ABSTRACT

Age-related brain disorders are associated with the decline in the ability of brain cells to cope with homeostatic challenge. Although all major brain cell types have the capacity to respond to homeostatic challenges, astrocytes are particularly well-equipped to counteract these challenges. Here, we focused on Connexin 43 (Cx43) as a protein that is not only highly expressed in astrocytes, but whose expression is critical to inter-cellular communication that in turn, can influence cell viability. Most studies to date have focused on the expression (i.e., abundance) of Cx43. However, a critical limitation of these studies is that they did not thoroughly examine functionality of the Cx43 channels. In particular, there is a paucity of data describing the differential contributions of Cx43-containing hemichannels versus Cx43-containing gap junctions to cellular functions.

We hypothesized the astrocyte Cx43 hemichannel as a yet unreported target of androgens and estrogens based on three notions. First, our laboratory has determined that astrocytes are a relevant and important target of such gonadal steroid hormones as estrogens (e.g., 17β-estradiol (abbreviated herein as estradiol or E2)) and androgens (such as DHT), through which these hormones promote healthy brain cell function. Second, oxidative stress is associated with an increase in Cx43 opening. Finally, the Cx43 gene promoter contains functional estrogen response element (ERE) half sites, and estradiol, as well as other estrogenic compounds, decrease Cx43 channel opening in peripheral (non-CNS) tissue. Based on these notions, we hypothesized that gonadal androgens and estrogens will inhibit Cx43 hemichannel opening in cortical astrocytes as well.

My data revealed that while E2, dihydrotestosterone (DHT), and the estrogenic metabolite of DHT (3βdiol) all protect primary cortical astrocytes from the mixed metabolic/oxidative insult, iodoacetic acid (IAA), only DHT decreased astrocyte Cx43 mRNA expression. Consistent with their cytoprotective effects, however, all three steroids decrease astrocyte Cx43 hemichannel opening, and antagonized the increased opening of Cx43 hemichannels induced by IAA. In an effort to pursue the mechanism by which these steroids reduced Cx43 hemichannel opening, we evaluated the phosphorylation of Cx43 at two key residues, Ser 368 and Tyr 265. Phosphorylation at these residues is associated with channel closing, and as such, we predicted that the three hormones would increase the phosphorylation of Cx43 at one or both of these residues. Whereas Tyr265 phosphorylation was unaffected any of the three hormones, DHT significantly reduced the phosphorylation of Cx43 at Ser368. These observations may indicate that while all three steroids contribute to astrocyte protection through a mechanism that involves blocking astrocyte Cx43 hemichannel opening, DHT may induce molecular changes in the astrocytes that are distinct from those induced by estradiol or 3βdiol.

The knowledge gained through the experiments conducted not only enhance our understanding of how Cx43 hemichannels and Cx43 gap junctions influence astrocyte function and viability but also define Cx43 hemichannels as relevant targets of gonadal steroid hormone induced regulation of cell viability. Such knowledge may facilitate the development of more precise therapeutics (i.e., selectively targeting Cx43 hemichannels without activity at Cx43 gap junctions in the same cells or tissue), the benefit of which would be to better treat age-associated neurodegenerative disorders as well as disorders of peripheral tissue.

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CONNEXIN 43 CONTRIBUTES TO ESTROGEN PROTECTION
AGAINST OXIDATIVE STRESS IN
CORTICAL ASTROCYTES

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CONNEXIN 43 CONTRIBUTES TO ESTROGEN PROTECTION AGAINST OXIDATIVE STRESS IN CORTICAL ASTROCYTES

DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Sciences
University of North Texas
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for the Degree of

DOCTOR OF PHILOSOPHY

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3βdiol: 5-alpha-androstane-3-beta,17-beta-diol

AGA: 18-alpha-glycyrrhetinic acid

AR: androgen receptor

Cx43: connexin 43

DHT: dihydrotestosterone

E2: estradiol

ERE: estrogen response element

EtBr: ethidium bromide

GFAP: glial fribrillary acidic protein

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GJA1: gap junction protein family, alpha sub-family, first isoform cloned

GJIC: gap junction intercellular communication

IAA: iodoacetic acid

ICI: ICI-182,780

MAPK: matrix associated protein kinase

MPP: MPP dihydrochloride

PKA: protein kinase A

PKC: protein kinase C

PQ1: PQ1 Succinate

S368: serine 368

TNFα/IL1β: Tumor necrosis factor alpha / Interleukin 1 beta mixture

WT: wild type

Y265: tyrosine 265

ZO1: Zona Occludens 1

CHAPTER 1: INTRODUCTION

Background:

Age-related brain disorders are associated with the decline in brain cells' ability to cope with homeostatic challenge.(1) Although all major brain cell types have the capacity to respond to homeostatic challenges, astrocytes are particularly well-equipped to counteract these challenges. In the project described herein, we focused on astrocyte Connexin 43 (Cx43) as a protein that is not only highly expressed in astrocytes, but whose expression is critical to counteracting homeostatic challenges, including oxidative stress.(2-4) Oxidative stress is a well-studied type of disruptor of homeostasis, and increases as a function of age. There are known reciprocal, inhibitory interactions between oxidative stress and the expression of Cx43. For example, Cx43 expression is inhibited by oxidative stress and increasing endogenous Cx43 expression increases resistance to toxic insults including oxidative stress.(5,6) Furthermore, estrogen increases Cx43 expression in several peripheral tissues.(7,8) These studies all focused on expression, and suggest that increased Cx43 expression is protective. However, an important limitation of these studies is that they did not discriminate between the role of Cx43-containing hemichannels and Cx43-containing gap junctions. A critical contribution of my work to the field is to infer that expression of Cx43, in and of itself, does not sufficiently inform its functional impact or consequence. Our data suggest that there is indeed a difference between the two (gap junctions versus hemichannels) and, there is a critical need to tease apart the roles of the Cx43 hemichannels and the Cx43 gap junctions in biological systems. Moreover, the work presented in this dissertation focuses on the hypothesis

that gonadal androgens and estrogens, which have well described cytoprotective effects against oxidative stress, increase cell viability by regulating the function of Cx43 gap junctions and/or hemichannels. In fact, the experiments conducted extend the recent work from the Singh lab that implicated the estrogenic metabolite 3βdiol as the mediator of dihydrotestosterone (DHT)-induced cytoprotection. The series of studies we conducted evaluated the role of estradiol, DHT and 3βdiol in regulating Cx43 function within the brain. Moreover, a more thorough understanding of the contributions of Cx43 hemichannels and Cx43 gap junctions to astrocyte function may also facilitate the development of more precise therapeutics (i.e., selectively targeting Cx43 hemichannels without activity at Cx43 gap junctions in the same cells or tissue) to enhance the protective efficacy of estrogens and androgens in the brain. Given that estrogens and androgens have important effects on tissues outside the traditional confines of reproductive biology, coupled with the fact that alterations in Cx43 function have been associated with wide range diseases that present significant epidemiological and economic burden, including cancer, heart disease, ischemic stroke, anxiety, seizure disorders, depression, and Alzheimer's disease, the impact of this research could extend to the treatment of many of these diseases.(9-17)

Importance of Estrogens:

The higher incidence of chronic neurological disorders, such as Alzheimer's disease, after the menopause transition is directly correlated with significant decline in serum estrogen concentrations.(18) However, the molecular and cell type contributions to this higher incidence remain to be fully understood. It is well known that estradiol protects brain tissue against variety

of cytotoxic insults both *in vitro* and *in vivo*, including oxidative stress,(19) pro-inflammatory cytokines,(20) glutamate toxicity,(21) metabolic inhibition,(22) oxygen glucose deprivation,(23) and middle cerebral artery occlusion.(24)

From a mechanism of action perspective, estradiol acts on its target cell types through at least three distinct mechanisms. Through the first mechanism, estradiol enters the cell and associates with either cytosolic estrogen receptor (ER) alpha or beta, displacing the heat shock proteins otherwise associated with these receptors. After this interaction, the estradiol-ER complex translocates into the nucleus and interacts with estrogen response elements (EREs) located in the promoter region of target genes to increase their expression. Many of these genes are either survival promoting, such as anti-apoptotic members of the Bcl-2 family of proteins and anti-oxidant enzymes,(25,26) or pro-proliferative, such as beta-catenin, aquaporin 2, Ki-67, and PCNA. (27-29)

Through the second mechanism, estradiol interacts with putative membrane associated estrogen receptors to initiate non-genomic effects through augmenting the target cell's signaling cascade activities. Examples of this include activating the PKC, Src, and MAPK signaling pathways in target tissues.(30-32) Through the third mechanism, estradiol can act directly as a redox scavenger.(33) Given that estradiol has this multifaceted set of beneficial effects, it is reasonable that estrogen deprivation, such as that observed after menopause, is associated with increased prevalence of tissue dysfunction and chronic diseases.

While a significant focus of prior research on estrogen and its brain-protective effects has focused on neurons, we recognize that healthy brain function requires healthy neuronal and healthy

glial function. With regards to the latter, it is recognized that there is relatively less is known about the effects of estrogens and periods of estrogen deprivation on glial cells. As such, we chose to study this gap in the literature. More specifically, we chose to study the influence of estradiol and dihydrotestosterone (an androgen) on the transmembrane protein connexin 43 (Cx43), a protein highly expressed in astrocytes. Since impaired astrocyte Cx43 function has been implicated in chronic neurological disorders known to occur with greater prevalence in postmenopausal women compared to the general population such as Alzheimer's disease and ischemic stroke,(11,34) Cx43 seemed to be an even more relevant protein to study as an underreported component of either beneficial or detrimental sex steroid action on the brain.

Importance of Astrocytes:

While multiple classes of glia contribute to maintenance of overall healthy brain function, astrocytes contribute in a few distinct ways. Astrocytes facilitate dynamic intercellular trafficking of signaling metabolites and nutrients, secrete trophic factors, and actively participate in neuronal plasticity.(35-38) Furthermore, astrocytes are the cell type that occupies the greatest volume of the human brain.(39) However, some research indicates that the contributions of astrocytes to healthy brain functions are likely to be brain region specific,(40) and this brain region variation in astrocyte function is associated with differential expression of cell surface molecular markers on astrocytes, such as brain region dependent differences in astrocyte cell surface CD protein isoform expression.(41,42)

Astrocytes contribute to healthy brain function in three distinct ways at the cellular level. In the first way, astrocytes found adjacent to synapses facilitate detoxification of excess extracellular calcium and glutamate and assist in maintaining optimal extracellular milieu for healthy neuron firing, efficient synaptic neurotransmitter processing, and maintaining an efficient synaptic microenvironment.(43,44) Contrary to previous assumptions regarding this astrocyte function, the role of perisynaptic astrocytes is not only to benefit neurons, but also that the interaction between the neurons and the perisynaptic astrocytes is bidirectional such that synaptic activity regulates the function of perisynatic astrocytes as well as perisynaptic astrocytes regulating the function of neurons.(45,46) These bidirectional interactions of astrocytes and neurons at the level of the synapse have been noted in multiple regions of the central nervous system (i.e. in the cerebral cortex and hippocampus).(47,48)

Through a second cellular mechanism, clusters of astrocytes also connect to each other and to neighboring cells, such as neurons or other glial cells, through multiple types of junctions including gap junctions and tight junctions. Recent studies have shown that astrocytes, and more specifically astrocyte gap junctions, mediate the functional coupling of astrocytes among themselves, and through doing so, facilitate bidirectional dynamic exchange of signaling molecules and nutrients between the brain vasculature and the neurons.(49,50) Based on this function, the astrocyte gap junction has been studied as an a potential therapeutic target for a variety of brain disorders. Specifically, research has revealed that manipulations in astrocyte coupling by way of manipulating astrocyte connexin, the building block of gap junctions, impacts

subjects' behavior, including the regulation of spatial short term memory, hippocampal-dependent learning, and fear consolidation.(51-53)

Through the third cellular mechanism, astrocytes interact with other glial cell types, most notably endothelial cells, as well as the neurovasculature, to form the blood brain barrier. Although connexin proteins are not thought to directly contribute to this function, it is known that Cx43 directly interacts with zona occludens 1 through protein-protein interactions.(54) Zona occludens 1 is a tight junction protein that is a major constituent of the blood brain barrier,(55) and whose function is disrupted in both experimental models of blood brain barrier leakiness(56) and experimental models of diseases in which blood barrier is disrupted, such as ischemic stroke.(57) While this information supports the putative role of astrocyte Cx43 is maintaining BBB integrity, additional research is needed to further support this interpretation.

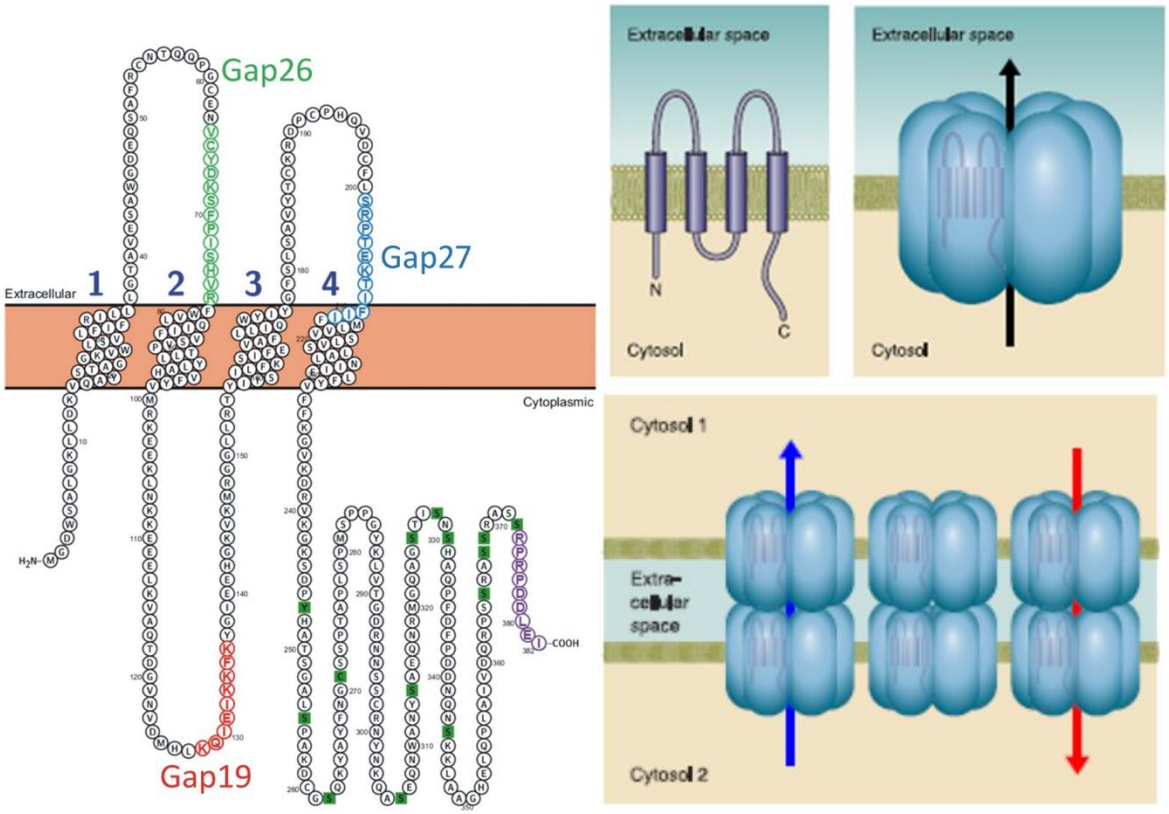


Figure 1: Connexin 43 Protein Structure

(Left): The amino acid sequence of Cx43 is shown. The protein structure of Cx43 has cytosolic animo- and carboxy-terminal ends, contains 4 membrane spanning domains, two extracellular loops, one intracellular loop, and an extensive carboxy-terminal domain that is intrinsically disordered and readily associates with many cytosolic signaling pathway proteins. The carboxy-terminal domain contains several regulatory sites, such as serine phosphoregulatory sites (indicated in green "S", such as serine 368), cysteine regulatory sites which form disulfide bridges (indicated in green "C"), and tyrosine phosphoregulatory sites (not indicated, but an example is tyrosine 265). Adapted from Abudara *et al.*, 2015 and Tabernero *et al.*, 2016. (Right): Once translated, multiple connexin 43 peptides aggregate together to form hemichannels (Top Right), which when in close proximity to each other on neighboring cells, form gap junctions (Bottom Right). Adapted from review by Bosco, Haefliger, & Meda, 2010.(7,54,58)

Cx43 Function & Regulation:

Connexin 43 (Cx43) is a transmembrane protein highly expressed in astrocytes,(2,3) where it contributes to protection against oxidative stress by mediating intercellular transfer of ions and small signaling molecules, as well as short linear peptides and nucleotides.(59-63) While it is known that astrocytes *in vivo* co-express connexin isoforms Cx43 and Cx30,(64) astrocyte expression of Cx30 does not manifest until later in development (around postnatal day 21), whereas Cx43 is highly expressed in astrocytes throughout development. Furthermore, astrocytes *in vitro* do not readily express Cx30 unless the astrocytes are co-cultured with neurons, in which case astrocyte Cx30 expression in upregulated in those astrocyte processes closest to neuronal soma.(48) Since the mice used to generate the astrocytes for this dissertation's experiments were sacrificed on postnatal day 2, we did not anticipate a significant contribution of the connexin 30 isoform to any of our experimental results. Therefore, this background is focused on the connexin 43 isoform as the only isoform expressed in early postnatal mouse astrocytes derived from the cerebral cortex.

Cx43's contributions to cell functions are primarily regulated by three factors: Cx43 expression, Cx43 phosphorylation, and Cx43 channel opening.(65-69) Regulation of these three aspects of Cx43 in biological systems has been characterized in both the context of healthy tissue physiology and pathophysiology. Multiple transgenic mouse models have been developed in which Cx43 gene expression is disrupted to study the functional roles of Cx43 in healthy tissue, but only one transgenic model has been developed to study this notion in astrocytes: GFAP cre directed Cx43 knockout. Behavioral characterizations of these transgenic mice have shown that

they are viable and fertile, but display increased exploratory behavior in elevated plus maze, a noticeable anxiolytic-like phenotype in open field task, impaired nociception, and disrupted sleep-wake cycles.(70-72)

Regulation of Cx43 Expression:

The Cx43 gene promoter (gene name: "GJA1," GeneBank ID: 2697) has estrogen response element half sites and AP1 sites. Consistent with this observation, it is known that estradiol increases the expression of Cx43 in reproductive, heart, and bone tissue.(10,73-77) In contrast, testosterone and DHT decrease Cx43 expression.(76,78) Indeed, all previous studies assessing androgen regulation of Cx43 expression have shown androgens decrease Cx43 expression in peripheral tissue unless prior androgen-to-estrogen conversion occurs in that tissue.(10,73,74,78-81) Cx43 expression in both heart tissue and astrocytes is decreased in the presence of hydrogen peroxide- or metabolic inhibition-induced oxidative stress.(6,82) Given that estrogen has been shown to be neuroprotective in a variety of experimental models that use oxidative/metabolic stress as the "insult,"(23,24,83,84) we inferred from the above referenced research that manipulating Cx43 expression or function may be a meaningful therapeutic strategy for enhancing the neuroprotective efficacy of protective neurosteroids such as estradiol and DHT. Given that estrogen receptors can be activated not only by estradiol but also by 3βdiol, the estrogenic metabolite of DHT,(85-90) we chose to compare all three of these steroid hormones at equimolar concentrations (100 nM) to assess two questions. The first question was whether estrogens or androgens alter Cx43 function (defined later as either at the level of altering Cx43 expression,

Cx43 phosphorylation, or Cx43 channel opening). The second question asked whether protection against oxidative stress induced by either estradiol, DHT, or 3βdiol could be enhanced by specifically altering Cx43 function through co-administration of pharmacological compounds acting on Cx43.

Regulation of Cx43 by Phosphorylation:

Connexin 43 was first proposed to be a phosphoprotein over 20 years ago.(91) Since then, studies demonstrated that the Cx43 protein contains more than 20 phosphoregulatory sites in its amino acid sequence, the vast majority of which are found in the cytoplasmic carboxy-terminal domain.(92-95) These sites are regulated by a variety of signal transduction proteins.(66,96-98) The two most extensively studied Cx43 carboxy-terminal phosphoregulatory sites are tyrosine 265 and serine 368.

Tyrosine 265 (Y265) of the Cx43 is a phosphorylation site for Src, a non-receptor tyrosine kinase.(54,99-101) Src phosphorylates Cx43 on Y265, generating a SH2 domain docking site for Src and other SH2 interacting proteins.(102) This Cx43-Src interaction has several important intracellular consequences *in vitro* and *in vivo,* such as reducing Cx43 intracellular trafficking to the plasma membrane, increasing the interaction of Cx43 to the scaffolding protein zona occludens 1 (ZO-1), and promoting ubiquitin-mediated degradation of Cx43.(55,103-105) Based on these findings, Y265 phosphorylation could potentially have a variety of secondary effects on regulating Cx43 opening mediated through scaffolding or protein-protein interactions. Although these findings suggest the interaction between Cx43 and Src may be important for cellular response to

cytotoxic insult and regulation of this protein-protein interaction may impact cell survival, it remains to be seen if DHT, 3βdiol, or estradiol can modulate Y265 phosphorylation of Cx43.

