ABSTRACT

Title of Document: EFFICACY OF ESTROGEN TREATMENT IN
                            A MURINE MODEL OF ALZHEIMER'S
                            DISEASE.

                            Anna Elise Schlappal, Doctor of Philosophy,
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Directed By: Professor, Mary Ann Ottinger, Animal and
                            Avian Sciences

Clinically, Alzheimer's Disease (AD) presents with cognitive dysfunction, cell death,

and amyloid-beta (Aβ) plaque and neurofibrillary tangle (NFT) formation. Moreover,

age and gender are primary risk factors; women are at much higher risk for

developing AD compared to men. Estrogens may be neuroprotective; however,

clinical use in hormone replacement therapy (HRT) is controversial due to potential

adverse effects. Experiments were conducted using the APPswe/PS1dE9 (DTG) and

APPswe/PS1M146V/TauP301L (3xTgAD) transgenic mouse models to assess the

efficacy of an estrogen pro-drug, estradiol-quinol (E2Q). Treatment groups consisted

of vehicle, estradiol (E2), or E2Q in intact and ovariectomized (OVX) DTG females,

intact DTG males, and intact 3xTgAD females and males. The objectives of this

study were to 1) characterize AD progression in a double transgenic (DTG) murine

model and compare the efficacy of treatment with estradiol (E2) or E2Q in

ovariectomized (OVX) and intact females, 2) compare the effects of E2Q in males, 3)

determine if E2Q affects neurodegenerative disease progression in the triple transgenic (3xTgAD) murine model in both males and females, and 4) assess the effects of the neurodegenerative disease progression on mitochondrial function and determine if E2Q affects these endpoints. E2Q did not stimulate uterine tissue and proved to be an effective intervention; treated DTG mice had better cognitive behavior, decreased amyloid precursor protein (APP), and amyloid beta (Aβ) protein levels. Taken together, these data suggest that E2Q has potential as a therapeutic for AD patients.

EFFICACY OF ESTROGEN TREATMENT IN A MURINE MODEL OF
ALZHEIMER'S DISEASE.

By

Anna Elise Schlappal

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Advisory Committee:
Dr. Mary Ann Ottinger, Chair
Dr. Rosemary Schuh
Dr. Edward Orlando
Dr. Marco Colombini
Dr. Hey Kyoung Lee

Dedication

To my parents, Gary and Kathleen Schlappal, and my sister Elizabeth Schlappal. Thank you for your encouragement, and constant love and support.

To Paul Tschiffely, for everything you have done for me, your awesome pep talks and your never-ending love and patience.

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Abbreviations

Abbreviation	Clinical Term or Disease Defined
3xTgAD	Triple Transgenic
Aβ	Amyloid-Beta
AβAD	Aβ Alcohol Dehydrogenase
ADAM	A Disintegrin And Metalloprotease
AD	Alzhiemer's Disease
AICD	APP Intracellular Domain
APOE4	Apolipoprotein 4
APP	Amyloid Precursor Protein
BACE	Beta Amyloid Cleavage Enzyme
CAT	Catalase
CDK5	Cyclin-dependent Kinase-5
COX	Cytochrome Oxidase
CSF	Cerebrospinal Fluid
DTG	Double Transgenic
E2	Estradiol
E2Q	Estradiol-Quinol
ETC	Electron Transport Chain
ERK	Extracellular Related Signaling Kinase
FAD	Familial Alzheimer's Disease
GC	Gas Chromatography
GDX	Gonadectomy
GSK3	Glycogen Synthase Kinase-3
HRT	Hormone Replacement Therapy
MAP	Microtuble Associated Protein
MAPK	Mitogen-Activated Protein Kinase
MS	Mass Spectrometry
MWM	Morris Water Maze
NEP	Neprilysin
NFT	Neurofibrillary tangles
NTG	Non-Transgenic
OVX	Ovariectomy
PDH	Pyruvate Dehydrogenase
PSEN	Presenilin
p-Tau	Phosphorylated Tau
PTP	Permeability Transition Pore
RAM	Radial Arm Maze
RAWM	Radial Arm Water Maze
RCR	Respiratory Control Ratio
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
TBARS	Thiobarbituric Acid Reactive Substances
TOM	Translocase of the Outer Mitochondrial Membrane

Chapter 1: Introduction

<u>Introduction</u>

Alzheimer's disease (AD) is a progressive neurodegenerative disease impacting 5.2 million Americans that are 65 years of age and older. In the next 40 years the prevalence of AD in the American population is expected to more than double from 5.2 million in 2010 to 13.2 million in 2050 (Alzheimer's Association 2012). The recent surge of diagnoses is associated in large part with the aging baby boomer population who will all be 65 years or older by 2029. Alzheimer's disease (AD) primarily affects women, with 3.4 million women affected compared to 1.8 million men. Accordingly the role of decreasing gonadal steroids, particularly estradiol (E2) has been suspect as a critical factor in the onset and progression of AD. Studies have focused on the role of estradiol (E2) neuroprotection and mitochondrial deficiencies related to AD. This review will survey pertinent literature on the potential role of estrogen as a neuroprotective hormone, mitochondrial deficiencies associated with AD, and efficacy of Estradiol-Quinol (E2Q) as a potential therapeutic for AD.

<u>Alzheimer's Disease</u>

Chronology of Alzheimer's Disease in Humans Clinically, AD presents initially with evidence of cognitive impairment. As the neurodegenerative disease progresses, there is atrophy of the hippocampal formation, a decreased number of neuronal cell bodies, and formation of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs) (Walsh and Selkoe 2004). While age is the leading risk factor for the development of

AD, genetic mutations as in the case of Familial Alzheimer's Disease (FAD), can increase the chance of developing the disease. Multiple genetic variants have been identified in contributing to AD pathology, such as mutations in the amyloid precursor protein (APP), the γ-secretase enzymes (presenilin1 and 2), and the Tau protein. These genetic variants are commonly used in transgenic mouse models to study AD neuropathology and potential interventions.

Amyloid Precursor Protein Production of amyloid-beta (Aβ), the plaque forming protein, develops from cleavage and breakdown of the amyloid precursor protein (APP). APP is a transmembrane protein, proteolytically cleaved by the β-secretase enzyme producing the soluble APPβ fragment and the β-stub. The β-stub is cleaved by the gamma secretase enzyme complex to produce Aβ40 or Aβ42 and the APP intracellular domain (AICD; Figure 1). Alternatively α-secretase can also cleave full length APP generating the soluble APPα fragment and the α-stub. The α stub can be cleaved to produce the P3 fragment (LaFerla 2002, Figure 1). The Aβ fragments have been studied extensively due to the key role of the Aβ42 isoform in formation of plaques (Roher 1993). The Aβ42 isoform has hydrophobicity, allowing for quicker oligomerization, aggregation, and plaque formation (Saito 2011). While various Aβ fragment isoforms have been identified, recent identification of a third isoform, the Aβ43 isoform was found as frequently as Aβ42 variant in AD patients. The importance of this additional isoform solicits questions regarding isoforms and their potential role in the plaque formation and pathology leading to AD.

Figure 1. APP processing. Adapted from LaFerla 2002. Cleavage pathway associated with processing of the amyloid precursor protein. Formation of Aβ 42 variant is important in AD pathology.
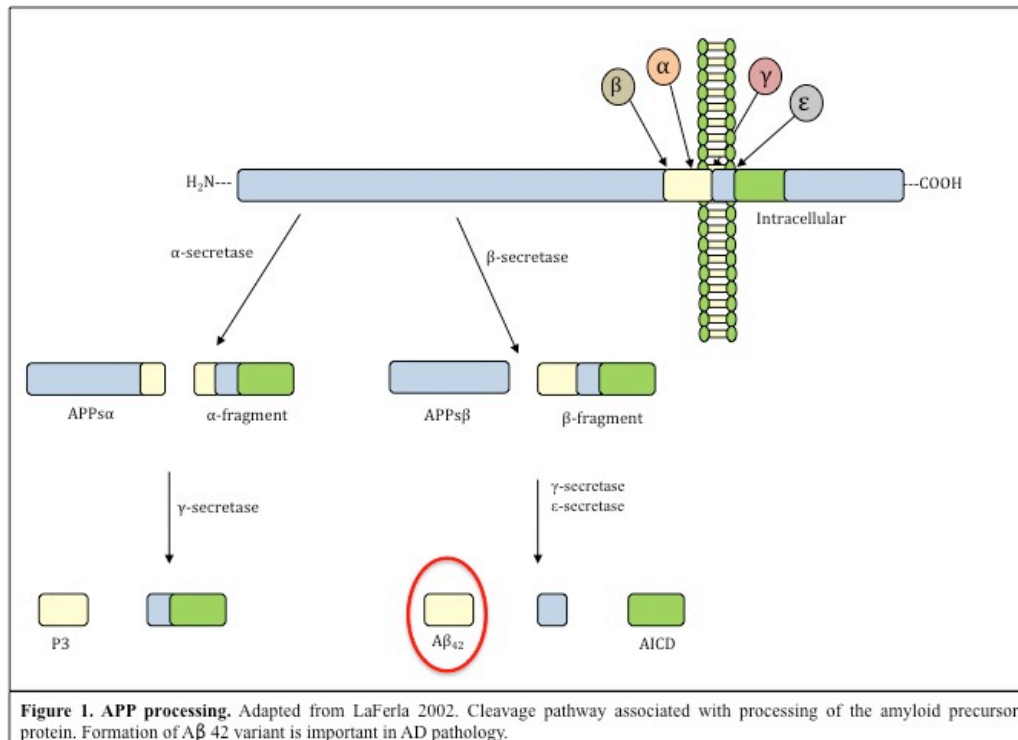
Presenilin Enzymes Three main secretase enzymes, α, β, and γ-secretase (Figure 1) play a role in AD. The secretase enzymes cleave APP at various points resulting in numerous fragment products. The α-secretases (ADAM 17, 10 and 9) form sAPPalpha and α-fragments while the β-secretase (BACE1) form APPsβ (N-terminal) and β-fragments (C-terminal) (Vetrivel 2007, Figure 1). α− and β-secretase compete equally for the APP substrate and therefore mutations that alter their activity can impact AD pathology. The γ-secretase is a complex of proteins that work together to break up proteins by cleaving the C terminal fragments after the alpha and beta secretase have cleaved APP. Gamma secretase cleavage results in p3 (cleavage of α-fragments) or in Aβ cleavage, which occurs between 40-41 or 42-43 (cleavage of the β -fragment)(Figure 1). Presenilin enzymes of the γ-secretase complex include presenilin 1 (PSEN1/PS1) and presenilin 2 (PSEN2/PS2). Both are membrane

proteins that function as the catalytic subunit of the γ-secretase complex (Vetrivel 2007). FAD mutations in PS1 or PS2 selectively elevate levels of Aβ42 (Sherrington 1996). These mutations are autosomal dominant and cause the most aggressive forms of AD, beginning as early as 30 years of age. Variations of this enzyme have been investigated, using mouse models to alter the levels of amyloid fragments produced (Oddo 2003, Jankowsky 2004).

Tau Protein Tau protein is a microtubule-associated protein (MAP), which upon hyperphosphorylation, aggregates forming neurofibrillary tangles (NFTs). There are various sites at which tau can be phosphorylated; regulated by a balance between tau kinase and phosphatase activity (Martin 2012). Glycogen synthase kinase-3 (GSK3), cyclin-dependent kinase-5 (CDK5), and mitogen-activated protein kinases (MAPK) have been considered as potential therapeutic targets for AD due to their role in tau phosphorylation (Martin 2012). As with the other AD associated proteins, there is also a tau mutation (TauP301L), which contributes to increased hyperphosphorylation of tau leading to a higher incidence of NFTs especially in the presence of Aβ (Götz 2001, Oddo 2003). In an adult population of AD patients, CSF tau concentrations significantly increased as compared to healthy, non-symptomatic patients (Tapiola 2000). Measurement of the phosphorylated tau (p-tau) levels in combination with CSF Aβ concentrations provides a potential diagnostic tool for AD, which was more predictive than the presence of the apoE e4 allele (*APOE4*) alone (Buerger 2002). This correlation indicates the important role tau plays in AD pathology, thereby

suggesting the use of CSF p-tau levels as a sensitive measure of tau pathology (Buerger 2002).

The role of tau versus Aβ in the progressive pathology of AD is a question of balance and synergy; specifically, do these processes interact and occur together temporally? In human subjects, Aβ production and accumulation occurs at the same time as tau hyperphosphorylation and aggregation. Vossel (2010) reported no changes in total Tau were observed after injection of Aβ 1-42 by lentivirus, with a significant increase in Tau phosphorylation at the Ser-396, Thr-231, and Ser-199/202 sites. These data suggest that Aβ may not directly increase Tau protein levels; rather it promotes phosphorylation of Tau thereby allowing for the hyperphosphorylated form of Tau to form and aggregate into tangles. Tau protein is a component of the AD pathology that appears to work with Aβ to instigate neuronal deficiencies (Vossel 2010).

AD Models With the high visibility of AD research many models have been developed to study the pathology of the disease. Models including cellular (Thinakaran 1996, Wang 2006), mouse (Oddo 2003, Jankowsky 2004, Lord 2006), and canine (Martin 2011, Dowling 2012) have been established. While all of these models allow for examination of the disease, they each have their own strengths and weaknesses. The cellular model is easily manipulated but too far removed from human systems to provide sufficient systemic answers. Transgenic mice provide an excellent investigational tool, but they can be difficult to assess behaviorally, particularly for AD associated cognitive decline. Accordingly, investigators have

developed a number of behavioral tests that take advantage of a spectrum of transgenic mouse models with AD pathology. The Swedish amyloid precursor protein chimeric mouse/human variant (APP$_{SWE}$) and the exon-9-deleted variant of the human γ-secretase presenilin1 (PS1dE9) were used to generate the APP/PS1 double transgenic mouse model (Jankowsky 2004). The APP$_{SWE}$ variant, identified in a Swedish familial AD (FAD) case has two point mutations, K594N/M595L3, within the APP sequence, and the PS1dE9 variant is missing exon 9 within the presenilin1 sequence. The APP$_{SWE}$ variant was also used to create the triple transgenic mouse model containing the APP$_{SWE}$ variant, the PS1 (PS1M146V) variant, and a Tau (TauP301L) variant (Oddo 2003).

Alzheimer's Disease and Estrogens

Estrogen Neuroprotection Estrogens provide various routes of neuroprotection including direct estrogen receptor activation of the mitogen activated protein kinase/extracellular signal-related kinase (MAPK/ERK) pathway and non-receptor mediated antioxidant activity (Brinton 2001; Simpkins 2012). This antioxidant characteristic is due to the free phenolic hydroxyl group found on the A-ring of estradiol (Badeau 2005). Estrogen treatment has shown to up-regulate genes associated with neuronal survival such as *bcl-2*(+), *IGFBP-5*(+), Calmodulin(+), *CaMKIIα*(+), *Mef2C*(+), *Connexin43*(-), and *Jak1* and *2*(+ and -)(Manthey and Behl 2006). Additionally, Manthey and Behl (2006) identified that ERα and β receptor transfected cells can positively and negatively regulate genes affiliated with AD such as *APP*(-), *PS1*(+ and -), and *ADAM10*(+). This suggests not only a potential effect of

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estrogen on APP at the gene level but also reveals a role specific receptor subtypes may play in altering gene expression. While estradiol (E2) is the key hormone examined regarding estrogen neuroprotection, recent evidence demonstrated that estriol (E3), which is less biologically active than estradiol, also inhibited Aβ oligomer formation in vitro (Morinaga 2011). This suggests that other estrogens may play a role in neuroprotection. Additionally, estradiol treatment following surgical menopause prevented memory deficits (Phillips and Sherwin 1992). As suggested by Resnick and colleagues (1997), there appears to be a critical time period for efficacy when using hormone replacement therapy (HRT); women who started HRT 1 year post- menopause did not regain their memory capacity, while women who started HRT at menopause retained their memory capacity (Resnick 1997). Temporal studies must be conducted to determine optimal timing for any hormonal treatments. Based on this and other research, estrogenic compounds are potentially viable therapeutic candidates for AD associated deficits as well as for menopause related symptoms.

Androgens and Progesterone While many studies focus on estrogens, other hormones such as progesterone and androgen have also shown promise in ameliorating AD related pathologies. Progesterone has been shown to protect against a variety of excitotoxic insults such as glutamate toxicity, Aβ toxicity, and glucose deprivation (Goodman 1996). Allopregnanolone increased the number of BrdU labeled cells (measure of new cells) and decreased 6E10 staining in the hippocampus of the 3xTg mouse model of AD (3xTgAD)(Chen 2011). These data indicate allopregnanolone may increase neurogenesis and decrease APP/Aβ levels. Additionally, testosterone

reduced AD pathology in the triple transgenic mouse model (Rosario 2012). In gonadectomized (GDX) male 3xTgAD mice, testosterone treatment at the time of GDX prevented a plaque load increase seen with GDX mice (Rosario 2012). Additionally, dihydrotestosterone (DHT), a metabolite of testosterone, increases expression of neprilysin (NEP) (Yao 2008). In GDX male rats NEP levels were decreased, however when treated with DHT expression of NEP increased to levels comparable to that of intact rats. This is important because NEP is a major part of the Aβ-catabolizing pathway (Iwata 2000) and in this regard, androgens may be beneficial by increasing the amount of NEP available to degrade amyloid plaque.

Alzheimer's Disease, Estrogens, and Mitochondria

Mounting evidence implicates mitochondrial dysfunction as a critical element in AD associated neurodegeneration (Mutisya 1994, Bonda 2001, Müller 2010). Mitochondria are a major site for reactive oxygen species (ROS) production (CITATION). Since, estrogen is an antioxidant, it may play a therapeutic role in reducing ROS production and ameliorating oxidative stress in neurological diseases. A key discovery in this field was the finding that estradiol receptor (β isoform) localizes to the mitochondria, in primary rat neurons, primary cardiomyocytes, and a murine hippocampal cell line (Yang 2004). This indicates a potential direct role of estrogen in impacting mitochondria. The AD "mitochondrial cascade" hypothesized by Swerdlow and Khan (2004) focused on the decline in mitochondrial function with age as a catalyst for sporadic AD pathology and symptoms. Specifically, their hypothesis states that baseline mitochondrial function and durability is inherited,

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which influences how mitochondria change with age, and there is a threshold of change at which AD pathology commences (Swerdlow and Kahn 2004).

AD and Mitochondria Differences in mitochondrial function have been shown in the human AD population. Their specific connection to the neuropathology of AD is still unclear. However, there is evidence that some mitochondrial enzymes have altered activity in AD, such as pyruvate dehydrogenase (PDH) and cytochrome C oxidase (COX) (Swerdlow and Kish 2002, Cardoso 2004). In AD patient populations, reduced COX activity occurs in platelets (Swerdlow 2007); accompanied by decreased brain mitochondria complex I and IV activity and increased reactive oxygen species (ROS) level (Navarro and Boveris 2007). There is an interaction between the Aβ protein and mitochondria found in both mouse models and humans. In the TgAPP mouse model there was an increase in Aβ42 in the synaptic fraction of isolated mitochondria at 12 months of age (Du 2010). Additionally, the TgAPP model has a reduced respiratory control ratio (RCR) at 4 months of age (Du 2010). Combined, these results describe early mitochondrial deficits and suggest a relationship to increased Aβ production. In a separate study, the triple transgenic (3xTgAD) mice had decreased respiration, decreased metabolic enzyme expression and activity, and increased oxidative stress compared to age-matched control mice (Yao 2009). These data indicate an early deficiency of cellular respiration potentially linked to AD. Interestingly, Aβ has been shown to directly associate with and is taken up by, mitochondria (Hansson-Petersen 2008). Furthermore, the enzyme, Aβ alcohol dehydrogenase (AβAD), has been shown to directly associate with Aβ (Pickrell 2009). This interaction of AβAD with

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Aβ results in inhibition of mitochondrial function, increased ROS, and cytochrome C release which initiates cell death (Pickrell 2009). Aβ also interacts with cyclophilin D (CypD), a subunit of the permeability transition pore (PTP) . The proper function of CypD is required for normal mitochondrial function. Together these studies suggest a direct physical interaction of Aβ with various mitochondrial structures and related proteins impacting their function.

Estrogen and Mitochondria In the ovariectomized (OVX) 3xTgAD mouse, females treated with estradiol at the time of OVX showed a recovery of mitochondrial respiration indicating estradiol may enhance cellular respiration in an OVX system (Yao 2011b). In another study, 24hr estradiol incubation in a primary hippocampal neuronal culture increased the mitochondrial respiratory capacity of the cells, indicating a beneficial role for estrogen at the mitochondrial level (Yao 2011a). These data provide further support for a role for estrogen in the mitochondrial respiratory deficits and as such, estrogen mimetics may hold promise for future therapeutic interventions.

Role of Tau in Mitochondria Tau overexpression has been implicated in mitochondrial dysfunction through disruption of trafficking (Kopeikina 2011). This interaction has been hypothesized to be due to the known role of Tau in cellular trafficking. Vossel and colleagues (2010) found Aβ 1-42 treatment inhibited axonal motility of mitochondria in wild type control hippocampal neurons, affecting anterograde transport more than retrograde transport. When Tau was reduced via a

partial or full knock-out, transport returned to baseline levels (Vossel 2010). These data implicate a role for Aβ in disruption of mitochondrial transport in the presence of Tau.

Purpose

The development of E2Q has allowed for the study of a new class of estradiol pro-drug therapeutics (Prokai 2001). As a pro-drug, E2Q, enters the system in the quinol form and is metabolized through NADPH reductase (Figure 2), into estrogen. This metabolism occurs in the brain at a rate 200x faster than in the body, allowing for more of the estrogen parent compound to accumulate in the brain than in the body (Gleason 2006). This characteristic is important in avoiding negative peripheral effects often seen with hormone replacement therapies (HRT).

Figure 2. Estrogen-Quinol Synthesis. Diagram of the reduction of estrogen to form the estrogen-quinol compound. In the E1 parent estrogen, R1 and R2 are ethyl groups (O) while in E2, R1 is a hydroxyl (OH) group and R2 is a hydrogen (H).
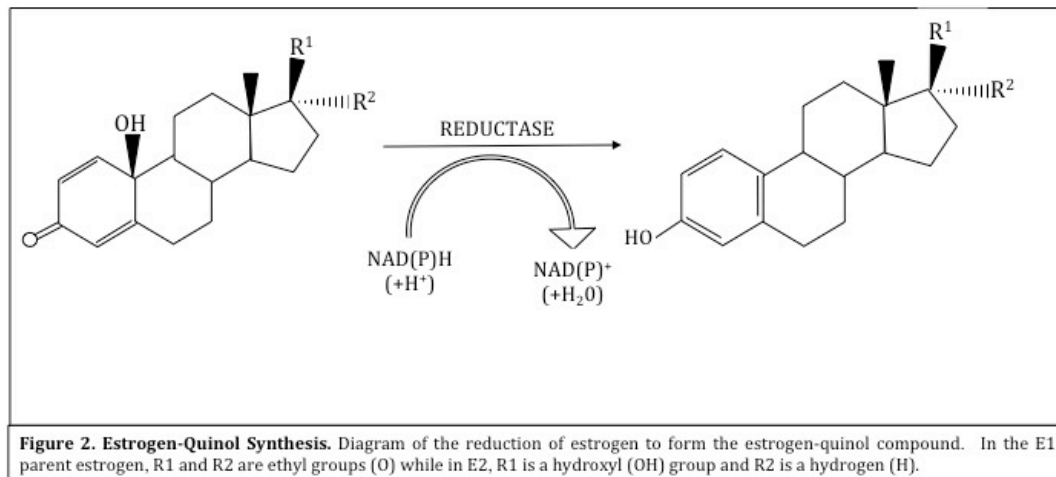
As such, the focus of this research is to test the efficacy of the pro-drug, E2-Quinol (E2Q), as a potential therapeutic using an AD-relevant mouse model. This was accomplished by comparing the effects of E2Q in ovariectomized (OVX) and intact female DTG mice. Additionally, E2Q effects were also examined in males to

determine if any gender related differences in responsiveness would emerge. Finally we investigated effects of E2Q in males and females of a different AD mouse model, the triple transgenic (3xTgAD) model, with a third transgene, TauP301L, to add another dimension of AD pathology. Our aim was to detect cognitive benefits, molecular changes at the protein level, and mitochondrial level changes in these models of AD as a result of treatment with the E2Q and compare these effects to estradiol.

Chapter 2: ESTROGEN PRO-DRUG EFFICACY IN A DOUBLE TRANSGENIC MURINE MODEL OF ALZHEIMER'S DISEASE

Introduction

Alzheimer's disease is characterized by cognitive and neuronal dysfunction associated with Amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs). Age is the leading risk factor for the development of sporadic AD; however, genetic mutations, as in the case of Familial Alzheimer's Disease (FAD), increase a person's chance of developing AD (Goate 1991; Kamino 1992; Mullan 1992). These human genetic variants have been used to create animal models enabling further study of the pathology leading to AD. In this study, we used the AD double-transgenic mouse (DTG) model of early onset human AD. The DTG model possesses a chimeric mouse/human APP Swedish gene ($APP_{SWE,}$ K594M/N595L) as well as the human PS1 delta-E-9 ($PS1_{dE9}$) gene (Jankowsky 2004). This particular model displays behavioral deficiencies at 7 months of age (Reiserer et al., 2007), which correlate temporally with the appearance of plaques (Jankowsky 2004).

Evidence supports the contention that estradiol hormones provide neuroprotection within the brain (Brinton 2001, Simpkins 2012); however, peripheral effects, including stimulation of estradiol-sensitive cancer and increased risk for stroke and heart disease, have diminished their clinical use as a therapeutic. Estradiol act on several targets including direct estradiol receptor activation of the mitogen activated protein kinase/extracellular signal-related kinase (MAPK/ERK) pathway as well as the non-receptor mediated antioxidant activity of estradiol thereby offering neuroprotection (Brinton 2001, Simpkins 2012). Badeau and colleagues

(2005) demonstrated that the antioxidant property of estrogenic compounds is associated with the free phenolic hydroxyl group found on the A-ring of the steroid. Estradiol treatment up-regulates expression of genes that are protective or associated with neuronal survival such as *bcl-2*, *CaMKIIα*(+), and *Jak1* and *2*(+ and -) (Manthey and Behl 2006). Additionally estrogen receptor alpha and beta (ERα/β) sub-types modulate gene regulation of *APP*, *PS1*, and *ADAM10*, genes affiliated with AD (Manthey and Behl 2006). This suggests not only a potential effect at the gene level, but also how different receptor subtypes may alter gene expression. Badeau and colleagues (2005) demonstrated that the antioxidant property of estrogenic compounds is due to the free phenolic hydroxyl group found on the A-ring of the steroid.

