shughrue et al. 1999; Dubal, Rau et al. 2006). In OVX females, the increase in ERα mRNA occurs in both oil and E2-treated groups, but is seen earlier after injury with E2 (Dubal, Rau et al. 2006). E2 treatment also attenuates the decrease in ERβ seen following injury in the oil treated females (Dubal, Rau et al. 2006). Interestingly in gonadally-intact males, ERα does not increase following injury. Since there are sex-specific changes in sex steroid hormone receptors in the intact brain following injury, I evaluated these sex steroid hormone receptors in my cortical explant model to determine if these changes will occur in a sex specific manner and are innate to the cortex.

The cortical explant model allowed us to investigate sex differences following injury in the cortex while maintaining the important neuron/glia connections and cross talk that other models lose by isolating and culturing either neurons or glia cells. Ischemia can be induced in cortical explants by applying

2DG/KCN to block glycolysis and oxidative phosphorylation, which are the two main areas that become disrupted during ischemic stroke. In my studies, I found that the time-course of cell death was different in female versus male cortical explants. Female cortical explants had significant injury with 2DG/KCN treatment at 24 hours. However, male cortical explants had significant injury at 12 hours, but the relative levels of injury were similar to females. Differences in the cell death time-course between females and males indicate males may be more susceptible to chemical induced ischemia than females. Interestingly, in a mouse model of traumatic brain injury (TBI), cell death occurs faster and at a higher magnitude in males than females (Roof, Duvdevani et al. 1993; Roof and Hall 2000; Bramlett and Dietrich 2001).

There was also a sex difference in the way that the explants responded to injury after the addition of E2. E2 attenuated cell death in female, but not male cortical explants. In contrast, following MCAO, male rats do experience additional protection with the addition of E2 following MCAO (Toung, Traystman et al. 1998; Saleh, Cribb et al. 2001; Saleh, Cribb et al. 2001). It would appear that these males were only protected by E2 when the cortex had inputs from other regions of the brain. It is also possible that the difference in the cell death in the two different paradigms may also account for the differences in response to cell death. These studies together indicate that the cortex may be organized differently in females and males, and that E2 may work via a sex-specific mechanism. In the female, E2 mediated protection is innate to the cortex, where E2 mediated protection in males is not and requires outside inputs.

Another possible reason for the sex difference in cell death with E2 pretreatment is that males require a higher concentration of E2 for protection. E2 mediated protection in females is at physiological concentrations and requires pretreatment for 7 days indicating that this protection may be through the classical genomic pathways for sex steroid hormones. However, since males are not protected at physiological concentrations and may required pharmacological concentrations to exert protection indicates that E2 in males may have antioxidant effects by reducing reactive oxygen, rather than receptor mediated effects.

Changes in ERα mRNA expression in cortical explants following injury

In female rat explants, E2 was protective following treatment with 2DG/KCN. These results are similar to the protective effect of E2 following MCAO. In the MCAO model, an increase in ERα is essential for E2-mediated protection in female rats (Suzuki, Brown et al. 2007). Interestingly, ERα also changed in female cortical explants following 2DG/KCN treatment, but E2 treatment prevented this increase. Not surprisingly, the increase in ERα mRNA expression occurred at a different time following injury in explants versus in the intact cortex. Following MCAO, ERα mRNA expression began to increase at 4 hours, where it peaked, and continued to remain significantly increased out to 24 hours. In female cortical explants, ERα mRNA expression was significantly increased at 8 hours, but had returned to baseline by 24 hours after injury. ERα mRNA also returns to baseline following MCAO (unpublished observations), but

it takes a longer period of time. These data suggest that ERα mRNA responses to injury may be innate to the cortex, although the time-course for expression is different in each model.

The difference in the time-course for changes in ERα mRNA could be attributed to the baseline expression of ERα before injury. Interestingly, ERα mRNA expression is virtually absent in the adult rodent cortex and increases following injury. However, in cortical explants there is a higher baseline expression of ERα mRNA expression at the time of injury and only the vehicle (EtOH)-treated female cortical explants respond to injury by increasing ERα mRNA expression. Because there was an increase in cell death in the vehicle-treated female explants (compared to E2) and an increase in ERα mRNA expression, it is possible that the E2 protects the female cortical explants by inhibiting an increasing in ERα mRNA expression. The levels of ERα mRNA are not different between EtOH and E2-treated female cortical explants across time in culture, specifically at the time of injury (PND10), suggests that E2 does not affect the baseline expression of ERα mRNA. However, E2 does inhibit ERα mRNA expression from increasing following injury in the cortical explant model, which is protective.