Serine 368 (S368) of Cx43 is a phosphorylation site for protein kinase C (PKC).(66,106,107) Depending on the target cell type in question, Cx43 S368 can be phosphorylated by either conventional PKC isoforms (such as PKCγ in lens epithelial cells),(108) novel PKC isoforms (such as PKCε in heart tissue),(109) or atypical PKC isoforms (such as PKCζ in anterior pituitary folliculostellate cells).(110) Decreased Cx43 channel opening following PKC induced phosphorylation at S368 is attributed to decreasing the cross-sectional area of the Cx43 channel pore.(111) Multiple indirect consequences of S368 phosphorylation have also been shown, such as increased ZO-1 associated Cx43 internalization from the plasma membrane and increased ubiquitin-mediated degradation of Cx43.(112-114) Phosphorylation at either residue (Y265 or S368) is associated with decreased Cx43 opening, and this mechanism is shared between Cx43 hemichannels and Cx43 gap junctions.(115,116) However, phosphorylation-induced reduction in Cx43 opening has only been observed for some Cx43 permeable molecules as indicated by decreased fluorescent dye conductance with no change observed for Cx43 ionic conductance.(116,117) One interpretation of these findings is that carboxy-terminal phosphorylation (at either Y265, S368, or other sites as well) is associated with reduced intercellular trafficking of higher molecular weight signaling molecules (such as miRNA) without decreasing trafficking vital low molecular weight molecules such as potassium, calcium, glutamate, and glucose. Since most of the miRNA identified to date as Cx43 channel permeable serve modulatory rather than vital functions in target cells,(118-122) this phosphorylation-induced

incomplete closure might be a compensatory mechanism of cells limiting spread of pro-death signaling molecules of higher molecular weight while preserving intercellular trafficking of ions and low molecular weight signaling molecules vital for cell survival. Taken together, these findings suggest Cx43 S368 phosphorylation by PKC promotes Cx43 internalization from the plasma membrane, and on the basis of the notion that more Cx43 translates to decreased cellular viability, the phosphorylation on S368 may be protective against some cytotoxic stimuli. However, it remains to be seen if DHT, 3βdiol, or estradiol can modulate S368 phosphorylation of Cx43, and whether these steroids impact Cx43 function through regulating S368 phosphorylation.

In addition to tyrosine 265 and serine 368, there are several other sites in the Cx43 cytoplasmic carboxy-terminal domain whose function is regulated by phosphorylation by PKA, MAPK, and other signaling pathways.(54,123-130) Whether other connexin isoforms are similarly regulated by the same signaling cascades as Cx43 remains to be assessed, but some evidence suggests the effects of phosphorylation on cell-to-cell coupling as well as channel permeability to specific molecules may be connexin isoform specific.(131-136) Further research is needed to assess the biological effects of regulating Cx43 phosphorylation and determining the clinical contexts for which examining Cx43 phosphorylation may be the most useful for development of therapeutics.

Lastly, there has been considerable research suggesting that estrogens and androgens exert relatively quick, nongenomic intracellular effects through multiple signaling pathways, such as Src and PKC.(137-139) Research has shown that Src kinase activity is increased following estradiol application in several tissues.(140-143) Similar effects have been observed in several tissues following androgen application.(144-147) Likewise, estrogens and androgens are known to

increase PKC activity.(19,31,148,149) Based on these findings, I designed experiments to assess whether DHT, 3βdiol, or estradiol treatment increased Cx43 phosphorylation at Y265 and S368.

Regulation of Cx43 Gap Junctions versus Hemichannels:

Cx43 channels exist as either monomeric hemichannel or dimeric gap junctions.(7) Gap junctions are the dimeric transmembrane channels that allow for direct transfer of ions and small signaling molecules among cells in contact with one another, while hemichannels, are transmembrane channels that allow passage of the same molecules between the cytoplasm and the extracellular space. Hemichannels are the monomer building blocks of the dimeric Cx43 gap junction and are regulated by many of the same mechanisms that regulate Cx43 gap junction function. Relatively little consideration has been given to the unique contributions of Cx43 hemichannels to cytotoxic drug treatments, such as targeted chemotherapy, or to cytoprotective interventions such as hormone replacement therapy, since many compounds regulate connexin gap junctions and hemichannels in very similar ways.(150,151) However, there are a few circumstances that promote preferential opening of hemichannels versus gap junctions, and these include reduced intracellular redox potential, reduced intracellular pH, and Cx43 dephosphorylation.(152-156) While there is substantial evidence supporting the cytoprotective roles of intercellular communication through Cx43 gap junctions, there is also contrary evidence suggesting that Cx43 gap junction-mediated intercellular transfer of ATP, calcium, and glutamate can, instead, exacerbate cytotoxic effects.(157-161) Blocking gap junction-mediated intercellular communication in cells exposed to chemotherapy or radiotherapy, for example, resulted in reduced

therapy-associated cell death,(162,163) and interventions that increased gap junction expression increased chemotherapy cytotoxicity.(164,165)

While Cx43 research is gradually acknowledging the separate contributions of Cx43 gap junctions and hemichannels to biological processes, more research is needed to achieve a more complete understanding of the roles of Cx43 in physiology. What is known is that metabolic inhibition by iodoacetic acid promotes cytotoxicity in part by selectively opening Cx43 hemichannels with minimal activity at Cx43 gap junctions.(82,166) Furthermore, several studies demonstrated selectively inhibiting Cx43 hemichannels as a novel therapeutic approach for cytoprotection.(69,167-170) In addition, selectively targeting astrocyte Cx43 hemichannels without simultaneously targeting astrocyte gap junctions has been shown in multiple studies to augment certain aspects of neurocognitive behavior. For example, one study demonstrated that selective blockade of Cx43 hemichannels impairs spatial short-term memory but not spatial working memory.(52) Another study demonstrated hippocampus-dependent learning can be disrupted by specifically inhibiting Cx43 hemichannel opening.(53) Lastly, another study demonstrated astrocyte Cx43 hemichannel opening is required for amygdala-dependent fear memory consolidation.(51) In light of these findings, additional research is needed to elucidate whether previously identified roles of Cx43 in biological processes would be more accurately described as either primarily gap junction associated, primarily hemichannel associated, or some combination of these two. The separate contributions of gap junctions and hemichannels was an important focus of my project.

Importance to Medicine:

Cancer:

Cx43 has been shown to be a mechanistic component of cancer pathology. High tumor Cx43 expression has a positive impact on prognosis for prostate, colorectal, pancreatic, non-small cell lung, breast, lung adenocarcinoma, glioblastoma, leukemia, head & neck squamous cell carcinoma, and liposarcoma.(171-181) However, high tumor Cx43 expression is associated with a negative prognosis in oral squamous cell carcinoma, non-muscle invasive urothelial bladder cancer, and esophageal squamous cell carcinoma.(182-184) Although these findings support Cx43 as a mechanistic component in cancer prognosis, additional research is needed to determine why Cx43 shows a positive prognostic effect for some types of cancer while negative effect on prognosis for other cancer types.

Cx43 has been shown to be a mechanistic component of cancer treatment in addition to impacting cancer prognosis. Several studies have shown Cx43 is reduced in cancer versus its endogenous expression in non-cancerous tissue, including breast cancer, glioma, and pancreatic cancer.(185-189) In particular, increasing Cx43 gap junction opening, often referred to as "increasing Cx43 based GJIC" or "enhancing Bystander Effect toxicity" has been shown to enhance chemotherapy efficacy in many studies. For example, increasing Cx43-based GJIC enhanced the chemotherapeutic efficacy of doxorubicin, etoposide, and taxol in human glioblastoma cells, cisplatin in mouse embryonic fibroblasts, tamoxifen in human breast cancer cells, temozolomide and vinchristine in human glioma cells, and adriamycin in four breast cancer cell lines.(190-194) In addition to enhancing chemotherapeutic efficacy by directly altering Cx43

based GJIC, several miRNAs that augment cancer progression and treatment efficacy by two main mechanisms involving Cx43 have been identified. These two mechanisms of Cx43 dependent intercellular transfer of miRNA are by directly decreasing Cx43 expression in a given cell, or by passing through Cx43 channels to spread its effects (of reducing Cx43 expression) to communicating cells. Several miRNA have been reported to directly suppress Cx43 expression, and through doing so, impact cancer cell survival. These miRNA include miR-221/222, miR-125b, and Let-7g-5p in glioma, miR-200a in breast cancer, and miR-20a in prostate cancer.(195-199) Based on the second mechanism, several studies have demonstrated functional transfer of miRNA between neighboring cells connected through Cx43 gap junctions with functional impacts on the recipient cells.(120-122) One study showed Cx43 gap junction dependent intercellular transfer of the exogenous miRNA cel-miR-67 between communicating rat gliosarcoma cells, which was blocked by the gap junction blocker, carbenoxolone.(118) Similarly, carbenoxolone also blocked transfer of astrocyte miRNAs to lung cancer metastases *in vivo*, where the transferred miRNA otherwise enhanced resistance to Paclitaxel chemotherapy.(200) Another study showed several anti-proliferative miRNAs could be transferred from bone marrow stroma to breast cancer cells through gap junctions between the two distinct cell types resulting in suppressed proliferation of the breast cancer metastases at distal sites.(119) Furthermore, gap junction dependent, bi-directional transfer of anti-angiogenic miR-145-5p was observed between SW480 colon cancer cells and human microvascular endothelial cells, resulting in decreased angiogenesis in the recipient cell type.(201) This group also demonstrated gap junction dependent transfer of the pro-invasive miR-5096 from U87 glioblastoma cells to recipient human microvascular endothelial

cells and human microvascular endothelial cells also use Cx43 gap junctions to transfer the anti-angiogenic miR-145-5p to recipient U87 glioblastoma cells.(202) Similarly, another study demonstrated that gap junctions mediated intercellular transfer of the anti-proliferative miR-124-3p among U87 glioblastoma cells and among C6 glioma cells.(203) These findings suggest that miRNA actions on Cx43 could occur through parallel mechanisms, one that directly suppresses Cx43 expression and another that uses Cx43 as intercellular channels to transfer miRNA among homogenous or heterogenous cell types. Future studies should consider the clinical utility of these mechanisms, as well as assess whether there are additional miRNAs that exert their effects through either of these mechanisms. Lastly, recent studies have demonstrated that cells within some astrocytomas are connected through networks of intercellular tunneling nanotubes that participate in resistance to anti-cancer therapy through mechanisms that remain poorly understood but that may involve Cx43 channels, as these tunneling nanotubes often express connexin 43.(204,205) Whether these Cx43-expressing intercellular tunneling nanotubes are equally capable of transferring miRNA among communicating cells remains to be demonstrated. Overall, these findings indicate that Cx43 is a key component of cancer pathology as well as anti-cancer therapies.

Neuroscience:

Ever since Rolf Dermietzel's and Christian Giaume's labs independently demonstrated astrocyte Cx43 expression in 1991,(2,3) data supporting astrocyte Cx43 as a shared mechanistic component of several neurological and neurodegenerative disorders has been published. These

disorders include Alzheimer's disease, epilepsy and seizure disorders, neuropathic pain disorders, and ischemic stroke. The following section will detail the findings supporting the potential roles of astrocyte Cx43 in these neurological disorders. Overall, these findings indicate the roles of Cx43 in brain health may not be exclusively protective or detrimental, and the exact role of Cx43 in brain health may be brain region specific, disease specific, experimental model specific, or cell type specific. It is of note that what makes Cx43 protective or detrimental in these various contexts may be a function of whether Cx43 exists as a gap junction or as a hemichannel, as is a central thesis of my research reported herein.

Alzheimer's disease:

Recent evidence suggests Alzheimer's disease is associated with changes in astrocyte connexin 43 function. Astrocyte Cx43 expression is increased in brains with Alzheimer's disease relative to healthy controls.(16) Additionally, PC12 cells transfected with the carboxy terminal fragment of the amyloid beta peptide displayed increased Cx43 expression and open Cx43 gap junctions compared to non-transfected controls.(206) Furthermore, Amyloid beta 1-42 peptide increased calcium wave propagation as well as increasing astrocyte Cx43 hemichannel-mediated glutathione, glutamate, and ATP release from the cytoplasm to the extracellular environment.(207-209) Amyloid beta 1-42 peptide also enhanced pro-inflammatory cytokine induced inhibition of gap junction intercellular communication *in vitro*.(210) This peptide directly decreased gap junction intercellular communication in cultured astrocytes *in vitro*, independent of the effect of pro-inflammatory cytokines.(17) Another study, using the CVN-AD transgenic mouse model of

Alzheimer's disease that co-expresses the Swedish and Dutch APP mutations with a knockout of iNOS, found that while no difference was observed between the CVN-AD transgenic mice brains and the WT controls in overall Cx43 expression, increased phosphorylation at Cx43 serine-368 was observed for the 24 month and 42 month old transgenic mice compared to WT controls.(211) However, in APP/PS1 transgenic mice, an age associated increase in Cx43 immunoreactivity was observed near beta-amyloid plaque aggregates starting in 4 month old mice and persisting with increased age.(212) Building off of these findings, inhibition of astrocyte Cx43 hemichannels is being assessed as a novel therapeutic approach in pre-clinical Alzheimer's disease studies.(169,170,213) However, additional research is needed to further characterize the therapeutic potential of targeting Cx43 in Alzheimer's disease.

Epilepsy & Seizure Disorders:

Recent evidence suggests the roles of astrocytes, and changes in astrocyte Cx43 function in particular, are associated with seizure disorders. One study demonstrated increased astrocyte Cx43 expression in the temporal lobe in patients suffering from seizure disorders.(214) However, subsequent studies partially refuted this notion by demonstrating no change in Cx43 expression in the amygdala or hippocampus following an epileptogenic stimulus, implying the previous finding may be a brain region specific observation.(215,216) Furthermore, increased astrocyte Cx43 expression is observed in multiple experimental models of seizure disorders, including those induced by pentylenetetrazole, pilocarpine, kainic-acid, and 4-aminopyridine.(13,217-219) In addition to changes in astrocyte Cx43 expression, changes in Cx43 GJIC, as well as altered Cx43

phosphorylation, have also been observed in seizure disorder models.(14,220,221) Finally, while increased Cx43 expression was associated with increased seizure occurrence, seizure occurrence decreased following application of the connexin channel blocker carbenoxolone.(222-224) Additional research is needed to further assess the roles of astrocyte Cx43 in seizure disorders and to assess whether astrocyte Cx43 is a viable therapeutic target for seizure disorders.

Pain disorders:

Recent evidence suggests roles of astrocytes in neuropathic pain induction through regulation of astrocyte Cx43 function, specifically in spinal cord astrocytes. One study demonstrated increased spinal astrocyte Cx43 expression following chronic spinal constriction injury, and administering Cx43 selective RNAi reduced pain-associated behavior in rats.(225) Another study demonstrated central pain sensitization associated with medullary dorsal horn nociceptive neurons induced by mustard oil administration to tooth pulp in rats could be blocked by administering the connexin channel blocker carbenoxolone.(226) Another study demonstrated mechanical allydonia and heat hyperalgesia following weight drop induced spinal cord injury was observed in WT type control mice but absent in astrocyte directed (GFAP-cre directed) Cx43 KO transgenic mice.(71) It is not clear whether increasing or decreasing spinal astrocyte Cx43 expression enhances pain induction, as studies assessing Cx43 expression after treating spinal astrocytes with pro-inflammatory cytokines support either of these notions.(227-233) However, there is some evidence that decreasing Cx43-based GJIC may be a novel mechanism for reducing neuropathic pain induction. One study found that both TNF-alpha and IFN-gamma reduce Cx43

based GJIC by a JNK dependent mechanism,(234) while another study showed a synthetic, carbon monoxide releasing compound attenuated neuropathic pain induction by selectively inhibiting connexin hemichannel function.(235) These observations underscore the need to better tease apart the roles of Cx43 gap junctions from hemichannels as they pertain to the molecular mechanisms underlying the pathophysiology of neuropathic pain disorders.

Stroke:

Recent evidence suggests astrocytes play protective roles in ischemic stroke in part by changes in Cx43 function following ischemic injury. One study found elevated Cx43 expression in the penumbra region following ischemic injury in human brain slices, and the investigators speculated that the increased Cx43-based GJIC may serve a protective role after ischemic stroke.(236) Subsequent studies from the same group found elevated Cx43 expression in ischemic cores of human brain samples as well.(237) However, another study observed decreased Cx43 expression in mouse brain following ischemic insult achieved by the ischemia/reperfusion model, suggesting changes in Cx43 following ischemic injury may be partially dependent on the type of ischemic model used.(238) Interestingly, another study observed that astrocyte specific knockdown of Cx43 expression using Cx43 specific RNAi was detrimental to neurite outgrowth following oxygen glucose deprivation, an effect not observed in the control rats.(11) Furthermore, this study also demonstrated that neurite outgrowth following MCAO induction is facilitated by increased astrocyte Cx43 expression surrounding the ischemic lesion.

Since global knockout of Cx43 is neonatal lethal due to severe heart defects,(239) transgenic mouse models with either astrocyte targeted Cx43 knockout or specific point mutations in the Cx43 gene have been generated to assess the contributions of Cx43 to biological processes. Transgenic mice harboring the glial fibrillary acidic protein (GFAP) directed knockout of Cx43 in astrocytes showed increased infarct volume following middle cerebral artery occlusion compared to WT mouse littermates.(240) This finding was similarly demonstrated in transgenic mice harboring a *c*-terminal truncated form of Cx43,(241) as well as in Cx43 G60S heterozygous transgenic mice, which is a mouse model of rare genetic diseases in which Cx43 gap junctional coupling is significantly impaired (such as occulodentaldigital dysplasia).(242) These findings support the hypothesis that Cx43 is protective against cerebral ischemia, specifically through Cx43-mediated intercellular coupling composed of Cx43 gap junctions.

Contrary to the findings stated in the preceding paragraph, several studies also report enhanced Cx43 function as detrimental following cerebral ischemia. One group observed that enhanced Cx43 hemichannel open state following ischemic injury increases ischemia-associated injury.(243) Similarly, another study demonstrated that inhibiting Cx43 channel opening after ischemic injury enhanced the protective effects of leptin against ischemia-induced deficits.(244) Furthermore, blocking Cx43 channel function with carbenoxolone attenuated oxidative stress and reduced the percentage of apoptotic cells following ischemic injury,(245,246) further supporting the notion that increased Cx43 channel function is potentially detrimental following ischemic insult. Taken together, these findings indicate astrocyte Cx43 may be a potential therapeutic target for novel ischemic stroke treatments, but additional research is needed to determine how to

selectively utilize the protective aspects of Cx43 function while minimizing the detrimental aspects following ischemic injury.

Regulation by Medicinal Compounds*:*

Research to date on the effects of drugs used in medicine on Cx43 function has focused primarily on anesthetics, psychiatric compounds, and recreational drugs of abuse. To date, anesthetics' effects on Cx43 function has demonstrated a consensus—all anesthetics tested thus far (dexmedetomidine, halothane, isoflurane, ketamine, propofol, sevoflurane, and thiopental) reduce Cx43-based GJIC in the test tissues, the majority of which have been CNS derived tissue.(197,247-250) These findings suggest reducing Cx43-based GJIC may be a common mechanistic component of clinically used anesthetics. The notion that clinically used anesthetics act in part by reducing Cx43-based GJIC may be especially relevant for two aspects of anesthesia use: determining the most effective dose per patient and reducing the likelihood of anesthesia-associated side effects. Given that many patients undergoing medical procedures in which anesthesia is necessary are also using other drugs, prescriptions, or natural compounds that modulate Cx43-based GJIC, such drug combinations could influence the optimal anesthesia dose by additively influencing Cx43-based GJIC, among other mechanisms. Based on this notion, further research on the effects of anesthetic compounds on Cx43-based GJIC, as well as further characterization of drug-drug interactions on Cx43-based GJIC, could provide anesthesiologists with additional information for determining the most effective anesthesia dose per patient while reducing the likelihood of anesthesia-associated side effects. Similar to the findings regarding the

effects of anesthetics on Cx43 function, a consensus has been observed for the effects of drugs of abuse on Cx43 channel function. More specifically, cigarette smoke, ethanol, MDMA, methamphetamine, morphine, and delta-9-tetrahydrocannabinol have each been shown to decrease Cx43-based GJIC.(169,233,251-259) This is the first observation that these widely abused drugs share decreasing Cx43-based GJIC as a mechanistic component. Further research is needed to determine the clinical utility of this information. Furthermore, since drug abusers often abuse more than one of these drugs at the same time, future research needs to also consider the possibility that concomitant exposure to these drugs could result in synergistic negative effect on Cx43 function as a molecular component of drug-drug interactions between drugs of abuse.