Thus far, the clinical actions of estradiol treatment in aging has focused on post-menopausal women. The Women's Health Initiative (Rossouw 2002) investigated the impacts of a combination therapy of conjugated steroid (estradiol and progestin) hormone replacement therapy (HRT) on a variety of measures in postmenopausal women. The results suggested that HRT exacerbated the occurrence of heart disease and breast cancer, thus was halted three years early due to safety concerns (Rossouw 2002). Other studies have had more positive outcomes; for example, human non-AD subjects receiving estradiol following surgical menopause did not suffer memory deficits compared to patients without estradiol (Phillips and Sherwin 1992). However, there appears to be a critical time period for efficacy of HRT; women who waited to start hormone replacement therapy for 1 year post-menopause did not regain their memory capacity (Resnick 1997). Together, these

studies suggest a role for estradiol in neuroprotection, emphasize the likelihood of a strong temporal component for optimal treatment efficacy.

The development of E2-quinol (E2Q) has allowed for the study of a new class of estradiol pro-drug therapeutics (Prokai 2001). As a pro-drug, E2Q, enters the system in the quinol form and is metabolized through NADPH reducatse, into estrogen. This metabolism occurs in the brain at a rate 200x faster in the body, which allows for more of the parent compound to accumulate in the brain than in the body (Gleason 2006). This characteristic is important in avoiding negative peripheral effects often seen with hormone replacement therapies (HRT). The focus of our research was an investigation of the efficacy of the pro-drug, E2Q, as a potential therapeutic, using a transgenic Alzheimer's disease mouse model. The double transgenic mouse model (DTG) possesses a chimeric mouse/human APP Swedish gene (APP_{SWE}, K594M/N595L) as well as the human PS1 delta-E-9 ($PS1_{dE9}$) gene (Jankowsky 2004).

The purpose of this study was to determine the effect of E2Q on behavioral and biochemical endpoints in the AD mouse model. Behavioral responses were assessed using a radial arm water maze (RAWM), APP and Aβ protein levels, as well as overall plaque load in the brain tissue. This study was performed in intact as well as OVX animals to determine the effect of endogenous estrogens in the presence of our treatment paradigm.

<u>Materials and Method*s*</u>

Synthesis and Characterization of E2-quinol Compound   Estrogen-derived para-quinol 17β-dihydroxyestra-1,4-diene-3-one (E2-quinol, E2Q) was synthesized from estradiol (E2), as previously reported (Prokai 2001). Briefly, a stirred solution of estradiol (E2, 190mg, E8875, Sigma-Aldrich, St. Louis, MO, USA), 3-chloroperbenzoic acid (m-CPBA, 273031, Sigma-Aldrich, St. Louis, MO, USA), and benzoyl peroxide (PhCO)$_2$O$_2$, 33581, Sigma-Aldrich, St. Louis, MO, USA) in 40-60 ml dry carbon tetrachloride/Me$_2$CO (CCl$_4$/Me$_2$CO, 4:1 v/v) was heated to reflux while irradiated with 60 W tungsten lamp (Solaja 1996). Reaction completion was verified by thin layer chromatography. The residue was dissolved in CHCl$_3$ and washed with saturated NaHCO$_3$ to remove m-chlorobenzoic acid; the organic phase was evaporated and the remaining residue was divided into several portions to ensure manageability and then purified by column chromatography on silicagel. For purification the column was washed extensively with dichloromethane to remove any residual E2 followed by elution of E2Q with ethylacetate/dichloromethane mixture (CH$_3$COOCH$_2$CH$_3$/CH$_2$Cl$_2$, 9:1, v/v). The purity of the compound (white solid) was verified by high performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS). Purity of the final product was >99% with no trace amounts of the starting material.

Animal Husbandry   Transgenic APPswe/PS1dE9 (B6.Cg-Tg (APPswe, PSEN1dE9)85Dbo/J, Jackson Labs, Bar Harbor, ME, USA) mice were obtained at

3-4 months of age from the colony of Dr. Rosemary Schuh maintained at the Veterans Affairs Maryland Health Care System (Baltimore, MD). Animals were received and transferred following Institutional Animal Care and Use Committee (IACUC) approved protocols. The APPswe/PS1dE9 hemizygote genotype was maintained by crossing a female wild-type (C57BL/6, #000664, Jackson Labs, Bar Harbor, ME, USA) with a male APPswe/PS1dE9. Animals were purchased, bred on site and weaned at 21-25 days of age, then tail snipped and genotyped at 30-35 days of age. Animals were group housed by gender in an environmentally controlled animal facility on a 12-hour light/dark schedule. Food and water were provided *ad libitum* at the University of Maryland College Park. All animal care and experimental procedures were conducted under the University of Maryland, College Park IACUC approved protocols. To minimize any confound, due to estrogenic compounds in the diet, experimental animals were maintained on a phytoestrogen free diet (#AIN-93G, Bio-Serv, Frenchtown, NJ, USA), which was begun one week prior to initiating the study. Animals were individually housed following surgical procedures (see below for details).

Experimental Design

Treatments Female transgenic (DTG) and wild type mice (5.5-6 months) were treated with vehicle (propylene glycol, #P4347, Sigma-Aldrich, St. Louis, MO, USA), E2 (2ug/day, #, Sigma-Aldrich, St. Louis, MO, USA), or E2Q (2ug/day) (N = 7-9) (See Table 1). Animals were divided into cohorts containing individuals from each treatment group to conduct the study with smaller manageable numbers of animals

over the time required to complete the entire study. Further, this allowed for direct comparison of treatment effects on neural and behavioral responses. The long ½ life of E2Q allowed us to administer the treatment using Alzet osmotic minipumps (#2004, 0.25ul/min, Durect Corp., Cupertino, CA, USA). The minipumps delivered the appropriate doses over the 8-week period of treatment; pumps were replaced once at the 4-week time point. Release of the compound was confirmed by measuring circulating levels using an estradiol immunoassay. Compounds were dissolved in USP-grade propylene glycol (#P355, Fisher Scientific, Pittsburgh, PA, USA) in which both estrogen and E2Q are freely soluble and compatible with Alzet pumps. Animals were lightly anesthetized using isoflurane at an appropriate dose for mice according to the IACUC recommendation (IACUC 2012). After anesthetization, a small patch of hair was removed from the upper back of the mouse and the area disinfected. A small incision was made on the operative area and the Alzet osmotic pump implanted subcutaneously between the scapulae. The incision was closed, sutured and allowed to heal. Following surgery, animals recovered on a surgical grade (#DCT-15, Kent Scientific Instruments, Torrington, CT, USA) heating pad under continuous observation before being placed in a cage containing clean, dry bedding. No special post-operative care was generally required. Animals were monitored daily for possible infection and to ensure each individual maintained a healthy body weight, and there were no adverse effects.

Table 2.1. Treatments and number of females in each treatment group

	NTG		DTG	
	Intact	OVX	Intact	OVX
Vehicle	7	7	7	7

E2	9	7	8	8
E2-Quinol	9	7	7	7

Ovariectomy One-half of the females in each treatment group were surgically ovariectomized (OVX) immediately prior to initiating treatment. The surgical procedure was conducted as follows: an incision (1.5 cm) was used to open the intraperitoneal cavity exposing the muscle layers and the ovaries. The ovarian artery was clamped bilaterally and cut near the ovary. Clamps were removed after bleeding had ceased, and when necessary, absorbable suture (#J392H-RL 4-0 FS-2, Ethicon, Somerville, NJ, USA) used. The muscle layer was closed using a continuous stitch with absorbable suture, and the skin layer was closed using an interrupted stitch with non-absorbable suture (#8683G-RL 4-0 FS-2, Ethicon, Somerville, NJ, USA). Following surgery, animals recovered on a surgical grade-heating pad (#DCT-15, Kent Scientific Instruments, Torrington, CT, USA) and remained under continuous observation before being placed in a cage, on clean, dry bedding. No special post-operative care was generally required. Animals were monitored daily for possible infection and normal behavior.

Estradiol EIA An estradiol (E2) EIA 17β-Estradiol kit (#582251, Cayman chemicals, Ann Arbor, MI, USA) was validated for parallelism of measurements, sensitivity, accuracy, and precision. Standards and samples were prepared according to the manufacturer's instructions and samples were diluted using the assay buffer when necessary. Standards, blanks, and samples were incubated with tracer and antiserum for one hour at room temperature and then washed; Ellman's reagent was added to the

wells and plates were incubated for one hour at room temperature in the dark. Absorbance was read at 405nm (#680 Microplate Reader, Bio-Rad, Hercules, CA, USA) 60 minutes post development. Inter- and intra-assay variation were less than 5% and 10%, respectively (Data not shown). Charcoal stripped serum from male non-transgenic mice spiked with E2Q (10ug) showed less than 10% cross reactivity. The EIA cross reacts with estrone (12%) and estriol (0.30%). Serum estrogen levels were measured at 0, 4, and 8 weeks of treatment to measure estrogen levels to check for completeness of ovariectomy and for release of compounds by the implants.

Uterine Wet Weight Uterine wet weight was utilized to determine estrogen stimulation of uterine tissue. Uterine wet weight was measured at euthanasia by removal and gram weight measurement of the uterus. Body weight across treatment groups did not differ therefore uterine weight was not adjusted for body weight (Jefferson 2009).

Radial Arm Water Maze (RAWM) Behavioral Testing A radial-arm water maze test (Alamed 2006) was used to measure cognitive deficits in the mice post-treatment period. This test is designed as a water maze with 6 arms radiating out from the central pool area. The platform is placed at the end of the goal arm (specific for each mouse) for the animal to locate. A large plastic tub was spray-painted black and visual cues (3-D objects) were placed at the end of each arm on the wall of the pool. Mice received one day of training (Day 1) involving 12 trials alternating between a hidden and visible platform followed by 3 trials of all hidden platform training. Days

2 and 3 consisted of 15 hidden platform test trials. Each mouse was assigned one goal arm, which remained constant throughout the three testing days. The start arm changed for each trial and mice had 60 seconds to reach the platform. If they did not locate the platform within 60 seconds the mouse was gently directed to the platform and allowed to rest for 10 seconds before being removed from the pool. An error was recorded as an entry (all four paws) into an incorrect arm or entry into the goal arm without successful location of the platform. Latency was recorded as the time in seconds it took the animal to locate the platform correctly. All animals were scored by the same observer who stood in the same place during testing to eliminate themself as a visual cue.

Tissue Collection Following the RAWM protocol animals were euthanized by cervical dislocation and the brain was immediately removed and each half flash frozen for storage at -80°C. For analysis, the brain sample was homogenized using 1ml of homogenization buffer (225mM ultra pure mannitol, 75mM ultra pure sucrose, 5mM Hepes, 1mM EGTA, pH to 7.4 at 4°C, Sigma-Aldrich, St. Louis, MO, USA). Homogenate protein levels were measured using the standard Lowry (1951) protein assay (Protein Assay Reagent A (#500-0113) and Protein Assay Reagent B (#500-0114), Bio-Rad, Hercules, CA, USA). The other half of each brain was post-fixed in 4% paraformaldehyde and transferred to 30% sucrose after 24 hours. Tissue was allowed to sink in the sucrose solution ensuring full saturation of the tissue before further histological processing.

Histology

Processing and Immunochemistry for APP Serial sections of 50um thickness were collected using a freezing microtome (#860, American Optical AO, Buffalo, NY, USA) and sections stored in cryobuffer (30% Sucrose (#S5-3, Fisher Scientific, Fair Lawn, NJ, USA) 1% polyvinylpyrrolidone (#BP431, Fisher Scientific) 30% ethylene glycol (#102466, Sigma-Aldrich) until staining was performed. Tissue was washed in 1x TBS to remove cryobuffer residue and treated with 1% hydrogen peroxide (H_2O_2) (30 min, #325-100, Fisher Scientific) to block endogenous peroxidase activity. Tissue was then washed in 1x TBS and treated with 0.3% Triton X-100 in 1X phosphate buffered saline (PBS) (10min, #BP151, Fisher Scientific) followed by 5% Normal Goat Serum (NGS) (30 min, #S-1000, Vector Labs, Burlingame, CA, USA). Tissue was incubated with primary antibody overnight at 4°C while on a shaker. Amyloid isoforms and soluble APP were identified using antibodies against APP (6E10) (1:300, #39340, Covance, Princeton, NJ, USA). After incubation, tissue was washed and treated with the Vectastain ABC kit (90 min, #PK-6100, Vector Labs, Burlingame, CA, USA), washed again, then exposed to the secondary antibody (90min, biotinylated anti-Mouse IgG, #BA-9200, Vector Labs, Burlingame, CA, USA). For color development the tissue was treated with 3,3'-diaminobenzidine tetrahydrochloride (DAB, #D5905, Sigma-Aldrich, St. Louis, MO, USA) for 5 minutes. Tissue was immediately washed in 1x TBS, mounted on subbed slides (#OBSLD01CS, Fisher Scientific), and allowed to dry overnight on slides before being stained with nuclei stain cresyl violet, dehydrated, and coverslipped.

Semi-quantitative Measurement of Immunostaining Images were captured using an AxioCam MR camera and AxioVision image capture software (Zeiss, Houston, TX, USA). Quantification of plaque number was performed using ImageJ software (NIH, Bethesda, MD, USA). Five tissue sections spanning the hippocampus were analyzed in each individual. Measurements were taken at 2,5X magnification for the entire hippocampal region (CA1, CA2, CA3, and dentate gyrus) as well as the surrounding cortex, including arcuate nucleus, motor cortex, and somatosensory cortex. Color de-convolution was used to separate the DAB from the cresyl violet stain. Images were transformed to 8-bit gray scale images and stain threshold was adjusted to select for visually observed plaques. Computer generated number of plaques within one frame was recorded. The measurements from the 5 tissue slices analyzed were averaged for each animal.

Biochemical Measurements

Western Blot for APP Protein concentrations were measured on the brain homogenates (25ug total protein) by Western blot analysis. Western blots were performed using 10% Bis-Tris gel (#456-1046,Bio-Rad, Hercules, CA, USA) and samples were normalized to the housekeeping protein β-actin (#4967, Cell Signaling, Danvers, MA, USA) . Briefly, samples were boiled at 95 **°C** for 15 minutes in the presence of loading buffer ((25ul) β-mercaptoethanol (#M6250 Sigma) (475ul) Laemmli buffer (#161-0737, Bio-Rad)), vortexed and spun down before being loaded onto a 10% Bis-Tris gel. Samples were run at 200v for 30 minutes before being transferred to an Immobilon FL PVDF membrane (#IPFL00010, Millipore, Billerica,

MA, USA) using the transblot turbo transfer system (#170-4155, Bio-Rad) at 2.5A, 10v for 10 minutes. Membranes were blocked (60 min; #927-40000, LiCor, Lincoln, NE, USA) then incubated with primary antibodies against amyloid precursor protein (6E10, #39340, Covance, Princeton, NJ, USA) and the housekeeping protein β-actin overnight at 4°C. Membranes were then incubated with secondary anti-mouse (6E10) and anti-rabbit (β-actin) iRdye antibodies (#827-08366 and #926-32211, LiCor,) and imaged on the LiCor Odyssey system and densitometry was performed using Odyssey software (Licor,). Measurements were converted to a ratio of APP:Actin.

Amyloid Beta ELISA Total Aβ1-40 and Aβ1-42 was quantified using a commercially available ELISA kit ((40) #KHB3481 and (42) #KHB3441, Invitrogen, Grand Island, NY, USA). Briefly, standards provided by the company, and samples were measured using a monoclonal primary (rabbit) antibody specific for the NH2-terminus region of human Aβ 1-40 or 1-42. The bound primary antibody was detected using a horseradish peroxidase-labeled anti-rabbit antibody. Finally a stabilized chromagen solution was added which causes a color change directly proportional to the amount of human Aβ present in the sample. Absorbance was measured at 450nm using a Victor microplate reader (Wallac #1420, Perkin Elmer, Waltham, MA, USA). Final concentration values of Aβ were calculated as pg/mg protein.

Statistical Analyses

All statistical analyses were performed using the JMP software program (Version 10.0, SAS Institute, Inc., Cary, NC, USA).

Multivariate Analysis Behavioral results were analyzed using a multivariate, MANOVA, analysis.

One-Way ANOVA One-way ANOVA was performed for all analyses. Post-hoc analysis was performed using the Tukey Kramer HSD test to compare each treatment group to each other (Table 2).

Table 2.2. Statistical Comparisons were made between groups in each cell by Tukey HSD.

Tukey Test Comparisons	
Biologically Relevant Comparisons	*Non-Biologically Relevant Comparisons*
NTG VEH – DTG VEH	NTG VEH – DTG E2
NTG VEH – NTG E2	NTG VEH – DTG E2Q
NTG VEH – NTG E2Q	NTG E2 – DTG E2Q
NTG E2 – NTG E2Q	NTG E2 – DTG VEH
NTG E2 – DTG E2	
DTG VEH – DTG E2	
DTG VEH – DTG E2Q	
DTG E2 – DTG E2Q	
DTG E2Q – NTG E2Q	

Results

Estrogen levels measured by EIA Circulating estradiol levels were measured to confirm ovariectomy (OVX) and release of compounds from the osmotic pump (Figure 1). We did not attempt to quantify the concentration of E2Q released due to the low cross reactivity (<10%) of the estradiol antibody with the quinol compound. Results showed that OVX females had extremely low or non-detectable estradiol levels, verifying the complete removal of the ovaries. Serum was collected at 0, 4, and 8 weeks of treatment. The OVX females receiving the vehicle did not have

detectable levels of estrogen at 4 weeks of treatment as compared to intact vehicle treated females (NTG p = 0.0021, DTG p < 0.0001). Low endogenous estrogen due to OVX was also seen at 8 weeks of treatment (NTG p = 0.0084, DTG p < 0.0001) (Figure 1). OVX females receiving E2 or E2Q had detectable levels of estrogen, similar to those of intact vehicle treated females (Figure 1). Additionally intact females treated with E2 had significantly higher levels of estrogen at 8 weeks of treatment (NTG, p = 0.0009 DTG, p = 0.0002) (Figure 1), as did intact females treated with E2Q (NTG, p = 0.0084 DTG, p = 0.0180).

Uterine wet weight measurement Uterine wet weight was measured as gram weight of the uterine tissue. There was a significant difference (p<0.0001) between the vehicle and E2Q treated groups and the E2 groups (Figure 2). E2Q treated mice had wet weight measurements that were not significantly different from vehicle treated groups (Figure 2).

RAWM behavioral testing Initial differences in errors and latency between the NTG and DTG vehicle treated groups were observed.

     Intact females On day 1 the intact vehicle treated DTG females performed significantly more errors than the NTG vehicle treated females (p = 0.0002), DTG E2 treated females (p = 0.0119), and DTG E2Q treated females (p = 0.0321) (Figure 3A). There were no significant differences observed in the intact females across groups on day 2 of testing. On day 3 of testing, the intact vehicle treated DTG females performed significantly more errors than the DTG E2 treated

females (p = 0.0321) as well as the DTG E2Q treated females (p = 0.0119) (Figure 3A).

OVX females On day 1 the OVX vehicle treated DTG females performed significantly more errors than the NTG vehicle treated OVX females (p = 0.0019) (Figure 3B). Additionally DTG E2 treated OVX females performed significantly more errors than the NTG E2 treated OVX females (p = 0.0012). Interestingly, there was no significant difference in errors performed between the NTG E2Q and DTG E2Q treated OVX females. On day 2 of testing OVX vehicle treated DTG females still made more errors as compared to the NTG vehicle treated OVX females (p = 0.0026) (Figure 3B). Also the DTG E2 treated OVX females performed more errors than the NTG E2 treated OVX females (p = 0.0090). There was no significant difference observed between the NTG E2Q and DTG E2Q treated OVX females on day 2. On day 3 the DTG E2 (p = 0.0038) and DTG E2Q (p = 0.0147) treated OVX females performed less errors than the DTG vehicle treated OVX females (Figure 3B).

APP levels decrease in intact but not OVX females Baseline differences were apparent in the significantly higher level of full length APP in the DTG vehicle treated females as compared to the NTG vehicle treated females (p = 0.0248) (Figure 4A). APP levels significantly decreased in the DTG E2 (p = 0.0004) and E2Q (p < 0.0001) treated females as compared to the vehicle treated DTG intact females (Figure 4A). Additionally the NTG E2 treated intact females had a significantly lower amount of APP compared to the vehicle treated NTG intact females (p<0.0025)

(Figure 4A). There were no significant differences in APP levels observed across treatment groups in the OVX females (Figure 4B).

Aβ40 and 42 are reduced in E2 and E2Q treated females Aβ levels in E2 and E2Q treatment groups decreased in OVX females and intact females.

    Aβ40 In the DTG OVX females, Aβ40 was significantly decreased in the E2 treated females ($p < 0.0001$) and the E2Q treated females ($p < 0.0001$) as compared to the DTG vehicle treated OVX females (Figure 5A). In the intact females the amount of Aβ40 in the DTG E2 treated females ($p = 0.0002$) and DTG E2Q treated females ($p = 0.0014$) were significantly lower as compared to the DTG vehicle treated females (Figure 5B).

    Aβ42 In OVX females, Aβ42 in the DTG E2 treated females ($p < 0.0001$) and E2Q treated females ($p < 0.0001$) were significantly decreased as compared to DTG vehicle treated females (Figure 5A). Additionally, the amount of Aβ42 in OVX E2 treated DTG females was significantly higher than the E2Q treated DTG females ($p = 0.0398$) (Figure 5A). In the intact females, Aβ42 in the DTG vehicle treated females were significantly higher than the amount of Aβ42 in the E2 ($p = 0.0133$) and E2Q ($p = 0.0139$) treated DTG OVX females (Figure 5B).

Hippocampal and cortex amyloid plaque load Immunohistochemistry for measurement of the number of amyloid plaques occurring within the hippocampus and cortex of DTG mice showed no plaques in the NTG treatment groups (Figure 6). Plaque was present in the DTG groups however no differences were observed across

the DTG treatment groups, both in the intact and OVX females (Figure 6). There was no staining in the NTG treatment groups (Figure 6).

Discussion

Release and Peripheral Effects of E2Q A novel compound was tested for efficacy in providing neuroprotective effects of estrogen while avoiding adverse peripheral effects associated with estrogen. Although the EIA had limited cross-reactivity (<10%) with the quinol compound, there was sufficient E2Q measured in the plasma samples from treated females to demonstrate effective release of the compound from the minipumps. Uterine tissue was not stimulated by the E2Q compound. This confirms previous research (Prokai, Personal Communication) showing little or no stimulation of peripheral steroid sensitive tissues by E2Q. Importantly, no adverse effects were observed throughout the duration of the study.

Behavioral Effects of Estradiol and E2Q The DTG mouse model has shown cognitive deficits at 6 months of age in previous studies (Jankowsky 2005; Savonenko 2005). Our behavioral results mirrored this finding; an initial difference existed on day 1 of testing between the NTG vehicle treated females and the DTG vehicle treated females in both the intact and OVX groups. These initial differences have also been observed using a Barnes maze testing paradigm in this mouse model (Reiserer 2007). These initial deficits are rescued in the intact group by day 3 of testing in both the E2 and E2Q treated females. These positive results indicate that

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E2Q is beneficial in our mouse model of AD and thus may be a promising therapeutic to pursue in humans. Since these benefits were seen in the OVX animals, E2Q may be beneficial for menopausal women. Further investigation is needed to discern how E2Q acts on behavioral circuits to the alter response. One issue with the behavioral tests we conducted is that our test was a short-term test (3 days), a longer testing period may reveal other differences among the treatment groups. There are many behavioral tests focusing on hippocampal dependent functions, such as the classic Morris water maze (MWM), (Morris 1982) which is specific for hippocampal function and does not use food or water motivation (Bryan 2009). Alternatively the radial arm maze (RAM) tests short and long-term memory, but does use food or water as a reward. Additionally, fear conditioning tests contextual memory, a measure of amygdalar-hippocampal communication, which assesses hippocampal function that is different from the type of memory used in a spatial task (Bryan 2009). A battery of cognitive tests would be beneficial in obtaining information about short-term and long-term memory as well as amygdala related memory within this treatment paradigm.

Effect of E2Q on APP Our data showed that both the E2 and E2Q intact treated animals showed an overall decrease in APP levels measured in brain homogenate. This complements previous research, which showed that estrogen activation of the estrogen receptor (both α and β subtypes) down-regulates the *APP* gene (Manthey and Behl 2006). Based on this previous research, we can speculate that our current findings may be a result of a gene level effect, however further studies must be performed to determine the accuracy of that hypothesis. Potentially, the decrease in

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APP could be beneficial to an AD patient as a pathway to decrease overall Aβ production and subsequent plaque formation. It is important to remember, APP does have endogenous functions that are necessary for cellular process, and this fact must be considered when using a therapeutic that effects overall APP levels.

Impact on Aβ 40 and 42 As with APP, there was a decrease of Aβ 40 and 42 with E2 or E2Q treatment. With less APP we would expect less Aβ, but we cannot confirm if this decrease in Aβ is due to the decrease in APP or a result of Aβ degradation. Previously estriol (E3), which is less biologically active than estradiol, was shown to inhibit Aβ oligomer formation in vitro and may be a mechanism producing Aβ product (Morinaga 2011). Since plaque formation is apparent by 6 months in this DTG model, a decrease at this time point indicates the quinol may be useful as a therapeutic in the early stages of visible pathology.

Plaque Load in E2Q Treated Mice Overall plaque load did not decrease in number in either the E2 or E2Q treatment groups. These results indicate that while there was a decrease in APP and Aβ protein levels, neither E2 nor E2Q were effective in degrading plaques that had already formed by the time we initiated treatment. This suggests that these interventions may not be able to reverse already formed plaques but could affect further production or accumulation of APP protein, Aβ fragments, and Aβ plaques. Additionally in the OVX and intact animals we did see a slight difference in plaque load, which has been shown before (Yao 2012), however our data was not significant.