Changes in ERβ mRNA expression in cortical explants following injury

Following MCAO in female rat cortex, ERβ *mRNA* expression is initially elevated and then dramatically declines by 16 and 24 hours (Dubal, Rau et al. 2006). Pretreatment with E2 prevents the injury-induced decrease in ERβ at 16

and 24 hours after injury (Dubal, Rau et al. 2006). Here, ERβ mRNA expression did not change in either EtOH or E2-treated female or male cortical explants following treatment with 2DG/KCN. This difference in how ERβ responds to injury in the cortical explant model (*in vitro*) versus the MCAO model (*in vivo*) may reflect differences between the two injury paradigms or could indicate an influence of other brain regions that is lost in the explants. These data are very interesting because E2 does not decrease cell death following MCAO in ERα knock out mice, but does in ERβ knock out mice suggesting ERα is necessary to mediate E2 protection. However, it is possible that the two receptors are working together to mediate E2 neuroprotection when in homeostasis. Since ERβ may require feedback or connections from other brain regions it is possible that those regions are able to still send signaling cues even when ERβ is removed. However, since ERα appears to be regulated mainly with the cortex it is unable to compensate and E2 neuroprotection is lost in ERα knockouts.

Changes in AR mRNA expression in cortical explants following injury

There was a sex difference in the way AR mRNA expression responded to injury. In both EtOH and E2-treated female explants, AR mRNA was significantly increased at 8 hours following treatment with 2DG/KCN was decreased by 24 hours. There was no increase in AR mRNA expression in males at any time point following treatment with 2DG/KCN. These data are the first to demonstrate sex specific changes in AR in following injury. AR responds similarly to 2DG/KCN treatment in EtOH and E2 treated female explants, indicating that

changes in AR mRNA are in response to injury rather than E2 mediated protection. While changes in AR mRNA or protein have not been evaluated following injury, the role of AR in protection has been evaluated. Interestingly, males, not females (Kitano, Young et al. 2007), showed AR-dependent protection with isoflurane preconditioning following MCAO (Zhu, Wang et al. 2010). Flutamide, an AR antagonist, removed the protection of isoflurane precondition in male rats that have undergone MCAO (Zhu, Wang et al. 2010). AR has also been suggested to mediate the effects of androgens and neuroprotection (Uchida, Palmateer et al. 2009). There is a difference in the role AR plays in injury in the cortical explant model (*in vitro*) verse the MCAO model (*in vivo*). Flutamide decreased cell death in 2DG/KCN-treated female and male cortical explants depending on timing and concentration of the drug. However, Flutamide increased cell death in male rats following MCAO (Uchida, Palmateer et al. 2009)(Zhu, Wang et al. 2010). Since AR increases cell death following injury in the cortex when there are synapses and connections to other brain regions and decreases cell death when the cortex is isolated in culture could indicate that AR in the cortex are influenced from other regions of the brain.

Inhibiting ERα, ERβ and AR

If ERα, ERβ or AR is playing a role in E2 mediated protection then inhibiting that receptor should remove the protection. To evaluate the role of ERα, ERβ and AR selective antagonists were chosen to inhibit each receptor, MPP, PHTPP and Flutamide, respectively. Because we did not see the level of

cell death we anticipated when the inhibitors were added before the injury, we were concerned that the drug in the culture media before injury may be influencing the ability of 2DG/KCN to induce cell death. To eliminate this problem, we included two additional paradigms where inhibitor drugs were added following injury.

In female explants, only the vehicle (non antagonist) treated group had significant cell death when treated with 2DG/KCN. As expected, E2 attenuated this cell death in female cortical explants. 2DG/KCN did not induce a significant injury in any of the drug groups and depending on concentration and timing cell death was further reduced in the 2DG/KCN group. A tight regulation of sex steroid hormone receptors may be occurring because addition of the inhibitors alone caused additional cell death without 2DG/KCN treatment. These data suggest that if receptor function is too high or too low additional injury can result, indicated by the increase in cell death in the non-injured explants. These data indicate ERβ and AR, but not ERα, may play a role in the injury induced by 2DG/KCN in female cortical explants.