Unlike the findings regarding the effects of anesthetics and drugs of abuse on Cx43 channel function, there has not been a consensus observed for the effects of psychiatric drugs on Cx43 channel function. Some studies have observed that psychiatric drugs, such as amitriptyline, haloperidol, and lithium, decrease Cx43-based GJIC.(260,261) However, other studies have shown some psychiatric drugs, such as anandamide, clozapine, fluoxetine, and modafinil increase Cx43-based GJIC.(260,262-265) The lack of consensus observed for the effects of psychiatric drugs' effects on Cx43 function may be due to the findings being brain region specific, disease specific, drug mechanism specific, experimental model specific, or cell type specific, and additional research is needed to further address these considerations.

Regulation by Non-Medicinal Compounds:

Based on the notion that reduced Cx43 opening and reduced connexin expression support tumor formation and progression,(266,267) it was proposed in the early 2000's that screening environmental pollutants for their ability to reduce Cx43-based GJIC may be a useful technique for determining individual pollutant's carcinogenic potential.(268) Subsequent studies demonstrated that several types of environmental pollutants were indeed carcinogenic based on this notion. Pollutants characterized as carcinogenic to date using this approach include persistent organic pollutants (such as benzopyrenes, low molecular weight polycyclic aromatic hydrocarbons, and poly-chlorinated biphenyls (PCBs)),(269-286) endocrine disrupting pollutants (such as bisphenol A, ioxynil, perfluorooctanoic acid, and perfluorooctane sulfonate),(287-289) and pesticides (such as DDT, dieldrin, lindane, and toxaphene).(290-293) Collectively, the above studies demonstrated these pollutants may promote oncogenesis, at least in part, through decreasing Cx43-based GJIC, and likely do so in parallel with additional mechanisms that functionally converge on promoting oncogenesis. Furthermore, recent evidence suggests augmenting Cx43-based GJIC in the brain may impair brain health. Given that several of the pollutants listed above are already categorized by the Stockholm Convention as persistent organic pollutants with carcinogenic properties, subsequent studies assessing the effects of these compounds on brain health may support their additional classification as neurotoxins. Such findings would provide additional information regarding the health impacts of exposure to these compounds and further underscore the need to remove these compounds from the environment.

Based on this information, additional research is needed to characterize these pollutants with specific emphasis on their effects on brain health.

Similar to persistent organic pollutants, heavy metal toxicity has also been associated with reduced Cx43 gap junction opening correlating with adverse health consequences, such as increasing cancer risk, or increasing risk of neurological impairment. Examples of this observation include toxicity associated with exposure to arsenic, cobalt, lead, and zinc.(294-297) Collectively, this information suggests that Cx43 is a common molecular target of action for both persistent organic pollutants and heavy metal pollutants.

Summary of Literature Cited:

Cx43 is a molecular component that potentially underlies the pathophysiology of a wide variety of diseases and also mediates the negative consequences associated with exposure to a variety of chemical compounds. In terms of neurological disorders, Cx43 may serve more disease-specific functions such that in some diseases, increasing Cx43 opening may help alleviate symptoms, and in other diseases, decreasing Cx43 opening may help alleviate symptoms. With the exception of seizure disorders since seizures are associated with increased astrocyte Cx43 expression, there is no clear consensus whether increasing Cx43 opening is beneficial or detrimental in neurological disease treatment. Additional research is needed to assess whether augmenting Cx43 function in a particular phase of a disease, such as pre-symptomatic versus symptomatic, may be a useful treatment strategy. More specifically, whether selectively altering Cx43 gap junction opening versus hemichannel opening in experimental models of diseases

remains to be better understood. Likewise, there is no clear consensus regarding the role of Cx43 in neuropsychiatric disease treatment. While anesthetics and recreational drugs of abuse have been shown to reduce GJIC, anti-psychotic drugs do not show a clear pattern regarding their effects on augmenting Cx43 gap junction opening. Based on this information, additional research is needed to establish how Cx43 contributes to the mechanisms of action for each of these treatment categories, and whether there are different effects exerted by Cx43 hemichannels and gap junctions. Indeed, the contributions of astrocyte Cx43 to overall brain function appear to be brain region specific, disease type specific, disease drug mechanism specific, experimental model specific, and/or cell type specific.

One key path to clarifying the lack of clear association between Cx43 expression and functional impact is to more specifically consider the distinct contributions of Cx43 hemichannels and Cx43 gap junctions to changes in Cx43-expressing cell functions. Until recently, Cx43 targeted experimental tools, such as Cx43 antagonist peptides, Cx43 targeted antibodies, and channel blockers like carbenoxolone, all targeted both Cx43 hemichannels and gap junctions with relatively equal affinity and similar efficacy. Thus, it was difficult to discern the separate contributions of Cx43 hemichannels and Cx43 gap junctions to experimental observations. Several labs have worked to overcome this obstacle, including Dr. Thu Annelise Nguyen's lab characterizing a novel compound that selectively enhances Cx43 gap junction function (PQ1 Succinate),(192) the development Cx43 hemichannel-selective antibodies,(298,299) and Dr. Christian Giaume's lab characterizing a novel plant extract that selectively inhibits Cx43 hemichannel function without similar activity at Cx43 gap junctions (Boldine).(170) These

compounds have demonstrated pre-clinical efficacy in some experimental models to enhance breast cancer chemotherapy efficacy, decreasing breast cancer metastasis and decreasing hippocampal neuron dysfunction in Alzheimer's Disease transgenic mice.(170,192,300) These findings underscore the importance of future Cx43 research that considers the separate contributions of Cx43 hemichannels and gap junctions to experimental findings and drug development. In this way, one must take into consideration regulation of connexin expression, channel opening or closing, and connexin channel isoform composition to more clearly determine their roles in health and disease.(301,302) Through such understanding, Cx43 may become a viable therapeutic target for a wide variety of disease processes.

Goals of our Research:

Given that the brain has the highest endogenous Cx43 expression of any tissue in the body, it stands to reason that the brain is particularly vulnerable to alterations in Cx43 function. This is because astrocytes and their metabolic networks constructed through Cx43-based cell-to-cell coupling support optimal neuron firing, protect neurons against excitotoxicity, and these networks are reciprocally regulated by neighboring neurons' synaptic activity.(46,47,49,303,304) These notions are further supported by studies demonstrating a significant reduction in astrocyte-astrocyte coupling in GFAP directed Cx43 knockout mice compared to WT controls.(305,306) Clinically, alterations in Cx43 gap junction opening have been associated with a wide range of diseases that present significant economic and societal burden including cancer, heart disease, ischemic stroke, anxiety, seizure disorders, depression, and Alzheimer's

disease.(11,15,165,173,185,217,307) Even so, the mechanisms underlying impairments in Cx43 function remain to be completely understood in these different disease categories.

One important component of the beneficial actions shared between connexins, androgens and estrogens is their ability to protect tissues against oxidative stress. While the protective effects of gonadal steroid hormones has been extensively characterized in terms of steroid receptor-mediated mechanisms,(23,308-315) as well as the ability of estrogens to act as redox scavengers,(33,316-318) it is not known whether Cx43 is a vital component of these steroids' protective effects against oxidative stress. What is known is that androgens and estrogens regulate Cx43 expression in peripheral (non-CNS) tissue.(7,8,76,81) Furthermore, multiple estrogenic compounds decrease Cx43 channel gap junction opening in peripheral tissue.(83,319,320) However, whether estrogens exert a similar effect in the CNS, or whether androgenic compounds have similar effects on Cx43 remain unknown.

In support of the notion that Cx43 may be a target of estrogens, insofar as its protective effects against oxidative stress are concerned, are findings that oxidative stress results in decreased Cx43 expression and decreased Cx43 channel opening in peripheral tissue.(6,321) However, it remains largely unknown whether or not these effects are similarly observed in brain tissue. Given the well described protective effects of estrogens against oxidative stress,(308,310,322-324) this project proposes to demonstrate whether Cx43 is indeed a vital mediator/regulator of estradiol's protection against oxidative stress in astrocytes, and furthermore, will determine whether the non-aromatizable androgen, DHT, and DHT's estrogenic metabolite, 3βdiol, also target Cx43 as a mechanism for regulating astrocyte viability in the presence of oxidative stress. The latter is of

complementary significance since it is inferred that the protective effects of DHT are mediated by its estrogenic metabolite.

CHAPTER 2: GENERAL METHODS

Tissue Acquisition and Culture:

Primary cortical astrocytes were derived from female postnatal day 2 C57Bl/6 mice as described previously with some modifications.(325,326) Briefly, pups were anesthetized by hypothermia, followed by cardiac puncture as the method of euthanasia. Following craniotomy, the brain was removed and microdissected to isolate the total cerebral cortex. The resulting cortical tissue was then mechanically and enzymatically dissociated into individual cells, which were then centrifuged and filtered (to remove cellular debris), and finally plated and cultured until reaching confluence, with media changes occurring every other day. In order to obtain astrocyte enriched cultures, culture flasks were incubated with horizontal shaking for 72 hours after cells reached confluence to detach microglia.(327) The resulting cells were then subcultured and plated in either 96-well black bottom plates (Thermo Fisher Scientific, Rochester, NY; cat# 165305) for cell viability assays and hemichannel permeability assays, or clear bottom 6-well plates (VWR North American, Radnor, PA; cat# 10062-892), for the purposes of assessing mRNA and protein expression, at a ratio of 1 75 cm^2 flask per either 96-well or 6-well plates. Astrocytes enriched cells cultures were grown in Astrocyte media (Dulbecco's Modified Eagle Media containing 110 mg/L Sodium Pyruvate, Gibco Life Technologies, Grand Island, NY; cat# 11995-065) with 10% v/v Fetal Bovine Serum (Atlanta Biologicals, Flowery Beach, GA; cat# S11550) and 1% v/v PenStrep 10,000 units/mL (HyClone, South Logan, UT cat# SV30010). All experimental procedures involving animals were approved by the UNT Health Science Center's Institutional Animal Care and Use Committee (IACUC).

Pharmacologic Treatments:

17-β estradiol (E2, Sigma, St. Louis, MO; cat# E2758), dihydrotestosterone (DHT, Steraloids, Newport, RI; cat# A2570-000), and 5α-androstane-3β, 17β-diol (3βdiol, Steraloids, Newport, RI; cat# A1220-000) were dissolved in DMSO, and applied to the cells at a final concentration of 100 nM (where the DMSO concentration was < 0.1%). This concentration was selected based on dose response and time course optimizations that indicated 100 nM Estradiol when applied for either 3 or 18 hours protected against an iodoacetic acid (IAA) induced 50% reduction in cell viability by MTT assay. Equimolar concentrations of DHT and 3βdiol were determined to be similarly as effective at protecting against IAA toxicity after dose response assessments were conducted with these two steroids. Figure 1 in Chapter 3 demonstrates that 100 nM of each of these steroids are similarly protective against otherwise cytotoxic iodoacetic acid exposure. Iodoacetic acid (IAA, final concentration 25 μM, Sigma, St. Louis, MO; cat# I4386), was dissolved in de-ionized water, sterile filtered, aliquoted, and used at the final concentrations indicated in the results and figure legends. Experimental concentration of IAA was determined by dose response optimizations to identify the concentration of IAA that when applied to confluent astrocytes for 6 hours, reduced their cell viability by approximately 50 percent. TNFα/IL-1β (final concentration 10 ng/mL for each, both mouse recombinant peptides (EMD Millipore (now Millipore Sigma), Darmstadt, Germany; cat# 654245 and IL014 respectively), Amyloid β (25-35) peptide Ala 28 (final concentration 50 μM, Sigma, St. Louis, MO; cat# SCP0041), and boldine (final concentration 100 μM, Sigma (now Millipore Sigma), Darmstadt, Germany; cat# B3916) were prepared and aliquoted based on the manufacturer's recommendation. ER antagonists ICI-

182,780, (500 nM, cat# 1047/1), MPP Dihydrochloride, (100 nM, cat# 1991/10) and PHTPP (500 nM, cat# 2662/10) were from Tocris (Tocris Bioscience, Minneapolis, MN). Both the gap junction opening compound PQ-1 Succinate (100 nM PQ1, cat# SML 0431)(130) and gap junction closing compound 18-α-Glycyrrhetinic acid (100 μM AGA, cat# G8503)(328) (both from Sigma, St. Louis, MO) were also dissolved in sterile DMSO at a stock concentration of 10 mM, and sterile filtered prior to application to the cells at the stated final concentrations. TNFα/IL-1β and Amyloid β 25-35 peptide Ala 28 were dissolved in de-ionized water while boldine was dissolved in sterile DMSO, and sterile filtered prior to application to the cells at the stated final concentrations.

RTPCR:

Semi-quantitative, real-time PCR was conducted using 20 ng of template per reaction to assess treatment induced changes in astrocyte mRNA expression for Cx43 (primer: Mm01179639_s1 from Applied Biosystems, Pleasanton, CA) and GAPDH (primer: Mm99999915_g1 from Applied Biosystems, Pleasanton, CA). RNA was extracted from astrocytes using the RNeasy Mini Kit (Qiagen, Hilden, Germany, cat# 74106), converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Vilnius, Lithuania, cat# 4368814), and analyzed on an Applied Biosystems 7300 Real Time PCR System Thermocycler (Foster City, CA). Target mRNA levels were evaluated in triplicate and averaged to obtain average cycle threshold (Ct) values for each sample's Cx43 and GAPDH expression. Fold change values were calculated using the $2^{-\Delta\Delta Ct}$ method,(329) followed by transforming the data to percent of

DMSO control (serving as the experimental reference group). The resulting averages were compared for statistical significance by One-Way ANOVA followed by Tukey's *post hoc* analysis to determine statistical significance between treatment groups in GraphPad Prism Version 5 software.

Western Blot:

Western blots were conducted as previously described,(330) to assess relative changes in Cx43 protein expression following treatment with estradiol, DHT, and 3βdiol (all 100 nM, 3 and 18 hour vehicle or steroid treatment). In brief, astrocyte cultures were lysed in brain cell type optimized protein lysis buffer containing protease and phosphatase inhibitors.(331) After homogenization, samples were centrifuged at 100,000 x g for 30 min at 4° C. 40 µg of total protein (quantified using the Bio-Rad DC assay (Rio-Rad, Hercules, CA; cat# 5000116) per lane was separated by SDS-PAGE and subsequently transferred onto polyvinylidene fluoride membranes (PVDF, Bio-Rad Laboratories) by electroblotting. Membranes were blocked with 3% bovine serum albumin (BSA) in tris-buffered saline containing 0.2% Tween 20 (TBS-T) for 1hr at room temperature, followed by overnight incubations with primary antibodies at 4°C. The following antibodies were used: Cx43 / GJA1 rabbit polyclonal antibody (Abcam, Cambridge, MA; cat# ab11370, 1:5000 in TBST), GAPDH rabbit polyclonal antibody (Cell Signaling, Danvers, MA; cat# 2118, 1:1000 in TBST), and HRP-conjugated mouse anti-rabbit monoclonal secondary antibody, light chain only (Jackson ImmunoResearch, West Grove, PA; cat# 211-032-171, 1:

10,000 in 3% v/v BSA in TBST). Additionally, phosphospecific antibodies were used to assess treatment induced changes in Cx43 phosphorylation at amino acid residues Y265 (Abcam, Cambridge, MA; cat# ab197598, 1:1000 in TBST) and S368 (Abcam, Cambridge, MA; cat# ab194928, 1:1000 in TBST). Immunoreactive bands were visualized with the ECL detection system (ThermoFisher Scientific) and were captured using a luminescent image analyzer (Alpha Innotech). Densitometric analyses were conducted using ImageJ (National Institutes of Health) software.

MTT Viability Assay:

The Methyltetrazolium (MTT, Bio Vision, Milpitas, CA; cat# 2809) viability assay was conducted in a 96-well plate format, with 8 wells allocated per treatment group. Iodoacetic acid (IAA) was chosen as the oxidative insult because it recapitulates *in vitro* age-associated decreased glycolysis and increased oxidative stress,(82,332) as well as opening Cx43 hemichannels without similar effects at Cx43 gap junctions.(166) Preliminary iodoacetic acid (IAA) dose response MTT assay revealed that 25 μM IAA for 6 hr was sufficient to reduce cell viability by 50%. Steroid treated groups were treated for 18 hr prior to IAA treatment. The resulting treatment/insult-induced changes in MTT colorimetric absorbance were detected on a FilterMax F5 Multi-Mode Microplate Reader, (Molecular Devices, Sunnyvale, CA) 595 nm absorbance. Results for 8 wells were used to obtain treatment group average and variability per individual plate. Each experimental run (i.e., plate run at different times, where cells were derived from different animals) served as a single

biological replicate ("n"). Each data set presented represent at least three independent experiments. Between group differences were assessed using One-Way ANOVA followed by Tukey *post hoc* analyses in GraphPad Prism Version 5 software.

Ethidium Bromide Dye Uptake Assay to Assess Hemichannel Permeability:

The Ethidium Bromide Dye Uptake Assay was conducted using the Ca^{2+} free approach as described previously, and adapted for use with a 96-well fluorescence plate reader.(58) Hemichannel permeability in cultured astrocytes was induced either by exposing the cells to a Ca^{2+} free solution, TNFα/IL-1β or Amyloid β 25-35 peptide. Ethidium bromide (EtBr, VWR North American, Radnor, PA; cat# X328) was applied for 10 min at a final concentration of 4 μM and incubated at 37°C for 10 minutes. Cells were then washed with Hank's balanced salt solution (HBSS, Corning Cellgro, Manassas, VA; Ref: 21-022-CM) supplemented with 1.2 mM $CaCl_2$ (HBSS-Ca^{2+}). Treatment-induced changes in red fluorescence were then assessed on the FilterMax F5 Multi-Mode Microplate Reader, using the following excitation/emission parameters: 520 nm excitation and 610 nm emission. Resulting between group differences were assessed using One-Way ANOVA followed by Tukey *post hoc* analyses in GraphPad Prism Version 5 software.

Statistical Analysis:

For all data generated (RTPCR, Western Blot, EtBr hemichannel permeability assay, and MTT cell viability assay), resulting between treatment group differences were assessed using One-Way ANOVA followed by Tukey's *post hoc* analyses. RTPCR, EtBr hemichannel permeability assay, and MTT cell viability assay was analyzed using GraphPad Prism Version 5 software. Normalized Western Blot densitometry values were analyzed by single degree of freedom F test, followed by Tukey's *post hoc* analyses for group differences using Systat (Systat Software, Inc., San Jose, CA). For each experiment, each flask of astrocyte culture was considered an independent replicate since each flask of astrocytes were derived from separate mouse pups. As an example, if one litter generated 8 female pups, this would translate to four culture flasks, and thus four independent replicates. In order to determine the statistical power of each data set, we input the data generated into a power analysis software (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html) set to two tailed power calculation to detect a mean difference of 50%. While most of our data sets had a statistical power of 80% or greater, our only data set not meeting this threshold was our phosphospecific Y265 Western Blot data, with a statistical power of 77%. This observation was likely due to the large variability within this data set compared to our other data sets.

CHAPTER 3: EFFECTS OF CX43 FUNCTION ON E2, DHT, AND 3BDIOL INDUCED PROTECTION OF ASTROCYTES

ABSTRACT

Cx43 is a transmembrane protein highly expressed in astrocytes, where it contributes to protection against oxidative stress. In order to interrogate the contributions of Cx43 to protection against oxidative stress, we assessed the separate contributions of Cx43 gap junctions and Cx43 hemichannels. We hypothesized that in mouse cortical astrocytes, both Cx43 gap junctions and hemichannels contribute to protection against oxidative stress conferred by estradiol, dihydrotestosterone (DHT), or 5-α-androstane-3β,17β-diol (3βdiol). Using primary cortical astrocytes from neonatal female C57/Bl6 mice, we assessed changes in cortical astrocyte viability following treatment with 100 nM estradiol, DHT, or the DHT metabolite, 5-α-androstane-3-β,17-β-diol (3βdiol). Furthermore, we applied the steroids in combination with multiple estrogen receptor (ER) antagonists, a Cx43 gap junction opener compound (PQ1), a Cx43 gap junction closing compound (18-AGA), and Cx43 hemichannel closing compound (boldine). These studies were designed to tease apart not only the role of the two classical ERs in the effects elicited by the steroid hormones, but also the contributions of either of the two forms of Cx43 channels to estradiol, DHT, and 3βdiol protection against oxidative stress in cortical astrocytes. Neither selectively opening astrocyte Cx43 gap junctions nor selectively closing astrocyte Cx43 gap junctions with the pharmacological compounds listed above conferred protection against oxidative stress. Instead, the open state of astrocyte Cx43 gap junctions exacerbated IAA-induced cytotoxicity. In contrast, closing Cx43 hemichannels conferred protection against oxidative stress.