In summary, the E2 and E2Q treatments were beneficial in a number of endpoints. We observed decreased number of errors in the DTG E2Q treated

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groups by the third day of testing as well as decreased APP and Aβ protein, all without any uterine tissue stimulation. These beneficial effects on cognitive and molecular measures without the characteristic of uterine stimulation makes the E2Q a potentially useful therapeutic tool without the deleterious effects of estradiol. As such, E2Q as an intervention, certainly warrants further study.
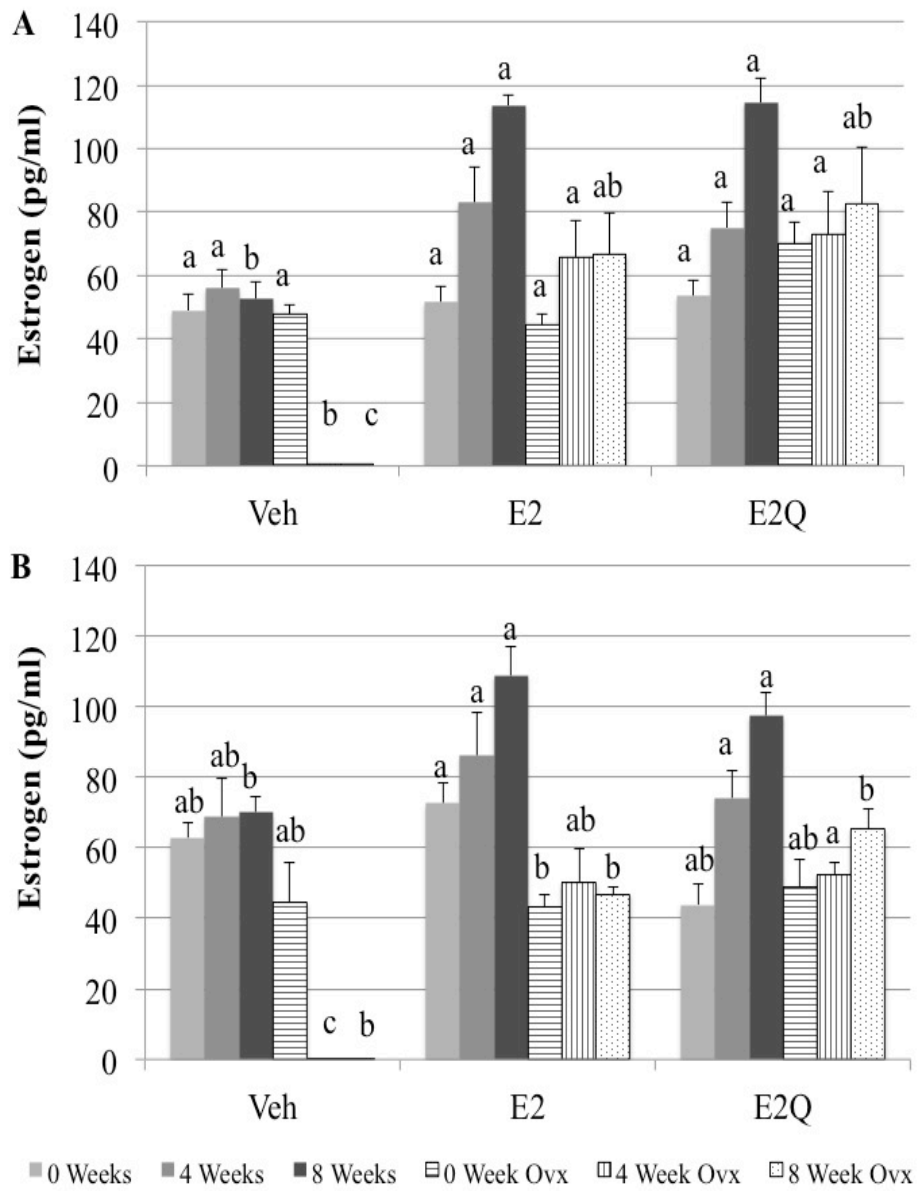
Figure 2.1 Estrogen levels measured in treated females. A. Estrogen levels in NTG intact females as measured at 0, 4, and 8 weeks of treatment **B**. Estrogen levels in DTG intact females as measured at 0, 4, and 8 weeks of treatment. Data represented as pg/ml ± SEM. N=6, ($P \leq 0.05$)
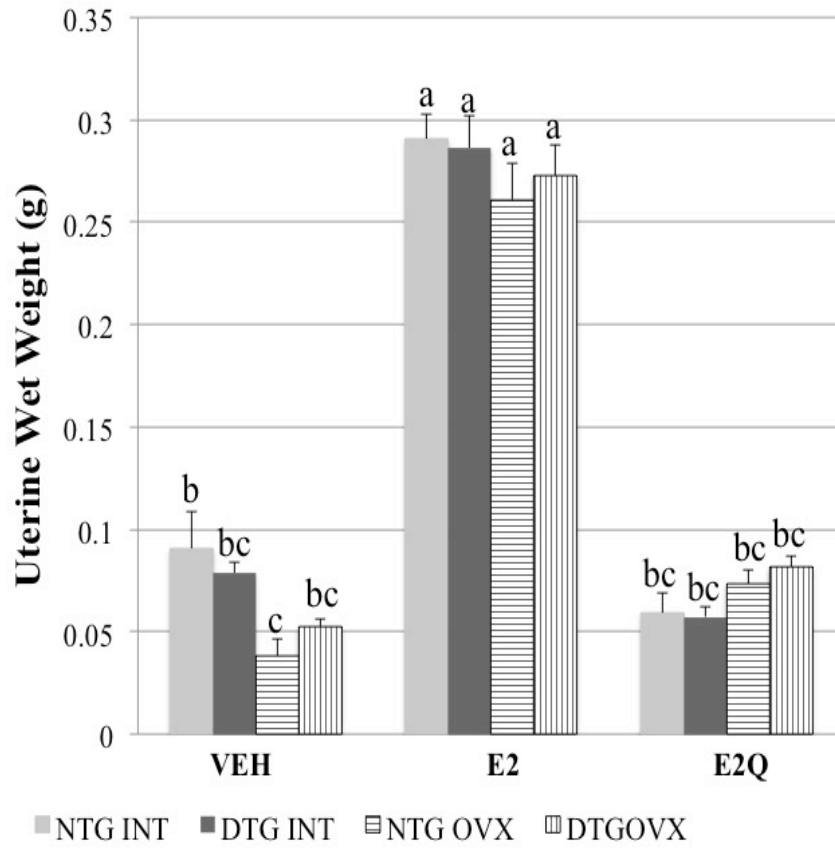
Figure 2.2 Uterine wet weight in treated females shows E2Q does not stimulate uterine tissue. Uterine wet weight as measured by gram weight in females. Data represented as gram weight of tissue ± SEM. N = 5, (P ≤ 0.05)
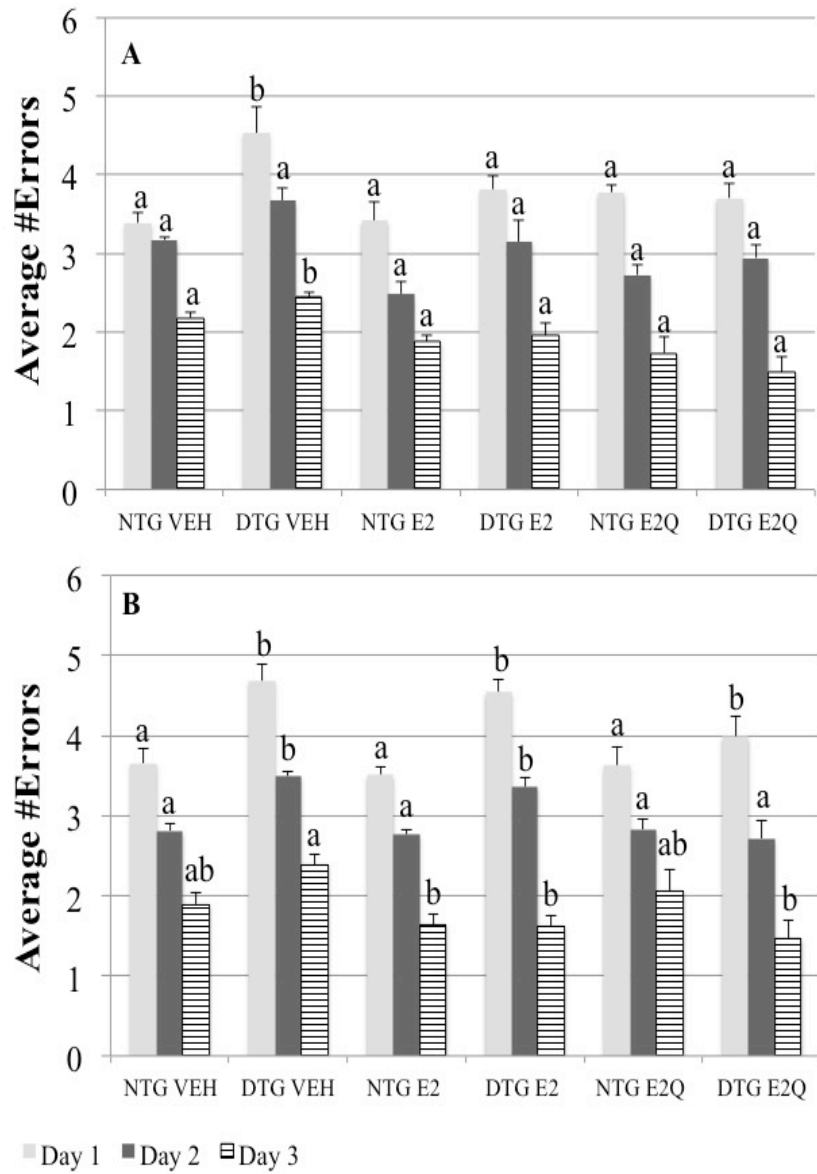
Figure 2.3. Behavioral differences observed as a result of E2Q treatment. Radial Arm Water Maze results expressed as average number of errors performed by intact (A) Day 1, Day 2, Day 3 and OVX (B) Day 1, Day 2, Day 3 treated females. Data expressed as average for each day $\pm$ SEM. N= 7-9, (P $\leq$ 0.05).
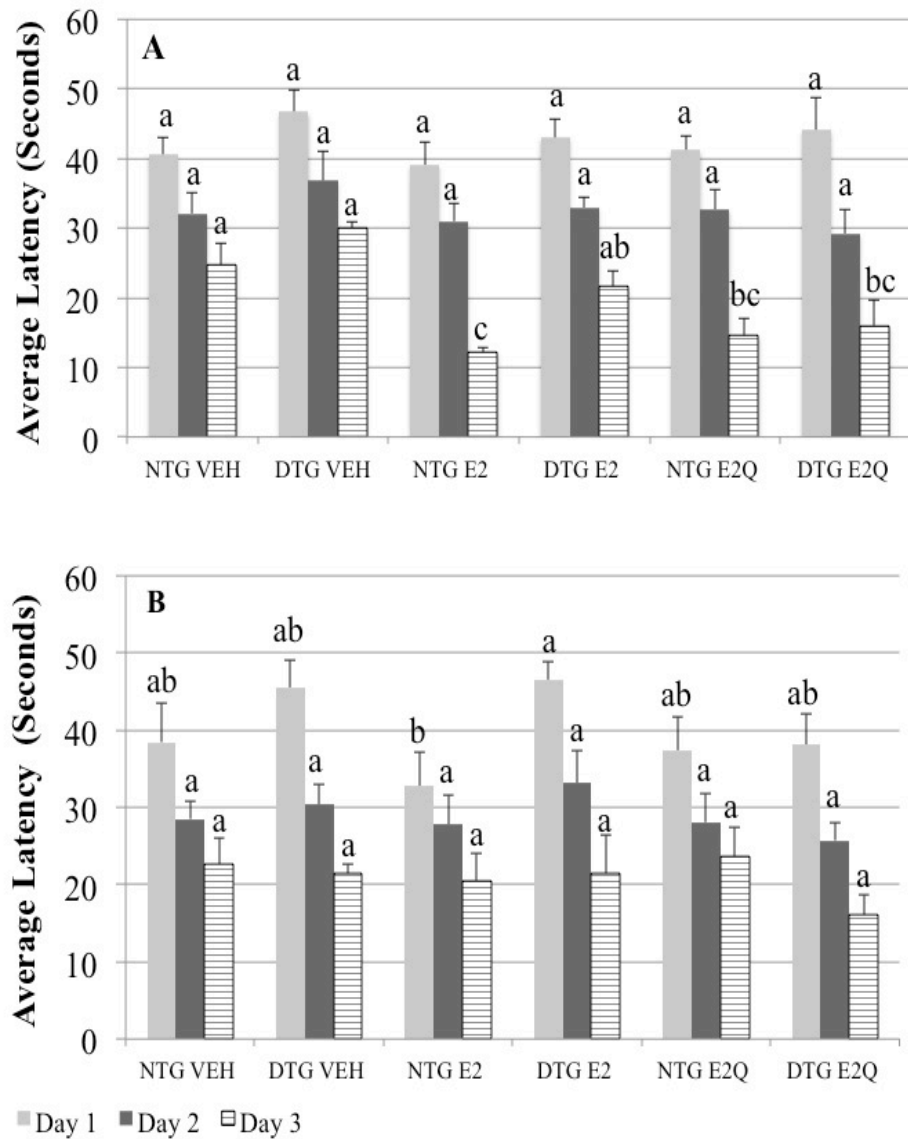
Figure 2.4. Latency to goal arm observed in behavioral task. Radial Arm Water Maze performance expressed as average latency in seconds performed by intact (A) Day 1, Day 2, Day 3 and OVX (B) Day 1, Day 2, Day 3 treated females. Data expressed as average for each day ± SEM. N = 7-9, (P ≤ 0.05).

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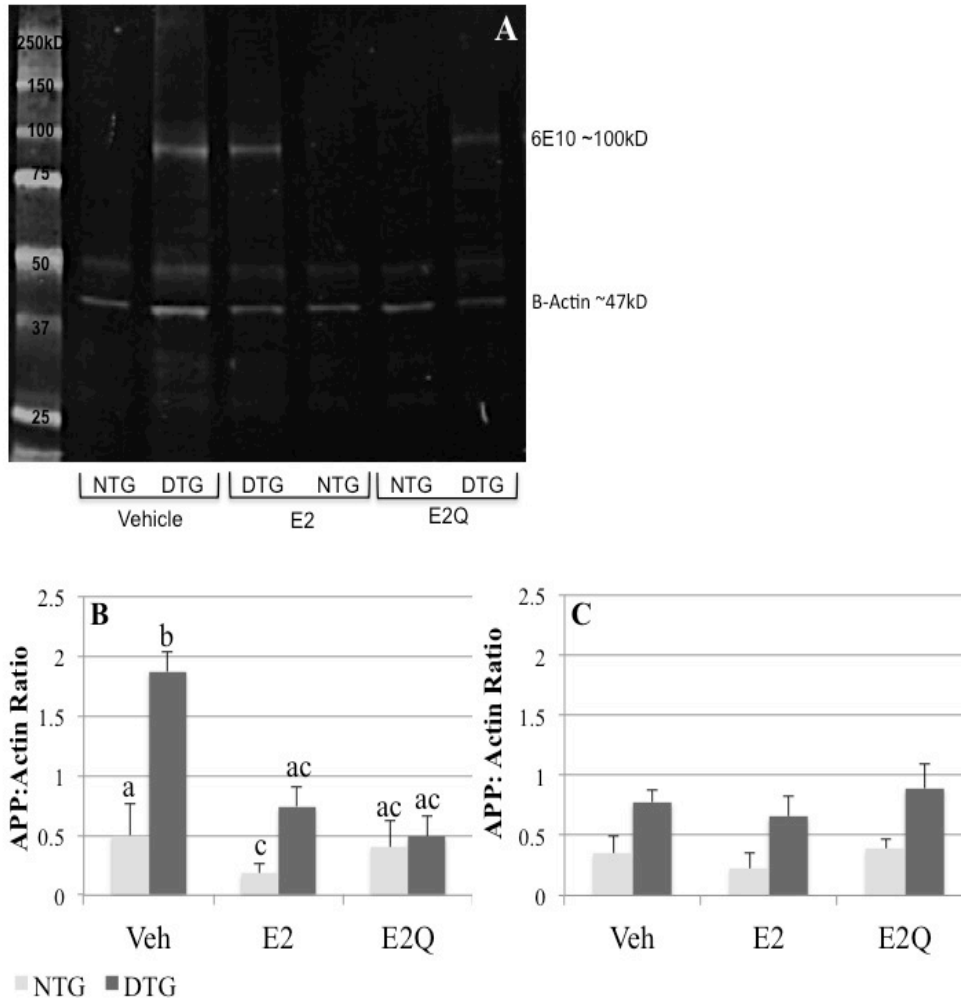
Figure 2.5. APP (6E10) levels are altered in intact but not OVX females. Representative image of western blot (A) APP levels as measured with western blot technology using the 6E10 antibody in intact (B) and OVX (C) females. Protein levels expressed as ratio of β-actin housekeeping protein ± SEM. N=5-8, (P ≤ 0.05).
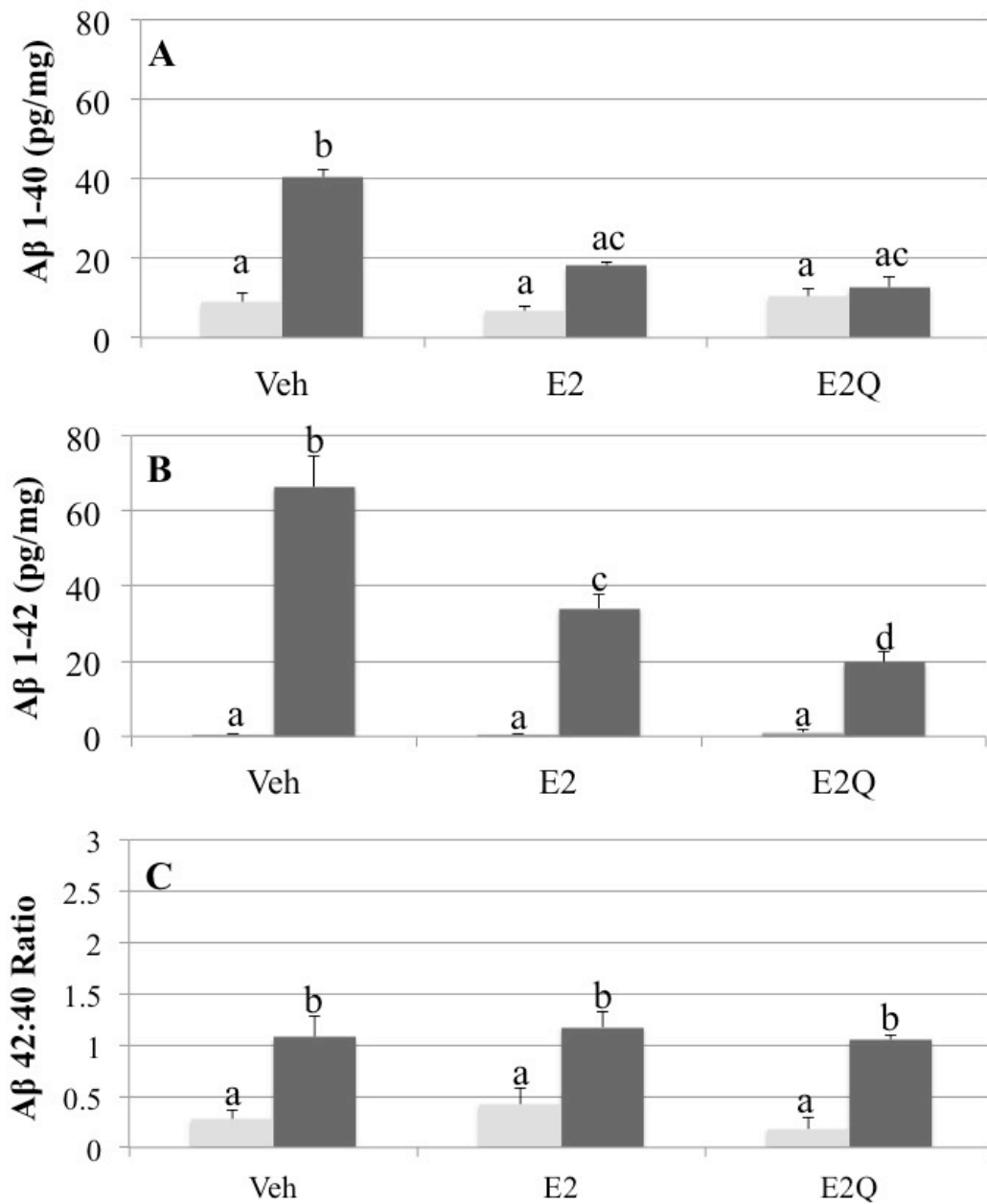
Figure 2.6 (A-C). Amyloid beta levels as measured by ELISA. Protein level of amyloid-beta 1-40(A), 1-42(B), and 42:40 ratio(C) measured in OVX females and amyloid-beta 1-40(D), 1-42(E), and 42:40 ratio(F) measured in intact females. Protein levels are expressed as pg/mg ± SEM. Ratios are expressed as ratio ± SEM. N=6, (P ≤ 0.05).
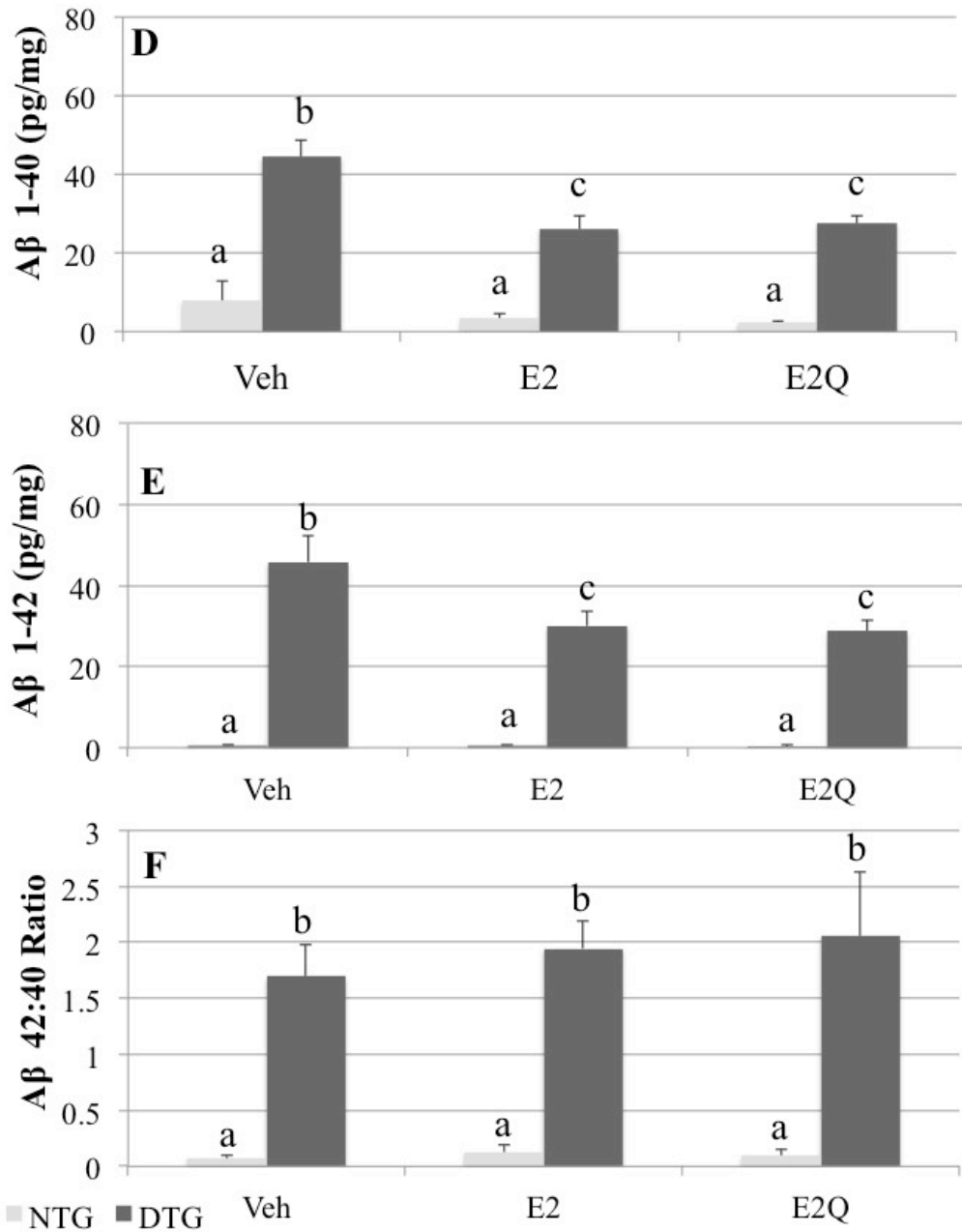
Figure 2.6 (D-F). Amyloid beta levels as measured by ELISA. Protein level of amyloid-beta 1-40(A), 1-42(B), and 42:40 ratio(C) measured in OVX females and amyloid-beta 1-40(D), 1-42(E), and 42:40 ratio(F) measured in intact females. Protein levels are expressed as pg/mg ± SEM. Ratios are expressed as ratio ± SEM. N=6, (P ≤ 0.05).
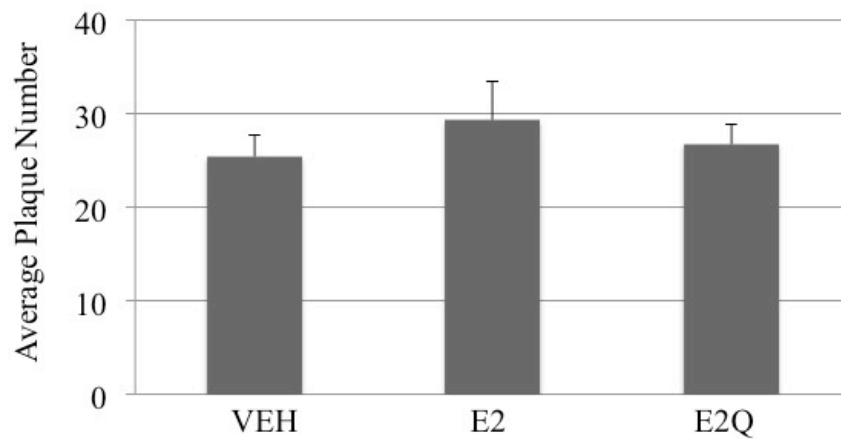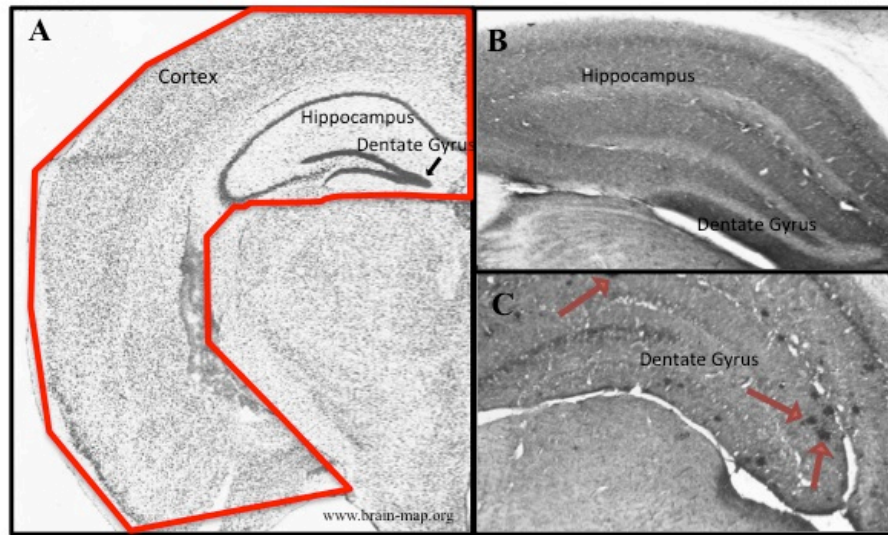
Figure 2.7. Semi-quantitative measurement of plaque load in hippocampus and surrounding cortex. Representative image of area quantified (A), NTG tissue (B), and DTG tissue (C) from treated animals; Red arrows indicate plaque as stained using 6E10 antibody. Plaque load as measured in DTG intact and OVX females (D). Intact and OVX tissue quantification was pooled due to no significant differences seen across treatment groups. Data are expressed as average number of plaques ± SEM. N=12, (P ≤ 0.05).