The male cortical explants also only had significant cell death with 2DG/KCN treatment in the vehicle group. E2 did not attenuate cell death in male cortical explants. 2DG/KCN did not induce a significant injury in any of the drug groups and depending on concentration and timing cell death was further reduced in the 2DG/KCN group. These data indicate that ERα, ERβ and AR may play a role in the injury induced by 2DG/KCN in male cortical explants.

The initial goal of determining which receptor was responsible for the E2-mediated protection was not obtained. In fact, significant cell death was not obtained when the explants were treated with 2DG/KCN in the presence of an antagonist for sex steroid hormone receptors. These antagonists may cause off target effects that are not directly related to blocking the specific sex steroid receptor. Off target effects have been reported with other antagonist in clinical trials (Miyazawa 2011). Specific off target effects have not been reported with these antagonists; however, sex steroid hormone membrane receptors or ion channels could have been activated without our knowledge. These off target effects can be beneficial or detrimental and may lead to misinterpretation of experimental results.

MPP, ERα antagonist, is a selective silent antagonist that blocks the activity of ERα at the receptor level and does not have any intrinsic activity to activate the ERα receptor. This receptor antagonist may affect other signaling pathways or proteins with in the cell. Since 2DG/KCN treatment did not cause significant cell death when MPP, along with PHTPP and Flutamide, were present these antagonist may activate cell survival pathways that disrupts 2DG/KCN's ability to cause cell death. These antagonists may also disrupt the cell death pathways by disrupting the cells ability to undergo apoptosis, possibly by decreasing cytochrome c or disrupting caspase cell death pathways.

The pathways of cell death may be different for females and males. In females, the caspase cascade, a family of cysteine proteases that are involved in apoptotic cell death, is believed to play a major role in cell death following injury

(Renolleau, Fau et al. 2007). Inhibition of this cascade attenuates cell death in female, but not male rodents following MCAO (Renolleau, Fau et al. 2007). However, the poly (ADP-ribose) polymerase-1, an enzyme involved in DNA repair, inhibition in males is protective, but not in females following MCAO (Hagberg, Wilson et al. 2004). The difference in cell death pathways that occur *in vivo*, following MCAO, may add insight to sex difference in cell death in the cortical explant model. It is important to note that these two models are different types of ischemic injuries. Cell death in the cortex following MCAO is largely secondary to the primary death that occurs in the striatum. 2DG/KCN injury is directly applied to the cortex and this injury is considered primary.

From these data we can correlate that there is an effect of sex steroid hormone receptors in injury because we could not reach a significant injury with 2DG/KCN in drug groups. Only treatment with 2DG/KCN induced a significant injury within the vehicle group. Studies have shown that ERα, ERβ (Solakidi, Psarra et al. 2005; Pedram, Razandi et al. 2006; Arnold and Beyer 2009; Vasconsuelo, Pronsato et al. 2011) and AR (Solakidi, Psarra et al. 2005) are present in the mitochondria. KCN in our injury inhibits oxidative phosphorylation by inhibiting cytochrome c oxidase in complex IV of the electron transport chain (ETC). E2 mediated protection in another injury models, hemorrhagic shock, was mediated through ERβ in the mitochondria by upregulating the genes of complex IV in the ETC (Hsieh, Yu et al. 2006). Disruption of complex IV by cyanide (CN-) treatment inhibited the E2 mediated protection (Hsieh, Yu et al. 2006). E2 also increased expression of ERα and ERβ in the mitochondria

(Chen, Delannoy et al. 2004; Chen, Eshete et al. 2004). Since E2 increases expression of ER in the mitochondria where there is a balance of normal cellular respiration from the ETC, in our vehicle model the balance of hormone receptors is such that when E2 is present and the explants are injured with CN- that E2 is able to compensate for the inhibition of cytochrome c oxidase and mediate protection.