However, this selective closing of Cx43 hemichannels did not enhance estradiol, DHT, or 3βdiol's protection against oxidative stress. Given the selective opening or closing of Cx43 gap junctions conferred no added benefit to steroid induced protection, we focused our efforts on assessing the contributions Cx43 hemichannels to steroid induced protection. Since all three steroids blocked IAA-induced opening of Cx43 hemichannels in the EtBr Dye uptake assay, we inferred that all three steroids can exert protection against oxidative stress in astrocytes by closing Cx43 hemichannels. This was supported by the viability assay data which showed that closing hemichannels in the absence of steroid versus applying either of the three steroids were equally protective against IAA-induced cytotoxicity. Taken together, this data is the first to characterize the interaction of estradiol, DHT, or 3βdiol and astrocyte Cx43 with implications for protecting astrocytes against oxidative stress. Our results indicate Cx43 hemichannels as a previously underappreciated target of estradiol, DHT, and 3βdiol protection in astrocytes, and characterizing these effects at the cellular level remain on-going.

INTRODUCTION

Cx43 is highly expressed in astrocytes, where its expression and function (e.g., opening) can influence cell viability. However, the mechanisms by which astrocyte Cx43 expression contributes to protection against oxidative stress remains to be more completely characterized. In terms of Cx43 serving as a target of such gonadal steroid hormones as estradiol (E2) and dihydrotestosterone (DHT), there are converging lines of evidence, primarily from peripheral cells (e.g., reproductive, heart, and bone tissue.(10,73-77)), supporting the possibility that androgens and estrogens regulate astrocyte Cx43 function. Given the reported role Cx43 expression/function can have on cell viability, coupled with the fact that the Cx43 gene promoter has estrogen response element half sites, we hypothesized that the protective effects of both estradiol and DHT are ER dependent, emphasizing the importance of conversion of DHT to 3βdiol, an ER-beta preferring DHT metabolite, in mediating the protective effects of DHT.(333,334) Further, given the limited understanding of how Cx43 gap junctions versus hemichannels influence estradiol, DHT and 3βdiol–induced protection, we determined how pharmacological regulators of each type of Cx43 channel would affect not only IAA-induced cytotoxicity but estradiol, DHT and 3βdiol induced cytoprotection as well. More specifically, we hypothesized that estradiol would protect against IAA-induced cytotoxicity and that protection would be enhanced by opening of Cx43 gap junctions, since opening gap junctions can facilitate the intracellular transfer of pro-viability signaling molecules.(335) Based on the same logic, we hypothesized that pharmacologically-induced closing of Cx43 gap junctions would not further enhance estradiol mediated protection against oxidative stress in astrocytes. With regards to investigating the role of Cx43 hemichannels,

given that multiple cannabinoids inhibit insult-induced cell damage by closing (reducing Cx43 channel opening) of astrocyte Cx43 hemichannels,(169,265) we hypothesized that boldine, a selective inhibitor of hemichannel opening would enhance the cytoprotective efficacy of estradiol, DHT, and 3βdiol.

METHODS

Tissue Acquisition and Culture:

Primary cortical astrocytes were derived from female postnatal day 2 C57Bl/6 mice as described previously with some modifications.(325,326) Briefly, pups were anesthetized by hypothermia, followed by cardiac puncture as the method of euthanasia. Following craniotomy, the brain was removed and microdissected to isolate the total cerebral cortex. The resulting cortical tissue was then mechanically and enzymatically dissociated into individual cells, which were then centrifuged and filtered (to remove cellular debris), and finally plated and cultured until reaching confluence, with media changes occurring every other day. The resulting cells were then subcultured and plated in either 96-well black bottom plates (Thermo Fisher Scientific, Rochester, NY; cat# 165305) for cell viability assays and hemichannel permeability assays, or clear bottom 6-well plates (VWR North American, Radnor, PA; cat# 10062-892), for the purposes of assessing mRNA and protein expression, at a ratio of 1 75 cm^2 flask per either 96-well or 6-well plates. Astrocytes were cultured in Astrocyte media (Dulbecco's Modified Eagle Media containing 110 mg/L Sodium Pyruvate, Gibco Life Technologies, Grand Island, NY; cat# 11995-065) with 10% v/v Fetal Bovine Serum (Atlanta Biologicals, Flowery Beach, GA; cat# S11550) and 1% v/v PenStrep 10,000 units/mL (HyClone, South Logan, UT cat# SV30010). Initial MTT viability assay optimization studies were conducted for cortical astrocyte cultures obtained from male only mouse pups and separate optimizations were conducted for female only cortical astrocytes. No statistical difference was observed in E2 induced protection in either male-only derived astrocytes or female-

only derived astrocytes, so moving forward from those experiments, we chose to use female only astrocytes. This design circumvented the possible confounding effect that pooling male and female astrocytes in the same culture flask (e.g. astrocytes from two males, two females, or one male and one female in the same flask) might have on our experimental results. All experimental procedures involving animals were approved by the UNT Health Science Center's Institutional Animal Care and Use Committee (IACUC).

Pharmacologic Treatments:

17-β estradiol (E2, Sigma, St. Louis, MO; cat# E2758), dihydrotestosterone (DHT, Steraloids, Newport, RI; cat# A2570-000), and 5α-androstane-3β, 17β-diol (3βdiol, Steraloids, Newport, RI; cat# A1220-000) were dissolved in DMSO, and applied to the cells at a final concentration of 100 nM (where the DMSO concentration was < 0.1%). This concentration was selected based on dose response and time course optimizations that indicated 100 nM Estradiol induced a significant increase in Cx43 mRNA in astrocytes and 100 nM Estradiol when applied for either 3 or 18 hours protected against an iodoacetic acid induced 50% reduction in cell viability by MTT assay. Iodoacetic acid (IAA, final concentration 25 μM, Sigma, St. Louis, MO; cat# I4386), was dissolved in de-ionized water, sterile filtered, aliquoted, and used at the final concentrations indicated in the statement of Results and figure legends. Experimental concentration of IAA was determined by dose response optimizations to identify the concentration of IAA that when applied to confluent astrocytes for 6 hours, reduced their cell viability by approximately 50 percent. Boldine (final concentration 100 μM, Sigma (now Millipore Sigma),

Darmstadt, Germany; cat# B3916)(170) were prepared and aliquoted based on the manufacturer's recommendation. ER antagonists ICI-182,780, (500 nM, cat# 1047/1), MPP Dihydrochloride, (100 nM, cat# 1991/10) and PHTPP (500 nM, cat# 2662/10) were from Tocris (Tocris Bioscience, Minneapolis, MN), each concentration being at least 10 times the respective compound's EC50. Both the gap junction opening compound PQ-1 Succinate (PQ1, cat# SML 0431) and gap junction closing compound 18-α-Glycyrrhetinic acid (AGA, cat# G8503) (both from Sigma, St. Louis, MO) were also dissolved in sterile DMSO at a stock concentration of 10 mM, and sterile filtered prior to application to the cells at the stated final concentrations (PQ1 final concentration 100 nM(130) and 18-AGA final concentration 100 µM).(328) Boldine was dissolved in sterile DMSO, and sterile filtered prior to application to the cells at the stated final concentrations.

RTPCR:

Semi-quantitative, real-time PCR was conducted using 20 ng of template per reaction to assess treatment induced changes in astrocyte mRNA expression for Cx43 (primer: Mm01179639_s1 from Applied Biosystems, Pleasanton, CA), 3BHSD7 (primer: (Mm00505894_g1 from Applied Biosystems, Pleasanton, CA), ERα (primer: Mm00433149_m1 from Applied Biosystems, Pleasanton, CA), ERβ (primer: Mm00599821_m1 from Applied Biosystems, Pleasanton, CA), and GAPDH (primer: Mm99999915_g1 from Applied Biosystems, Pleasanton, CA). RNA was extracted from astrocytes using the RNeasy Mini Kit (Qiagen, Hilden, Germany, cat# 74106), converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Vilnius, Lithuania, cat# 4368814), and analyzed on an Applied Biosystems 7300 Real Time PCR System Thermocycler (Foster City, CA). Target mRNA levels

were evaluated in triplicate and averaged to obtain average cycle threshold (Ct) values for each sample's Cx43 and GAPDH expression. Fold change values were calculated using the $2^{-\Delta\Delta Ct}$ method,(329) followed by transforming the data to percent of DMSO control (serving as the experimental reference group). The resulting averages were compared for statistical significance by One-Way ANOVA followed by Tukey's *post hoc* analysis to determine statistical significance between treatment groups in GraphPad Prism Version 5 software.

MTT Viability Assay:

The Methyltetrazolium (MTT, Bio Vision, Milpitas, CA; cat# 2809) viability assay was conducted in a 96-well plate format, with 8 wells allocated per treatment group. Iodoacetic acid (IAA) was chosen as the oxidative insult because it recapitulates *in vitro* age-associated decreased glycolysis and increased oxidative stress,(82,332) as well as opening Cx43 hemichannels without similar effects at Cx43 gap junctions.(166) Preliminary iodoacetic acid (IAA) dose response MTT assay revealed that 25 μM IAA for 6 hr was sufficient to reduce cell viability by 50%. Steroid treated groups were treated for 18 hr prior to IAA treatment. The resulting treatment/insult-induced changes in MTT colorimetric absorbance were detected on a FilterMax F5 Multi-Mode Microplate Reader, (Molecular Devices, Sunnyvale, CA) 595 nm absorbance. Results for 8 wells were used to obtain treatment group average and variability per individual plate. Each experimental run (i.e., plate run at different times, where cells were derived from different animals) served as a single biological replicate ("n"). Each data set presented represent at least three independent replicates.

Between group differences were assessed using One-Way ANOVA followed by Tukey *post hoc* analyses in GraphPad Prism Version 5 software.

Statistical Analysis:

For all MTT viability assay data generated, resulting between treatment group differences were assessed using One-Way ANOVA followed by Tukey's *post hoc* analyses using GraphPad Prism Version 5 software. For each experiment, each flask of astrocyte culture was considered an independent replicate since each flask of astrocytes were derived from separate mouse pups. As an example, if one litter generated 8 female pups, this would translate to four culture flasks, and thus four independent replicates. In order to determine the statistical power of each data set, we input the data generated into a power analysis software (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html) set to two tailed power calculation to detect a mean difference of 50%. All data sets in this section had a statistical power of 80% or greater.

Statistical Power Results:

Figure	**Number of replicates**	**Statistical Power (%)**
Figure 1	4	100
Figure 2	3	100
Figure 4	3	100
Figure 5	3	100
Figure 6	3	100
Figure 7	3	100

RESULTS

E2, DHT, 3βdiol protect cortical astrocytes against oxidative stress

We assessed whether estradiol, DHT, or 3βdiol protected cortical astrocytes against oxidative stress. As shown in Figure 1, the MTT viability assay revealed that iodoacetic acid (IAA)-induced oxidative and metabolic stress significantly reduced cortical astrocyte viability. Estradiol, DHT, and 3βdiol (at a concentration of 100 nM) protected cortical astrocytes against iodoacetic acid (IAA)-induced oxidative stress. As shown in Figure 2, DHT protection against IAA-toxicity is dependent upon prior conversion to 3βdiol.

Cortical Astrocytes Express both Cx43 and Estrogen Receptors

Using semi-quantitative RTPCR, we assessed the baseline mRNA expression of Cx43, and estrogen receptor (ER)-alpha and -beta, in non-treated cortical astrocytes. As shown in Figure 3, cortical astrocytes show high baseline expression for the full length Cx43 mRNA transcript (as evidenced by the low Ct value) and relatively low, but detectable expression of both ER-alpha and –beta.

Both DHT protection and 3βdiol protection against oxidative stress are ER dependent

Cortical astrocytes express estrogen receptors but their expression of classical androgen receptor is equivocal.(336) In our hands, primary cortical astrocytes are devoid of the classical androgen receptor (data not shown). As such, we hypothesized that both DHT- and 3βdiol-induced protection against IAA-induced oxidative stress are mediated through estrogen receptors in cortical

astrocytes, likely through DHT being first converted to 3βdiol. As shown in Figure 4, the ER antagonist ICI-182,780 blocked both DHT and 3βdiol protection against oxidative stress in cortical astrocytes, supporting our hypothesis that the protective effects of DHT are mediated by an estrogenic metabolite acting at the estrogen receptor. Furthermore, selective antagonism of ER-beta by PHTPP blocked 3βdiol induced protection against oxidative stress but failed to block DHT induced protection.

Effects of E2 and Opening Cx43 Gap Junctions on Astrocyte Viability

Given that pharmacologically enhanced opening of Cx43 gap junctions has been directly associated with enhanced cytotoxic efficacy in several chemotherapy studies,(192,337-339) but estrogenic compounds have been shown to close Cx43 gap junctions in reproductive tissue,(320) we initially hypothesized that opening gap junctions would increase the cytotoxicity of IAA, and estradiol would protect against this exacerbated cytotoxicity. As shown in Figure 5, the gap junction opener compound, PQ1, exacerbated IAA-induced cytotoxicity (from about 40% reduction in cell viability to about a 75% percent reduction). In the absence of oxidative stress, neither PQ1 alone, or in combination with estradiol pre-treatment, significantly altered cell viability. Unexpectedly, instead of protecting against combined PQ1+IAA enhanced cytotoxicity, applying PQ1+IAA after estradiol pre-treatment further enhanced cytotoxicity (from about 75% reduction in cell viability to about a 90% percent reduction).

Effects of E2 and Closing Cx43 Gap Junctions on Astrocyte Viability

Given that closing of Cx43 gap junctions has been directly associated with enhanced protection in several chemotherapy studies, we next hypothesized that closing gap junctions would increase the protective efficacy of estradiol. As shown in Figure 6, the gap junction closing compound on its own induced a significant reduction in cell viability, and exacerbated IAA-induced toxicity. Closing astrocyte Cx43 gap junctions, however, did not enhance estradiol-induced protection against IAA-induced oxidative stress.

Closing Astrocyte Cx43 Hemichannels protects Astrocytes against Oxidative Stress

Since neither opening nor closing astrocyte Cx43 gap junctions conferred protection against IAA-induced oxidative stress (although both influences on Cx43 gap junctions enhanced IAA-induced cell death), we chose to evaluate if pharmacological reduction of Cx43 hemichannel opening would confer protection against IAA-induced oxidative stress. We hypothesized that estradiol, DHT, or 3βdiol protects against oxidative stress in astrocytes by attenuating IAA-induced astrocyte Cx43 hemichannel opening. As shown in Figure 7, estradiol, DHT, and 3βdiol each protected cortical astrocytes against IAA-induced cell death. Boldine (the selective inhibitor of Cx43 hemichannel opening) also blocked IAA-induced cell death, and there was no additive protective interaction between boldine and either of the steroid hormones applied, potentially suggesting a common mechanisms of action (i.e., inhibition of Cx43 hemichannel opening).

E2, DHT, 3βdiol protect cortical astrocytes against oxidative stress

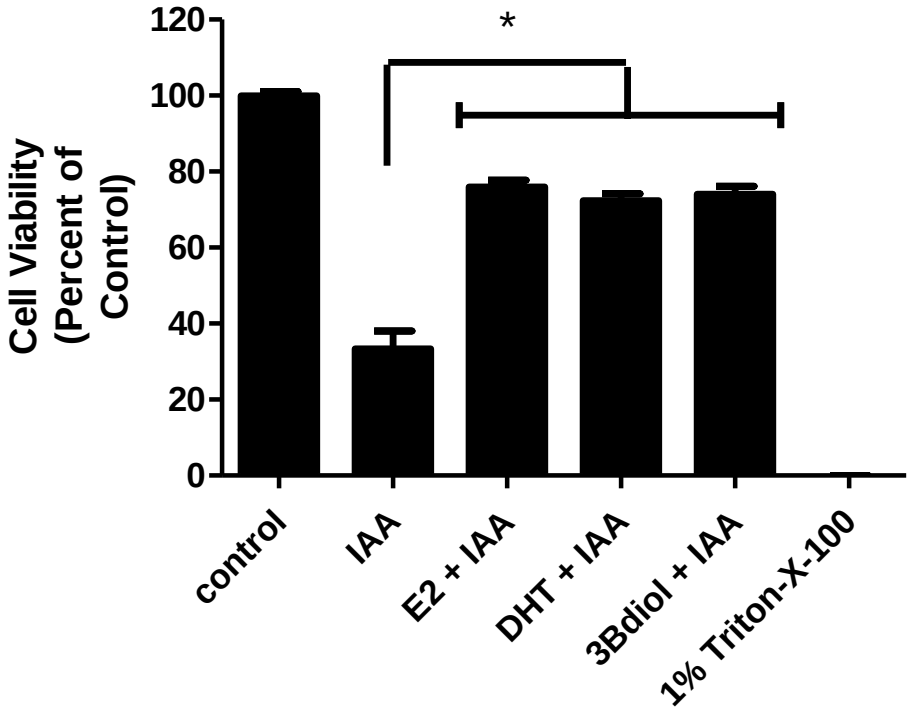


Figure 1: Estradiol (E2), dihydrotestosterone (DHT) and 3βdiol protected primary cortical astrocytes from iodoacetic-acid induced cell death.

Primary cortical astrocytes were treated with either estradiol (E2), dihydrotestosterone (DHT) or 3beta-diol (3Bdiol) for 18 hours prior to treatment with the oxidative/metabolic insult, IAA, for an additional 6 hr. Cell viability, as assessed by the MTT viability revealed cell death following IAA, while estradiol, DHT and 3Bdiol were protective. Triton X-100 (1%) was used as a control to induce maximum cell death. Data were normalized to the vehicle-treated control, and are presented as average ± SEM. * = different from IAA alone with $p < 0.05$, n= 4 independent replicates.

DHT protection is Blocked when DHT to 3βdiol Conversion is Blocked

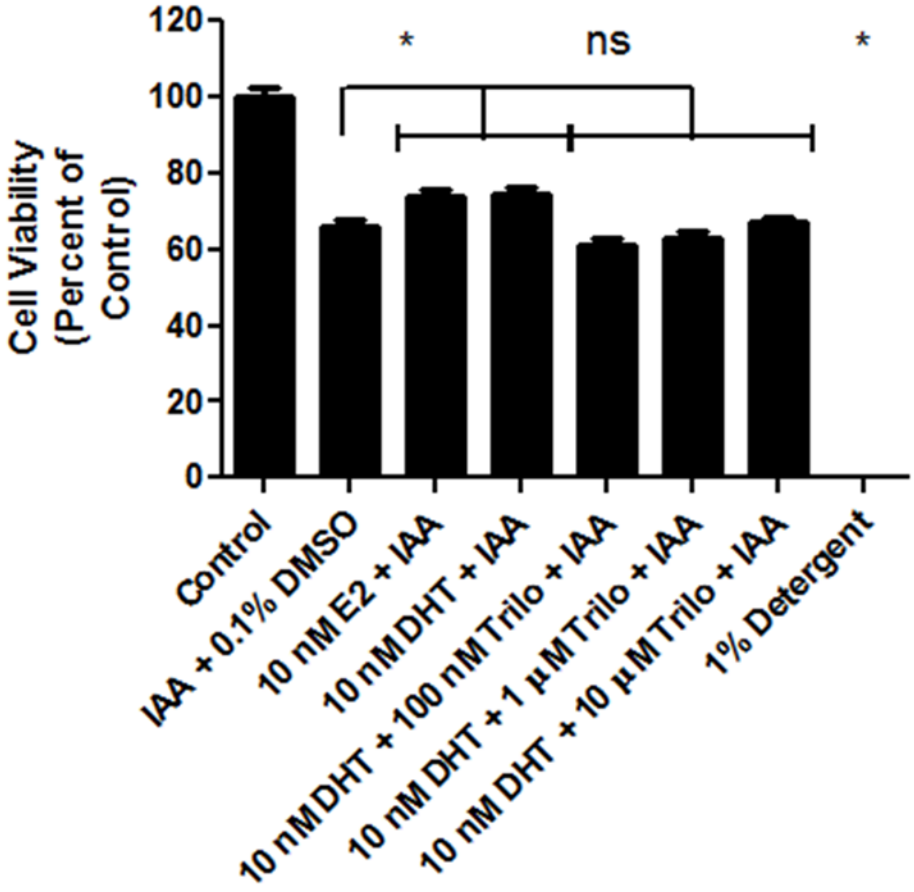


Figure 2: DHT-mediated protection in astrocytes is dependent upon prior conversion to 3βdiol

Primary cortical astrocytes were treated with either estradiol (E2) or dihydrotestosterone (DHT) with or without co-application of Trilostane (an inhibitor of the enzyme that converts DHT to 3βdiol) for 18 hours prior to treatment with the oxidative/metabolic insult, IAA, for an additional 6 hr. Cell viability, as assessed by the MTT viability revealed cell death following IAA, while estradiol, DHT and 3Bdiol were protective. Triton X-100 (1%) was used as a control to induce maximum cell death. Data were normalized to the vehicle-treated control, and are presented as average ± SEM. * = different from IAA alone with $p < 0.05$, n= 4 independent replicates.