Chapter 3: **MITOCHONDRIAL RESPONSE TO AN ESTROGEN PRO-**

DRUG IN A MURINE MOUSE MODEL OF ALZHEIMER'S DISEASE

Introduction

Recent research has implicated mitochondrial dysfunction as an early consequence as well as a primary cause in neurodegenerative disease. The "mitochondrial cascade" hypothesis originally introduced by Swerdlow and Kahn (2004) points to mitochondrial decline with age as a catalyst for sporadic Alzheimer's disease (AD) pathology and symptoms. This hypothesis states that 1) inheritance determines mitochondrial integrity, 2) baseline integrity changes with age, and 3) when these changes reach a threshold AD pathology commences (Swerdlow and Kahn 2004). While research has helped to elucidate the mechanisms at work in the mitochondrial pathway, questions remain regarding the mechanistic connections between mitochondrial dysfunction and AD pathology.

Mitochondrial deficiencies and increased reactive oxygen species (ROS) have been documented in human AD (Swerdlow and Kish 2002, Cardoso 2004, Navarro and Boveris 2007). AD patients produce excessive ROS and a deficiency of mitochondrial respiratory complex IV (cytochrome c oxidase, COX); thereby, suggesting a deficiency in mitochondrial function (Cardoso 2004). Additionally, lipid peroxidation can result from excess ROS within a cell. In patients diagnosed with mild cognitive impairment (MCI), increased lipid peroxidation was identified as an early event in dementia related degenerative diseases (Markesbery 2005). Additionally, the same study saw increased lipid peroxidation levels in patients with late-stage AD

(Markesbery 2005). Together, these studies demonstrate a prevalence of disruptions in AD patients that may play a role in mitochondrial dysfunction. Furthermore, healthy cell functioning requires appropriate activity of ROS degrading enzymes including superoxide dismutase (SOD) and catalase (CAT). In AD patients, an increase in the cytosolic copper-zinc SOD (CuZn-SOD) protein levels were observed while the activity of the CuZn-SOD remained unchanged (Puertas 2012). In this same study CAT activity decreased while the protein level was unchanged (Puertas 2012). These findings indicate the relative importance of functional activity versus protein levels, which must be defined in the AD studies. These differences may prove to be very important in determining the underlying mechanisms of cellular respiration disruptions.

Researchers are exploring the link between mitochondrial function and neurodegenerative diseases such as the interaction of amyloid precursor protein (APP), amyloid-beta (Aβ), and mitochondria. It is known that mitochondria take up Aβ via the translocase of the outer mitochondrial membrane (TOM) (Hansson Petersen 2008). While transport of APP occurs through the TOM protein, APP has been found to accumulate within the TOM channel (Lin and Beal 2006), potentially disrupting mitochondrial function. Additionally the Avadhani lab (2006) showed that amyloid precursor protein could be succesfully targeted to mitochondria isolated from an AD transgenic mouse model as well as human AD brain (Anandatheerthavarada 2003, Devi 2006). Taken together these data suggest a role for APP and Aβ in direct interaction with mitochondria and potential to disrupt cell processes at the organelle level.

Estrogens have been shown to effect mitochondria (CITATION), more importantly, it is known that the estrogen receptor beta sub-type (ERβ) localizes to the mitochondria (Simpkins 2004). While estrogens are known to be neuroprotective, their negative peripheral effects (Van Gorp and Neven 2002, Rossouw 2002) have limited their use as a therapeutic in neurodegenerative diseases. Estrogens provide various routes of neuroprotection such as direct estrogen receptor activation of the MAPK/ERK pathway and non-receptor mediated antioxidant activity (Brinton 2001 and Simpkins 2012). Estradiol has been shown to improve depressed mitochondrial respiration in a 3xTgAD OVX system (Yao 2011b). A separate study showed administration of estradiol increased mitochondrial respiratory capacity, again indicating a beneficial role for estrogen in relation to mitochondria (Yao 2011a). Based on animal studies, therapeutics using estrogen mimetics may provide interventions, however, within the AD population therapeutic studies aimed at mitochondrial endpoints have yet to be rigorously assessed.

In this study the effect of E2-Quinol (E2Q), a pro-drug, was assessed for electron transport chain protein levels, lipid peroxidation, and antioxidant levels in ovariectomized (OVX) and intact females of the DTG mouse model. The development of E2Q has allowed for the study of a new class of estradiol pro-drug therapeutics (Laszlo 2001). As a pro-drug, E2Q, enters the system in the quinol form and is metabolized through NADPH reductase, into estrogen. This metabolism occurs in the brain at a rate 200x faster than in the body (Gleason 2006). This high rate of metabolism allows for more of the estrogen parent compound to accumulate in the brain than in the body. This characteristic is important in avoiding negative peripheral

effects often seen with hormone replacement therapies (HRT) (Van Gorp and Neven 2002 and Rossouw 2002). The purpose of this study was to determine the effect of E2Q on various mitochondrial endpoints in the DTG AD mouse model. We measured protein levels of the five electron transport chain protein complexes (I-V), total superoxide dismutase activity, catalase activity, and lipid peroxidation. This study was performed in intact as well as OVX animals to determine the effect of endogenous estrogens in the presence of our treatment paradigm. We report that E2Q treatment was successful in both intact and OVX animals, with no uterine tissue stimulation in E2Q treated animals. Additionally there was increase in complex III and IV protein levels in the OVX DTG E2Q treated animals. These results indicate E2Q may be beneficial for electron transport chain protein levels in an OVX system.

Materials and Methods

Synthesis and Characterization of E2-quinol Compound Estrogen-derived para-quinol 17β-dihydroxyestra-1,4-diene-3-one (E2-quinol, E2Q) was synthesized from estradiol (E2), as previously reported (Prokai 2001). Briefly, a stirred solution of estradiol (E2, 190mg, E8875, Sigma-Aldrich, St. Louis, MO, USA), 3-chloroperbenzoic acid (m-CPBA, 273031, Sigma-Aldrich, St. Louis, MO, USA), and Benzoyl peroxide $(PhCO)_2O_2$, 33581, Sigma-Aldrich, St. Louis, MO, USA) in 40-60 ml dry carbon tetrachloride/Me_2CO (CCl_4/Me_2CO, 4:1 v/v) was heated to reflux while irradiated with 60 W tungsten lamp (Solaja 1996). Reaction completion was verified by thin layer chromatography. The residue was dissolved in $CHCl_3$ and washed with saturated $NaHCO_3$ to remove m-chlorobenzoic acid; the organic phase was

evaporated and the remaining residue was divided into several portions to ensure manageability and then purified by column chromatography on silicagel. For purification the column was washed extensively with dichloromethane to remove any residual E2 followed by elution of E2Q with ethylacetate/dichloromethane mixture ($CH_3COOCH_2CH_3/CH_2Cl_2$, 9:1, v/v). The purity of the compound (white solid) was verified by high performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS). Purity of the final product was >99% with no trace amounts of the starting material.

Animal Husbandry Transgenic APPswe/PS1dE9 (B6.Cg-Tg (APPswe, PSEN1dE9)85Dbo/J, Jackson Labs, Bar Harbor, ME, USA) mice were obtained at 3-4 months of age from the colony of Dr. Rosemary Schuh maintained at the Veterans Affairs Maryland Health Care System (Baltimore, MD). Animals were received and transferred following IACUC approved protocols. The APPswe/PS1dE9 hemizygote genotype was maintained by crossing a female wild-type (C57BL/6, 000664, Jackson Labs, Bar Harbor, ME, USA) with a male APPswe/PS1dE9. Animals were purchased, bred on site and weaned at 21-25 days of age then tail snipped and genotyped at 30-35 days of age. Animals were group housed by gender in an environmentally controlled animal facility on a 12-hour light/dark schedule. Food and water were provided *ad libitum* at the University of Maryland College Park. All animal care and experimental procedures were conducted under the University of Maryland, College Park Institutional Animal Use and Care Committee (IACUC)

approved protocols. To minimize any confounding factor of estrogenic compounds in the diet, one week prior to experimental treatment animals were placed on a phytoestrogen free diet (AIN-93G, Bio-Serv, Frenchtown, NJ, USA) to eliminate dietary estrogen sources. Following surgical procedures animals were individually housed.

Experimental Design

Treatments Female Transgenic and wild type mice (5.5-6 months) were treated with vehicle (propylene glycol, P4347, Sigma-Aldrich, St. Louis, MO, USA), E2 (2ug/day), or E2Q (2ug/day) (N = 7-9) (See Table 1). Animals were divided into cohorts where all treatment groups were represented for behavioral testing and to eliminate potential seasonal effects Due to the long ½ life of E2Q, the Alzet osmotic minipumps (#2004, 0.25ul/min, Durect Corp., Cupertino, CA, USA) were used to deliver the appropriate dose over the 8-week treatment period. Pumps were replaced once, at the 4-week time point. Compound release was confirmed by measuring circulating levels of estradiol using an enzyme immunoassay. Compounds were dissolved in USP-grade propylene glycol (P355, Fisher Scientific, Pittsburgh, PA, USA) in which both estrogen and E2Q are freely soluble and compatible with Alzet pumps. Animals were lightly anesthetized using isoflurane at an appropriate dose for mice according to the IACUC recommendation (IACUC 2012). After anesthetization, a small patch of hair was removed from the upper back of the mouse and the area disinfected. A small incision was made on the operative area and the Alzet osmotic pump implanted subcutaneously between the scapulae. The incision was closed,

sutured and allowed to heal. Following surgery, animals recovered on a surgical grade (DCT-15, Kent Scientific Instruments, Torrington, CT, USA) heating pad under continuous observation before being placed in a cage containing clean, dry bedding, atop a heating pad. No special post-operative care was generally required. Animals were monitored daily for possible infection and to ensure wounds remained clean, the animals maintained a healthy body weight, and there were no adverse effects.

Table 1. Treatments and number of females in each treatment group

	NTG		DTG	
	Intact	OVX	Intact	OVX
Vehicle	7	7	7	7
E2	9	7	8	8
E2-Quinol	9	7	7	7

Ovariectomy One-half of the females in each treatment group were surgically ovariectomized (OVX) immediately prior to treatment. The surgical procedure was conducted as described in chapter 2. Briefly, ovarian tissue was removed and the ovarian arteries clamped until bleeding ceased. When necessary, absorbable suture (J392H-RL 4-0 FS-2, Ethicon, Somerville, NJ, USA) was used. The skin layer was closed using non-absorbable suture (8683G-RL 4-0 FS-2, Ethicon). Following surgery, animals were monitored daily for possible infection and normal behavior.

Estradiol EIA An estradiol (E2) EIA 17β-Estradiol kit (582251, Cayman chemicals, Ann Arbor, MI, USA) was validated and used to measure estradiol in serum samples as previously reported (Chapter 2).

Uterine Wet Weight Uterine wet weight was previously reported (Chapter 2).

Tissue Collection Following Animals were euthanized by cervical dislocation. Brain was immediately removed and half of each brain was flash frozen for storage at -80C. For analysis, the brain sample was homogenized using 1ml of homogenization buffer (225mM ultra pure mannitol, 75mM ultra pure sucrose, 5mM Hepes, 1mM EGTA, pH to 7.4 at 4˚C, Sigma-Aldrich, St. Louis, MO, USA). Homogenate protein levels were measured using the standard Lowry protein assay (Protein Assay Reagent A (500-0113) and Protein Assay Reagent B (500-0114), Bio-Rad, Hercules, CA, USA) (Lowry 1951). The other half of each brain was post-fixed in 4% paraformaldehyde and transferred to 30% sucrose after 24 hours. Tissue was allowed to sink in the sucrose solution ensuring full saturation of the tissue before being processed for histology.

Biochemical Measurements

Western Blot for Mitochondrial Proteins Complex I-V Protein levels were measured on brain homogenates by Western blot analysis. Western blots were performed using a 10% Bis-Tris gel (#456-1046,Bio-Rad) and samples were normalized to 25ug protein. Briefly, samples were boiled at 95 °C for 15 minutes in the presence of loading buffer ((25ul) Beta-Mercaptoethanol #M6250 Sigma, St. Louis, MO, USA and (475ul) Laemmli Buffer 161-0737, Bio-Rad), vortexed and spun down before being loaded onto the gel. Samples were processed through the gel at 200v for 30 minutes. Gels were then transferred to an Immobilon FL PVDF membrane

(IPFL00010, Millipore, Billerica, MA, USA) using the transblot transfer system (170-4155, Bio-Rad) at 2.5A, 10v for 10 minutes. Membranes were blocked ((60 min) 927-40000, LiCor, Lincoln, NE, USA) before being incubated with primary antibodies against one subunit of each of the five proteins of the electron transport chain, complexes I-V (OxPhos Cocktail, MS604, Mitosciences, Eugene, OR, USA) and the housekeeping protein beta-actin (4967, Cell Signaling, Danvers, MA, USA). Membranes were then exposed to secondary anti-mouse (OxPhos) and anti-rabbit (beta-actin) IRdye antibodies (827-08366 and 926-32211, LiCor). Membranes were imaged using the LiCor Odyssey system and densitometry performed using the Odyssey software (Licor).

Superoxide Dismutase (SOD) Enzyme Activity SOD activity was measured using a commercially available kit according to manufacturer's protocol (#706002, Cayman Chemicals). Briefly, brain tissue homogenates were collected and protein values measured using the Lowry assay as described above. Supernatant from brain homogenates were combined with a tetrazolium salt solution and xanthine oxidase then incubated for 20 minutes at room temperature. Absorbance was read at 450nm on a Victor microplate reader (Wallac 1420, Perkin Elmer, Waltham, MA, USA).

Catalase Enzyme Activity Catalase enzyme activity was measured according to manufacturer's protocol (#707002 Cayman Chemicals). Briefly, brain tissue homogenates were collected and protein value measured using Lowry assay as described above. Supernatant from brain homogenates were combined with hydrogen

peroxide for 20 minutes at room temperature before being incubated with potassium hydroxide and chromagen for 10 minutes at room temperature. Samples were then incubated with potassium periodate for 5 minutes at room temperature. Absorbance was measured at 405nm using a Victor microplate reader (Wallac 1420, Perkin Elmer).

Lipid Peroxidation Levels Lipid peroxidation levels were measured using a thiobarbituric acid reactive substances (TBARS) commercially available kit according to the manufacturer's protocol (#10009055, Cayman Chemicals). Briefly, brain tissue homogenates were collected and protein value measured using Lowry assay as described above. Briefly, supernatant from brain homogenates were combined with sodium dodecyl sulfate (SDS) and color reagent (thiobarbituric acid, sodium hydroxide, and acetic acid) and then boiled for one hour. Samples were placed on ice for 10 minutes and centrifuged at 14,000g for 10 minutes. Absorbance was measured at 540nm using the SpectraMax microplate reader (190, Molecular Devices, Sunnyvale, CA, USA).

Statistical Analyses

All statistical analyses were performed using the JMP software program (Version 10.0, SAS Institute, Inc., Cary, NC, USA).

Multivariate Analysis Behavioral results were analyzed using a multivariate analysis of variance (MANOVA), analysis.

One-Way Analysis of Variance (ANOVA) One-way ANOVA was performed for all analyses. Post-hoc analysis was performed using the Tukey Kramer Honestly Significant Difference (HSD) test to compare each treatment group to each other (Table 2).

Table 2. Statistical Comparisons were made between groups in each cell by Tukey HSD.

Tukey Test Comparisons	
Biologically Relevant Comparisons	*Non-Biologically Relevant Comparisons*
NTG VEH – DTG VEH	NTG VEH – DTG E2
NTG VEH – NTG E2	NTG VEH – DTG E2Q
NTG VEH – NTG E2Q	NTG E2 – DTG E2Q
NTG E2 – NTG E2Q	NTG E2 – DTG VEH
NTG E2 – DTG E2	
DTG VEH – DTG E2	
DTG VEH – DTG E2Q	
DTG E2 – DTG E2Q	
DTG E2Q – NTG E2Q	

Results

Estrogen levels measured by EIA Circulating estradiol levels were measured to confirm ovariectomy (OVX) and release of compounds from the osmotic pump as previously reported (Chapter 2). We did not attempt to quantify the concentration of compound released due to low cross reactivity (<10%) of the estradiol antibody with the quinol compound. Results showed that OVX females had low or non-detectable estradiol levels, verifying the complete removal of the ovaries. Serum was collected at 0, 4, and 8 weeks of treatment. Additionally at sacrifice uterine wet weight was

measured as gram weight of the uterine tissue. Estradiol levels as well as uterine wet weight measurement were previously reported in chapter 2.

Complex III and IV protein levels increased in OVX E2Q treated females Protein levels of the five electron transport chain complexes (I-V) were measured in intact and OVX females (Figure 3.2). In the intact treated females, NTG females had an increase in complexes III protein levels in the E2 treated group (p = 0.0008) and E2Q treated group (p = 0.0009) as compared to the vehicle control (Figure 3.1). Additionally there was an increase in complex II protein levels in the intact E2Q treated group as compare to the vehicle treated group (p = 0.0004) and the E2 treated group (p = 0.0005) (Figure 3.1). In the OVX treatment groups there was an increase in complex III protein levels in the DTG E2Q treated groups compared to the vehicle treated group (p = 0.0045) and the E2 treated group (p = 0.0054) (Figure 3.2). Additionally, there were increased complex IV protein levels in both the NTG and DTG E2Q treated groups as compared to the vehicle controls (NTG, p < 0.0001, DTG p < 0.0001) and the E2 (NTG, p < 0.0001, DTG, p < 0.0001) treatment groups (Figure 3.2).

Lipid peroxidation (TBARS) Measurement of lipid peroxidation using the TBARS assay showed a significant increase in the NTG OVX E2Q treated group as compared to the NTG OVX vehicle group (p = 0.0081) (Figure 3.3). However the DTG OVX E2Q treated group were not significantly different as compared to the vehicle DTG OVX females (Figure 3.3).

Superoxide dismutase activity was not altered in treated females Total superoxide dismutase (SOD) activity was measured using supernatant collected from brain homogenates (Figure 3.4). There were no observable differences in the levels of SOD in either the intact treated females. Interestingly, the DTG OVX females showed significantly lower ($p = 0.0276$) SOD levels in the vehicle and E2 treatment groups as compared to the NTG OVX E2 treatment groups (Figure 3.4).

Catalase activity Catalase (CAT) activity and was measured in supernatant collected from brain homogenates. No significant differences were observed among all treatment groups for the CAT activity levels (Figured 3.5).

Discussion

Release and Effects of E2Q As was determined in our previous study (Chapter 2) there was sufficient E2Q measured in the plasma samples from treated females to demonstrate effective release of the compound from the minipumps. Additionally, it was previously reported that uterine tissue was not stimulated by the E2Q compound (Chapter 2). This confirms previous research (Prokai, Personal Communication) showing little or no stimulation of peripheral steroid sensitive tissues by E2Q. No adverse effects were observed throughout the duration of the study.

ETC Protein Complex Levels Vary In the intact treated females, NTG females had an increase in protein complexes II and III when comparing the vehicle and E2Q treated groups. This is interesting, since compared to the DTG intact females the NTG

intact females did have significantly lower levels of complexes II and III. In the OVX treatment groups there was an increase in protein complexes III and IV when comparing the E2 and E2Q treatment groups in both the NTG and DTG genotype. The increase of complex IV may be beneficial in humans with AD, since studies have demonstrated deceased levels of complex IV protein in the AD population (Cardoso 2004). Additionally, it should be noted that complex II is not a know site of ROS production, and thus changes in the availability of these complexes should not increase ROS production capabilities of the mitochondria.

Lipid Peroxidation (TBARS) Lipid peroxidation is a measurement of macromolecule oxidation due to reactive oxygen species (ROS). The amount of lipid peroxidation, is related to the formation of malondialdehyde acid (MDA) in the assay we used. MDA formation increased in the NTG E2Q OVX female group and this could be due to a lack of endogenous estrogens. Since the E2Q doesn't act like estrogen until it is metabolized in the system, the lack of endogenous estrogen could leave the system vulnerable to increased ROS.

SOD Activity Total superoxide dismutase (SOD) activity was measured in supernatants collected from brain homogenates. In the NTG animals we did not observe any differences in the levels of SOD in either the intact or OVX treated females. Interestingly, the DTG OVX females showed significantly lower SOD levels in the vehicle and E2 treatment groups as compared to the NTG OVX E2 treatment groups. Previous studies have determined that in humans with AD, cytosolic

Cu/ZnSOD was increased at the protein level, but no change was observed at the activity level (Puertas 2012). While our assay measured total SOD, a difference may likely exist between Cu/ZnSOD and MnSOD. Mitochondrial MnSOD activity and protein level of MnSOD would be an important test that could elucidate any differences between cytosolic and mitochondrial changes in AD. Additionally, total SOD and Cu/ZnSOD protein levels should be measured as well to determine if the amount of enzyme available is different among treatment groups.

CAT Activity Catalase (CAT) activity overall did not show any significant differences among treatment groups. However, assessment of total CAT protein levels could be beneficial because while the enzyme activity was not different, this could be due to the amount of CAT protein available to the system. Other research has shown that changes in activity and protein level vary in AD patients, and thus both aspects should be considered (Puertas 2012).

In summary, the E2 and E2Q treatments appeared to be beneficial for a number of endpoints relating to oxidative damage and mitochondrial function. The increase of protein complex IV levels in the OVX E2 and E2Q treated females could be extremely beneficial in regards to humans with AD since there was decrease level of the complex IV protein in the AD population (Cardoso 2004). Additionally, the increase of protein complex IV in the OVX AD system is a positive result since this demonstrates that OVX insult can be overcome in some aspects with the E2 or E2Q treatments. However

the increase seen in protein complexes II and III levels could also benefit overall cell viability.

Complex I

Complex II

Complex III

Complex IV
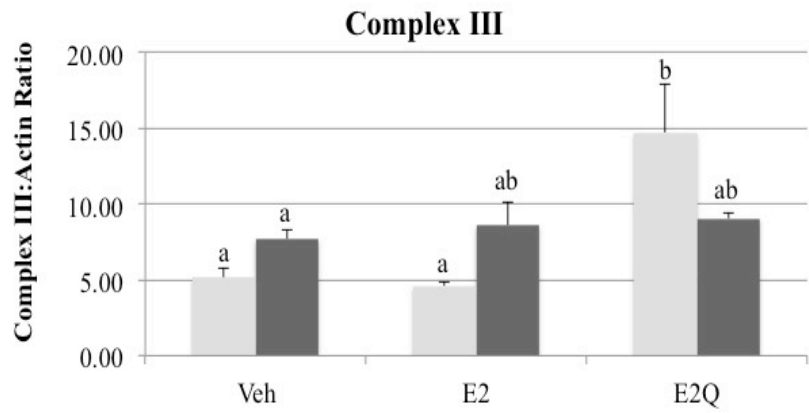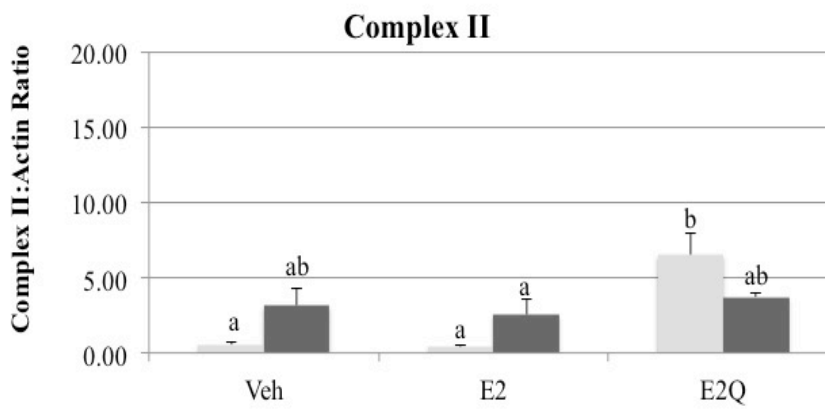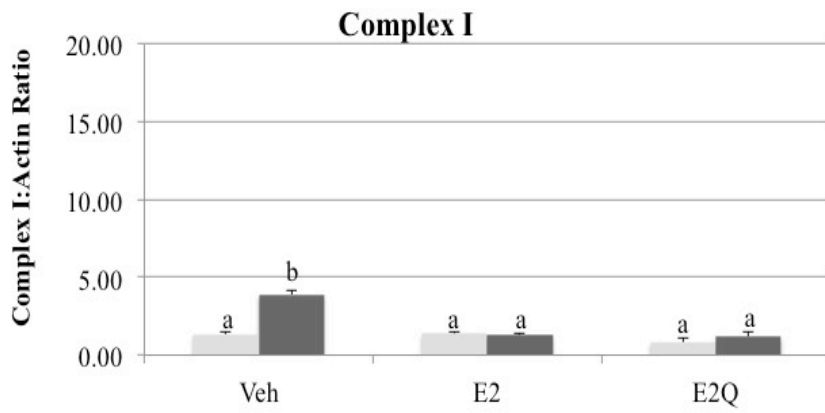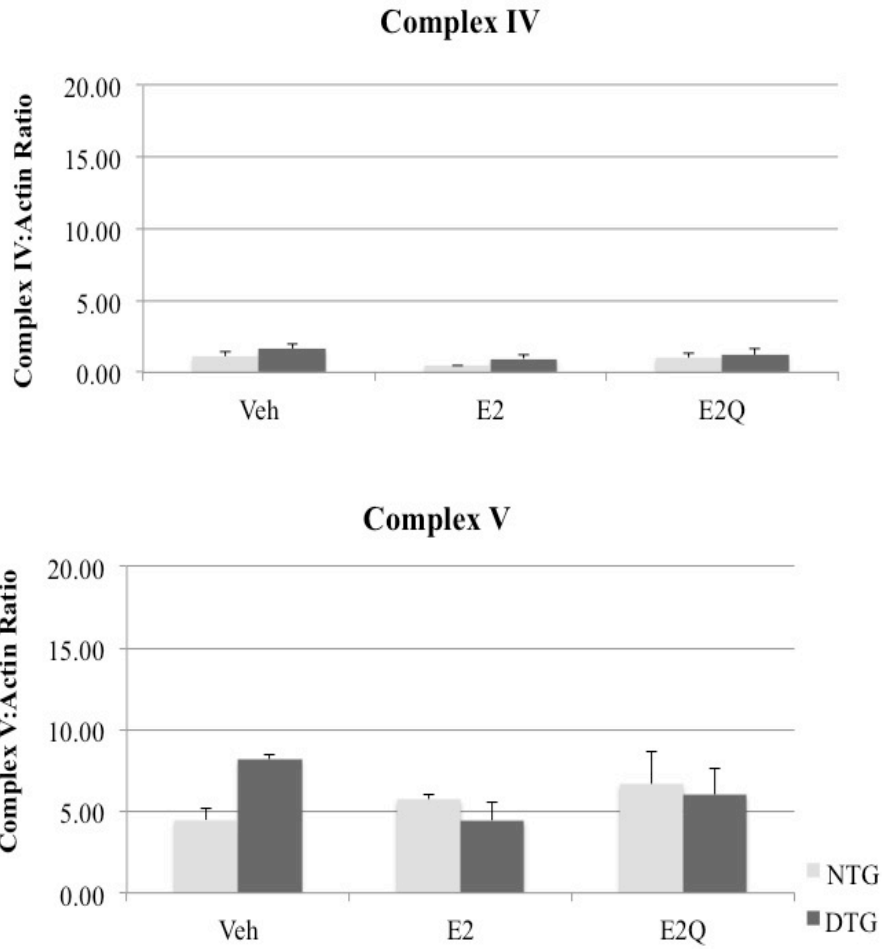
Complex V

Figure 3.1. Complex I-V Protein Levels in intact females. Protein levels for electron transport chain complexes I-V measured using the Total OxPhos Cocktail antibody. Data presented as ratio of complex:actin. N=5-6, ($P \leq 0.05$).