The disruption of the activity level of some of these sex steroid hormone receptors in the mitochondria caused an increase in cell death without the addition of 2DG/KCN. The increase in cell death in the non-injured female and male explants could indicate that these receptors are needed at a certain activity level to maintain a healthy explant. Another possible reason for the additional damage is that the inhibitor cocktail itself is damaging to the explant and some how prevents additional injury when the explants are treated with 2DG/KCN. However, because there effect was dependent on the concentration and timing of treatment of the antagonist, it is unlikely the inhibitor cocktail cause damage but rather a base line activity level of receptors are needed.

ERα, ERβ and AR mRNA expression did not change in male cortical explants following 2DG/KCN treatment. ERα and ERβ mRNA expression also did not change in response in injury in the male brain *in vivo* (Broughton, Brait et al. 2012). AR mRNA has not been evaluated following injury in the cortex. However, another estrogen receptor, GPR30, has been recently evaluated following a 30 minutes transient MCAO in OVX females and gonadally intact female and male mice (Broughton, Brait et al. 2012). Interestingly, in male mice

there is an increase in GPR30 4 hours following MCAO that returned to baseline at 24 hours (Broughton, Brait et al. 2012). In the same studies, there were no significant changes in ERα or ERβ at 4 or 24 hours post injury (Broughton, Brait et al. 2012). These data suggest that GPR30 responds to injury in the male, but not the female cortex. Here we did evaluate changes in GPR30 mRNA expression 24 hours following 2DG/KCN treatment in both female and male cortical explants. We did not see any change in GPR30 mRNA expression at 24 hours following injury (unpublished observations), but it is possible that there was a GPR30 response that we missed by only looking at 24 hours. It is possible that the increase occurred early was only transient (as was seen with ERα and AR in females).

Potential Models

In our 2DG/KCN cortical explant model, cell damage is caused by inhibiting glycolosis and also disrupting oxidative phosphorylation in the mitochondria, which may increase cytochrome c release activating an intrinsic pathway of cell death. Cell death can occur extrinsically or intrinsically in the mitochondria. The extrinsic cell pathway, also called the “death receptor pathway,” is activated when “death receptors” such as tumor necrosis factor (TNF) receptor or the TNF-related apoptosis inducing ligand (TRAIL) receptors become activated and signal Caspase-8 (Kroemer, Galluzzi et al. 2007). Caspase-8 can activate additional caspases that lead to apoptosis, such as Caspase-3 to 6 to 7, or can activate a pro-apoptotic Bcl-2 protein (BID) (reviewed

in (Kroemer, Galluzzi et al. 2007). BID can induce the intrinsic pathway by interacting with Bax, another Bcl-2 protein, causing mitrochondrial outer membrane permeabilization (Wang, Yin et al. 1996). This permeabilization releases cytochrome c and other pro-apoptotic factors that induce additional caspases, which leads to cell death (Figure 5.1). The intrinsic pathway can also be activated by DNA or damage to the cell (Kroemer, Galluzzi et al. 2007).

In our cortical explant model of ischemia, E2 does not appear to act via the classic receptor mediated mechanism of protection. E2 can activate many organelles in the cell, such as the cytoplasm, nucleus and the mitochondria. One mechanism of E2-mediated protection is through steroid hormone receptors. However, when we added antagonist to inhibit these receptors in our model, 2DG/KCN was not able to cause significant cell death that we normally see when no antagonist is added. In the vehicle group (no antagonist), 2DG/KCN induced significant injury in both female and male cortical explants and E2 attenuated this cell death in females. These data suggest that E2 is not working through ERα, ERβ or AR to mediate protection. Our proposed model of E2 mediated protection in cortical explants is diagramed in Figure 5.2. E2 can bind to receptors on the membrane, such as GPR30, tyrosine kinase receptors, or growth factor receptors. These receptors become activated and signal to downstream signaling pathways to promote cell survival. For example, E2 has been shown to activate AKT by phosphorylation, pAKT (Wilson, Liu et al. 2002). pAKT can signal down stream to increase cell survival proteins. pAKT could also

activate transcription of genes to produce more membrane receptors, GPR30. E2 binds to GPR30, which activates more PI3K that phosphorylates more AKT.