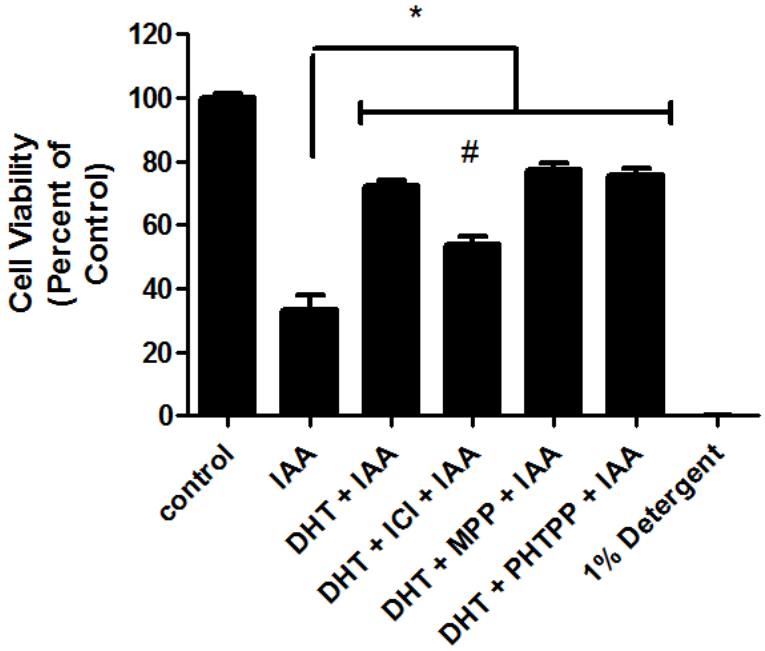
Cortical Astrocytes Express both Cx43 and Estrogen Receptors

Primer	**Relative mRNA Expression**
GAPDH	++++
Cx43 (GJA1, Full Length)*	+++
ERα	+
ERβ	+

Figure 3: Non-treated cortical astrocytes show high baseline expression for Cx43 mRNA and low, but detectable mRNA expression for both classical Estrogen Receptors alpha and beta.

Primary cortical astrocytes were grown to confluency, total RNA was extracted, converted to cDNA, and quantified. Average baseline mRNA expression was binned on resulting cycle threshold (Ct) values based on the following cut-offs, where lower Ct values reflect higher mRNA expression: "++++" = 18-21 Ct, "+++" = 22-25 Ct, "++" = 26-30 Ct, "+" = 31-35 Ct. * = Different RTPCR primers can be used to distinguish between the mRNA transcript coding for the full length 43 kDa protein coding product versus the mRNA transcript coding for the 20 kDa splice variant.

Both DHT protection and 3βdiol protection against oxidative stress are ER Dependent



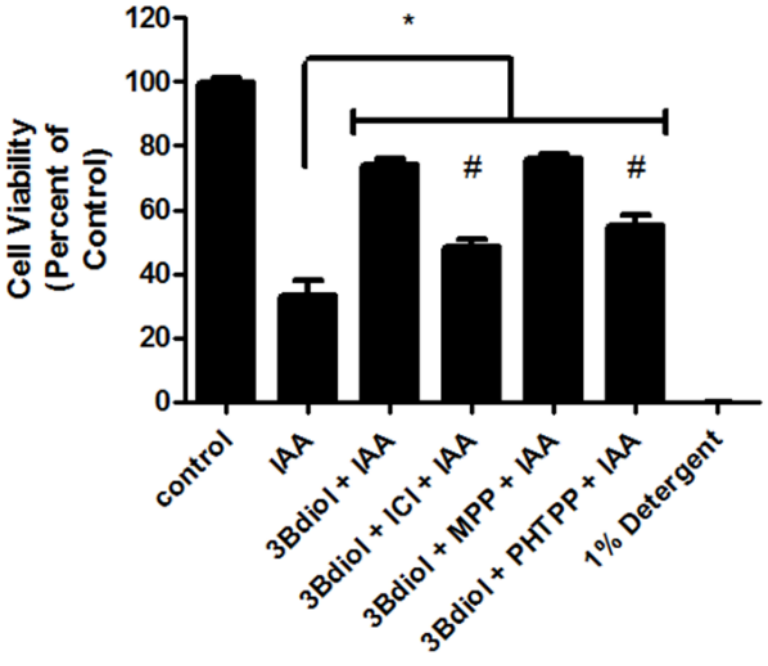


Figure 4: ER Antagonists Block DHT and 3βdiol protection against oxidative stress in cortical astrocytes.

Primary cortical astrocytes were treated with either dihydrotestosterone (DHT) or 3βdiol (3Bdiol) for 18 hours prior to treatment with the oxidative/metabolic insult, IAA, for an additional 6 hr. In the indicated groups, the specified antagonists were included and were co-applied with the indicated steroid. For MPP (ER alpha selective antagonist) 100 nM was used. For both ICI-182,780 (ER subtype non-selective antagonist) and PHTPP (ER beta selective antagonist) 500 nM was used. Cell viability, as assessed by the MTT viability revealed cell death following IAA, while estradiol, DHT and 3Bdiol were protective. Data were normalized to the vehicle-treated control and are presented as average ± SEM. * = different from IAA alone, $p < 0.05$, # = different from steroid + IAA, $p < 0.05$, and n= 4 independent replicates for all groups.

Effects of E2 and Opening Cx43 Gap Junctions on Astrocyte Viability

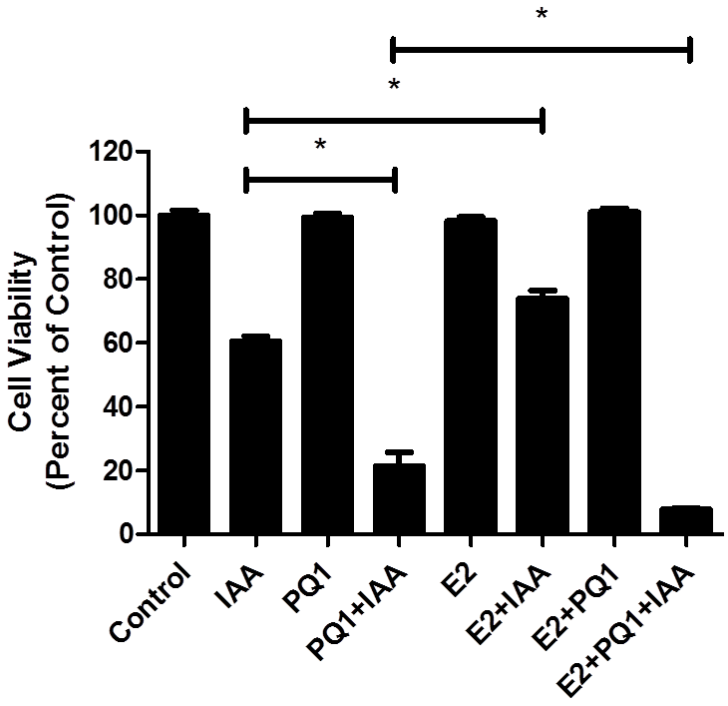


Figure 5: Estradiol induced astrocyte protection against oxidative stress is blocked by opening astrocyte Cx43 gap junctions

MTT viability data indicated the Cx43 gap junction opener compound PQ1 enhanced IAA-induced cytotoxicity. Furthermore, while estradiol protected against IAA-induced cytotoxicity, this effect was lost when gap junctions were opened with PQ1. Additionally, estradiol pre-treatment significantly enhanced PQ1 associated increase in IAA induced toxicity. Data represents 8 wells per group, and 3 independent replicates. "*" = $p < 0.05$.

Effects of E2 and Closing Cx43 Gap Junctions on Astrocyte Viability

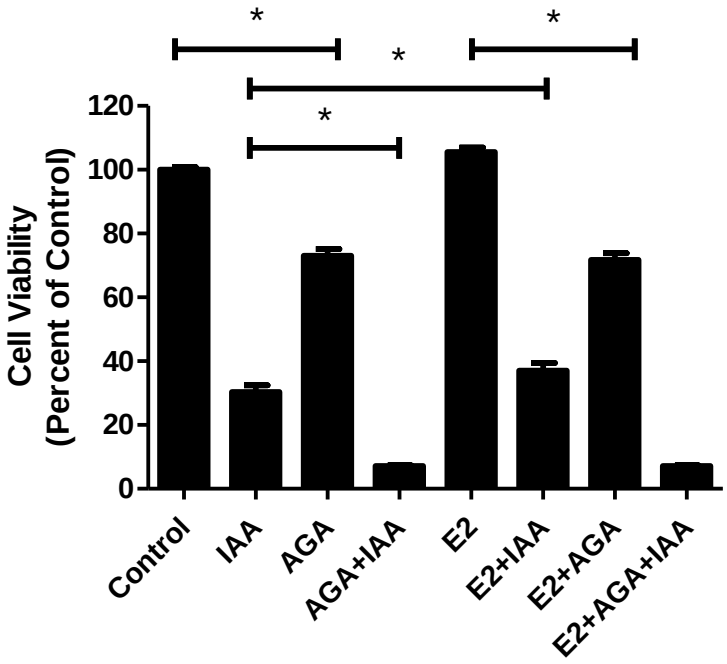
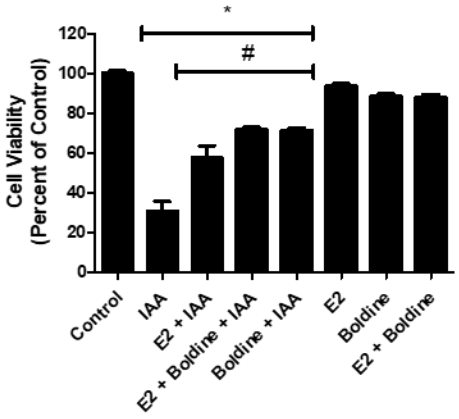
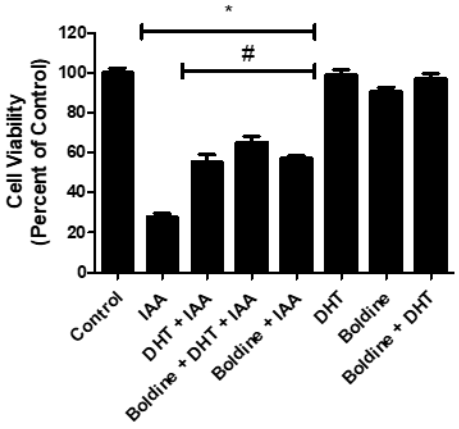


Figure 6: Estradiol induced astrocyte protection against oxidative stress is not altered by closing astrocyte Cx43 gap junctions.

MTT viability data indicated the Cx43 gap junction closing compound 18-alpha-glycyrrhetinic acid (AGA, 100 μM) enhanced IAA-induced cytotoxicity. Furthermore, while estradiol protected against IAA-induced cytotoxicity, this effect was lost when gap junctions were closed with AGA. Data represents 8 wells per group, and 3 independent replicates. "*" = $p < 0.05$.

Closing Astrocyte Cx43 Hemichannels protects Astrocytes against Oxidative Stress





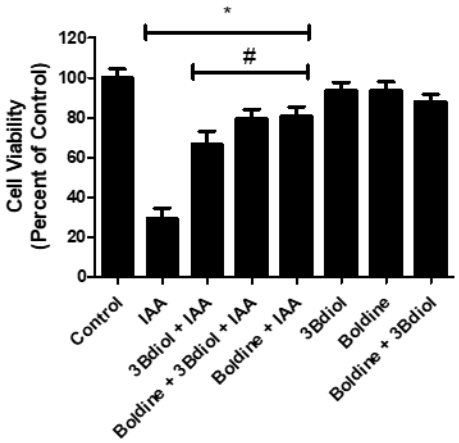


Figure 7: Boldine does not enhance the cytoprotective effects of E2, DHT or 3βdiol beyond the effect of boldine alone.

The combined treatment of either estradiol (top panel), DHT (middle panel) or 3βdiol (3Bdiol, bottom panel) with boldine (100 µM) did not result in any further augmentation of cell viability, when compared with the administration of either hormone alone or boldine alone. Data were normalized to the vehicle-treated control, and are presented as average ± SEM, with n= 3 independent replicates. * = different from control with $p < 0.05$, # = different from IAA alone with $p < 0.05$.

DISCUSSION

The first portion of this study (Figures 1-4) was designed to assess whether or not estradiol, DHT, and 3βdiol protect cortical astrocytes from IAA-induced cytotoxicity, and furthermore, to establish whether DHT or 3βdiol protection of astrocytes against oxidative stress was dependent upon estrogen receptor activation. While there is evidence that metabolic inhibition by IAA reduces astrocyte viability,(82) and estradiol protects against similar metabolic inhibition in heart tissue,(83) similar protective efficacy against IAA-induced metabolic inhibition in astrocytes has yet to be shown for the three steroids being assessed here. Our hypothesis that estradiol will exert protection in astrocytes is based on our preliminary data and previous findings that estradiol protected astrocytes against cytotoxicity induced by oxygen and glucose deprivation/reperfusion by an estrogen receptor dependent mechanism.(23) Our hypothesis that DHT will exert a similar effect as 3βdiol is based on 3βdiol being a direct metabolite of DHT, active at estrogen receptors.(333,334)

As shown in Figure 1, all three steroids were significantly protective against IAA induced oxidative stress. We initially proposed that DHT or 3βdiol protection against oxidative stress are estrogen receptor dependent in cortical astrocytes. Figure 2 supports this interpretation given that DHT protection against IAA-toxicity was blocked in astrocytes exposed to Trilostane, an inhibitor that blocks conversion of DHT to its estrogenic metabolite 3βdiol. As shown in Figure 3, the cortical astrocytes we used in these studies showed low but detectable mRNA expression for both estrogen receptors alpha and beta. We then assessed whether the protective efficacy of DHT or 3βdiol could be blocked by ER selective antagonists. As shown in Figure 4, DHT or 3βdiol

protection against oxidative stress could be blocked by ER selective antagonist ICI-182,780, but not the ER alpha selective antagonists. Protection induced by 3βdiol was also blocked by the ER beta selective antagonist, PHTPP, while a similar effect was not observed for DHT induced protection. While these results could indicate that protection by these steroids is ER dependent in cortical astrocytes such that activation of both ER alpha and ER beta were required for steroid-mediated protection, these results may also suggest the role of such splice variants of the classical androgen receptor as AR 45,(340) which can have separate effects on the cell type in question, such as classical ERs increasing transcription of proliferative genes through activating EREs,(138) whereas AR45 can dimerize with cARs to inhibit activation of AREs, and through doing so, inhibits the transcription of proliferative genes, such as beta-catenin.(341) There are no reports in any tissue whether Cx43 function can be regulated by steroid receptor splice variants. However, previous findings demonstrate that sex steroids can regulate Cx43 expression and permeability in reproductive tissue(76) where steroid receptor splice variants have also been observed.(340) Based on our findings that DHT protection in astrocytes was associated with reduced Cx43 expression and S368 phosphorylation and that the AR45 splice variant has been observed in brain tissue,(340) additional studies are warranted to further elucidate whether steroid receptor splice variants contribute to androgen regulation of Cx43 in astrocytes.

Building off of the previous findings, we next interrogated which forms of the Cx43 protein (gap junctions versus hemichannels) mostly likely contributed to estradiol, DHT and 3βdiol induced protection in cortical astrocytes. Using different Cx43 targeting compounds in parallel with administration of either estradiol, DHT, and 3βdiol, the effects of selectively opening or

closing astrocyte Cx43 hemichannels or Cx43 gap junctions on protection induced by these steroids were assessed. These findings indicated that either opening or closing Cx43 gap junctions failed to enhance estradiol induced protection against oxidative stress. This observation is consistent with previous cancer radiotherapy literature in which enhanced cytotoxicity was observed in cells co-treated treated with cytotoxic insult and treatments known to enhance gap junction opening.(157,159,342) However, gap junction integrity is dependent upon intact disulfide bridge formation between the extracellular loops of adjacent hemichannels(343) and oxidative stress disrupts this type of amino acid interaction, resulting a lower net expression of gap junctions in the presence of oxidative stress at a constant level of total Cx43 expression. Based on this notion, it is reasonable that compounds acting selectively on gap junctions (either to open or close them) would have a minimal impact on the astrocytes since there are potentially less gap junctions available for these compounds to act on in the presence of oxidative stress. In contrast, closing Cx43 hemichannels seemed to be more effective at promoting cytoprotection. The findings from these experiments demonstrate for the first time that estradiol, DHT, and 3βdiol protect against oxidative stress in astrocytes by a mechanism that may include astrocyte Cx43 hemichannels. While there may have been a mild additive interaction between steroid efficacy and closing Cx43 hemichannels, the differences were not statistically significant for any of the three steroids.

CHAPTER 4: THE EFFECTS OF E2, DHT, AND 3BDIOL ON ASTROCYTE CX43 FUNCTION

ABSTRACT

Cx43 is a protein highly expressed in astrocytes where it contributes to several beneficial functions through its two distinct conformations, hemichannels and gap junctions. These beneficial functions include mediating intercellular metabolite shuttling,(59) intercellular signaling molecule trafficking,(72) intercellular miRNA transfer,(200) promoting exosome adhesion to target cells,(344) and interactions with known scaffold proteins.(345) Through these channels, Cx43 contributes to protection of neighboring cells from cytotoxic insults. In peripheral (non-central nervous system (CNS) tissue, Cx43 gap junction expression and gap junction opening are regulated by neurosteroids including 17β-estradiol (estradiol) and dihydrotestosterone (DHT). However, steroid hormone regulation of Cx43 in the brain has yet to be reported. Here, we tested the hypothesis that DHT, 3βdiol (an estrogenic metabolite of DHT), and estradiol protect cortical astrocytes against oxidative stress through their regulation of the expression and/or opening of Cx43 channels. Our results demonstrate for the first time that estradiol, DHT, and 3βdiol each regulate the opening of astrocyte Cx43 hemichannels in primary cortical astrocytes. DHT, but not estradiol or 3βdiol, decreased astrocyte Cx43 mRNA expression. In addition, DHT reduced Cx43 phosphorylation on S368. This influence on Cx43 hemichannel opening was correlated with the effect of these steroids on astrocyte cell viability and was comparable to the effect of boldine, a pharmacological inhibitor of Cx43 hemichannels. Whereas DHT, but not estradiol or 3βdiol, transiently decreased astrocyte Cx43 mRNA expression, all three steroids decreased astrocyte

Cx43 hemichannel opening, an effect that antagonized the increased hemichannel opening associated with the metabolic and pro-oxidative insult, iodoacetic acid (IAA). This influence on Cx43 hemichannel opening was correlated with the effect of these steroids on astrocyte hemichannel opening and was comparable to the effect of boldine, a pharmacological inhibitor of Cx43 hemichannels. Taken together, these data advance our understanding of how astrocytes respond to oxidative stress, a feature of biological aging and certain neurodegenerative diseases, and implicates Cx43 hemichannels as a potential target of the cytoprotective effects of androgens and estrogens in the brain, and specifically identify DHT induced changes in astrocyte Cx43 expression and phosphorylation state as understudied components of DHT's molecular actions on brain tissue.

INTRODUCTION

Cx43 is highly expressed in astrocytes, but whether its expression in astrocytes is regulated by gonadal androgens or estrogens remains unknown. However, there is converging lines of evidence to support the possibility that gonadal estrogens regulate astrocyte Cx43 expression. The Cx43 gene promoter has estrogen response element half sites, and estradiol increases the expression of Cx43 in reproductive, heart, and bone tissue through estrogen receptor dependent mechanisms.(10,73-77) Whereas studies in peripheral tissue demonstrated that testosterone and DHT decreased Cx43 expression,(76,78) we hypothesized that DHT, through its estrogenic metabolite, 3βdiol, would increase Cx43 expression in astrocytes, through 3βdiol activating estrogen receptors in astrocytes. Indeed, all previous studies assessing androgen regulation of Cx43 expression have shown androgens decrease Cx43 expression in peripheral tissue unless androgen-to-estrogen conversion occurs in that tissue.(10,73,74,78-81)

In an effort to complement the studies that evaluate changes in the expression of Cx43 in response to estradiol, DHT, and 3βdiol, we also assessed the effects of estradiol, DHT, and 3βdiol on astrocyte Cx43 phosphorylation, specifically at amino acid residues tyrosine 265 (Y265) and serine 368 (S368), both located in Cx43's carboxy-terminal end. These two phosphorylation sites were chosen based on the association of phosphorylation at these amino acid residues and Cx43 channel opening. Phosphorylation of Cx43 at either Y265 or S368 are associated with reduced Cx43 channel opening and promotes Cx43 channel internalization from the plasma membrane,(115-117,346) Y265 phosphorylation also reduces Cx43 protein-protein interactions with tubulin and the tight junction scaffolding protein zona occludens 1 (ZO-1), which reduces

Cx43 intracellular trafficking towards the plasma membrane and indirectly supports Cx43 internalization form the plasma membrane and degradation.(54,55,103,112,347) Conceptually, these changes in Cx43 availability at the plasma membrane support Cx43's proposed roles in conferring protection to cytotoxic insult.(348-352) While there is evidence that estradiol protection against metabolic inhibition in heart tissue is associated with estradiol-induced increases in Cx43 S368 phosphorylation,(83) it remains to be seen if this effect is equally true in astrocytes, and furthermore, whether or not DHT or 3βdiol exert similar effects on Cx43 phosphorylation at Y265 or S368.