Complex I

Complex II
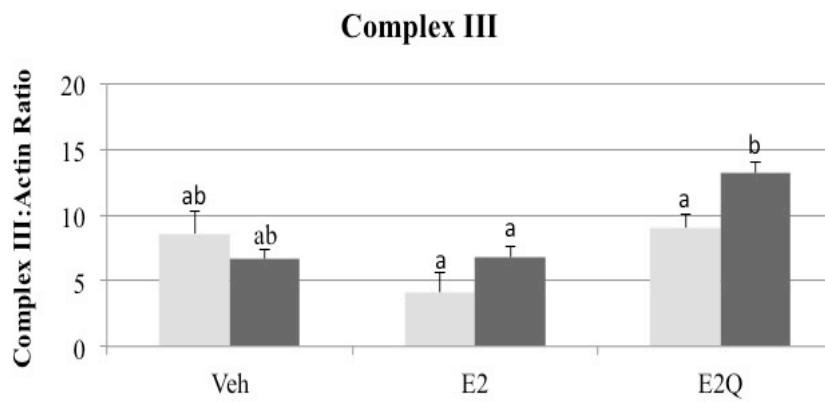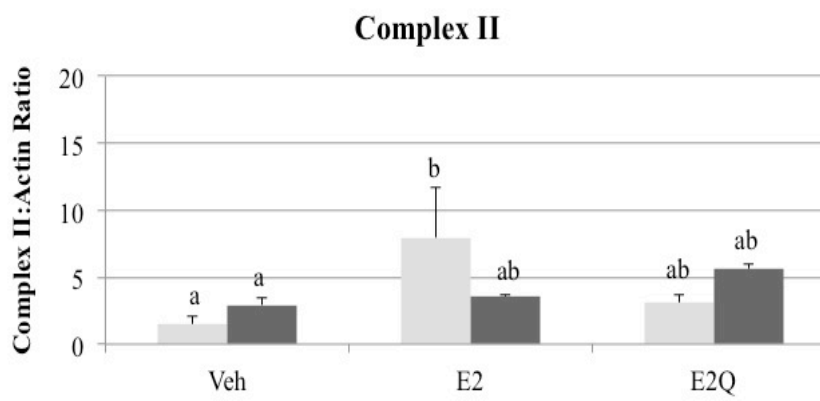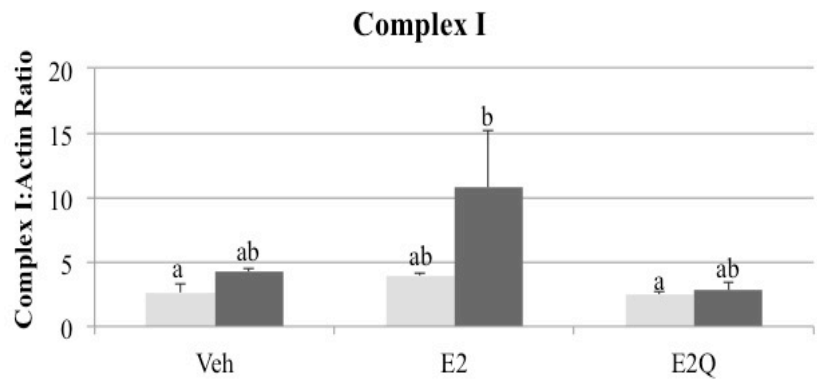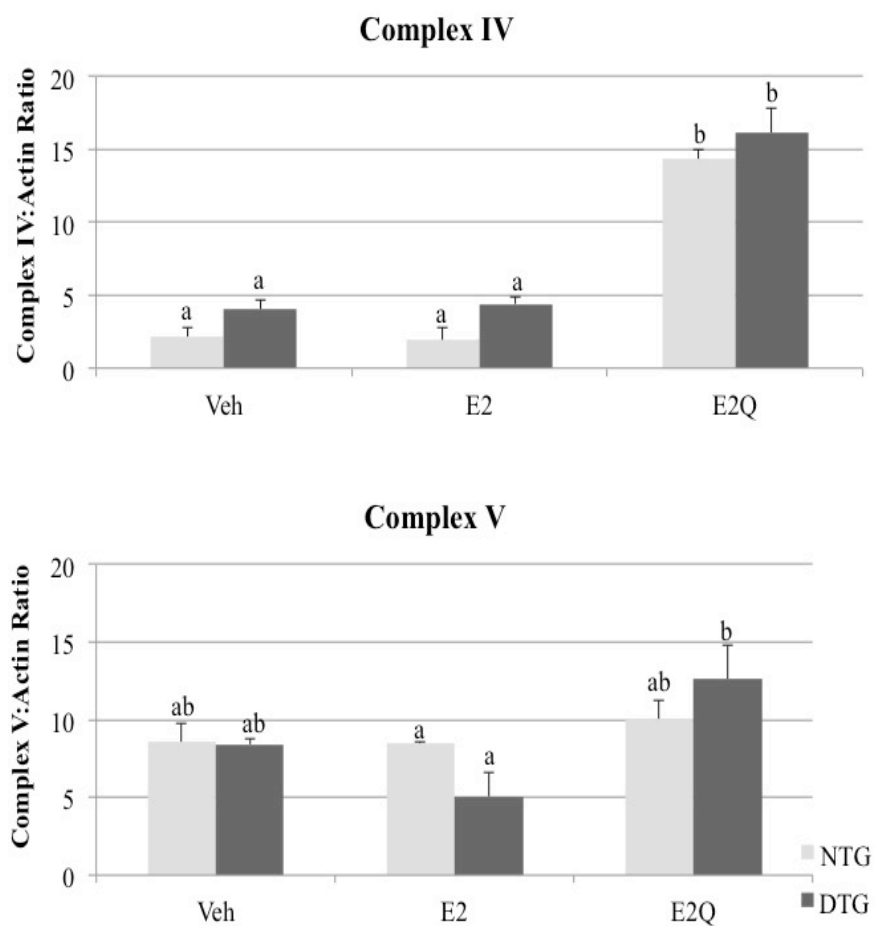
Complex III

Figure 3.2. Complex I-V Protein Levels in OVX females. Protein levels for electron transport chain complexes I-V measured using the Total OxPhos Cocktail antibody. Data presented as ratio of complex:actin. N=5-6, (P $\leq$ 0.05).
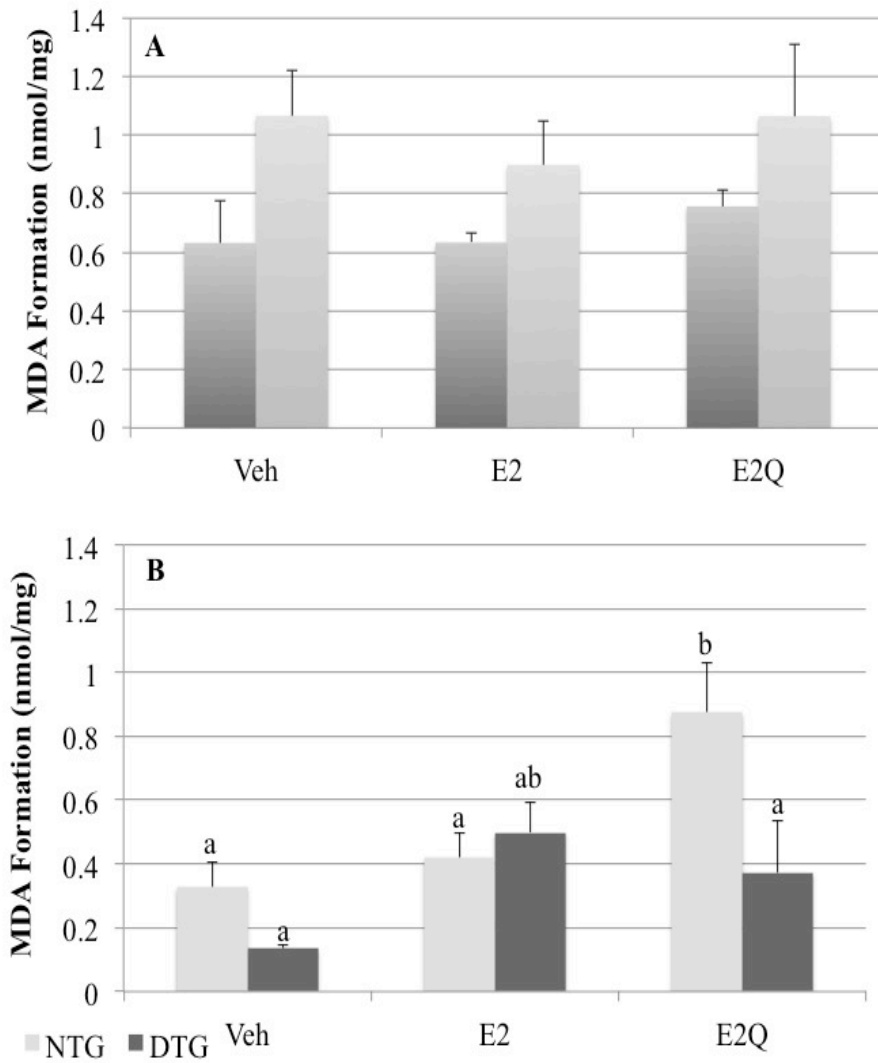
Figure 3.3. MDA Formation as measurement of Lipid Peroxidation. MDA formation in intact (A) and OVX (B) females. Data shown as nmol/mg ± SEM. N=5-8, (P ≤ 0.05)
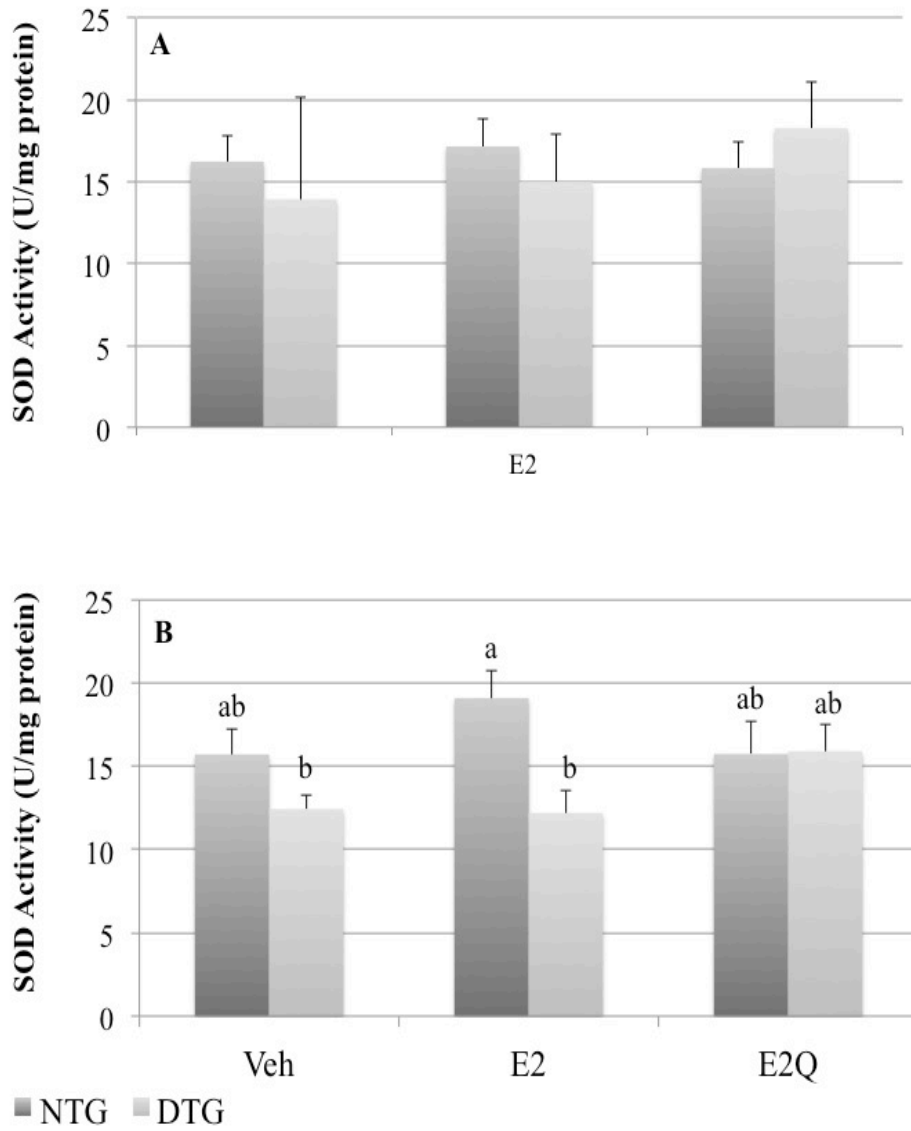
Figure 3.4. SOD Activity. SOD activity in intact (A) and OVX (B) females. Data presented as U/mg ± SEM. N=5-7, (P ≤ 0.05).
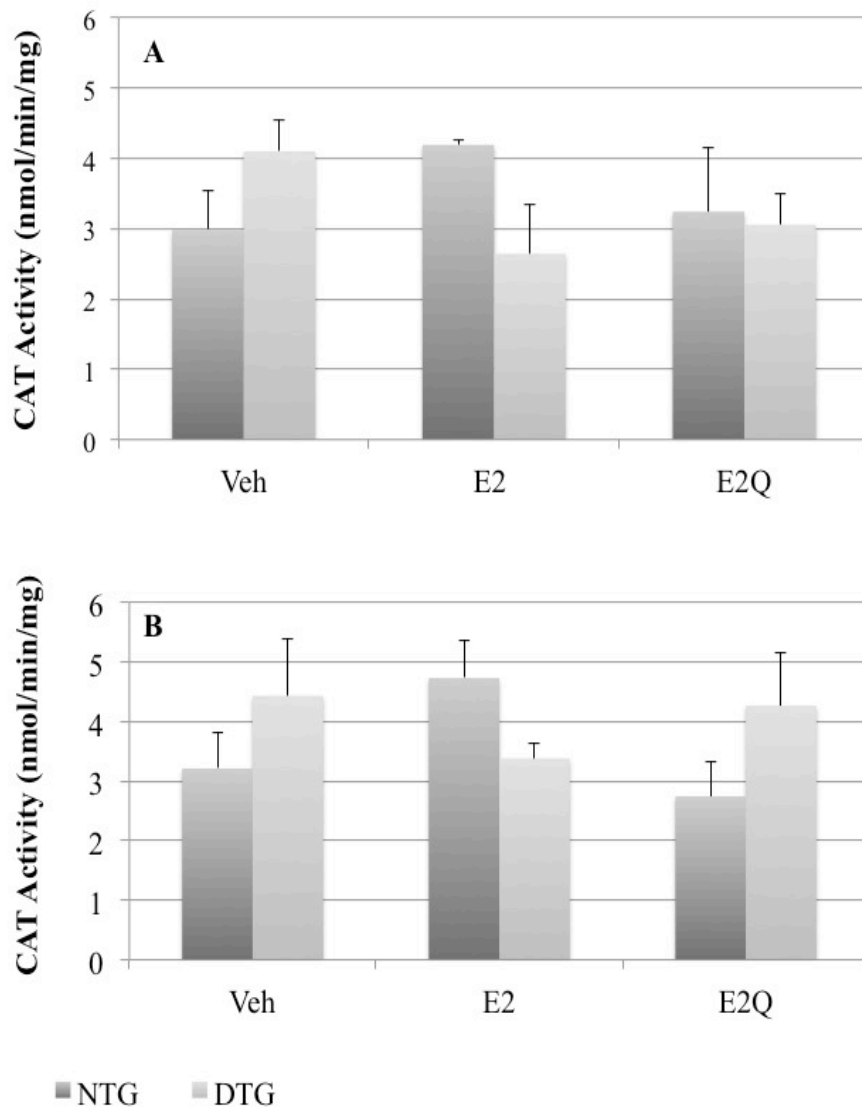
Figure 3.5. CAT Activity. CAT activity in intact (A) and OVX (B) females. Data presented as nmol/min/mg ± SEM. N=5-8, (P ≤ 0.05).

Chapter 4: EFFICACY OF AN ESTROGEN PRO-DRUG IN MALES: STUDIES IN A MURINE MODEL OF ALZHEIMER'S DISEASE

Introduction

Alzheimer's disease is characterized by cognitive and neuronal dysfunction associated with Amyloid-Beta (Aβ) plaques and neurofibrillary tangles (NFTs). Age is the leading risk factor for the development of sporadic AD, however genetic mutations, as in the case of Familial Alzheimer's Disease (FAD), increase a person's chance of developing AD (Goate 1991, Kamino 1992, Mullan 1992,). These human genetic variants have been used to create animal models enabling further study of the pathology of AD. In this study we used an AD double-transgenic mouse (DTG) model of early onset human AD. This DTG mouse strain possesses a chimeric mouse/human APP Swedish gene (APP695$_{SWE}$) as well as the human PS1 delta-E-9 (PS1$_{dE9}$) gene (Jankowsky 2004). This particular model displays behavioral deficiencies at 7 months of age (Reiserer et al., 2007), that correlate temporally with the appearance of plaques (Jankowsky 2004). Here we utilized an estrogen pro-drug, E2-Quinol as a potential therapeutic within the DTG mouse model.

Estrogen and Neuroprotection in Males It has been established that females develop Alzheimer's disease more often than males (Andersen 1999, Lobo 2000, Callahan 2001, Irvine 2012). Evidence supports the contention that estrogen hormones provide neuroprotection within the brain (Brinton 2001 and Simpkins 2012); however

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peripheral effects, including stimulation of estrogen-sensitive cancer and exacerbation of risk for stroke and heart disease have diminished their clinical use as a therapeutic. Estrogens act on several targets including direct estrogen receptor activation of the MAPK/ERK pathway as well as the non-receptor mediated antioxidant activity of estrogen (Brinton 2001 and Simpkins 2012) thereby offering neuroprotection. Researchers know that estrogen treatments up-regulate expression of genes that are themselves protective or associated with neuronal survival such as bcl-2, CaMKIIα(+), and Jak1 and 2(+ and -) (Manthey and Behl). Additionally estrogen receptor alpha and beta (ERα/β) sub-types can positively and negatively influence gene regulation of APP, PS1, and ADAM10, genes affiliated with AD (Manthey and Behl). This suggests not only a potential gene level effect, but also points to how different receptor subtypes may alter gene expression. Badeau and colleagues (2005) demonstrated that the antioxidant property of estrogenic compounds is due to the free phenolic hydroxyl group found on the A-ring of the steroid.

Thus far, the effect of estradiol in human populations has been focused around menopausal women. However, estrogen therapies in men are an important area of research showing promising results. Researchers have shown that in a triple transgenic AD mouse model (3xTgAD), gonadexctomized males (GDX) treated with E2 prevented Aβ accumulation seen in the GDX mice (Rosario 2010). These data provide rationale to believe E2 might be equally beneficial in males and these studies should be further explored.

Purpose In the present study we assessed the effect of E2-Quinol (E2Q), a pro-drug

in males of a DTG mouse model. The development of E2Q has allowed for the study

of a new class of estradiol pro-drug therapeutics (Laszlo 2001). As a pro-drug, E2Q,

enters the system in the quinol form and is metabolized through NADPH reductase,

into estrogen. This metabolism occurs in the brain at a rate 200x faster than in the

body (Gleason 2006). This high rate of metabolism allows for more of the estrogen

parent compound to accumulate in the brain than in the body. The purpose of this

study was to determine the effect of E2Q on behavioral, biochemical, and

mitochondrial endpoints in a DTG AD mouse model. We measured behavior, APP

and Aβ protein levels, plaque load, electron transport chain protein complex (I-V)

levels, superoxide dismutase activity, catalase activity, and lipid peroxidation. This

study was performed in intact males. We report the E2Q treated males made less

errors compared to the vehicle treated males and had lower levels of APP and Aβ

protein levels. Additionally mitochondrial complex proteins I, II and IV were altered

with E2 and E2Q treatment.

Materials and Methods

Synthesis and Characterization of E2-quinol Compound Estrogen-derived para-quinol

17β-dihydroxyestra-1,4-diene-3-one (E2-quinol, E2Q) was synthesized from estradiol

(E2), as previously reported (Prokai 2000). Briefly, a stirred solution of estradiol (E2,

190mg, E8875, Sigma-Aldrich, St. Louis, MO, USA), 3-chloroperbenzoic acid (m-

CPBA, 273031, Sigma-Aldrich), and Benzoyl peroxide $(PhCO)_2O_2$, 33581, Sigma-

Aldrich) in 40-60 ml dry carbon tetrachloride/Me_2CO (CCl_4/Me_2CO, 4:1 v/v) was

heated to reflux while irradiated with 60 W tungsten lamp (Solaja 1996). Reaction completion was verified by thin layer chromatography. The residue was dissolved in $CHCl_3$ and washed with saturated $NaHCO_3$ to remove m-chlorobenzoic acid; the organic phase was evaporated and the remaining residue was divided into several portions to ensure manageability and then purified by column chromatography on silicagel. For purification the column was washed extensively with dichloromethane to remove any residual E2 followed by elution of E2Q with ethylacetate/dichloromethane mixture ($CH_3COOCH_2CH_3/CH_2Cl_2$, 9:1, v/v). The purity of the compound (white solid) was verified by high performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS). Purity of the final product was >99% with no trace amounts of the starting material.

Animal Husbandry Transgenic APPswe/PS1dE9 (B6.Cg-Tg (APPswe, PSEN1dE9)85Dbo/J, Jackson Labs, Bar Harbor, ME, USA) mice were obtained at 3-4 months of age from the colony of Dr. Rosemary Schuh maintained at the Veterans Affairs Maryland Health Care System (Baltimore, MD). Animals were received and transferred following IACUC approved protocols. The APPswe/PS1dE9 hemizygote genotype was maintained by crossing a female wild-type (C57BL/6, 000664, Jackson Labs, Bar Harbor, ME, USA) with a male APPswe/PS1dE9. Animals were purchased, bred on site and weaned at 21-25 days of age and then tail snipped and genotyped at 30-35 days of age. Animals were group housed by gender in an environmentally controlled animal facility on a 12-hour light/dark schedule.

Food and water were provided *ad libitum* at the University of Maryland College Park. All animal care and experimental procedures were conducted under the University of Maryland, College Park Institutional Animal Use and Care Committee (IACUC) approved protocols. To minimize any confounding factor of estrogenic compounds in the diet, one week prior to experimental treatment animals were placed on a phytoestrogen free diet (AIN-93G, Bio-Serv, Frenchtown, NJ, USA) to eliminate dietary estrogen sources. Following surgical procedures animals were individually housed.

Experimental Design

Treatment Groups Male APPswe/PS1dE9 mice (5.5-6 months) were treated with vehicle (propylene glycol, P4347, Sigma), E2 (2ug/day), or E2-quinol (2ug/day) (N = 7-9). Due to the long ½ life of the quinol compound in the body, the Alzet osmotic minipumps (2004, 0.25ul/min, Durect Corp., Cupertino, CA) were used to deliver the appropriate dose over the 8 week period of treatment. Pumps were replaced once, at the 4 week time point. Compounds were dissolved in USP-grade propylene glycol, a substance in which both estrogen and the E-quinol compound are freely soluble and which is compatible with Alzet pumps. Animals were lightly anesthetized using isoflurane at an appropriate dose for mice according to the AALAS recommendation. After anesthetization, a small patch of hair was removed from the upper back of the mouse and the area disinfected. A small incision was made on the operative area and the Alzet osmotic pump implanted subcutaneously between the scapulae. The incision was closed, sutured and allowed to heal. Following surgery, animals recovered on a

surgical grade (DCT-15, Kent Scientific Instruments, Torrington, CT) heating pad under continuous observation before being placed in a cage containing clean, dry bedding, atop a heating pad. No special post-operative care was generally required. Animals were monitored daily for possible infection and to ensure wounds remained clean, the animal maintained a healthy body weight, and there were no adverse effects.

Table 4.1. Treatments and number of males in each treatment group

	NTG	DTG
Vehicle	8	8
E2 (2ug/day)	9	7
E2-Quinol (2ug/day)	9	9

Estrogen Measurements

Estradiol EIA An estradiol (E2) EIA 17β-Estradiol kit (582251, Cayman chemicals, Ann Arbor, MI, USA) was validated and used to measure estradiol in serum samples as previously reported (Chapter 2).

Radial Arm Water Maze (RAWM) Behavioral Testing A radial-arm water maze (Alamed 2006) was used to measure cognitive deficits of the mice post-treatment period. The details of this paradigm were previously reported (Chapter 2).

Tissue Collection Following RAWM protocol animals were euthanized by cervical dislocation. Brain was immediately removed and half of each brain was flash frozen for storage at -80C. For analysis, the brain sample was homogenized using 1ml of homogenization buffer (225mM ultra pure mannitol, 75mM ultra pure sucrose, 5mM

Hepes, 1mM EGTA, pH to 7.4 at 4°C, Sigma). Homogenate protein levels were measured using the standard Lowry protein assay (Protein Assay Reagent A (500-0113) and Protein Assay Reagent B (500-0114), Bio-Rad, Hercules, CA, USA) (Lowry 1951). The other half of each brain was post-fixed in 4% paraformaldehyde and transferred to 30% sucrose after 24 hours. Tissue was allowed to sink in the sucrose solution ensuring full saturation of the tissue before being processed for histology.