E2 mediated protection was not removed when ERα, ERβ or AR was inhibited. In fact, treatment with these inhibitors decreased cell death in the 2DG/KCN treated explants. This decrease in cell death with the inhibitors was not affected by the presence of E2. Since inhibiting the receptors decreased cell death, it is possible that KCN may be interacting with sex steroid hormones receptors, Figure 5.1. ERα and ERβ can be found in the mitochondria, but very little is know about their location or function. KCN acts by disrupting complex IV of the ETC in the mitochondria, I hypothesize that there are sex steroid hormone receptors near that complex that can interfere with how KCN disrupts complex IV. Since very little is know about the location of these receptors and there function in the mitochondria, they could not only aide in regulating mitochondria genes necessary for the electron transport chain but could also affect how cytochrome C is released to trigger cell death.

These data are the first to show that E2 mediated protection following 2DG/KCN injury is not mediated through ERα, ERβ or AR. In fact, depending on concentration and timing of the antagonist, ERβ and AR antagonist could reduce cell death following treatment with 2DG/KCN without affecting the non-injured explants. These data demonstrate that cortical responses to injury are innate and preprogrammed based on sex and hormonal background. The role of sex steroid hormone receptors in injury needs to be further evaluated along with mechanism of sex differences in cell death.

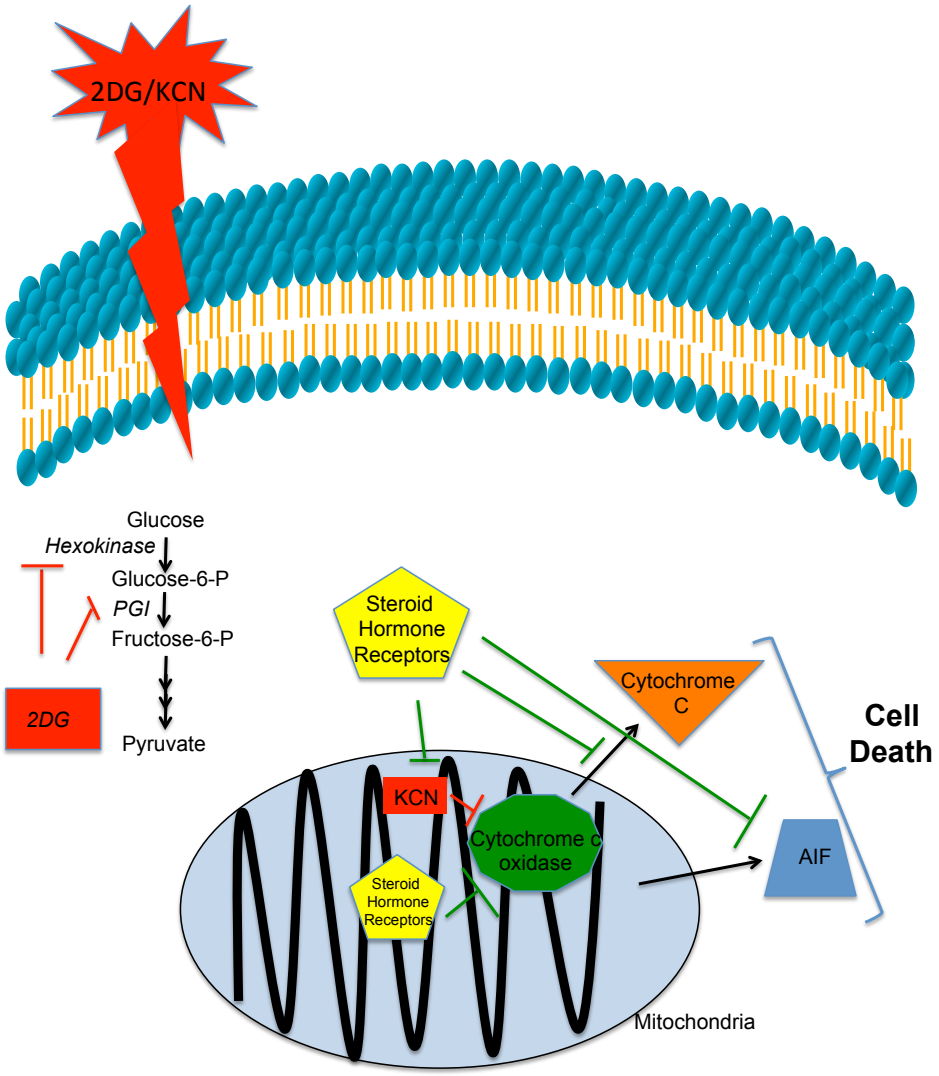


Figure 5.1 Diagram of 2DG/KCN injury in cortical explants. 2DG/KCN blocks glycolyosis and oxidative phosphorylation. 2DG is a glucose molecule that has a hydrogen in place of a hydroxyl group. Hexokinase traps this modified glucose molecule in the cell by converting 2DG to 2DG-P. This molecule cannot continue in glycolysis because phosphoglucose isomerase (PGI) cannot use 2DG-P as a substrate (Brown 1962; McComb and Yushok 1964; Parniak and Kalant 1985; Karczmar, Arbeit et al. 1992). KCN inhibits cytochrome c oxidase in the mitochondrial electron transport chain by binding to the iron in cytochrome c so that cytochrome c oxidase is unable to transfer electrons to oxygen. This disruption inhibits the ETC from producing ATP.