Lastly, we directly assessed whether estradiol, DHT, and 3βdiol altered astrocyte Cx43 channel opening. Given the paucity of information regarding the contributions of Cx43 hemichannel opening to the regulation of cell viability versus the contributions of regulating gap junctions, the analysis of steroid-induced alteration in Cx43 hemichannel opening is deemed to be critical. Our hypotheses regarding estradiol's effects on Cx43 hemichannel opening are based on previous studies showing that the estrogenic compounds, estradiol, genistein, and diethylstilbestrol reduce Cx43 gap junction opening in peripheral tissue.(83,319,320)

There is also a gap in knowledge regarding the effects of androgens on Cx43 channel opening. In this study, we proposed to evaluate the effect of DHT on Cx43. The rationale for our expectation that DHT will mimic estrogenic influences on Cx43 is based on prior work from our lab that supports the conversion of DHT to 3βdiol as an important mechanism underlying DHT-induced protection of hippocampal HT-22 cells. As such we hypothesized that the (protective) effects of DHT are mediated by 3βdiol, and as such, any influence of DHT on Cx43 will operate

through this estrogen receptor-targeting mechanism.(334) The information gained from these studies will help advance the current understanding of how estradiol, DHT, and 3βdiol act on their target tissues to exert beneficial effects in the central nervous system (CNS).

METHODS

Tissue Acquisition and Culture:

Primary cortical astrocytes were derived from female postnatal day 2 C57Bl/6 mice as described previously with some modifications.(325,326) Briefly, pups were anesthetized by hypothermia, followed by cardiac puncture as the method of euthanasia. Following craniotomy, the brain was removed and microdissected to isolate the total cerebral cortex. The resulting cortical tissue was then mechanically and enzymatically dissociated into individual cells, which were then centrifuged and filtered (to remove cellular debris), and finally plated and cultured until reaching confluence, with media changes occurring every other day. The resulting cells were then subcultured and plated in either 96-well black bottom plates (Thermo Fisher Scientific, Rochester, NY; cat# 165305) for hemichannel permeability assays, or clear bottom 6-well plates (VWR North American, Radnor, PA; cat# 10062-892), for the purposes of assessing mRNA and protein expression, at a ratio of 1 75 cm^2 flask per either 96-well or 6-well plates. Astrocytes were cultured in Astrocyte media (Dulbecco's Modified Eagle Media containing 110 mg/L Sodium Pyruvate, Gibco Life Technologies, Grand Island, NY; cat# 11995-065) with 10% v/v Fetal Bovine Serum (Atlanta Biologicals, Flowery Beach, GA; cat# S11550) and 1% v/v PenStrep 10,000 units/mL (HyClone, South Logan, UT cat# SV30010). All experimental procedures involving animals were approved by the UNT Health Science Center's Institutional Animal Care and Use Committee (IACUC).

Pharmacologic Treatments:

17-β estradiol (E2, Sigma, St. Louis, MO; cat# E2758), dihydrotestosterone (DHT, Steraloids, Newport, RI; cat# A2570-000), and 5α-androstane-3β, 17β-diol (3βdiol, Steraloids, Newport, RI; cat# A1220-000) were dissolved in DMSO, and applied to the cells at a final concentration of 100 nM (where the DMSO concentration was < 0.1%). This concentration was selected based on dose response and time course optimizations that indicated 100 nM estradiol induced a significant increase in Cx43 mRNA in astrocytes and 100 nM estradiol when applied for either 3 or 18 hours protected against an iodoacetic acid induced 50% reduction in cell viability by MTT assay. Iodoacetic acid (IAA, final concentration 25 μM, Sigma, St. Louis, MO; cat# I4386), was dissolved in de-ionized water, sterile filtered, and aliquoted. Experimental concentration of IAA was determined by dose response optimizations to identify the concentration of IAA that when applied to confluent astrocytes for 6 hours, reduced their cell viability by approximately 50 percent. TNFα/IL-1β (final concentration 10 ng/mL for each, (1:1 mixture used previously to induced Cx43 hemichannel opening in astrocytes)(353) both mouse recombinant peptides (EMD Millipore (now Millipore Sigma), Darmstadt, Germany; cat# 654245 and IL014 respectively), Amyloid β (25-35) peptide Ala 28 (final concentration 50 μM (concentration used previously to open astrocyte Cx43 hemichannels)),(169) Sigma, St. Louis, MO; cat# SCP0041), and boldine (final concentration 100 μM, Sigma (now Millipore Sigma), Darmstadt, Germany; cat# B3916)(170) were prepared and aliquoted based on the manufacturer's recommendation. TNFα/IL-1β and Amyloid β 25-35 peptide Ala 28 were dissolved in de-ionized water while boldine

was dissolved in sterile DMSO, and sterile filtered prior to application to the cells at the stated final concentrations.

RTPCR:

Semi-quantitative, real-time PCR was conducted using 20 ng of template per reaction to assess treatment induced changes in astrocyte mRNA expression for Cx43 (primer: Mm01179639_s1 from Applied Biosystems, Pleasanton, CA) and GAPDH (primer: Mm99999915_g1 from Applied Biosystems, Pleasanton, CA). RNA was extracted from astrocytes using the RNeasy Mini Kit (Qiagen, Hilden, Germany, cat# 74106), converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Vilnius, Lithuania, cat# 4368814), and analyzed on an Applied Biosystems 7300 Real Time PCR System Thermocycler (Foster City, CA). Target mRNA levels were evaluated in triplicate and averaged to obtain average cycle threshold (Ct) values for each sample's Cx43 and GAPDH expression. Fold change values were calculated using the $2^{-\Delta\Delta Ct}$ method,(329) followed by transforming the data to percent of DMSO control, which served as the experimental reference group. The resulting averages were compared for statistical significance by single degree of freedom F test followed by Tukey's *post hoc* analysis to determine statistical significance between treatment groups in Systat software (Systat Software, Inc., San Jose, CA) with assistance from Dr. Nathalie Sumien.

Western Blot:

Western blots were conducted as previously described(330) to assess relative changes in Cx43 protein expression following treatment with estradiol, DHT, and 3βdiol (all 100 nM, 3 and 18 hour vehicle or steroid treatment). In brief, astrocyte cultures were lysed in brain cell type

optimized protein lysis buffer containing protease and phosphatase inhibitors.(331) After homogenization, samples were centrifuged at 100,000 x g for 30 min at 4° C. 40 µg of total protein (quantified using the Bio-Rad DC assay (Rio-Rad, Hercules, CA; cat# 5000116) per lane was separated by SDS-PAGE and subsequently transferred onto polyvinylidene fluoride membranes (PVDF, Bio-Rad Laboratories) by electroblotting. Membranes were blocked with 3% bovine serum albumin (BSA) in tris-buffered saline containing 0.2% Tween 20 (TBS-T) for 1hr at room temperature, followed by overnight incubations with primary antibodies at 4°C. The following antibodies were used: Cx43 / GJA1 rabbit polyclonal antibody (Abcam, Cambridge, MA; cat# ab11370, 1:5000 in TBST), GAPDH rabbit polyclonal antibody (Cell Signaling, Danvers, MA; cat# 2118, 1:1000 in TBST), and HRP-conjugated mouse anti-rabbit monoclonal secondary antibody, light chain only (Jackson ImmunoResearch, West Grove, PA; cat# 211-032-171, 1: 10,000 in 3% v/v BSA in TBST). Additionally, phosphospecific antibodies were used to assess treatment induced changes in Cx43 phosphorylation at amino acid residues Y265 (Abcam, Cambridge, MA; cat# ab197598, 1:1000 in TBST) and S368 (Abcam, Cambridge, MA; cat# ab194928, 1:1000 in TBST). Immunoreactive bands were visualized with the ECL detection system (ThermoFisher Scientific) and were captured using a luminescent image analyzer (Alpha Innotech). Densitometric analyses were conducted using ImageJ (National Institutes of Health) software. Once raw densitometric values were obtained between treatment groups, the total Cx43 densitometry values were normalized to GAPDH values. In a separate set of experiments, the phosphospecific Cx43 Y265 densitometry values were normalized to the corresponding total Cx43 values for each treatment condition. The phosphospecific Cx43 S368 densitometry values were

normalized to the corresponding total Cx43 values for each treatment condition. The resulting averages were compared for statistical significance by single degree of freedom F test followed by Tukey's *post hoc* analysis to determine statistical significance between treatment groups in Systat software (Systat Software, Inc., San Jose, CA) with assistance from Dr. Nathalie Sumien.

Ethidium Bromide Dye Uptake Assay to Assess Hemichannel Permeability:

The Ethidium Bromide Dye Uptake Assay was conducted using the Ca^{2+} free approach as described previously, and adapted for use with a 96-well fluorescence plate reader.(58) Hemichannel permeability in cultured astrocytes was induced either by exposing the cells to a Ca^{2+} free solution, TNFα/IL-1β or Amyloid β 25-35 peptide. Ethidium bromide (EtBr, VWR North American, Radnor, PA; cat# X328) was applied for 10 min at a final concentration of 4 μM and incubated at 37°C for 10 minutes. Cells were then washed with Hank's balanced salt solution (HBSS, Corning Cellgro, Manassas, VA; Ref: 21-022-CM) supplemented with 1.2 mM $CaCl_2$ (HBSS-Ca^{2+}). Treatment-induced changes in red fluorescence were then assessed on the FilterMax F5 Multi-Mode Microplate Reader, using the following excitation/emission parameters: 520 nm excitation and 610 nm emission. Resulting between group differences were assessed using One-Way ANOVA followed by Tukey *post hoc* analyses in GraphPad Prism Version 5 software.

Statistical Analysis:

For all data generated (from RTPCR, Western Blot, and EtBr hemichannel permeability assay) resulting between treatment group differences were assessed using One-Way ANOVA followed by Tukey's *post hoc* analyses. EtBr hemichannel permeability assay data was analyzed using GraphPad Prism Version 5 software. Through consulting with Dr. Nathalie Sumien,

normalized RTPCR and Western Blot densitometry values were analyzed by single degree of freedom F test, followed by Tukey's *post hoc* analyses using Systat software. In order to determine the statistical power of each data set, we input the data generated into a power analysis software (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html) set to two tailed power calculation to detect a mean difference of 50%. While most of our data sets had a statistical power of 80% or greater, our only data set not meeting this threshold was our phosphospecific Y265 Western Blot data, with a statistical power of 77%. This observation was likely due to the large variability within this data set compared to our other data sets.

Statistical Power Results:

Figure	**Number of replicates**	**Statistical Power (%)**
Figure 1	4	98
Figure 2	6	80
Figure 3	3	77
Figure 4	3	91
Figure 5	3	86
Figure 6	3	84

RESULTS

DHT decreased astrocyte Cx43 mRNA expression but not protein expression

We assessed whether estradiol, DHT, or 3βdiol regulated Cx43 mRNA and protein expression. As shown in Figure 1, RTPCR analysis revealed that DHT, but not estradiol or 3βdiol, decreased astrocyte Cx43 mRNA expression at both timepoints assessed. In contrast to the mRNA data, analysis of Cx43 protein expression (Figure 2) demonstrated that estradiol, DHT, and 3βdiol did not alter Cx43 protein expression in cortical astrocytes when assessed 3 or 18 hours after steroid application.

E2, DHT, and 3βdiol did not change astrocyte Cx43 phosphorylation at Tyrosine 265

We assessed whether estradiol, DHT, or 3βdiol altered phosphorylation on Cx43 protein at amino acid residues Y265 as a potential component of these steroid's mechanisms of exerting protection against oxidative stress in cortical astrocytes. As shown in Figure 3, neither estradiol, DHT, nor 3βdiol altered Cx43 tyrosine 265 phosphorylation in cortical astrocytes compared to vehicle controls.

DHT, but not estradiol or 3βdiol decreased astrocyte Cx43 phosphorylation at Serine 368

We assessed whether estradiol, DHT, or 3βdiol altered phosphorylation on Cx43 protein at amino acid residue S368 as a potential component of these steroid's mechanisms of exerting protection against oxidative stress in cortical astrocytes. As shown in Figure 4, DHT, but not

estradiol or 3βdiol decreased Cx43 serine 368 phosphorylation in cortical astrocytes compared to vehicle controls, but only at the 18 hour timepoint.

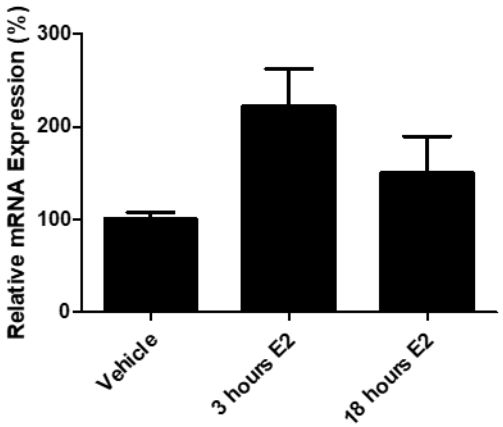
Oxidative stress opens astrocyte Cx43 hemichannels

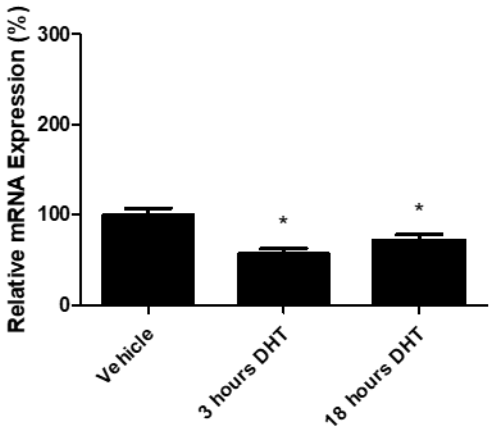
We assessed whether oxidative stress altered opening of astrocyte Cx43 hemichannels. As shown in Figure 5, IAA elicited Cx43 hemichannel opening, as indicated by increased uptake of EtBr from the extracellular media, compared to non-treated controls. Furthermore, the IAA-induced increase in dye uptake was similar to positive controls used, which were overnight exposure of cells to TNF-alpha/Il-1beta, overnight exposure of cells to ABeta 25-35 peptide, or short-term exposure of cells to Calcium free washing buffer.

E2, DHT, and 3βdiol inhibit oxidative stress-induced opening of astrocyte Cx43 hemichannels

Next, we assessed whether estradiol, DHT, or 3βdiol prevented or reduced IAA-induced opening of astrocyte Cx43 hemichannels. Figure 6 shows that estradiol, DHT, or 3βdiol each blocked IAA-induced Cx43 hemichannel opening, an effect comparable to that of boldine, the Cx43 hemichannel selective permeability blocker. No additive interaction could be discerned as boldine and the three steroid hormones each were maximally effective at blocking IAA-induced Cx43 astrocyte opening.

DHT decreased astrocyte Cx43 mRNA expression





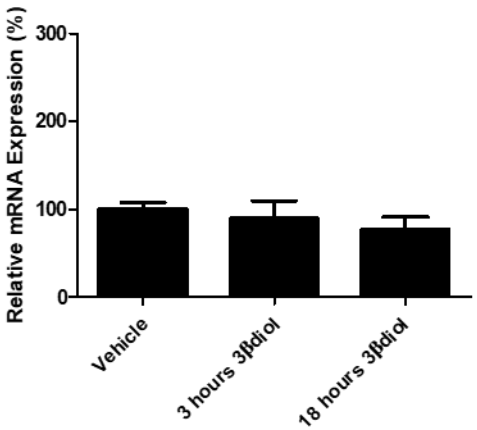


Figure 1: Dihydrotestosterone (DHT), but not Estradiol (E2) or 3βdiol, decreased Cx43 mRNA expression in primary cortical astrocytes.

Cx43 mRNA expression was assessed following 3 and 18 hour treatment with estradiol (top panel), DHT (middle panel), or 3βdiol (bottom panel), or 0.1% DMSO serving as the vehicle control). DHT elicited a statistically significant decrease in Cx43 expression at both timepoints assessed. Neither estradiol nor 3βdiol (3Bdiol) altered the expression of Cx43 mRNA. Data were normalized to the vehicle-treated control, and are presented as average ± SEM, with n= 4 independent replicates, and * = different from DMSO vehicle control with $p < 0.05$.

E2, DHT, and 3βdiol did not change astrocyte Cx43 protein expression

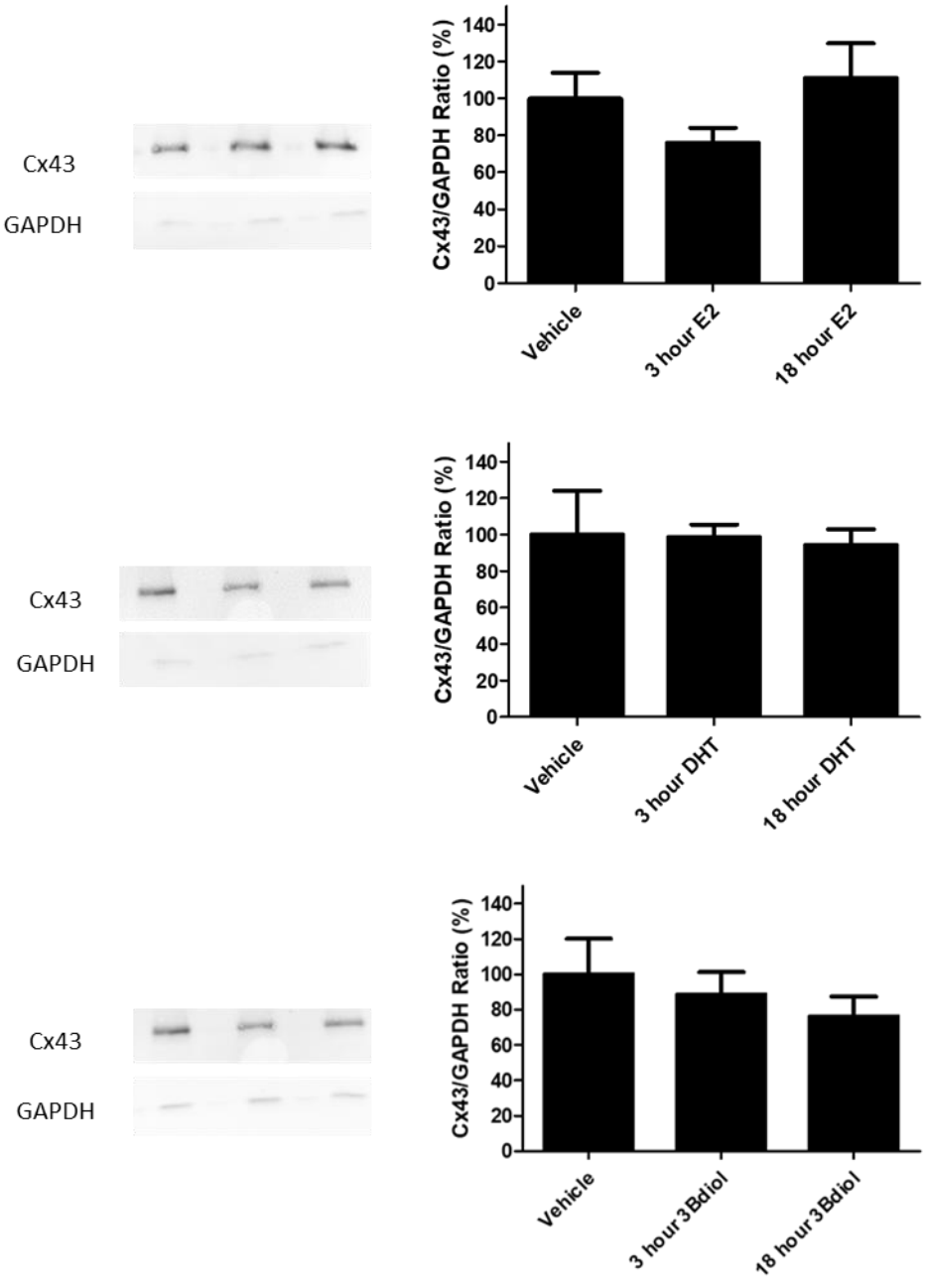


Figure 2: Neither Estradiol (E2), dihydrotestosterone (DHT) nor 3βdiol altered Cx43 protein expression in primary cortical astrocytes.