Biochemical Measurements

Western Blot for APP Protein levels were measured on the brain homogenates by Western blot analysis. Western blots were performed using a 10% Bis-Tris gel (456-1046,Bio-Rad) and samples were normalized to 25ug protein. Briefly, samples were boiled at 95 °C for 15 minutes in the presence of loading buffer ((25ul) Beta-Mercaptoethanol M6250 Sigma and (475ul) Laemmli Buffer 161-0737, Bio-Rad), vortexed and spun down before being loaded onto the gel. Samples were processed through the gel at 200v for 30 minutes. Gels were then transferred to an Immobilon FL PVDF membrane (IPFL00010, Millipore, Billerica, MA, USA) using the transblot transfer system (170-4155, Bio-Rad) at 2.5A, 10v for 10 minutes. Membranes were blocked ((60 min) 927-40000, LiCor) before being exposed to primary antibodies against amyloid precursor protein (6E10, 39340, Covance, Princeton, NJ, USA) and the housekeeping protein beta-actin (4967, Cell Signaling, Danvers, MA, USA) overnight at 4C. Membranes were then exposed to secondary anti-mouse (6E10) and anti-rabbit (beta-actin) iRdye antibodies (827-08366 and 926-32211, LiCor, Lincoln,

NE, USA). Membranes were imaged using the LiCor Odyssey system and densitometry performed using the Odyssey software (Licor, Lincoln, NE, USA).

Amyloid Beta ELISA Aβ1-40 and Aβ1-42 variants were quantified using commercially available ELISA kit ((40) KHB3481 and (42) KHB3441, Invitrogen, Grand Island, NY, USA). Briefly, standards provided by the company, and samples were measured using a monoclonal primary (rabbit) antibody specific for the NH2-terminus region of Human Aβ 1-40 or 1-42. The bound primary antibody was detected with the use of a horseradish peroxidase-labeled anti-rabbit antibody. Finally a stabilized chromagen solution was added which causes a color change directly proportional to the amount of Human Aβ present in the sample. Absorbance was measured at 450nm using the Victor microplate reader (Wallac 1420, Perkin Elmer, Waltham, MA, USA). Final concentration values of Aβ were calculated as pg/mg.

Western Blot for OxPhos Complex I-V Protein levels were measured on the brain homogenates by Western blot analysis. Western blots were performed using a 10% Bis-Tris gel (456-1046,Bio-Rad, Hercules, CA, USA) and samples were normalized to 25ug protein. Briefly, samples were boiled at 95 **°C** for 15 minutes in the presence of loading buffer ((25ul) Beta-Mercaptoethanol M6250 Sigma and (475ul) Laemmli Buffer 161-0737, Bio-Rad), vortexed and spun down before being loaded onto the gel. Samples were processed through the gel at 200v for 30 minutes. Gels were then transferred to an Immobilon FL PVDF membrane (IPFL00010, Millipore, Billerica, MA, USA) using the transblot transfer system (170-4155, Bio-Rad) at 2.5A, 10v for

10 minutes. Membranes were blocked ((60 min) 927-40000, LiCor) before being exposed to primary antibodies against complexes I-V (OxPhos Cocktail, MS604, Mitosciences, Eugene, OR, USA) and the housekeeping protein beta-actin (4967, Cell Signaling, Danvers, MA, USA). Membranes were then exposed to secondary anti-mouse (OxPhos) and anti-rabbit (beta-actin) iRdye antibodies (827-08366 and 926-32211, LiCor). Membranes were imaged using the LiCor Odyssey system and densitometry performed using the Odyssey software (Licor).

Superoxide Dismutase (SOD) Enzyme Activity SOD activity was measured using a commercially available kit (706002, Cayman Chemicals). Briefly, tissue homogenates were collected and protein value measured using Lowry assay as described above. Homogenate supernatant was combined with a tetrazolium salt solution and xanthine oxidase and allowed to incubate for 20 minutes at room temperature. Absorbance was read at 450nm on a Victor microplate reader (Wallac 1420, Perkin Elmer).

Catalase Enzyme Activity Catalase enzyme activity was measured according to manufacturer's protocol (#707002 Cayman Chemicals). Briefly, tissue homogenates were collected and protein value measured using Lowry assay as described above. Homogenate supernatant was combined with hydrogen peroxide for 20 minutes at room temperature before being incubated with potassium hydroxide and chromagen for 10 minutes at room temperature. Finally samples were incubated with potassium periodate for 5 minutes at room temperature. Absorbance was measured at 405nm using a Victor microplate reader (Wallac 1420, Perkin Elmer).

Lipid Peroxidation Levels Lipid peroxidation levels were measured using a commercially available kit (10009055, Cayman Chemicals). Briefly, tissue homogenates were collected and protein value measured using Lowry assay as described above. Briefly, homogenate supernatant was combined with sodium dodecyl sulfate (SDS) and Color reagent (thiobarbituric acid, sodium hydroxide, and acetic acid) and boiled for one hour. Samples were then placed on ice for 10 minutes and centrifuged at 14,000g for 10 minutes. Absorbance was measured at 540nm using the SpectraMax microplate reader (190, Molecular Devices, Sunnyvale, CA, USA).

Statistical Analyses

All statistical analyses were performed using the JMP software program (Version 10.0, SAS Institute, Inc., Cary, NC, USA).

Multivariate Analysis Behavioral results were analyzed using a multivariate, MANOVA, analysis.

One-Way ANOVA One-way ANOVA was performed for all analyses. Post-hoc analysis was performed using the Tukey Kramer HSD test to compare each treatment group to each other (Table 2).

Table 4.2. Statistical Comparisons were made between groups in each cell by Tukey HSD.

Tukey Test Comparisons	
Biologically Relevant Comparisons	*Non-Biologically Relevant Comparisons*
NTG VEH – DTG VEH	NTG VEH – DTG E2

NTG VEH – NTG E2	NTG VEH – DTG E2Q
NTG VEH – NTG E2Q	NTG E2 – DTG E2Q
NTG E2 – NTG E2Q	NTG E2 – DTG VEH
NTG E2 – DTG E2	
DTG VEH – DTG E2	
DTG VEH – DTG E2Q	
DTG E2 – DTG E2Q	
DTG E2Q – NTG E2Q	

Results

Estrogen levels measured by EIA Circulating estradiol levels were measured to confirm release of compounds from the osmotic pump (Figure 4.1). We did not attempt to quantify the concentration of compound released due to low cross reactivity (<10%) of the estradiol antibody with the quinol compound. Results showed that males had low or non-detectable estradiol levels at week 0. Serum was collected at 0, 4, and 8 weeks of treatment. The males receiving the vehicle did not have detectable levels of estrogen at 4 weeks of treatment as compared to intact vehicle treated males (NTG $p < 0.0001$, DTG $p < 0.0001$) as well as at 8 weeks of treatment (NTG $p < 0.0001$, DTG $p < 0.0001$) (Figure 4.1). Males receiving E2 or E2Q had detectable levels of estrogen at 4 weeks (NTG $p < 0.0001$, DTG $p < 0.0001$) as well as 8 weeks of treatment (NTG $p < 0.0001$, DTG $p < 0.0001$) (Figure 4.1). Additionally, while E2 and E2Q treated males did not significantly differ from each other at 4 weeks, the E2Q treated males had significantly higher levels of estrogen at 8 weeks of treatment ($p = 0.0029$) (Figure 4.1).

RAWM behavioral testing Initial differences in errors and latency between the NTG and DTG vehicle treated groups were observed. On day 1 the NTG vehicle treated

group performed significantly less errors than the DTG vehicle treated group (p = 0.0460)(Figure 4.2). Interestingly, the DTG E2Q treated group performed fewer errors than the DTG vehicle treated group (p = 0.0064) and the DTG E2 treated group (p = 0.0006)(Figure 4.2). On day 2 of testing the DTG E2Q treated group again performed better than the DTG vehicle treated group (p = 0.0056) as well as the DTG E2 treated group (p = 0.0030) (Figure 4.2). On day 3 of testing the DTG E2Q treated group again performed better than the DTG vehicle treated group (p = 0.0001) and the DTG E2 treated group (p = 0.0242)(Figure 4.2). Additionally, the DTG E2Q treated group was not significantly different from all NTG treated groups.

APP protein levels The DTG vehicle treated group had significantly higher levels of full length APP compared to the NTG vehicle treated group (p = 0.0142) (Figure 4.3). However, the APP levels were significantly decreased in both the DTG E2 treated group (p = 0.0065) and also in the DTG E2Q treated group (p = 0.0195)(Figure 4.3). DTG E2 and E2Q treated groups did not differ significantly from any of the NTG treatment groups (representative image Chapter 2).

Reduced Aβ Levels Aβ levels were measured for the 1-40 and 1-42 variants. Across groups there were no significant differences in the amount of Aβ 1-40 (Figure 4.4). However, the DTG vehicle treated group had significantly higher levels of Aβ 1-42 as compared to DTG E2 treated (p < 0.0001) and DTG E2Q treated (p < 0.0001) groups. There was no significant difference observed in the ratio of 42:40 (Figure 4.4).

Superoxide dismutase (SOD), Catalase (CAT) activity, and lipid peroxidation (TBARS) did not change SOD (Figure 4.6) and CAT (Figure 4.7) activity were not significantly different across treatment groups. Additionally no differences were observed for the measure of lipid peroxidation using the TBARS endpoint (Figure 4.5).

ETC complex protein levels Protein levels of the five electron transport chain complexes (I-V) were measured in male brain homogenates (Figure 4.8). There was a significant increase in the protein levels of complex I ($p = 0.0079$), complex II ($p < 0.0001$) and complex IV ($p = 0.0087$) in the DTG vehicle treatment group as compared to the DTG E2Q treated group (Figure 4.8). Additionally the level of complex IV protein in the DTG E2 and E2Q treated groups did not significantly differ from any of the NTG treated groups (Figure 4.8) (representative image Chapter 3).

Discussion

Estrogen levels show delivery of compound Our assay did not detect baseline levels of estrogen in males. Males receiving E2 or E2Q had detectable levels of estrogen at 4 weeks and 8 weeks of treatment, which confirms the delivery of the compound. E2 and E2Q treated males did not significantly differ from each other at 4 weeks, however the E2Q treated males had higher levels of estrogen at 8 weeks of treatment. This is interesting and may have to do with a difference of the metabolism of the E2Q compound within the male system.

Cognitive benefits seen in behavioral testing Initial differences in errors between the NTG and DTG vehicle treated groups were rescued in the E2 and E2Q treated groups by day 3 of testing. Additionally, the DTG E2Q treated group performed fewer errors the DTG E2 treated group as well as the vehicle treated group, indicating the E2Q treatment provided more benefit than the E2.

APP levels decreased with E2 and E2Q treatment Initially we saw the expected difference between the NTG and DTG vehicle treated groups; the DTG vehicle group had significantly higher levels of APP compared to the NTG vehicle treated group. However, with E2 treatment the APP levels of the DTG group decreased, as did the APP levels of the DTG E2Q treated group. DTG E2 and E2Q treated groups did not differ significantly from each other or any of the NTG treatment groups. This indicates the E2 as well as the E2Q were able to bring the level of APP protein in the system down to a level equal to that of the NTG animals. This could provide tremendous benefits in the way of reducing overall APP in patients who carry genes causing them to overexpress APP. However, caution is needed due to the endogenous activity of APP relating to synaptogenesis and synaptic plasticity; reducing APP too much could also be a problem.

Aβ levels were reduced in E2 and E2Q treated groups Across groups there were no significant differences in the amount of Aβ 1-40, which indicates that in the males of the DTG model, E2 and E2Q were not effective at decreasing Aβ 1-40. However, the DTG vehicle treated group had significantly higher levels of Aβ 1-42 as compared

to DTG E2 treated DTG E2Q treated groups. Therefore, E2 and E2Q were effective at decreasing Aβ 1-42. Since Aβ 1-42 is the more hydrophobic, and thus plaque forming, form of Aβ this is a great benefit to the animals. By decreasing overall levels of Aβ E2 as well as E2Q could potentially minimize the amount of senile plaques formed. It follows that we have decreased APP and thus have decreased Aβ, however there is also the possibility for the E2 or E2Q to be effecting formation of Aβ 1-42 through a different pathway.

ETC complex I, II, and IV protein levels increased Protein levels of the five electron transport chain complexes (I-V) were measured in treated males. Our data showed an increase with E2Q treatment of protein complexes I, II and IV. The increase in all these protein levels could be beneficial to the cellular respiration of the cell, thus increasing overall cell viability. Specifically, complex IV is seen in the human literature to be deficient in AD patients, and thus increasing the amount of this protein could be a great benefit to the overall health of their mitochondrial system.

No differences seen in SOD or CAT activity levels No differences were observed in the SOD or CAT enzyme activity levels across treatment groups. While this assay measured total SOD, a difference may likely exist between Cu/ZnSOD and MnSOD. Cu/ZnSOD is cytosolic while MnSOD is specific to mitochondria so looking at the activity and level of MnSOD would be an important test that could elucidate any differences in activity of cytosolic versus mitochondrial SOD. Additionally, total

CAT, SOD, Cu/ZnSOD, and MnSOD enzyme protein levels should be measured to determine if the amount of enzyme available differs among treatment groups.

TBARS levels were not affected TBARS, the measure of lipid peroxidation we used, did not show any differences among treatment groups. Lipid peroxidation is associated with an increased level of ROS in a system. While there is a trend in our data of a higher level of TBARS in the DTG vehicle treated group and a decrease with E2Q treatment, these data were not significant. This may be due to an overall lower level of ROS present in males. Alternatively, this lack of significance is likely due to the large variance within our groups and increasing the number of animals per group may elucidate significant difference.

In summary, the behavioral responses seen in the males is promising because these data show the E2Q compound may beneficial for males as well as females for whom E2Q provided a behavioral benefit as previously reported (Chapter 2). Additionally, the reduction of Aβ fragments, as well as APP as a result of E2 or E2Q treatment indicates E2Q is acting in manner similar to that of E2, and may be acting through the same mechanisms as E2. It has been shown that Aβ release is activity dependent (Cirrito 2008) and so it would interesting to explore possible correlations that may exist between behavioral benefit and reduction of Aβ fragments. Regarding mitochondrial changes, the lack of many significant changes is disappointing, however as discussed other measurements may give us further insight into the mechanisms of E2Q. One promising finding was the

increase of complex IV, which is often deficient in AD patients, and a therapeutic which increases this protein may be beneficial to mitochondrial respiration. Overall, the behavioral benefit and Aβ fragment changes are promising and warrant further research into the mechanisms behind E2Q.
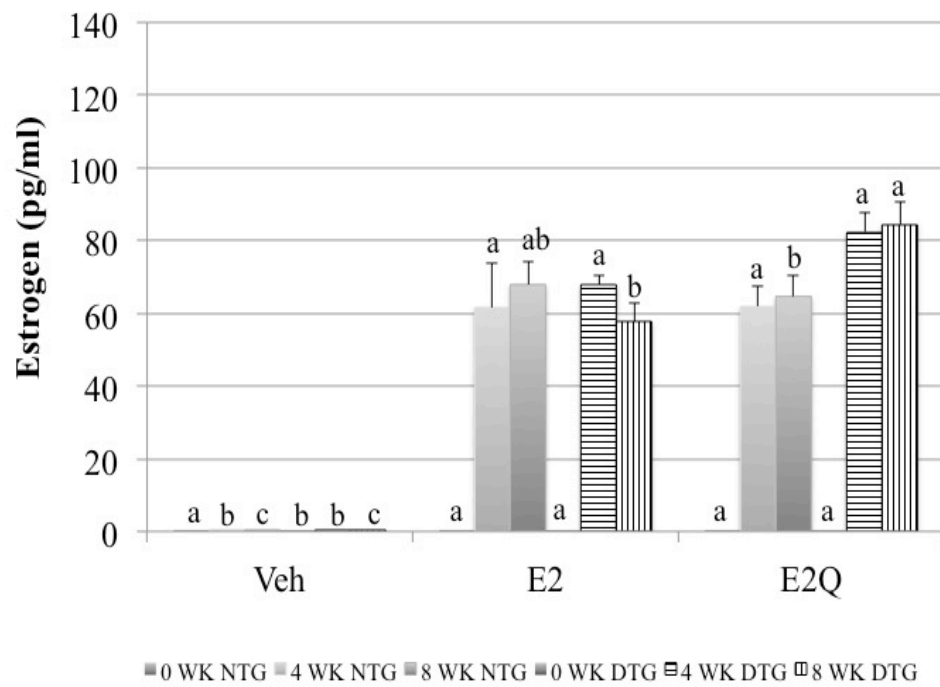
Figure 4.1. Estrogen levels measured using EIA in treated males. Estrogen levels in NTG and DTG males as measured at 0, 4, and 8 weeks of treatment. Data represented as pg/ml ± SEM. N=6 (P ≤ 0.05)

A

B

■ Day 1 ■ Day 2 ▨ Day 3
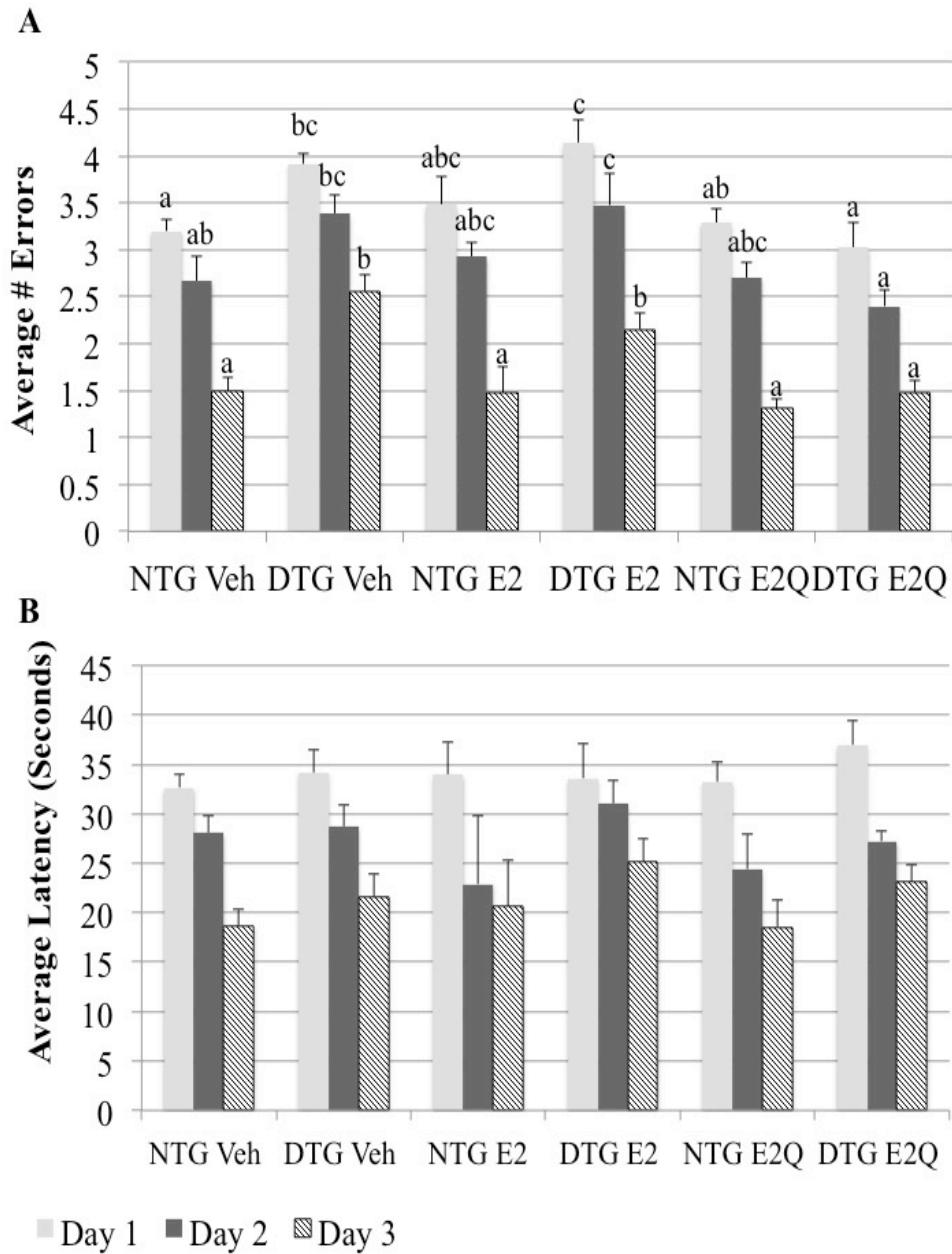
Figure 4.2. Behavioral differences are evident in the E2Q treated group. Radial Arm Water Maze results expressed as average number of errors (A) and average latency to goal (B). Expressed as average for each day ± SEM. ($P \leq 0.05$)
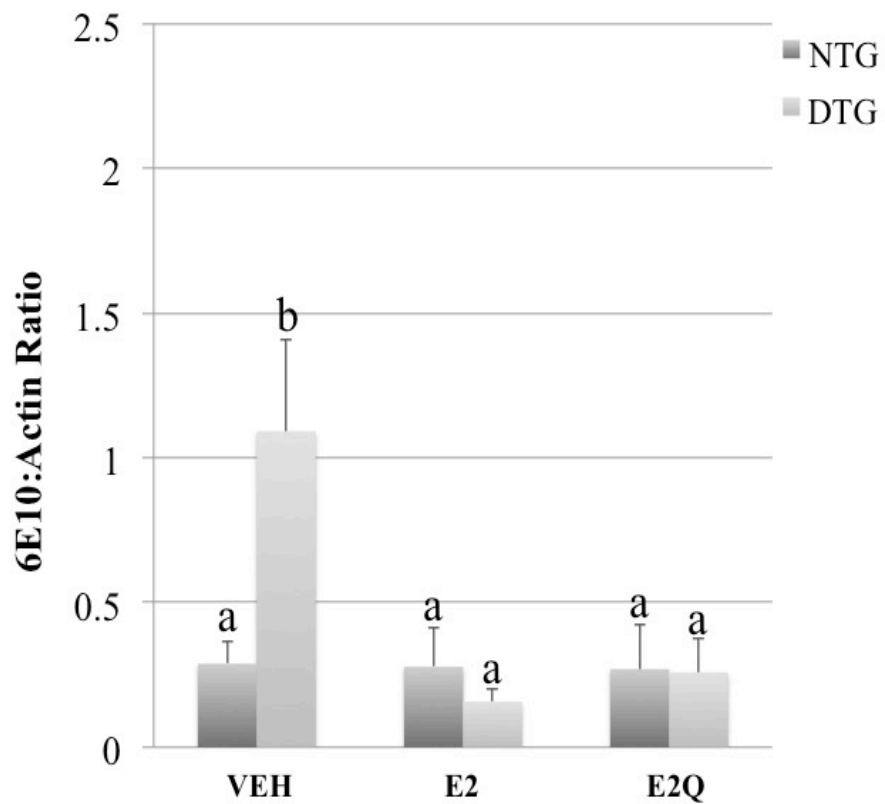
Figure 3. Amyloid Precursor Protein (APP) Levels (Western Blot).
APP (6E10) levels were decreased in the E2 and E2Q treated animal
groups. Protein levels expressed as ratio of β-actin housekeeping
protein ± SEM. N=6-8 (P < 0.001).
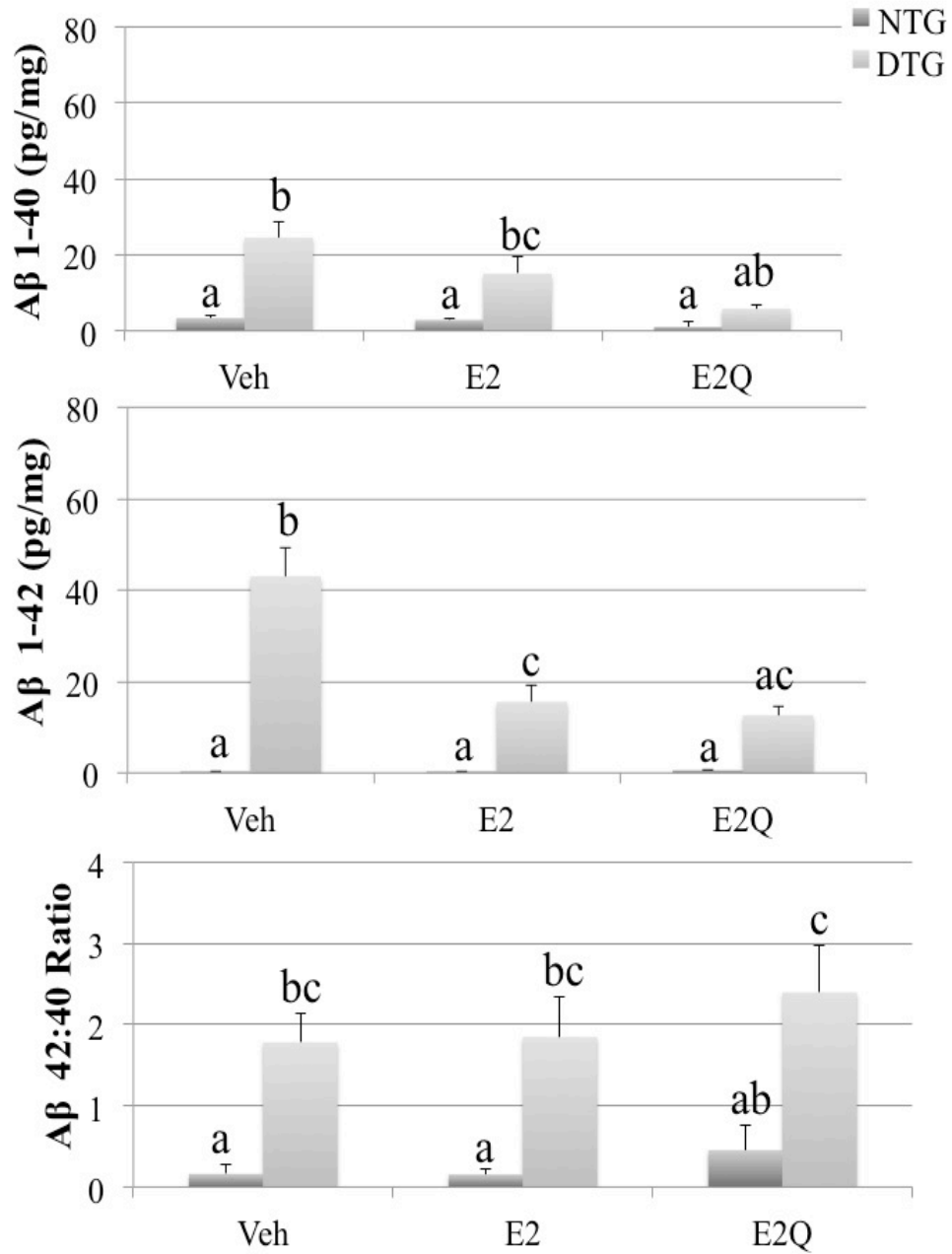
Figure 4. Amyloid-Beta levels decreased in E2 and E2Q treated groups (ELISA). Aβ 1-40 (A) 1-42 (B) and Ratio of 42:40 (C) measured using ELISA. N=6-8 (P < 0.001).
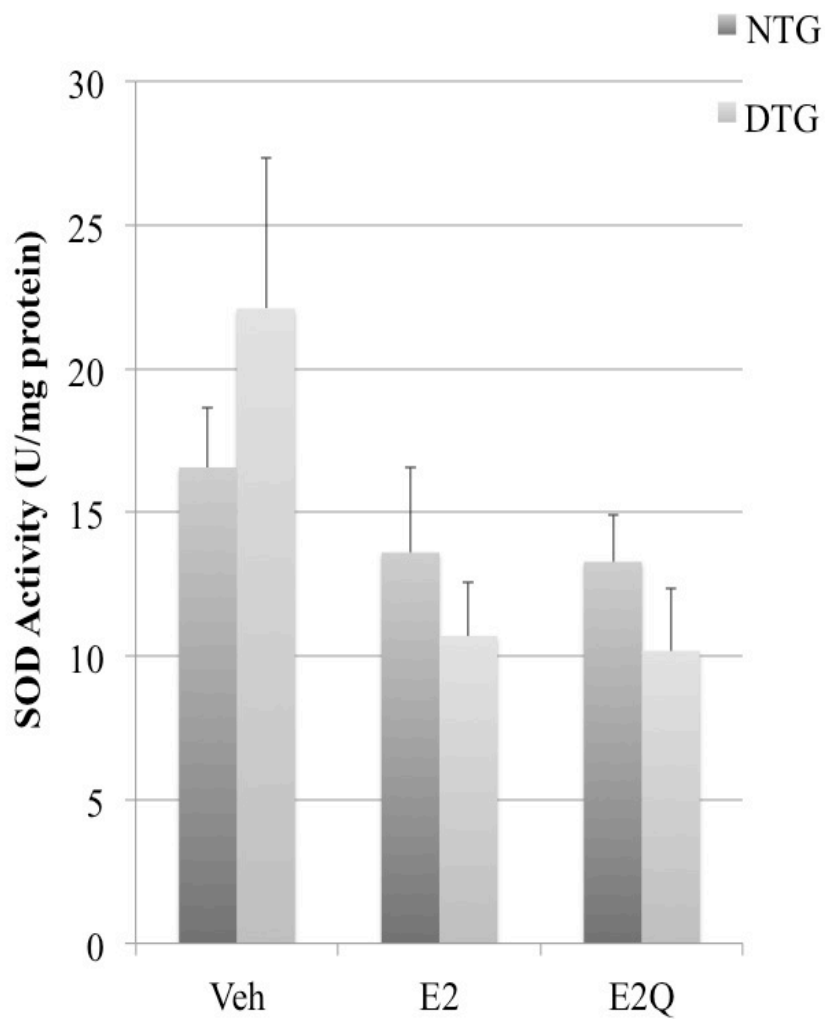
Figure 5. Quinol treatment does not impact SOD activity. Enzyme activity measured using enzymatic assay. Data expressed as activity unit/mg protein ± SEM. N = 6-8.
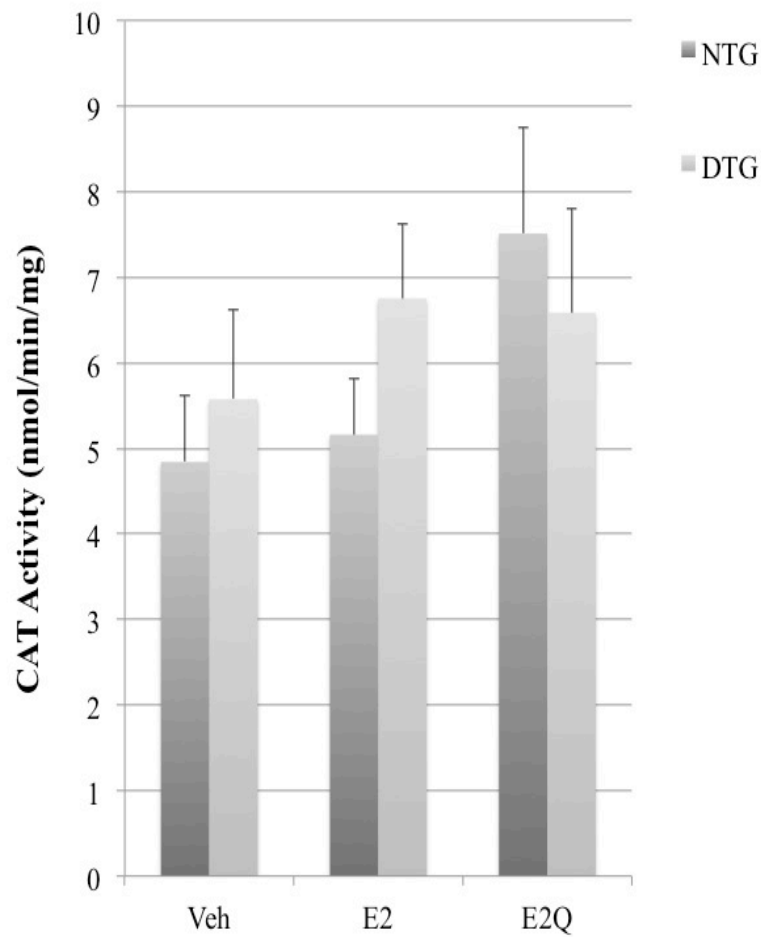
Figure 6. Quinol treatment does not impact CAT activity. Enzyme activity measured using enzymatic assay. Data expressed as activity unit/mg protein ± SEM. N = 7-9.
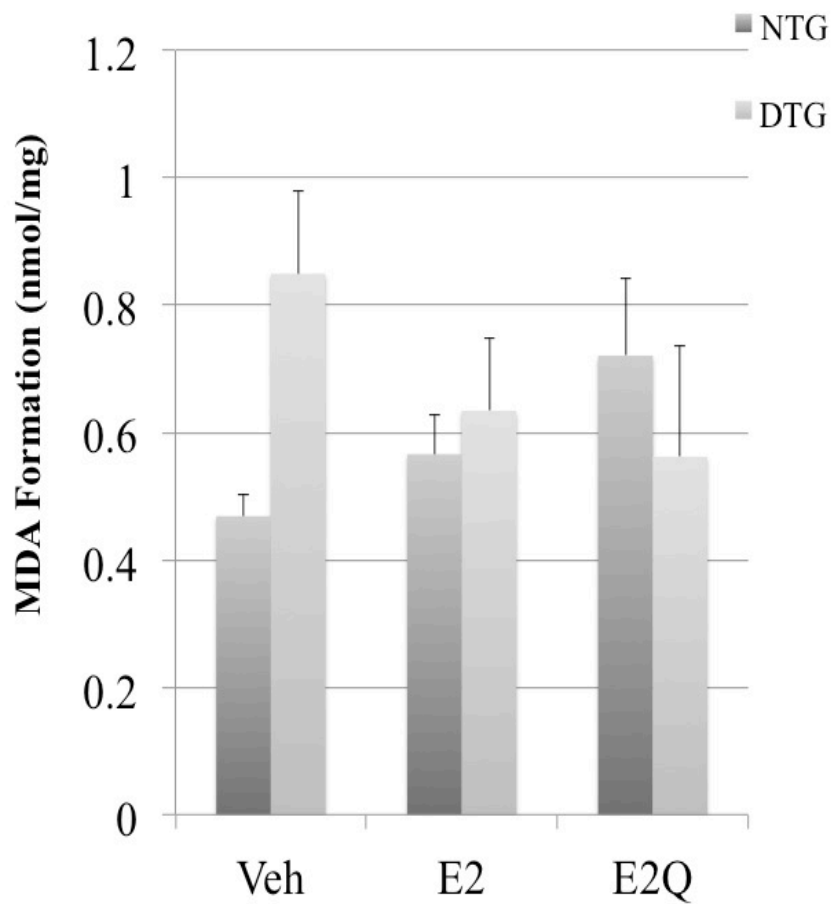
Figure 7. Quinol treatment does not impact lipid peroxidation. Enzyme activity measured using enzymatic assay. Data expressed as activity unit/mg protein ± SEM. N = 7-9.