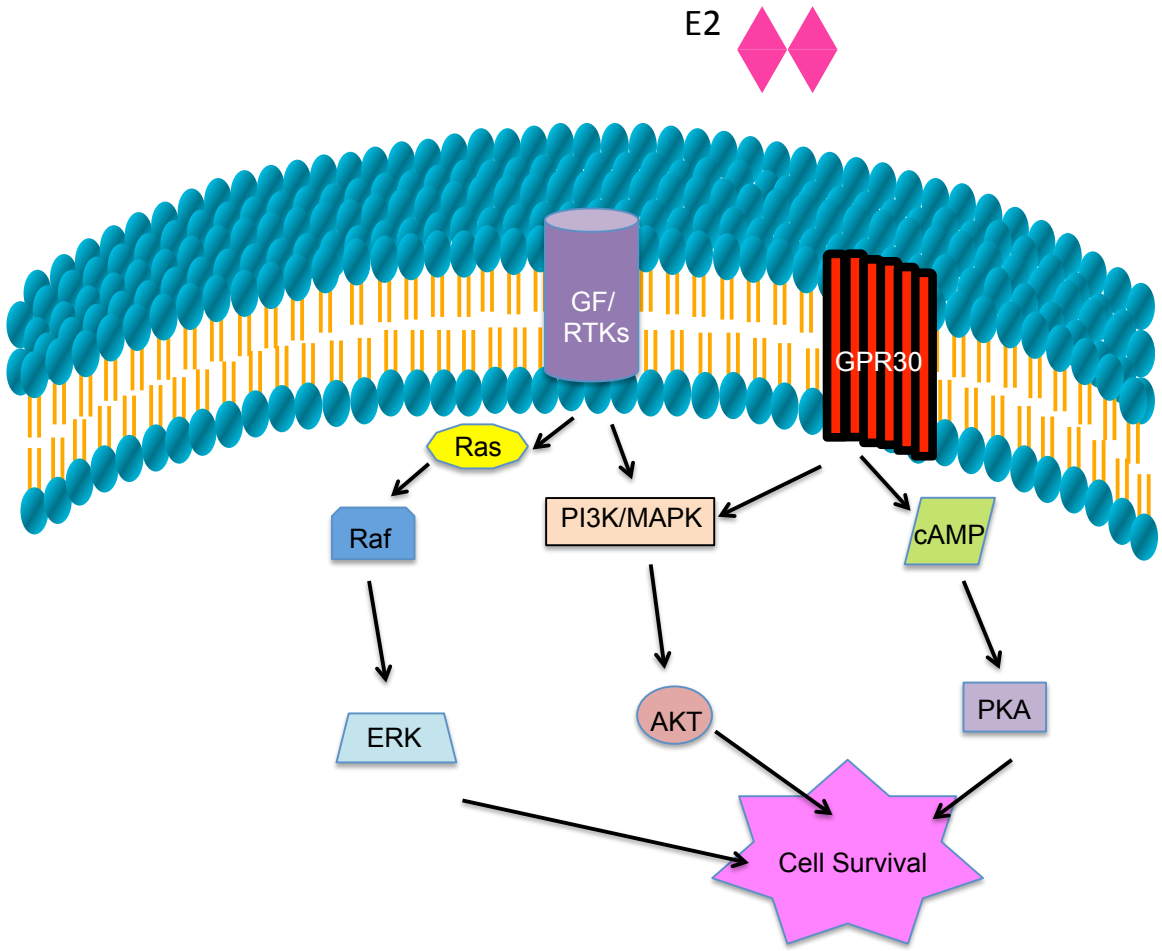


Figure 5.2. E2-mediated protection in cortical explants. E2 activates GPR30, G protein-coupled receptor 30, or a growth factor, GF, receptor. These receptors activate a kinase cascade that up-regulate cell survival proteins.

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Amanda L. Trout

EDUCATION

2005- 2012	**University of Kentucky, Lexington, KY** PhD Program (Physiology) Advisor: Melinda Wilson, PhD Dissertation Topic: Sex differences following ischemic injury in cortical explants
1998-2003	**Northern Kentucky University, Highland Heights, KY** B.S. Chemistry B.S. Biology

RESEARCH/WORK EXPERIENCE

2005- Present	**University of Kentucky. Lexington, KY** **Graduate Research Assistant**
2004-2005	**Meridian Bioscience Inc. Cincinnati, OH** **Research Assistant 1**
2003	**Advance Testing Laboratories. Cincinnati, OH** **Microbiologist**
2001-2002	**Northern Kentucky Univ. Water lab, Highland Heights, KY** **Research Assistant**

PROFESSIONAL AFFILIATIONS

2007-Present	Society for Neuroscience
2006-Present	Bluegrass Chapter Society for Neuroscience
2005-Present	University of Kentucky Forum For Reproductive Sciences and Women's Heath
1999-present	Beta-Beta-Beta Member of Biology National Honor Society,

HONORS AND AWARDS

Travel Award to Society for Neuroscience 2011. The Graduate School, University of Kentucky. Washington D.C.

Travel Award to Society for Neuroscience 2010. The Graduate School, University of Kentucky. San Diego, CA.

Travel Award to Society for Neuroscience 2009. The Graduate School, University of Kentucky. Chicago, IL.

CINSAM- Research Scholar, Northern Kentucky University 2001-02.