Relative Cx43 protein expression was assessed was assessed following 3 and 18-hour treatment with estradiol (top panel), DHT (middle panel), or 3βdiol (bottom panel), or 0.1% DMSO serving as the vehicle control). No statistically significant differences were observed. Data were normalized to the vehicle-treated control, and are presented as average ± SEM, with n= 6 independent replicates.

E2, DHT, and 3βdiol did not change astrocyte Cx43 phosphorylation at Tyrosine 265

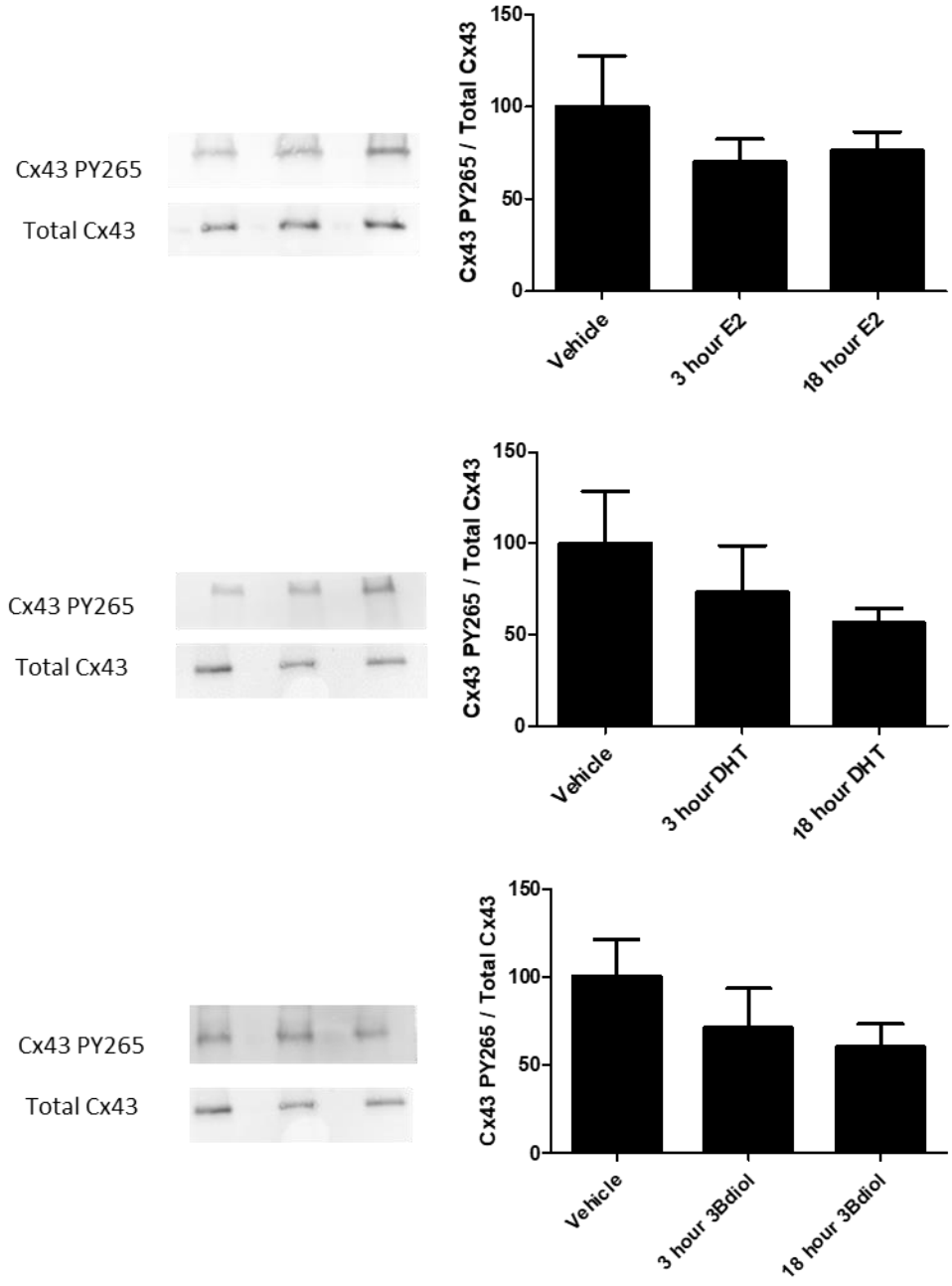


Figure 3: Neither Estradiol (E2), dihydrotestosterone (DHT) nor 3βdiol increased Cx43 Tyrosine 265 phosphorylation in primary cortical astrocytes.

Cx43 Tyrosine 265 phosphorylation relative to total Cx43 protein expression was assessed was assessed following 3 and 18 hour treatment with estradiol (top panel), DHT (middle panel), or 3βdiol (bottom panel), or 0.1% DMSO serving as the vehicle control). No statistically significant differences were observed. Data were normalized to the vehicle-treated control, and are presented as average ± SEM, with n= 3 independent replicates.

DHT decreased astrocyte Cx43 phosphorylation at Serine 368

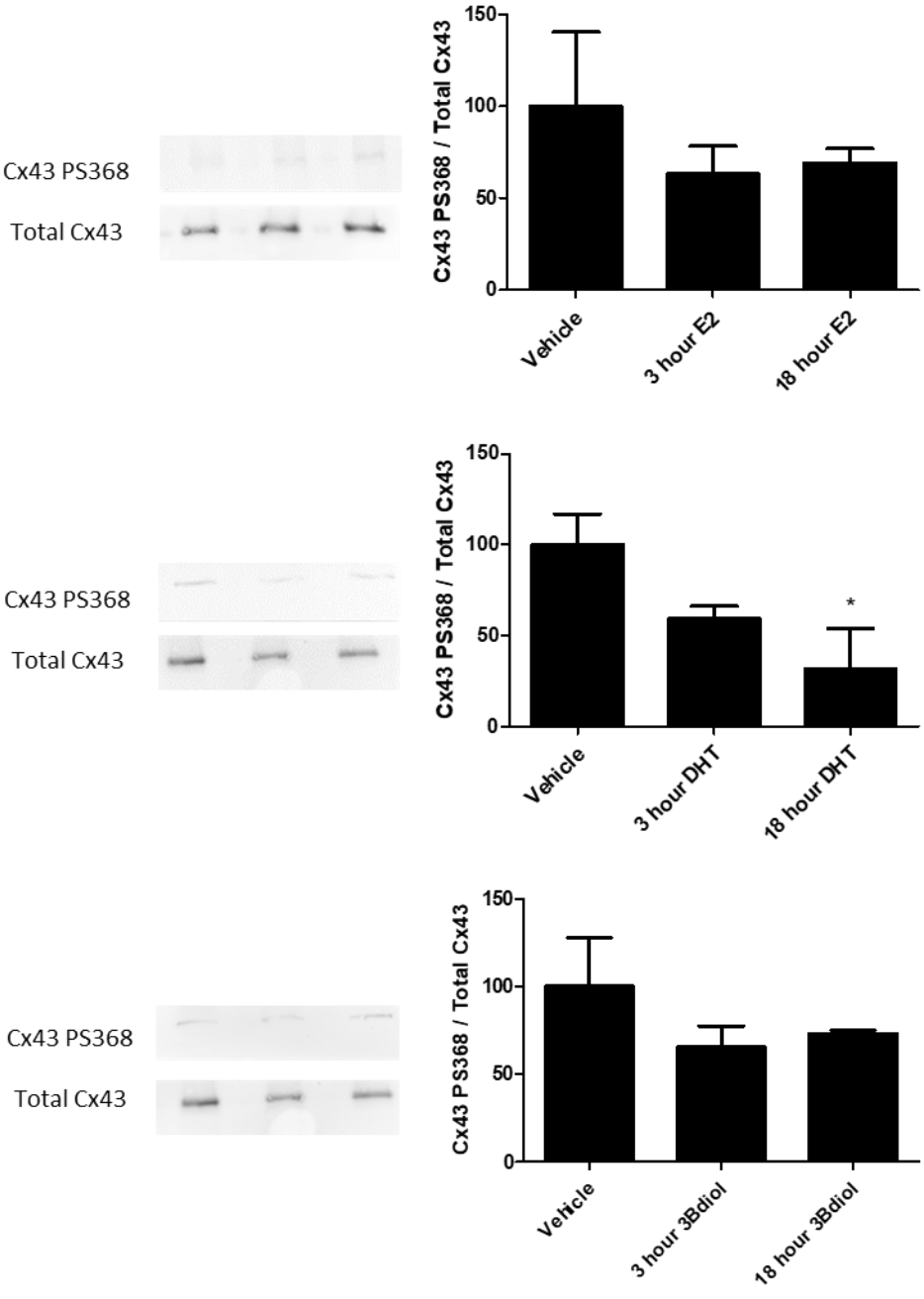


Figure 4: Dihydrotestosterone (DHT), but not Estradiol (E2) or 3βdiol decreased Cx43 Serine 368 phosphorylation in primary cortical astrocytes.

Cx43 Serine 368 phosphorylation relative to total Cx43 protein expression was assessed following 3 and 18 hr treatment with estradiol (top panel), DHT (middle panel), or 3βdiol (bottom panel), or 0.1% DMSO serving as the vehicle control). No statistically significant differences were observed for estradiol or 3βdiol treated astrocytes. Cortical astrocytes only showed a statistically significant reduction in S368 phosphorylation 18 hours after DHT treatment, but not 3 hours. Data were normalized to the vehicle-treated control, and are presented as average ± SEM, * = different from DMSO vehicle control with $p < 0.05$, and n= 3 independent replicates.

Oxidative Stress Opens Astrocyte Cx43 Hemichannels

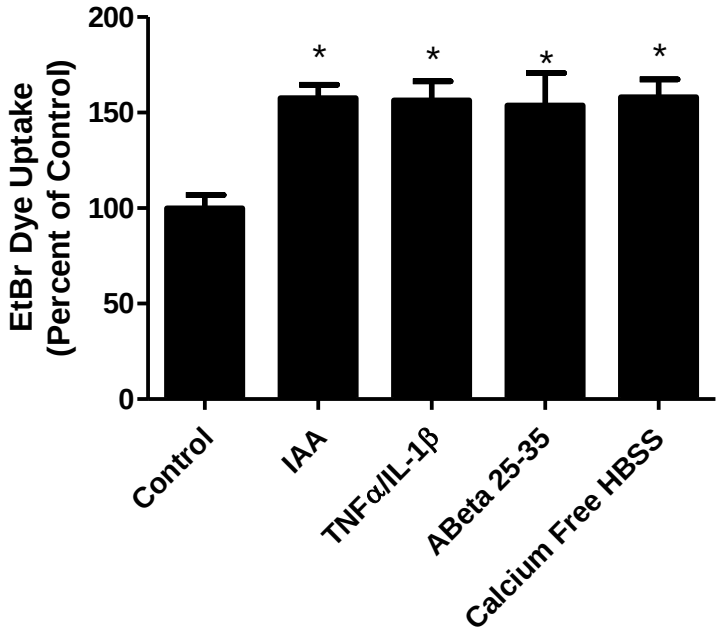
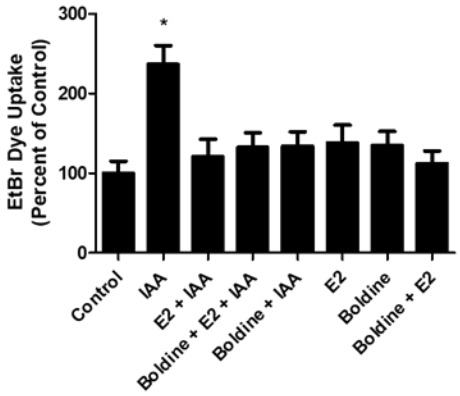
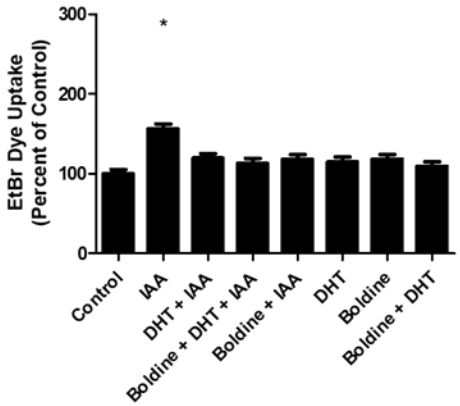


Figure 5: Treatment of Primary Cortical Astrocytes with iodoacetic acid (IAA) Opens Cx43 Hemichannels.

Oxidative stress was induced in cortical astrocytes by 6 hr IAA exposure. Treatment with IAA for 6 hr resulted in significant influx of EtBr into the cells, serving as a surrogate indicator of increased Cx43 permeability. TNFα/IL-1β and ABeta 25-35 peptide were used as pharmacological positive controls, while Calcium free HBSS was a methodological positive control. Data were normalized to the vehicle-treated control, and are presented as average ± SEM. * = different from control with $p < 0.05$, n= 3 independent replicates.

E2, DHT, and 3βdiol Block Oxidative Stress Induced Opening of Astrocyte Cx43 Hemichannels





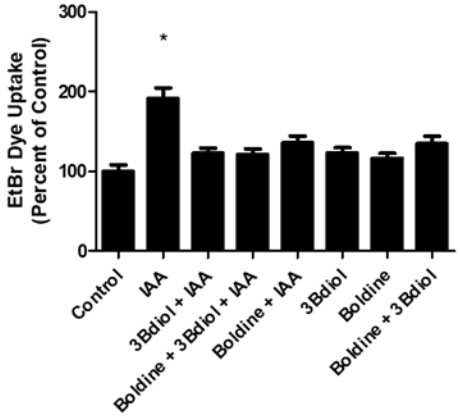


Figure 6: Boldine does not further inhibit Cx43 hemichannel opening beyond the effects of E2, DHT or 3βdiol alone.

The combined treatment of either estradiol (top panel), DHT (middle panel) or 3βdiol (3Bdiol, bottom panel) with boldine did not result in any further inhibition of Cx43 hemichannel opening relative to treatment with either hormone alone or boldine alone. Data were normalized to the vehicle-treated control, and are presented as average ± SEM, with n= 3 independent replicates. * = different from control with $p < 0.05$.

DISCUSSION

The overall purpose of this research was to determine whether or not Cx43 is a vital regulator/mediator of androgen and estrogen-mediated protection against oxidative stress in cortical astrocytes. Previous research indicated that Cx43 regulates astrocyte response to oxidative stress.(5,6,65) Moreover, sex steroids regulate Cx43 expression in peripheral tissue,(10,76) and regulating Cx43 channel opening independent of regulating overall Cx43 expression in cells impacts those cells' response to oxidative stress,(321) and this notion has been similarly demonstrated for estrogenic compounds in peripheral tissue.(320) Based on these observations, we proposed that Cx43 is a vital component of androgen and estrogen-mediated protection against oxidative stress in astrocytes. The proposed studies were the first to assess the effects of androgens and estrogens on Cx43 channel opening in astrocytes. Likewise, this study showed for the first time that estradiol, DHT, and 3βdiol close astrocyte Cx43 hemichannels. These findings support the interpretation that selectively targeting astrocyte Cx43 hemichannels with pharmacological interventions is a valid molecular approach for protecting the brain against neurotoxic insults.(213,265,354) These findings also provide new information about the relationship between steroid hormones and connexin 43 function, and this information may impact our understanding of the physiological consequences of changes in circulating serum androgen or estrogen concentrations, such as those observed following the menopausal transition.

In the first portion of this research, we assessed whether estradiol, DHT, and 3βdiol altered astrocyte Cx43 expression. With regard to our Cx43 expression data, the RTPCR results were consistent with findings from peripheral tissue, but the Western Blot findings were not. Our

findings indicated a decrease in cortical astrocyte Cx43 mRNA expression following 100 nM DHT exposure without a concomitant decrease in astrocyte Cx43 protein expression. Other reports noted an estradiol-induced increase in Cx43 expression in peripheral tissue.(10,81) This may be partially explained by the higher baseline classical androgen and classical estrogen receptor expression in peripheral tissue compared to the low but detectable estrogen receptor alpha and beta expression of cortical astrocytes we reported in our RTPCR findings here, which is consistent with a previous study from our lab of cell lines commonly used to study steroid neurobiology.(22,77,355) However, our observation that estradiol, DHT, and 3βdiol did not alter Cx43 protein expression in cortical astrocytes at either time-point is consistent with previous findings in cardiomyocytes.(83) In that study, estradiol failed to alter cardiomyocyte Cx43 expression in the absence of insult. With respect to androgens, however, DHT did induce a reduction in astrocyte Cx43 mRNA but not a change in astrocyte Cx43 protein expression. It is possible this difference is due to astrocytes not expressing the classical androgen receptor (cAR),(336,355) whereas tissue reported to decrease Cx43 expression following DHT exposure robustly express cAR.(76,356) However, this finding may also reflect DHT acting on cortical astrocytes through its estrogenic metabolite activating estrogen receptors, such as activating classical androgen receptors, putative membrane androgen receptors,(336,357) or classical androgen receptor splice variants.(340)

Given the nominal effects of the three steroid hormones on Cx43 expression, regulating Cx43 opening may be the more meaningful and perhaps under-appreciated mechanism that relates to cell viability. It is possible that the lack of observed effects of estradiol and 3βdiol on Cx43 protein expression may reflect transient elevations in protein expression that were missed at the 3

and 18 hour time points. Alternatively, the data may suggest differential regulation of transcripts versus protein. Likewise, the data may also suggest that there is indeed no influence of estradiol and 3βdiol on Cx43, and as such, suggests that the mechanism underlying estradiol- and 3βdiol-induced cytoprotection is independent of changing astrocyte Cx43 expression. As such, therapeutic interventions focused solely on regulating Cx43 expression may fall short of becoming a viable candidate for preserving brain cell function with age or in the face of a degenerative disease.

The focus on phosphorylation of Cx43 is based on the fact that phosphorylation at either the Y265 or S368 site reduces Cx43 channel opening and promotes Cx43 channel internalization from the plasma membrane.(111,115-117,191,346,358,359) Phosphorylation of Y265 phosphorylation also reduces Cx43 protein-protein interactions directly, and is associated with reduced Cx43 intracellular trafficking to the plasma membrane.(54,55,103,112,347) In our hands, the effects of estradiol, DHT, and 3βdiol treatment on phosphorylation of astrocyte Cx43 at the amino acid residue Y265 yielded no statistically significant effects of treatment. However, DHT induced a statistically significant decrease in Cx43 S368 phosphorylation observed at 18 hours after steroid application. Our original hypothesis that estradiol, DHT, and 3βdiol may exert protection through increasing phosphorylation at Cx43 amino acid residues Y265 or S368 was not supported by the above data. Based on other studies, an alternate interpretation is these steroids could exert protection against oxidative stress through altering phosphorylation at residues within the carboxy terminal domain of Cx43, other than Y265 and S368, by way of increasing the activity of signaling cascades that regulate phosphorylation of these residues, such as PKA and MAPK.(54,360,361) Another possibility is that our experimental findings may be completely

different if the additional treatment groups were added in which relative changes in Cx43 Y265 and S368 phosphorylation status were assessed in astrocytes exposed to oxidative stress with and without one of the three steroids we studied. This possibility is supported by one study in which cardiomyocytes were exposed to hypoxia in the presence or absence of estradiol pre-treatment, and it was observed that while estradiol on its own did not alter Cx43 S368 phosphorylation intensity, estradiol pretreatment blocked the hypoxia-induced decrease in Cx43 S368 phosphorylation.(83) Therefore, future studies could benefit from assessing the Cx43 phosphorylation status in both the presence and absence of oxidative stress, or other cytotoxic insult. Finally, it is also possible that neither 3 nor 18 hours are optimal durations to assess estradiol, DHT, or 3βdiol-induced changes in astrocyte Cx43 phosphorylation status. Future studies in the Singh lab will be aimed at evaluating such alternative possibilities.

The last portion of this research was designed to assess the separate effects of estradiol, DHT, and 3βdiol on astrocyte Cx43 hemichannel opening. To date, relatively little consideration has been given to the potential contributions of Cx43 hemichannels to cytotoxic drug treatments, such as targeted chemotherapy, or to cytoprotective interventions, such as hormone replacement therapy. As such, the bulk of the Cx43 research literature has focused on studying Cx43 gap junctions and their roles in health and disease. However, data from several recent studies support selectively inhibiting Cx43 hemichannels as a novel approach to promote cytoprotection.(69,167-170) Therefore, we used a fluorescence-based assay, the ethidium bromide dye uptake assay, to assess whether estradiol, DHT, and 3βdiol altered astrocyte Cx43 hemichannel opening. The findings from these experiments demonstrate for the first time that estradiol, DHT, and 3βdiol

blocked the opening of astrocyte Cx43 hemichannels elicited by mixed metabolic/oxidative stress. Given these findings, future studies assessing the effects of androgens and estrogens on tissues that highly express Cx43, such as brain, heart, bone, and reproductive tissue,(10,76,77,362) may benefit from being mindful of the distinct contributions of Cx43 gap junctions and hemichannels to treatment induced changes in these tissues. These findings build off the findings from the previous chapter in which preventing astrocyte Cx43 hemichannel opening promoted cell viability, under conditions of oxidative and metabolic stress. Furthermore, we observed preventing astrocyte Cx43 hemichannel opening is protective against oxidative stress to an extent comparable to application of either estradiol, DHT, and 3βdiol, supporting the interpretation that these steroids may exert protection through blocking oxidative stress-induced opening of astrocyte Cx43 hemichannels.