Figure 8. Quinol treatment altered electron transport chain protein levels. Western blot analysis of protein complexes I-V of the electron transport chain. Data is expressed as a ratio of housekeeping protein β-actin ± SEM. N=6-7.
P < 0.0089

Chapter 5: EFFICACY OF AN ESTROGEN PRO-DRUG IN A TRIPLE TRANSGENIC MURINE MODEL OF ALZHEMER'S DISEASE

<u>Introduction</u>

Alzheimer's disease (AD) is characterized by cognitive and neuronal dysfunction associated with Amyloid-Beta (Aβ) plaques and neurofibrillary tangles (NFTs). Age i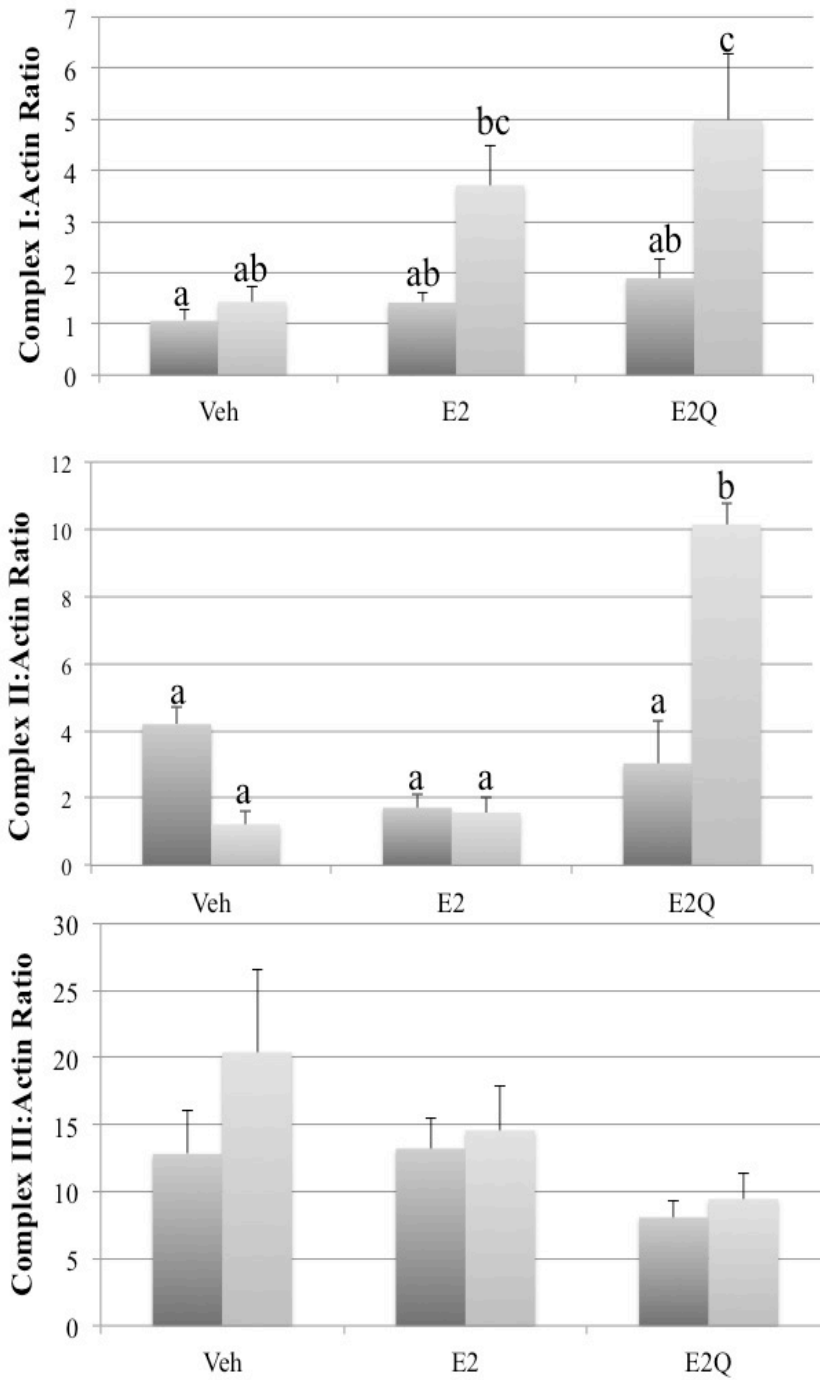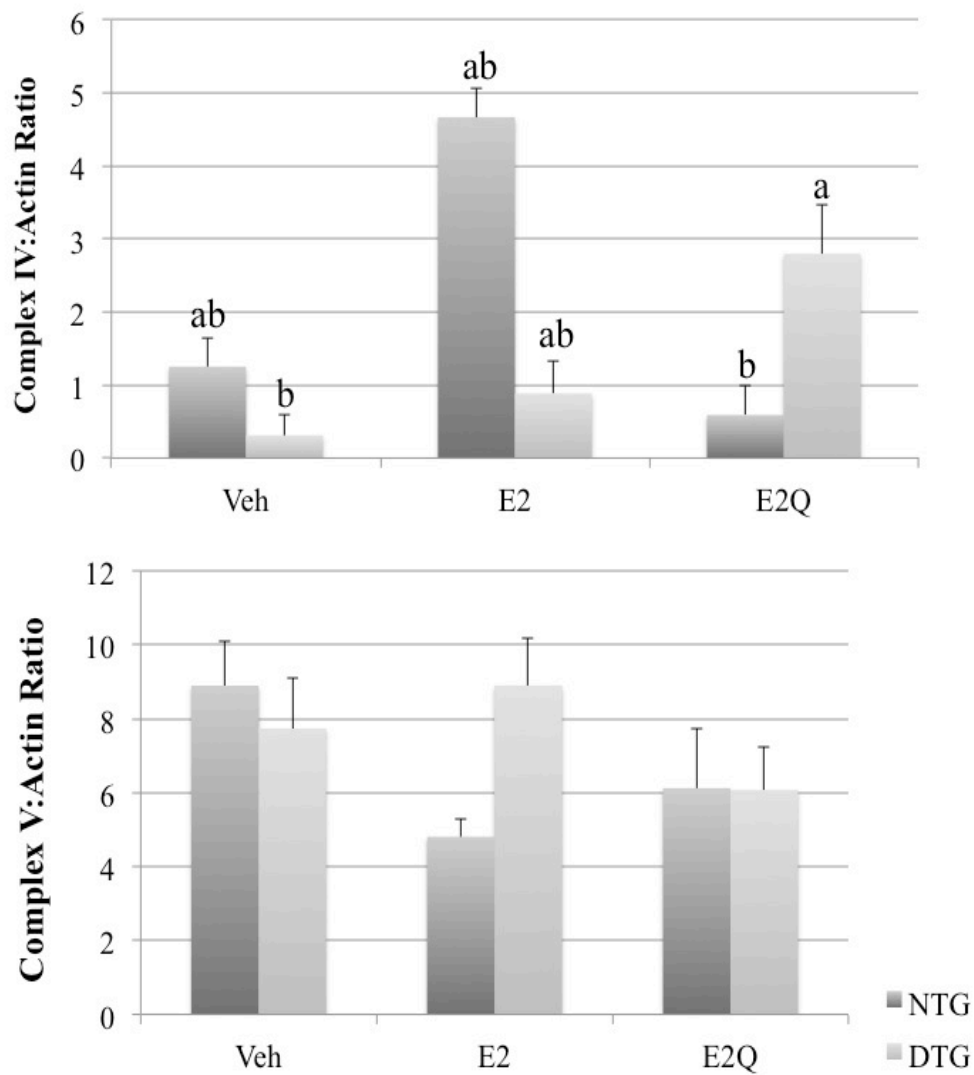s the leading risk factor for the development of sporadic AD, however genetic mutations can increase a person's chance of developing AD (Goate 1991, Kamino 1992, Mullan 1992). Some of these genetic variants have been used to create animal models enabling further study of the pathology of AD. In this study we used a triple-transgenic mouse (3xTgAD) model of early onset AD. The 3xTgAD model possesses three of the human genes for AD; APP Swedish (APP_{SWE}), PS1 M146V ($PS1_{M146V}$), and Tau (Tau_{P301L}) (Oddo 2003). This particular strain displays behavioral deficiencies at 12 months of age that correlate temporally with the appearance of amyloid plaques and neurofibrillary tangles.

Researchers have demonstrated the effect of estrogen within this 3xTgAD mouse model (Chen 2011, Rosario 2012). Estrogens provide various routes of neuroprotection such as direct estrogen receptor activation of the mitogen activated protein kinase/extracellular signal-related kinase (MAPK/ERK) pathway and non-receptor mediated antioxidant activity (Brinton 2001; Simpkins 2012). This antioxidant characteristic is due to the free phenolic hydroxyl group found on the A-ring of estradiol (Badeau 2005). Estrogen treatment has shown to up-regulate genes

associated with neuronal survival such as bcl-2(+), IGFBP-5(+), Calmodulin(+), CaMKIIα(+), Mef2C(+), Connexin43(-), and Jak1 and 2(+ and -)(Manthey and Behl 2006). Additionally, Manthey and Behl identified that ERα and β receptor transfected cells can positively and negatively regulate genes affiliated with AD such as APP(-), PS1(+ and -), and ADAM10(+) (Manthey and Behl, 2006). Other hormones have shown promise as therapeutics in the 3xTgAD model; Allopregnanolone increased the number of BrdU labeled cells (a measure of new cells) and decreased 6E10 staining in the hippocampus of the 3xTg mouse model of AD (3xTgAD)(Chen 2011). Additionally, testosterone reduced AD pathology in the triple transgenic mouse model (Rosario 2012). Gonadectomized (GDX) male 3xTgAD mice, testosterone treatment at the time of GDX prevented a plaque load increase seen with GDX mice (Rosario 2012). These data taken together provide strong evidence for hormone therapeutics to deliver benefits within the 3xTgAD model.

The E2-Quinol (E2Q) pro-drug has allowed for the study of a new class of estradiol pro-drug therapeutics (Laszlo 2001). E2Q, as a pro-drug, enters the system in the quinol form and is metabolized through NADPH reductase, into estrogen. This metabolism occurs in the brain at a rate 200x faster than in the body (Gleason 2006). This high rate of metabolism in the brain leaves more of the estrogen parent compound in the brain allowing for diminished negative peripheral effects.

The effects of E2Q treatment was investigated in the 3xTgAD model. We used the 3XTgAD mouse model because they have different variations of the APP and PS1 genes, and they develop plaques and cognitive dysfunction later than other mouse models. This study examines the effect of the E2Q pro-drug both

ovariectomized and intact animals of the 3xTgAD mouse model of AD. Specifically, we measured the levels estradiol delivered through the treatments, uterine wet weight, total protein level of APP, and total protein level of Aβ.

Materials and Methods

Synthesis and Characterization of E2-quinol Compound Estrogen-derived para-quinol 17β-dihydroxyestra-1,4-diene-3-one (E2-quinol, E2Q) was synthesized from estradiol (E2), as previously reported (Prokai 2000). Briefly, a stirred solution of estradiol (E2, 190mg, E8875, Sigma-Aldrich, St. Louis, MO, USA), 3-chloroperbenzoic acid (m-CPBA, 273031, Sigma-Aldrich, St. Louis, MO, USA), and Benzoyl peroxide $(PhCO)_2O_2$, 33581, Sigma-Aldrich, St. Louis, MO, USA) in 40-60 ml dry carbon tetrachloride/Me_2CO (CCl_4/Me_2CO, 4:1 v/v) was heated to reflux while irradiated with 60 W tungsten lamp (Solaja 1996). Reaction completion was verified by thin layer chromatography. The residue was dissolved in $CHCl_3$ and washed with saturated $NaHCO_3$ to remove m-chlorobenzoic acid; the organic phase was evaporated and the remaining residue was divided into several portions to ensure manageability and then purified by column chromatography on silicagel. For purification the column was washed extensively with dichloromethane to remove any residual E2 followed by elution of E2Q with ethylacetate/dichloromethane mixture ($CH_3COOCH_2CH_3/CH_2Cl_2$, 9:1, v/v). The purity of the compound (white solid) was verified by high performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS). Purity of the final product was >99% with no trace amounts of the starting material.

Animal Husbandry Triple-transgenic mice (3xTgAD) were rederived at the National Institutes of Aging (NIA, Baltimore, MD, USA) from the colony of Dr. Mark Mattson. Mice were received at 3-4 months of age and transferred following IACUC approved protocols. Animals were bred on site (3xTgAD x 3xTgAD), weaned at 21-25 days of age and tail snipped and genotyped at 30-35 days of age. Animals were group housed by gender in an environmentally controlled animal facility on a 12-hour light/dark schedule. Food and water were provided *ad libitum* at the University of Maryland College Park. All animal care and experimental procedures were conducted under the University of Maryland, College Park Institutional Animal Use and Care Committee (IACUC) approved protocols. To minimize any confounds due to estrogenic compounds in the diet, one week prior to experimental treatment animals were placed on a phytoestrogen free diet (AIN-93G, Bio-Serv, Frenchtown, NJ). Following surgical procedures animals were individually housed.

Experimental Design

Treatment Groups Male and female 3xTgAD mice (11.5-12 months) were used for this study to compare the different hormone profiles in intact animals of the same transgenic background. Animals were treated with vehicle (propylene glycol, P4347, Sigma, St. Louis, MO), E2 (2ug/day), or E2-quinol (2ug/day) (N = 5). Due to the long ½ life of the quinol compound in the body, the Alzet osmotic minipumps (2004, 0.25ul/min, Durect Corp., Cupertino, CA) were used to deliver the appropriate dose over the 8 week period of treatment. Pumps were replaced once, at the 4 week time

point. Compounds were dissolved in USP-grade propylene glycol, a substance in which both estrogen and the E-quinol compound are freely soluble and which is compatible with Alzet pumps. Animals were lightly anesthetized using isoflorane at an appropriate dose for mice according to the AALAS recommendation. After anesthetization, a small patch of hair was removed from the upper back of the mouse and the area disinfected. A small incision was made on the operative area and the Alzet osmotic pump implanted subcutaneously between the scapulae. The incision was closed, sutured and allowed to heal. Following surgery, animals recovered on a surgical grade (DCT-15, Kent Scientific Instruments, Torrington, CT) heating pad under continuous observation before being placed in a cage containing clean, dry bedding, atop a heating pad. No special post-operative care was generally required. Animals were monitored daily for possible infection and to ensure wounds remained clean, the animal maintained a healthy body weight, and there were no adverse effects.

Table 5.1. Treatments and number of males and females in each treatment group

	M	F
Vehicle	4	5
E2 (2ug/day)	5	5
E2-Quinol (2ug/day)	5	5

Estradiol EIA Validation An estradiol (E2) EIA was validated and serum samples were processed as previously reported (Chapter 2). Uterine wet weight was also measured in female mice as previously reported (Chapter 2).

Tissue Collection Following Animals were euthanized by cervical dislocation. Brain was immediately removed and half of each brain was flash frozen for storage at -80C. For analysis, the brain sample was homogenized using 1ml of homogenization buffer (225mM ultra pure mannitol, 75mM ultra pure sucrose, 5mM Hepes, 1mM EGTA, pH to 7.4 at 4°C, Sigma-Aldrich, St. Louis, MO, USA). Homogenate protein levels were measured using the standard Lowry protein assay (Protein Assay Reagent A (500-0113) and Protein Assay Reagent B (500-0114), Bio-Rad, Hercules, CA, USA). The other half of each brain was post-fixed in 4% paraformaldehyde and transferred to 30% sucrose after 24 hours. Tissue was allowed to sink in the sucrose solution ensuring full saturation of the tissue before being processed for histology.

Histology

Processing and Immunochemistry for APP Serial sections of 50um thickness were collected using a freezing microtome (860, American Optical AO, Buffalo, NY, USA) and sections stored in cryobuffer (30% Sucrose (S5-3, Fisher Scientific, Fair Lawn, NJ, USA) 1% Polyvinylpyrrolidone (BP431, Fisher Scientific) 30% Ethylene Glycol (102466, Sigma-Aldrich, St. Louis, MO, USA) until staining was performed. Tissue was washed in 1x TBS to remove cryobuffer residue and treated with 1% hydrogen peroxide (H2O2) (30 min, 325-100, Fisher Scientific) to block endogenous peroxidase activity. Tissue was then washed again in 1x TBS and treated with 0.3% Triton X-100 (10min, BP151, Fisher Scientific, Fair Lawn, NJ, USA) followed by 5% Normal Goat Serum (NGS) (30 min, S-1000, Vector Labs, Burlingame, CA, USA). Tissue was then exposed to the primary antibody overnight at 4C while on a shaker.

Amyloid isoforms and soluble APP were identified using antibodies against APP (6E10) (1:300, 39340, Covance, Princeton, NJ, USA). Tissue was then washed and treated with the Vectastain ABC kit (90 min, PK-6100, Vector Labs, Burlingame, CA, USA), washed again, and exposed to the secondary antibody (90min, Biotinylated anti-Mouse IgG, BA-9200, Vector Labs). For color development the tissue was then treated with 3,3'-Diaminobenzidine tetrahydrochloride (DAB, D5905, Sigma-Aldrich) for 5 minutes. Tissue was then immediately washed in 1x TBS and mounted on subbed slides. Tissue was allowed to dry overnight on slides before being processed through nuclei stain of Cresyl Violet, dehydrations, and coverslipped.

Semi-quantitative Measurement of Immunostaining Quanitification of plaque number and size was performed using ImageJ software (NIH, Bethesda, MD, USA). For each animal, 5 tissue slices ranging from early hippocampal to late hippocampal tissue were measured. Measurements were taken at 2,5X magnification and included the entire hippocampal region (CA1, CA2, CA3, and dentate gyrus) as well as the cortex areas (including arcuate nucleus, motor cortex, and somatosensory cortex) surrounding the hippocampus. Color de-convolution was used to separate the DAB stain from the cresyl violet nuclear stain. Images were then transformed to 8-bit gray scale images and stain threshold was adjusted to select for visually observed plaque. Computer generated number and size of plaque within one frame was reported. Measurements from the 5 tissue slices were averaged for each animal.