TriBeta (Biology National Honor Society) Regional Meeting 2002. First Place in Poster Competition.

Career Achievement Award. Northern Kentucky University) 2001.

PUBLICATIONS

Peer-Reviewed Publications

Westberry JM, **Trout AL**, Wilson ME. Epigenetic regulation of estrogen receptor beta expression in the rat cortex during aging. Neuroreport. 2011 Jun 22,22(9):428-32.

Wilson ME, Westberry JM, **Trout AL**. Estrogen receptor-alpha gene expression in the cortex: Sex differences during development and in adulthood. Horm Behav. 2010 Aug 14.

Westberry JM, **Trout AL**, Wilson ME. Epigenetic regulation of estrogen receptor alpha gene expression in the mouse cortex during early postnatal development. Endocrinology. 2010 Feb; 151 (2): 731-40.

Abstracts or posters at scientific meetings

Trout AL, Westberry JM, Sengoku T and Wilson ME (2012) Innate sex-specific responses in cortical explants following ischemia. University of Kentucky Leadership Center department of Physiology bi-annual retreat poster presentation. Jabez, KY.

Trout AL, Westberry JM, Sengoku T and Wilson ME (2012). Innate sex-specific responses in cortical explants following ischemia. University of Kentucky Forum For Reproductive Sciences and Women's Heath Symposiom. Lexington, KY.

Trout AL, Westberry JM, Sengoku T and Wilson ME (2011) Sex-specific regulation of cell death and steroid hormone receptors in cortical explants following 2DG/KCN treatment. Society for Neuroscience poster presentation, Washingtion DC.

Trout AL, Westberry JM, Sengoku T and Wilson ME (2011) Sex-specific regulation of cell death and steroid hormone receptors in cortical explants following 2DG/KCN treatment. University of Kentucky Forum For Reproductive Sciences and Women's Heath Symposiom. Lexington, KY.