CHAPTER 5: GENERAL DISCUSSION

The higher prevalence of chronic and progressive neurological disorders, such as Alzheimer's disease, after the menopause transition is directly correlated with significant decline in serum estrogen concentrations.(18) However, the molecular and cell-specific contributions to this higher incidence remain to be fully understood. Though healthy brain function requires optimal function of both neurons and glia, it is recognized that the focus of most research on the brain protective effects of gonadal steroid hormones, such as estrogens, has been on neurons. In an effort to partially address this gap in knowledge, we focused our studies, presented herein, on glial cells. More specifically, we studied the effect of these hormones on the transmembrane gap junction protein connexin 43 (Cx43), a protein highly enriched in astrocytes. Altered Cx43 function has been implicated in Alzheimer's disease pathology, and its expression is also noted in those tissues whose integrity/function is compromised following the menopause, and include the bone, heart, and reproductive tissue. Therefore, we postulated that studying how estrogens regulate Cx43 in the brain might also advance understanding of how estrogens might exert beneficial molecular actions in these peripheral tissues as well. By extension, this would advance the current knowledge regarding how impaired functioning of these tissue may develop during periods of estrogen deprivation, such as after the menopause transition.

Both Cx43 gap junctions and hemichannels contribute to astrocyte functions, but the mechanisms that regulate astrocyte Cx43 channels are not fully understood. The neurosteroids, estradiol and DHT, are known to regulate Cx43 gap junction expression and Cx43 channel opening in heart, bone, and reproductive tissue.(10,76,77) However, estradiol and DHT-mediated regulation

of astrocyte Cx43 channel expression and Cx43 channel opening has yet to be described. In addition, most studies of Cx43 often failed to consider the separate contributions of Cx43 associated gap junctions and hemichannels. Moreover, the studies which assessed DHT regulation of Cx43 function did not assess whether any of their observations were mediated by prior conversion of DHT to an estrogenic metabolite, such as 3βdiol. The body of work presented herein attempted to address these gaps in the literature. Specifically, the studies conducted were based on the testing of our hypothesis that estradiol, DHT, and 3βdiol protect astrocytes against oxidative stress through the regulation of expression and/or function of astrocyte Cx43 channels. Our findings indicate that estrogens and androgens regulate the Cx43 hemichannel opening, and that this effect correlated with their protective effects against the mixed metabolic/oxidative stress-inducing insult, IAA. To our knowledge, this is the first study to assess androgen or estrogen regulation of Cx43 expression or Cx43 channel opening in any brain tissue. Additionally, this is the first study to demonstrate DHT can promote astrocyte viability through an estrogen receptor-mediated mechanism, by way of the estrogenic DHT metabolite, 3βdiol. Finally, this is the first study to characterize how androgens and estrogens regulate astrocyte Cx43 phosphorylation and Cx43 channel opening and was the first study to reveal how these influences can impact the protective efficacy of DHT, estradiol, and 3βdiol against oxidative stress.

Studies have demonstrated that persistently open Cx43 hemichannels deplete intracellular concentrations of ions (such as potassium and calcium), ATP, glutamate, and other small signaling molecules, and by doing so, likely exacerbate cytotoxicity in neighboring cells.(151) Accordingly, preventing Cx43 hemichannel opening can be protective against such cytotoxic insults as oxidative

stress, increases in pro-inflammatory cytokines, and metabolic inhibition.(22,156,363) Our findings that estradiol, DHT, and 3βdiol inhibited IAA-induced opening of Cx43 hemichannels is therefore consistent with their cytoprotective effects. Furthermore, the protective efficacy of these steroids was equivalent to that elicited by boldine, a compound that selectively closes Cx43 hemichannels, and as such, bolstered our overall conclusion that Cx43 hemichannels are indeed contributors to (or targets of) androgen and estrogen protection of brain tissue.

With respect to our efforts to uncover the mechanism by which estradiol, DHT, and 3βdiol inhibited Cx43 hemichannel opening, we evaluated the phosphorylation of Cx43 at two residues linked to altered Cx43 channel opening, Cx43 Tyr 265 and Ser 368. We were somewhat surprised initially by the observation that DHT, but neither estradiol nor 3βdiol, decreased astrocyte Cx43 mRNA expression and Cx43 S368 protein phosphorylation. An important caveat in the interpretation of this observation is that the effects of the three steroids on Cx43 expression and phosphorylation were conducted under experimental conditions that omitted the insult, IAA. As such, under "healthy" conditions, DHT reduced phosphorylation of Cx43, at least on the Ser 368 residue, while estradiol and 3βdiol were without effect. On the surface, this would seem inconsistent with the data showing that all three steroid hormones were protective against IAA-induced cytotoxicity and inhibited IAA-induced increased opening of Cx43 hemichannels. However, it is possible that under conditions of hypo-phosphorylated Cx43, at least the estrogenic steroids (estradiol and 3βdiol) would help restore or hyper-phosphorylate Cx43, thereby facilitating its "closure" and mitigating the increased Cx43 channel opening triggered by IAA. Nevertheless, the decreased phosphorylation (at Ser368) of Cx43 following 18hr treatment with

DHT warrants further investigation. One possible explanation is that DHT acted directly (i.e., without prior conversion to an estrogenic metabolite) on a different molecular target compared to E2 and 3βdiol. One possible protein target that would explain this differential observation between DHT and the estrogenic steroids used is the putative membrane androgen receptor characterized previously by our laboratory, whose expression has been noted in cortical astrocytes.(336,357) Indeed, this putative membrane androgen receptor, when activated, has been shown to promote cell death in cortical astrocytes, and as such, may be consistent with our finding of Cx43 S368 hypophosphorylation elicited by DHT, which should increase Cx43 hemichannel opening.

The broader implication of our studies is that Cx43 hemichannels are a potentially viable target to exploit in promoting sex steroid induced cytoprotection. For example, long-term administration of the Cx43 hemichannel blocker boldine to a transgenic mouse model of Alzheimer's disease ($APP_{SWE}/PS\text{-}1_{(dE9)}$)(354) reduced beta-amyloid-induced neuronal damage. Furthermore, boldine administration also decreased the elevated ATP and glutamate release from astrocytes.(170) Similarly, other studies reported multiple cannabinoids inhibit insult-induced cell damage by inhibiting the opening of astrocyte Cx43 hemichannels.(169,265) These findings support the interpretation that protection, achieved through closing astrocyte Cx43 hemichannels, is not unique to boldine. Taken together, this information underscores astrocyte Cx43 hemichannels as an understudied target for brain protection, both *in vitro* and *in vivo*.

While the initial purpose of this study was to provide a greater understanding regarding the cellular changes that occur in brain cells exposed to mixed oxidative and metabolic stress as an *in vitro* model of the aging brain, the implications from this study's findings could have much broader

impact for biomedical sciences, noting that Cx43 has been implicated in cancer, neurological disorders, and variety of clinically significant drugs. Given that alterations in astrocyte Cx43 opening, in particular, has also been associated with exposure to clinically used anesthetics as well as several drugs of abuse, (247,249,250,259,261,364-366) it is possible that the findings from our studies will also advance the knowledge regarding how exposure to these compounds alter brain function at the molecular and cellular levels.

Another observation from the anesthesia/substance of abuse literature is that most studies assessed astrocyte Cx43 gap junction opening without considering how these widely used compounds might separately act on astrocyte Cx43 hemichannels. For example, exposing cortical astrocytes *in vitro* to either ethanol, amphetamine, or methamphetamine resulted in significantly reduced astrocyte Cx43 gap junction opening.(259,261,364,365) Similarly, *in vitro* exposure of cortical astrocytes to halothane, isoflurane, or propofol resulted in reduced astrocyte Cx43 gap junction opening.(247,249,250,366) Future studies specifically assessing the contribution of astrocyte Cx43 hemichannels to these compounds' mechanisms of action may provide new information for designing more effective anesthetics with fewer side effects and possibly also provide information for pharmacologically treating dependence on these drugs.

With regards to neurological disorders, previous studies directly implicated changes in astrocyte Cx43 expression and function in the development of pathology. In experimental models of Alzheimer's disease, astrocytes were observed to display higher total Cx43 expression compared to astrocytes obtained from WT controls. Furthermore, astrocytes from Alzheimer's transgenic mice showed increased Cx43 hemichannel opening and decreased Cx43 gap junction

opening.(208-210,212) These findings indicate that identifying therapeutics that either selectively decrease astrocyte Cx43 hemichannel opening or increase Cx43 gap junction opening may be a novel approach for identifying new interventions for Alzheimer's disease treatment. In experimental mouse models of amyotrophic lateral sclerosis, it was observed that transgenic mice displayed reduced astrocyte Cx43 expression compared to WT controls during the pre-symptomatic phase of the disease's progression, but then elevated astrocyte Cx43 expression compared to WT controls once symptoms manifested.(367-370) These findings indicate that identifying whether potential ALS therapeutics alter astrocyte Cx43 expression may be a worthwhile consideration for therapeutic efficacy and that astrocyte Cx43 may be mechanistically linked to the onset or progression of ALS symptoms. In experimental mouse models of seizure disorders, astrocyte Cx43 expression increased after seizure acquisition across multiple experimental seizure induction models. (217,219,371) These findings indicate astrocytes, and more specifically, astrocyte connexin channels, may be understudied molecular components of epilepsy and seizure disorder pathophysiology. In experimental mouse models of ischemic stroke, it was observed that higher astrocyte Cx43 expression facilitates post-stroke recovery, but higher Cx43 hemichannel opening increases ischemic damage.(11,23,245) These findings are consistent with our data which also suggest that selectively closing astrocyte Cx43 hemichannel may be a particularly useful to promote cytoprotection, and as such, may be the basis for designing future ischemic stroke therapeutics.

More intriguingly, in some studies, patent disclosures, and even on-going clinical trials, Cx43 channels are the specific therapeutic target of interest. The clinical outcomes of interest in

these patents include reduced arrhythmia risk,(372,373) enhancing chemotherapy induced apoptosis in an adjuvant manner,(374) improved tissue preservation for organ transplantation,(375) and improved wound healing.(376) Furthermore, there are two on-going phase III clinical trials (clinicaltrials.gov IDs: NCT02667327 and NCT02666131) using Granexin® (a selective stabilizer of Cx43 gap junctions without activity at hemichannels) to improve wound healing of diabetic foot ulcers.(377) While this consideration is distinct from my research focus on the Cx43 hemichannel at the cellular level, these findings underscore the central gap in knowledge this dissertation addresses: that there is indeed demonstrated clinical utility in selectively targeting Cx43 gap junctions versus Cx43 hemichannels.

Taken together, this literature alongside my experimental findings indicate astrocyte Cx43 is associated with diverse types of disorders and research to date does not always consider the likely distinct contributions of Cx43 hemichannels and Cx43 gap junctions to experimental findings. Future studies interrogating the pathophysiology of these diseases might benefit from taking into consideration this distinction. Furthermore, since studies have implicated estrogen deprivation as a contributor to increased incidence of neurological disorders after the menopause transition, it is possible that future studies would benefit from our overall finding of estradiol, DHT, and 3βdiol exerting protection through closing astrocyte Cx43 hemichannels rather than Cx43 gap junctions. More specifically, future studies assessing the molecular mechanisms underlying estrogen deprivation or estrogen replacement might consider the contribution of Cx43 channels to functional changes in the target tissue. Based on the literature and the experimental findings described here, it may be more prudent to consider estrogen regulated protective

mechanisms and modulation of Cx43 channel function as mechanistic considerations that may both mechanistically interact and functionally converge on the outcome of cytoprotection, but which may be differentially targeted by therapeutic interventions.

Our studies advance understanding of Cx43 biology within the brain, astrocytes in particular. Based on our findings, it is apparent that Cx43 channels play a role in regulating astrocyte viability, but it is also apparent that a more complete understanding of the contributions of Cx43 hemichannels are warranted. Such enhanced understanding of the neurobiology of these channels along with their interplay with steroid hormone neurobiology may also facilitate the development of more precise therapeutics (i.e., enhancing cytoprotective therapeutic efficacy by selectively targeting Cx43 hemichannels in the target tissue) to enhance the beneficial actions of estrogens and androgens in the brain. Given that estrogens and androgens have important effects on tissues outside the traditional confines of reproductive biology, coupled with the fact that alterations in Cx43 opening have been associated with wide range diseases that present significant epidemiological and economic burden, including cancer,(9) heart disease,(10) ischemic stroke,(11) anxiety,(12) seizure disorders,(13,14) depression,(15) and Alzheimer's disease,(16,17) the impact of this research could extend to the treatment of many of these other diseases beyond improving nervous system specific disorders.

FUTURE DIRECTIONS

Overall, our findings support our hypothesis and reveal that specifically blocking the opening of astrocyte Cx43 hemichannels, either through pharmacological means or through sex steroid treatment, can protect against oxidative/metabolic stress – induced cytotoxicity in cortical astrocytes. However, additional experiments could be conducted to further support this notion. First, a recently developed Cx43 antibody(298) that preferentially binds hemichannels and not gap junctions might demonstrate whether estradiol, DHT, and 3βdiol protect against oxidative stress by decreasing the expression of Cx43 hemichannels without a simultaneous change in the expression of Cx43 gap junctions. Alternatively, experiments assessing treatment induced changes in Cx43 channel opening could be conducted to more specifically interrogate the contributions of phosphorylation-induced partial channel pore closure to protection against oxidative stress and other cytotoxic insults using a variety of techniques, each of which have their own advantages and limitations.(378) More specifically, recent advances in fluorescence-based imaging techniques have allowed for enhanced spatial and temporal resolution when assessing the contributions of gap junctions to experimental questions regarding the nervous system. However, like many of the studies conducted to date in the connexin literature, these techniques are currently not able to separate the distinct contributions of Cx43 gap junctions and hemichannels to cellular systems without the incorporation of additional technical considerations into the experimental design.(379) Examples of these additional considerations are inclusion of pharmacological compounds that target either Cx43 gap junctions or Cx43 hemichannels without significant activity at the other target, such as the gap junction opener PQ1,(130) the gap junction closer 18-AGA,(380) the

hemichannel opener iodoacetic acid,(83) or the hemichannel closer boldine.(170) Furthermore, inclusion of antibodies that bind to epitopes more accessible on hemichannels than on gap junctions, such as the extracellular loop binding antibody previously characterized, may be useful in some studies.(128,381) Past Cx43 studies could also be re-evaluated using more modern techniques that are able to discern the separate contributions of Cx43 gap junctions and hemichannels to changes in the experimental system.

Additionally, recent studies have identified that in addition to high expression on the plasma membrane of Cx43 expressing cells, there is also some Cx43 expression on the mitochondria. In one study, mitochondria localized Cx43 associated with Bax, and through doing so, protected cells against apoptosis occurring through the mitochondrial apoptosis pathway in pancreatic cancer cells.(173) In another study, mitochondria localized Cx43 hemichannels facilitated calcium uptake into the mitochondria, and through doing so, contributed to cell death induced by ischemia/reperfusion injury in heart tissue.(382) However, it should be noted that no studies focused on mitochondria localized Cx43 have been conducted on brain tissue, nor have there been any studies conducted on mitochondria localized Cx43 that assessed sex steroid regulation of this function. Therefore, future studies could be conducted to fill in these gaps in knowledge as well.

Additionally, the overall molecular regulation of Cx43 protein "life cycle" remains to be more fully understood. While it is known that Tyr 265 and Ser 368 phosphorylation of the Cx43 protein decreases trafficking of the Cx43 to the plasma membrane and increases its degradation,(112-114) it is also known that several other phosphoregulatory sites in the Cx43 CTD

regulate Cx43 expression and channel opening.(54) Furthermore, it is not known whether these same changes increase trafficking of the protein to the mitochondria instead of the plasma membrane. Likewise, the processes that regulate coupling of Cx43 proteins on adjacent cells into gap junctions remains to be better understood. Some evidence indicates in the absence of insult, connexin hemichannels largely represent an intermediate pre-cursor state to gap junctions, in which hemichannels on neighboring cells first interact then functionally "associate" to form gap junctions. This description is supported by two different lines of evidence. First, pannexins, a class of proteins that are very similar to connexins in 3D shape and function, but which have low sequence homology to connexins, have additional glycosylations on their extracellular loops. As such, pannexins readily form hemichannels, but do not form pannexin gap junctions.(383,384) Second, cysteine residues on the extracellular loops of the Cx43 protein sequence have been identified as key residues in hemichannel docking to form gap junctions, a process dependent on formation of disulfide bridges among these six cysteine residues per Cx43 peptide.(343) Therefore, it is possible that in the presence of an insult that disrupts disulfide bridge formation,(363,385) such as oxidative stress, the relative preference of hemichannels on neighboring cells successfully docking to form functional gap junctions is decreased, and there is a resulting predominance of hemichannels versus gap junctions at relatively constant Cx43 expression in the presence of oxidative stress. Future studies could interrogate this possibility and whether protective compounds, like E2, DHT, or 3βdiol exert protection through such a mechanism.

LIMITATIONS

While this research advances the current understanding regarding how astrocytes respond to oxidative stress, how gonadal androgens and estrogens alter astrocyte function in the presence of oxidative stress, and specifically identifies Cx43 hemichannels as a novel molecular target of androgen and estrogen action in the brain, there are some limitations to the design of these experiments. First, only female derived cortical astrocytes were used in the data presented here. Future studies assessing androgen and estrogen regulation of astrocyte Cx43 function could assess whether the findings discussed here are the same or different in cultures containing cortical astrocytes from male only mice or cultures containing astrocytes from both males and females. Furthermore, past studies have assessed astrocyte Cx43 function in regions other than the cerebral cortex, such as hippocampus,(386) cerebellum, thalamus, caudate nucleus,(387) hypothalamus,(72) brain stem, olfactory bulb, and spinal cord.(41) Future studies could assess whether the findings observed in our experiments are similar or different in astrocytes obtained from these other areas of the nervous system. Additionally, our viability assays conducted here did not determine the effects of each of the ER antagonists on astrocyte viability in the absence of either IAA or steroid. While analogous optimization studies were conducted using C6 astrocytic cells and neither ICI-182,780, MPP, nor PHTPP resulted in significant C6 cell death in the absence of IAA, a lower IAA dose of 15 µM IAA for 6 hours was used in the C6 experiments (as opposed to the higher 25 µM IAA for 6 hours used in the cortical astrocyte viability assays.) Therefore, it may be worthwhile for future experiments to determine whether ICI-182,780, MPP, nor PHTPP alters baseline viability of cortical astrocytes in the absence of cytotoxic insult. The results from such

experiments would likely strengthen the interpretation of the viability assay findings we report earlier in this document.

Additionally, our tissue acquisition approach does not result in pure astrocyte cultures, but instead in cultures enriched for astrocytes. Subsequent studies could be conducted using tissue culture methods that isolate only astrocytes, such as by using flow cytometry assisted cell sorting set to isolate only those cells expressing an astrocyte specific protein marker or set of markers and assess whether the findings we obtained in our studies are equally true in pure astrocyte cultures. However, we anticipate the findings from such studies to be similar to our findings reported herein using astrocyte enriched cultures since the non-astrocyte cell types in our astrocyte-enriched cultures are most likely other glial cell types, such as oligodendrocytes or microglia, both of which are known to express connexin isoforms that form functional connexin gap junctions with astrocyte Cx43 (Cx43-Cx43 homotypic gap junctions between astrocytes and microglia and Cx43-Cx47 heterotypic gap junctions between astrocytes and oligodendrocytes).(388,389) Compared to the abundant literature regarding the molecular functions of Cx43 channels in Cx43 expressing cells, there is much less literature on the roles Cx47, a connexin isoform predominantly found on oligodendrocytes. While this dissertation does not intend to focus on connexin isoforms other than Cx43, formation of heterotypic gap junctions between astrocyte Cx43 and oligodendrocyte Cx47 is an established mechanism by which these two cell types communicate with each other. Therefore, it is possible that treatment induced changes in astrocyte Cx43 expression or channel permeability may indirectly impact the expression of channel opening of Cx43 and Cx47 on neighboring microglia and oligodendrocytes, respectively, although such mechanisms regulating

connexin proteins on multiple neighboring cell types remain to be better understood. Further interrogation of these considerations would strengthen the findings reported in this dissertation and through doing so, advance the current understanding of Cx43 in physiology.

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