Biochemical Measurements

Western Blot for APP Protein levels were measured on the brain homogenates by Western blot analysis. Western blots were performed using a 10% Bis-Tris gel (456-1046,Bio-Rad, Hercules, CA, USA) and samples were normalized to 25ug protein. Briefly, samples were boiled at 95 °C for 15 minutes in the presence of loading buffer ((25ul) Beta-Mercaptoethanol M6250 Sigma-Aldrich and (475ul) Laemmli Buffer 161-0737, Bio-Rad), vortexed and spun down before being loaded onto the gel. Samples were processed through the gel at 200v for 30 minutes. Gels were then transferred to an Immobilon FL PVDF membrane (IPFL00010, Millipore, Billerica, MA, USA) using the transblot transfer system (170-4155, Bio-Rad) at 2.5A, 10v for 10 minutes. Membranes were blocked ((60 min) 927-40000, LiCor, Lincoln, NE, USA) before being exposed to primary antibodies against amyloid precursor protein (6E10, 39340, Covance, Princeton, NJ, USA) and the housekeeping protein beta-actin (4967, Cell Signaling, Danvers, MA, USA) overnight at 4C. Membranes were then exposed to secondary anti-mouse (6E10) and anti-rabbit (beta-actin) iRdye antibodies (827-08366 and 926-32211, LiCor). Membranes were imaged using the LiCor Odyssey system and densitometry performed using the Odyssey software (Licor).

Amyloid Beta ELISA Aβ1-40 and Aβ1-42 variants were quantified using commercially available ELISA kit ((40) KHB3481 and (42) KHB3441, Invitrogen, Grand Island, NY, USA). Briefly, standards provided by the company, and samples were measured using a monoclonal primary (rabbit) antibody specific for the NH2-terminus region of Human Aβ 1-40 or 1-42. The bound primary antibody was detected with the use of a horseradish peroxidase-labeled anti-rabbit antibody. Finally a stabilized chromagen

solution was added which causes a color change directly proportional to the amount of Human Aβ present in the sample. Absorbance was measured at 450nm using the Victor microplate reader (Wallac 1420, Perkin Elmer, Waltham, MA, USA). Final concentration values of Aβ were calculated as pg/mg.

Statistical Analyses

All statistical analyses were performed using the JMP software program (Version 10.0, SAS Institute, Inc., Cary, NC, USA).

Multivariate Analysis Behavioral results were analyzed using a multivariate, MANOVA, analysis.

One-Way ANOVA One-way ANOVA was performed for all analyses. Post-hoc analysis was performed using the Tukey Kramer HSD test to compare each treatment group to each other (Table 2).

Table 5.2. Statistical Comparisons were made between groups in each cell by Tukey HSD.

Tukey Test Comparisons
Biologically Relevant Comparisons
3xTgAD VEH – 3xTgAD E2
3xTgAD VEH – 3xTgAD E2Q
3xTgAD E2 – 3xTgAD E2Q

Results

Estrogen levels measured by EIA Circulating estradiol levels were measured to confirm release of compounds from the osmotic pump (Figure 5.1). We did not attempt to quantify the concentration of compound released due to low cross

reactivity (<10%) of the estradiol antibody with the quinol compound. Results showed that males had low or non-detectable estradiol levels at week 0. Serum was collected at 0, 4, and 8 weeks of treatment. The males receiving the vehicle did not have detectable levels of estrogen at 0, 4, or 8 weeks of treatment (Figure 5.1). Males receiving E2 or E2Q had detectable levels of estrogen at 4 weeks ($p < 0.0001$) as well as 8 weeks of treatment ($p < 0.0001$) (Figure 5.1). Additionally, while E2 and E2Q treated males did not significantly differ from each other at 4 weeks or 8 weeks of treatment (Figure 5.1). Females receiving E2 had higher levels of estrogen than vehicle treated females at 4 weeks of treatment ($p = 0.0004$) as well as at 8 weeks of treatment ($p = 0.0001$) (Figure 5.1). Females receiving E2Q had higher levels of estrogen than vehicle treated females at 4 weeks of treatment ($p < 0.0001$) as well as at 8 weeks of treatment ($p = 0.0011$) (Figure 5.1).

Uterine wet weight confirms lack of peripheral tissue stimulation Uterine wet weight was measured as gram weight of the uterine tissue to confirm lack of uterine tissue stimulation with the E2Q compound (Figure 5.2). There was a significant difference ($p<0.001$) (Figure 5.2) between the vehicle, E2, and E2Q treated groups. E2Q treated mice did not show evidence of uterine tissue stimulation.

APP levels decrease in females APP levels in the E2 treated females was decreased in compared to both vehicle treated ($p = 0.0175$) and E2Q treated ($p = 0.0010$) groups (Figure 5.3). There was no statistical difference between the E2 and E2Q treated female groups (Figure 5.3). APP levels decreased with E2Q treatment in females but

not males (Figure 5.3). No significant differences were observed in the males treated with E2 or E2Q compared to vehicle treated males.

Aβ40 and 42 levels are reduced in E2Q treated males Aβ42 levels in E2Q treated males significantly decreased (P<0.001) (Figure 5.4) as compared to the vehicle treated group. While absolute protein levels were affected, the ratio of 42:40 remained the same for males. Female Aβ levels were not affected (Figure 5.4).

Hippocampal and cortex plaque load is not detected Immunohistochemistry for measurement of the number of plaques occurring within the hippocampus and cortex of 3xTgAD showed plaques were observed at this age (data not shown).

Discussion

Release and Effects of E2Q A novel compound was tested for efficacy in providing neuroprotective effects of estrogen while avoiding adverse peripheral effects associated with estrogen. Although the EIA had limited cross reactivity (<10%) with the quinol compound, there was sufficient E2Q measured in the plasma samples from treated males and females to demonstrate effective release of the compound from the minipumps. In the female treated animals uterine tissue was not stimulated by the E2Q compound. This confirms previous research (Prokai, personal communication) showing little or no stimulation of peripheral steroid sensitive tissues by E2Q. It is interesting to see the higher uterine wet weight in our vehicle treated females, which

we attribute to their age and the assumption that these mice are in a stage of constant estrus.

Effect of E2Q on APP Our data demonstrated that both the E2 and E2Q treated female 3xTgAD animals showed an overall decrease in APP levels measured in brain homogenate. In the male 3xTgAD group we did not see a decrease in APP. Our female data compliments previous research, which showed that estrogen activation of the estrogen receptor (both α and β subtypes) down-regulates the APP gene (Manthey and Behl 2006). Based on this previous research, we can speculate that our current findings may indeed be a result of a genetic effect, however further studies must be performed to determine the accuracy of that hypothesis. Certainly, the decrease in APP would be beneficial to an AD patient as a pathway to decrease overall Aβ production and subsequent plaque formation.

Impact on Aβ 40 and 42 As with APP, we saw a decrease of Aβ 40 and 42 with E2 or E2Q treatment. While it follows that with less APP we would expect less Aβ, we cannot confirm if this decrease in Aβ is due to the decrease in APP or a result of degradation of the Aβ itself. We know this DTG model begins forming plaques at 12 months, thus a decrease of Aβ protein at the 12 month time point indicates the quinol may be useful as a therapeutic after the onset of AD. Furthermore, the action of the Presenilin enzyme regarding APP cleavage and production of Aβ fragments may play an important role, especially in this particular 3xTgAD mouse model.

Aβ Plaque Load in E2Q Treated Mice Plaque was not detected using the 6E10 staining in our 3xTgAD mice. It has been shown in a previous data (Duffy, personal communication) that our 3xTgAD does not show detectable levels of plaque within

the hippocampus at the 12 -14 month age. Other regions of brain tissue should be examined to determine any changes in other areas such as the subbiculum, which do show plaques at the 12-14 month age (Duffy, personal communication).

In summary, the E2 and E2Q treatment groups appeared to be beneficial based on biochemical changes in the 3xTgAD mouse. The greatest benefit of E2Q compared to E2 is the lack of uterine tissue stimulation very clearly seen in our study. We also observed a marked decrease in APP and Aβ protein levels, which may benefit AD patients. Due to the age of pathology onset of this transgenic line, further studies are critical to determine overarching effects of E2Q. The positive effects of E2Q, combined with effectiveness similar to that of the E2, makes this compound potentially useful as a therapeutic intervention.
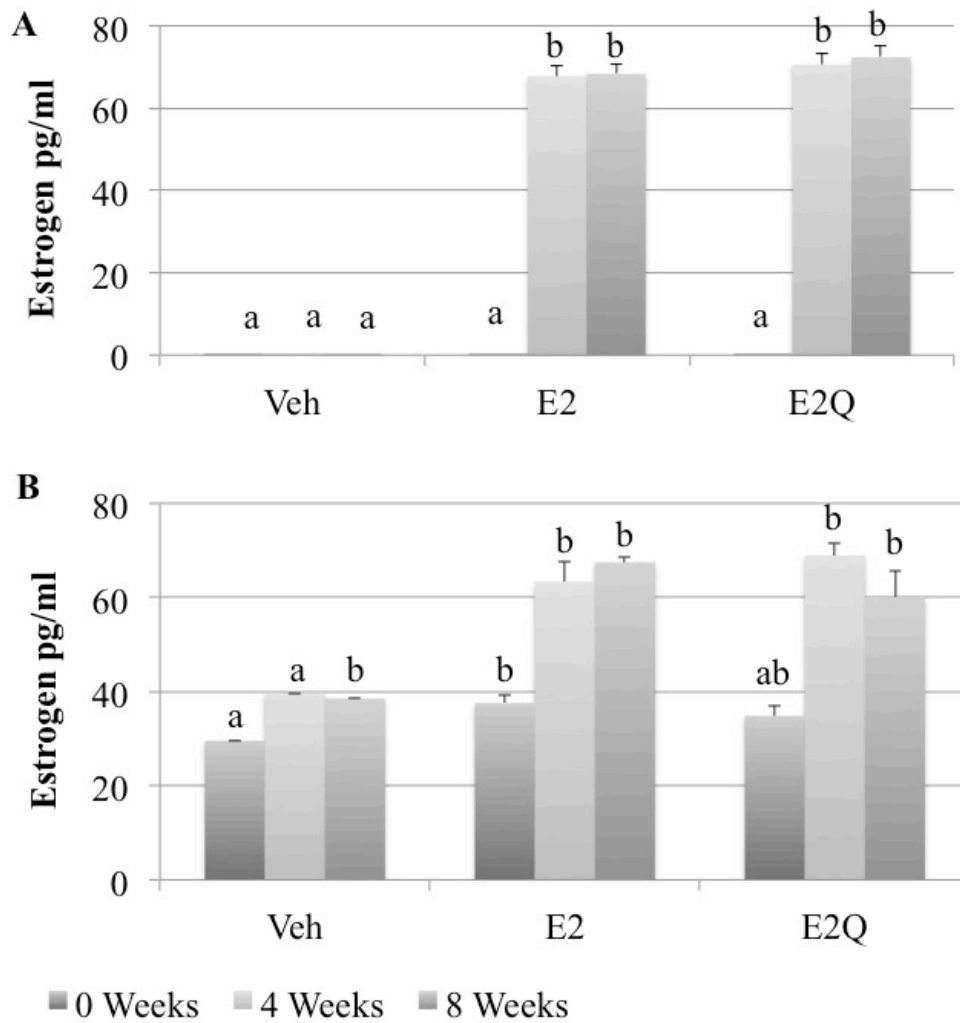
Figure 5.1. Estrogen levels measured using EIA in treated 12 month males and females. A. Estrogen levels in males as measured at 0, 4, and 8 weeks of treatment **B**. Estrogen levels in females as measured at 0, 4, and 8 weeks of treatment. Data represented as pg/ml ± SEM. N=5, (P ≤ 0.05).
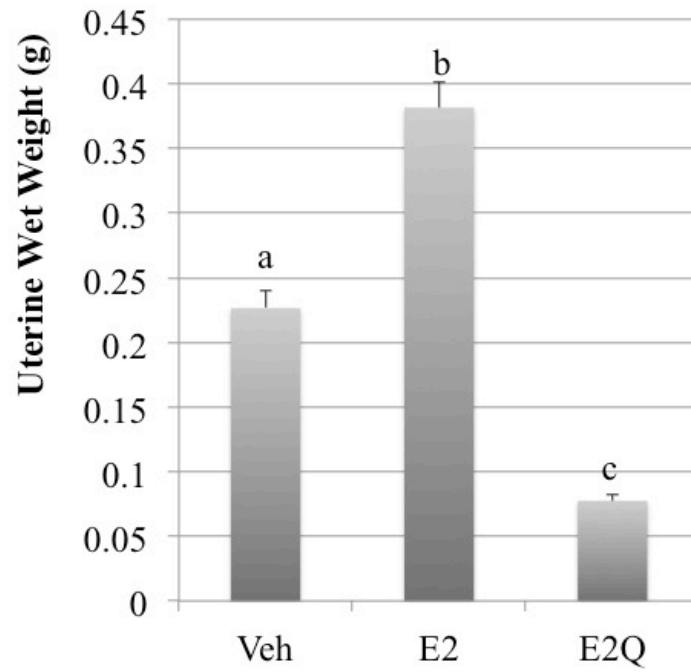
Figure 5.2. Uterine wet weight in treated 3xTgAD females shows E2Q does not stimulate uterine tissue. Uterine wet weight as measured by gram weight in 12-month old females. Data represented as gram weight of tissue ± SEM. N = 5, (P ≤ 0.05)
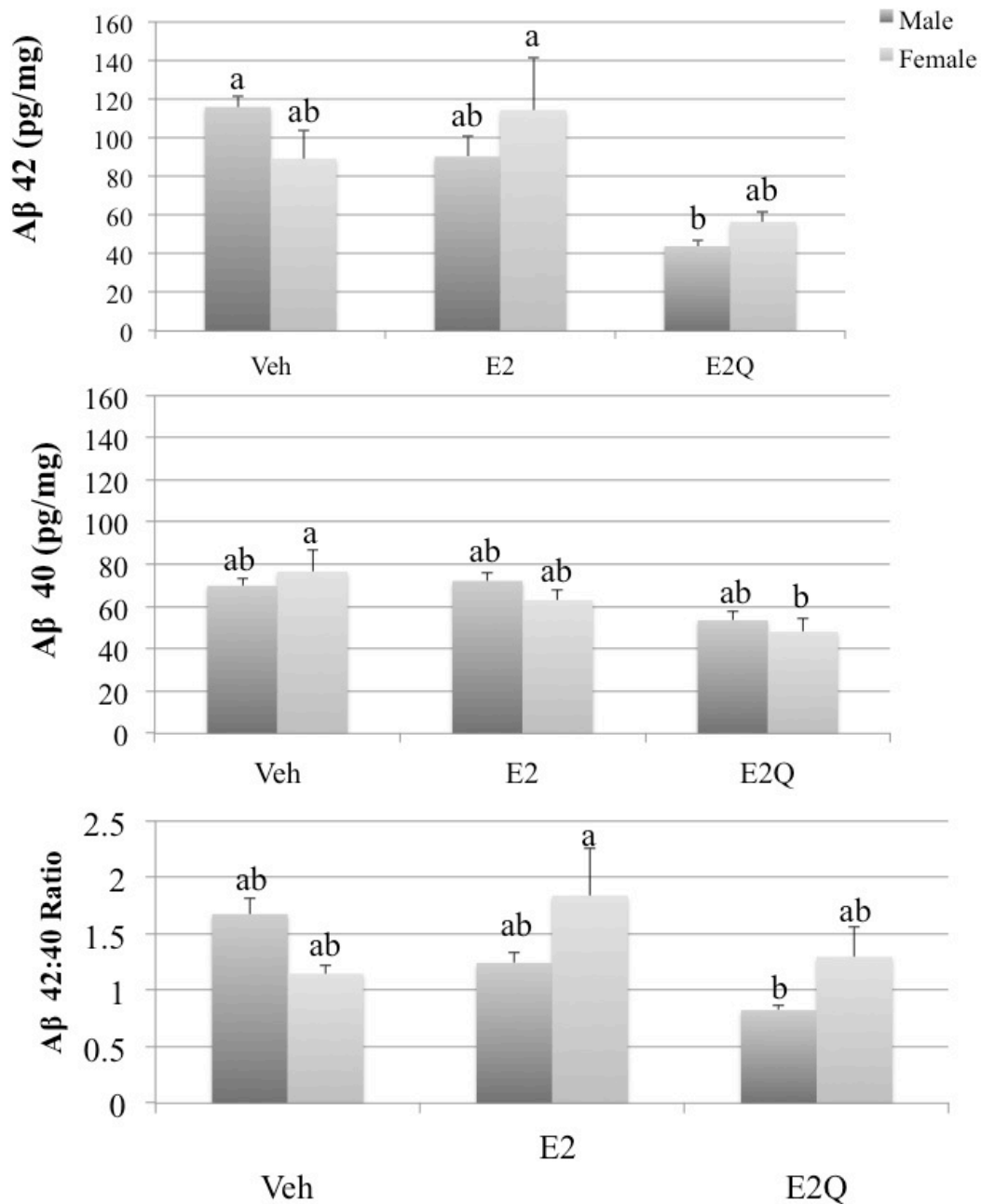
Figure 5.4. Amyloid beta levels as measured by ELISA. Protein level of amyloid beta 1-40, 1-42, and 42:40 ratio measured 3xTgAD in 12-month old males and females. Protein levels are expressed as pg/mg ± SEM. Ratios are expressed as ratio ± SEM. N=6, (P ≤ 0.05).
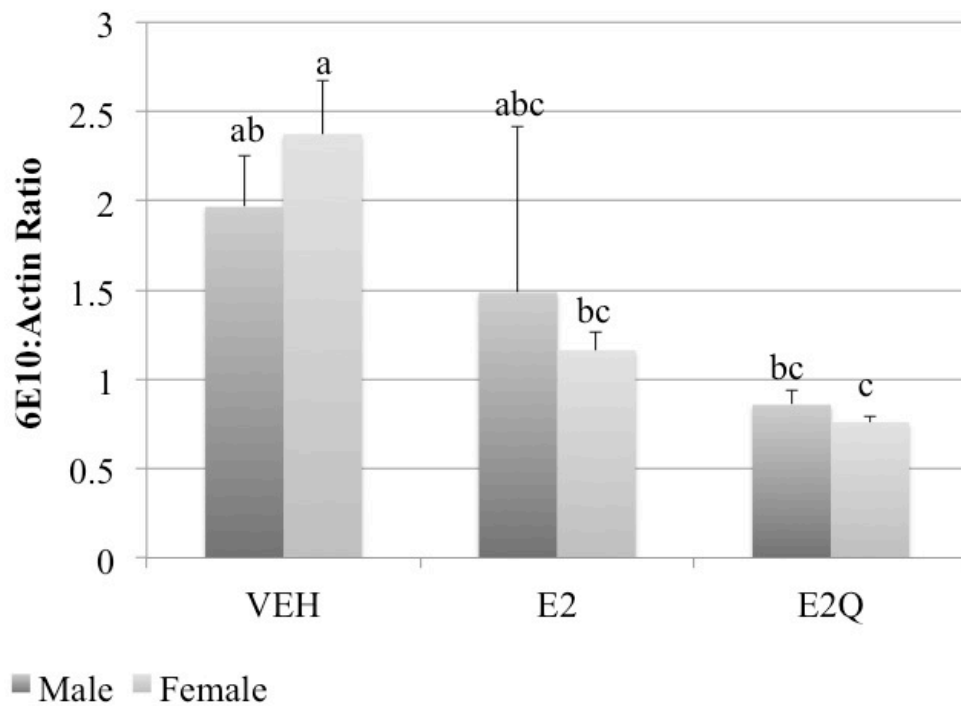
Figure 5.3. APP (6E10) levels are altered in females. APP levels as measured with western blot using the 6E10 antibody in 12-month old males and females. Protein levels expressed as ratio of β-actin housekeeping protein ± SEM. N=4-5, (P ≤ 0.05).

Chapter 6: Concluding Remarks

Concluding Remarks

The beneficial effects of estrogens are often over shadowed by their negative peripheral effects (Resnick 1997; Rossouw 2002). Here we show the quinol compound provides a benefit at both the cognitive and molecular level, but does not stimulate uterine tissue like estradiol (E2).

In the first study we assessed the proper delivery of the estradiol-quinol (E2Q). The estradiol EIA we used recognized the E2Q in the treated animals enabling us to confirm appropriate delivery of the compound. However, there was no stimulation of the uterine tissue, which is an important component of these studies. This lack of uterine stimulation will allow E2Q to be used in situations where estrogen sensitive tissue stimulation is a concern. We also saw a decrease in errors performed by both intact and OVX females of the E2Q treatment group. These data demonstrate that E2Q is beneficial at ameliorating the cognitive deficits seen in the DTG AD mouse model. Additionally, overall levels of APP and Aβ were decreased in the E2 and E2Q treated animals. These data compliment previous research, which showed that estrogen activation of the estrogen receptor (both α and β subtypes) down-regulates the *APP* gene (Manthey and Behl 2006). Based on this previous research, we can speculate that our current findings may be a result of a gene level effect, however further studies must be performed to determine the accuracy of that hypothesis. In general though, a decrease in APP and Aβ protein level would be beneficial to an AD patient as a pathway to

decrease overall Aβ production and subsequent plaque formation. Our data did not show a significant difference in the overall plaque load. This may be due to the fact that E2Q is not able to degrade plaques already formed, and instead may decrease further production of plaque by impacting levels of the APP and Aβ proteins.

In the second study, we focused on electron transport chain complexes, lipid peroxidation, and antioxidant activity. Estradiol levels were the same as in the first study, including the lack of uterine tissue stimulation. Our data showed there was an increase of protein complex IV in the OVX E2 and E2Q treated females, which could be extremely beneficial in regards to humans with AD since decreased complex IV protein levels have been demonstrated in the AD population (Cardoso 2004). Additionally, the increase of protein complex IV in the OVX AD system is a positive result since this shows us the OVX insult can be overcome in some aspects with the E2 or E2Q treatments. We also saw an increase of protein complexes II and III, which could also be beneficial in overall cell viability by increasing the cell's capacity to perform oxidative phosphorylation. Additionally we examined superoxide dismutase (SOD) activity levels and found DTG OVX females showed significantly lower SOD levels in the vehicle and E2 treatment groups as compared to the NTG OVX E2 treatment groups. These data indicate there are initial differences in the DTG OVX groups and regardless of treatment SOD activity levels were not increased by E2 or E2Q. Similarly lipid peroxidation, which is a measure of MDA formation relating to overall reactive oxygen species (ROS) increased in the NTG E2Q OVX group (Table 6.1). This response could be due to a lack of endogenous estrogens. Since the E2Q would primarily affect the brain, the lack of circulating estrogen could leave the

peripheral system vulnerable to increased ROS. Differential effects of E2 and E2Q are shown in table 6.1.

In all treatment groups we saw the proper delivery of each compound including vehicle, E2, and E2Q. The estradiol EIA we used recognized the E2Q in the treated animals and thus we were able to confirm delivery of the compound. One of the most important findings was that uterine wet weight was not stimulated by E2Q. All OVX females treated with E2Q had low uterine wet weights, similar to those of the vehicle treated females, indicating that E2Q did not stimulate the uterine tissue as was seen with E2 treatment. In the E2Q treated 3xTgAD females, uterine wet weights were actually lower than vehicle treated females. This is an interesting finding and one reason for this difference between the DTG and 3xTgAD findings may be the age of the 3xTgAD (Table 6.2) females (12 months) at treatment.

In the behavioral testing we saw a cognitive benefit in vehicle treated DTG animals compared to the E2Q treated animals for intact, OVX, and male groups. All three groups made fewer errors in the E2Q treated DTG groups on the third day of testing as compared to the vehicle treated DTG controls. This benefit was seen across all three treatment paradigms and indicates the strong benefit of E2Q in both males and females.

Overall total APP protein level was altered in all treatment groups (DTG and 3xTgAD) except in the OVX DTG females. This decrease in APP levels is consistent with previous research, which showed estrogen activation of estrogen receptors decreased APP gene levels (Manthey and Behl 2006). However, this decrease was not observed in the OVX treated females in any treatment groups. It is possible the

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surgical OVX procedure was stressful. The osmotic pump insertion was performed immediately following OVX and established circulating E2 levels similar to intact females. Therefore, we would expect the E2 treated animals to look similar to the vehicle treated intact females, however, this is not the case. These results indicate that OVX may have additional effects. At this point, the reason or their difference is unclear.

Amyloid-beta (Aβ) levels were decreased with E2 and E2Q and it logically follows that there would be lower APP levels. However, other mechanism may be involved here because there were decreased Aβ levels in the OVX treated females, without decreased APP levels. Possibilities include the alteration of presenilin activity due to the estrogen treatments, or a change in neprilysin levels as a result of the estrogen treatments. Neprilysin acts on Aβ and would not have effects on APP levels.

Overall, positive changes were observed in behavioral responses, decreases in APP protein level and Aβ levels with E2Q. These changes combined with the lack of uterine tissue stimulation make E2Q unique in that it combines the positive benefits of estradiol without the negative peripheral characteristics of stimulation of estrogen sensitive tissues. Further studies should aim at determining optimal dose and treatment period. Additionally, the role of the presenilin enzymes and tau protein in response to the changes seen here are important factors to be assessed.

Table 6.1 Summary of findings from chapter 2 and 3.

		Wet Weight	RAWM	Histology	AB ELISA	APP	Complexes*	TBARS	SOD	CAT
Intact	NTG Veh	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ
	DTG Veh	NΔ	-	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ
	NTG E2	+ +	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ
	DTG E2	+ +	++	NΔ	-	-	NΔ	NΔ	NΔ	NΔ
	NTG E2Q	NΔ	NΔ	NΔ	NΔ	NΔ	+ **III, II**	NΔ	NΔ	NΔ
	DTG E2Q	NΔ	++	NΔ	-	-	+ **I**	NΔ	NΔ	NΔ
OVX	NTG Veh	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ
	DTG Veh	NΔ	-	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ
	NTG E2	+ +	NΔ	NΔ	+ +	NΔ	+ **II**	NΔ	NΔ	NΔ
	DTG E2	+ +	+	NΔ	+ +	NΔ	+ **I**	NΔ	NΔ	NΔ
	NTG E2Q	NΔ	NΔ	NΔ	NΔ	NΔ	+ **IV, V**	++	NΔ	NΔ
	DTG E2Q	NΔ	++	NΔ	NΔ	NΔ	+ **IV, V**	NΔ	NΔ	NΔ

- *Roman numeral indicates complex level that changed
- + or – signs indicate positive or negative changes respectively
- NΔ = no change

	RAWM	AB ELISA	APP	Complexes *	TBARS (MDA)	SOD	CAT
N V	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ
D V	-	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ
N E2	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ
D E2	-	-	- -	+ I	NΔ	NΔ	NΔ
N E2Q	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ	NΔ
D E2Q	+ +	- -	- -	+ I, II, IV	NΔ	NΔ	NΔ

Table 6.3 Summary of findings from chapter 5.

	Uterine Wet Weight	AB ELISA	APP	Histology
TTG Veh F	NΔ	NΔ	NΔ	NΔ
TTG E2 F	++	NΔ	-	NΔ
TTG E2Q F	-	-	- -	NΔ
TTG Veh M	NM	NΔ	NΔ	NΔ
TTG E2 M	NM	NΔ	NΔ	NΔ
TTG E2Q M	NM	-	- -	NΔ

- *Roman numeral indicates complex level that changed
- + or – signs indicate positive or negative changes respectively
- NΔ = no change
- NM = measurement not taken for this group

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