Trout AL, Westberry JM, Sengoku T and Wilson ME (2010) Sex-specific responses in cell death and estrogen receptor expression in cortical explants following 2-deoxyglucose and potassium cyanide treatment. Society for Neuroscience poster presentation, San Diego, CA.

Westberry JM, **Trout AL** and Wilson ME (2010) Hormone-specific regulation of estrogen receptor alpha by methylation following middle cerebral artery occlusion. Society for Neuroscience poster presentation, San Diego, CA.

Trout AL, Westberry JM, Sengoku T and Wilson ME (2010) Gender-specific regulation of cell death and estrogen receptors in cortical explants following 2DG/KCN treatment. University of Kentucky College of Medicine and Physiology 50th Anniversary poster presentation. Lexington, KY.

Trout AL, Westberry JM, Sengoku T and Wilson ME (2010) Gender-specific regulation of cell death and estrogen receptors in cortical explants following 2DG/KCN treatment. Bluegrass Chapter Society for Neuroscience poster presentation. Lexington, KY.

Trout AL, Westberry JM, Sengoku T and Wilson ME (2009) Gender-specific regulation of cell death and estrogen receptors in cortical explants following 2DG/KCN treatment. Society for Neuroscience poster presentation, Chicago, IL.

Westberry JM, **Trout AL** and Wilson ME (2009) Changes in Promoter Methylation in the Rat Cortex Following Middle Cerebral Artery Occlusion. Society for Neuroscience poster presentation, Chicago, IL.

Trout AL, Westberry JM, Jasper DK and Wilson ME (2009) Gender-specific Regulation of ER alpha in cortical explants following ischemic injury. Bluegrass Chapter Society for Neuroscience poster presentation. Lexington, KY.

Westberry JM, **Trout AL** and Wilson ME (2009) Regulation of estrogen receptor beta gene expression in the Rodent cortex during aging. Bluegrass Chapter Society for Neuroscience poster presentation.

Trout AL, Westberry JM, Jasper DK and Wilson ME (2009) Gender-specific Regulation of ER alpha in cortical explants following ischemic injury. 28th Annual UK Symposium in Reproductive Sciences and Women's Heath.

Trout AL, Westberry JM, Jasper DK and Wilson ME (2008) Regulation of Estrogen Receptor Alpha in Cortical Explants. Society for Neuroscience poster presentation, Washington, DC.

Trout AL, Westberry JM, Jasper DK and Wilson ME (2008) Gender-Specific Regulation of ERα in Cortical Explants Following Ischemic Injury. University of Kentucky Leadership Center department of Physiology bi-annual retreat poster presentation. Jabez, KY.

Wilson ME , Prewitt AK, Bisotti AJ, **Trout AL**, Rosewell AN, Jasper DK and Allred KF. Developmental Regulation of ER-alpha mRNA Involves Differential Promoter Usage. Presented to the Keystone Symposia, British Columbia, 2006.

Prewitt AK, Bisotti AJ, **Trout AL**, Rosewell AN, Jasper DK, Allred KF and Wilson ME. Developmental regulation of estrogen receptor-alpha mRNA involves differential promoter usage. Presented to Neuroscience Day Bluegrass Chapter-Society for Neuroscience, Lexington KY, 2006.

Prewitt AK, Bisotti AJ, **Trout AL**, Rosewell AN, Jasper DK, Allred KF and Wilson ME. Developmental regulation of estrogen receptor-alpha mRNA involves differential promoter usage. Presented to Neuroscience Day Bluegrass Chapter-Society for Neuroscience, Lexington KY, 2006.

PRESENTATIONS

Sex-specific regulation of cell death and steroid hormone receptors in cortical explants following 2DG/KCN treatment. (April 2011). 30th Annual UK Symposium in Reproductive Sciences and Women's Heath.

Gender-specific regulation of cell death and estrogen receptors in cortical explants following 2DG/KCN treatment. (March 2010). University of Kentucky Forum For Reproductive Sciences and Women's Heath.

Regulation of Estrogen Receptor Alpha in the Cortex Following Ischemic Injury. (April 2008). University of Kentucky Physiology department seminar.