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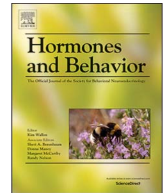

An exploratory investigation of brain-selective estrogen treatment in males using a mouse model of Alzheimer's disease

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ABSTRACT

Estrogens are neuroprotective, and studies suggest that they may mitigate the pathology and symptoms of Alzheimer's disease (AD) in female models. However, central estrogen effects have not been examined in males in the context of AD. The purpose of this follow-up study was to assess the benefits of a brain-selective 17β-estradiol estrogen prodrug, 10β,17β-hydroxyestra-1,4-dien-3-one (DHED), also in the male APPswe/PS1dE9 double-transgenic mouse model of the disease. After continuously exposing 6-month old animals to DHED for two months, their brains showed decreased amyloid precursor and amyloid-β protein levels. The DHED-treated APPswe/PS1dE9 double transgenic subjects also exhibited enhanced performance in a cognitive task, while 17β-estradiol treatment did not reach statistical significance. Taken together, data presented here suggest that DHED may also have therapeutic benefit in males and warrant further investigations to fully elucidate the potential of targeted estrogen therapy for a gender-independent treatment of early-stage AD.

1. Introduction

Alzheimer's disease (AD) is characterized by cognitive and neuronal dysfunctions associated with amyloid-beta (Aβ) plaques and neurofibrillary tangles. AD affects men and women differently (Laws et al., 2016; Irvine et al., 2012). So far, there has not been clear explanation for the gender differences in AD, or in other central nervous system-related disorders (Musicco, 2009; Carter et al., 2012; Zagni et al., 2016). Therefore, it is important to include both sexes in basic science studies, especially if preclinical drug candidates are evaluated in animal models of neurodegenerative diseases.

Due to the prevalence of AD in females (Lobo et al., 2000; Callahan et al., 2001; Irvine et al., 2012), most studies concentrate on the use of estrogen-deprived female animals owing to the logical correlation between loss of endogenous estrogens and increased incidence of neurodegeneration in women (Baum, 2005). As such, only a few studies have been devoted to the potential beneficial effect of estrogens in males, especially in the context of AD (Rosario et al., 2010; Carroll and Rosario, 2012). Estrogens have long been shown to be neuroprotective *via* a variety of mechanisms pertinent to the neuropathology of neurodegenerative diseases, including AD (Lan et al., 2015; Davey, 2017). Additionally, estrogens may also act as neuroprotective antioxidants (Prokai et al., 2003; Prokai-Tatrai et al., 2008, 2013) to ameliorate some effects of oxidative stress-related injury and redox dysregulation that have been implicated in the initiation and/or progression of neurodegenerative diseases (Von Bernhardi and Eugenin, 2012). In spite of evidence for neuroprotection, the use of the main human estrogen, 17β-estradiol (E2), in AD as a preventative or therapeutic agent is yet to be fully justified in clinical setting (Wharton et al., 2009; Correia et al., 2010).

While the rate of occurrence in men is lower than in women, genetic mutations, as in familial AD, also can increase the probability of developing AD in males (Bird, 2008; Bekris et al., 2010). Particular genetic variants have been utilized in developing animal models to mimic

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pathology of AD. Others and our laboratory have been using the APPswe/PS1dE9 double-transgenic AD model (abbreviated below as DTG), which possesses a chimeric mouse/human amyloid precursor protein (APP) Swedish gene ($APP695_{SWE}$) and the human PS1 delta-E-9 ($PS1_{dE9}$) gene (Heikkinen et al., 2004; Jankowsky et al., 2004; Tschiffely et al., 2016). This mouse model displays behavioral deficiencies at seven months of age (Reiserer et al., 2007) that correlate temporally with the appearance of plaques (Jankowsky et al., 2004) making it suitable to study early apperance of the disease.

Previously we had shown that treatment with 10β,17β-dihydroxyestra-1,4-dien-3-one (DHED), a brain-selective prodrug of E2 that produces the hormone only in the brain (Prokai et al., 2015; Merchenthaler et al., 2016), decreased Aβ levels in the brain of ovariectomized female DTG mice and, consequently, these animals had higher cognitive performance than the untreated control group (Tschiffely et al., 2016). This beneficial effect was similar to those treated with the parent E2. However, the distinguishing feature of chronic DHED administration was the lack of peripheral E2 formation, indicating therapeutic safety. As a continuation of this previous study, the present investigation focused on assessing the AD-therapeutic potential of DHED in males of the same DTG mouse model in terms of slowing down the progression of AD characteristics onset, including the reduction of Aβ formation and protecting against cognitive impairment. We hypothesized that administration of DHED would provide therapeutic benefit against early-stage AD mimicked by the selected animal model of the disease in a gender-independent fashion.

2. Materials and methods

2.1. Chemicals

17β-dihydroxyestra-1,4-diene-3-one (DHED) was synthesized from E2, as reported before (Prokai et al., 2015). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

2.2. Animals

Transgenic APPswe/PS1dE9 mice (obtained from The Jackson Laboratory, Bar Harbor, ME, USA) were bred and maintained through the laboratory of Dr. Rosemary Schuh (Veterans Affairs Maryland Health Care System, Baltimore, MD). At the age of 3–4 months, they were transferred from Dr. Schuh's colony to the University of Maryland College Park according to Institutional Animal Care and Use Committee (IACUC) approved protocols. Mice were then bred at the University of Maryland and additional animals were transferred from the colony as needed for the duration of the study. The APPswe/PS1dE9 hemizygote genotype was maintained by crossing a female C57BL/6 mouse (The Jackson Laboratory) with a male APPswe/PS1dE9. Animals were bred on site and weaned at 21–25 days of age, tail snipped and genotyped at 30–35 days of age. They were group-housed by sex in an environmentally controlled animal facility on a 12/12 h light/dark schedule. Food and water were provided *ad libitum*. All animal care and experimental procedures were conducted under the University of Maryland, College Park IACUC approved protocols. To minimize any confounding factor of estrogenic compounds in the diet, one week prior to initiating treatments all animals, including controls, were placed on a phytoestrogen free diet (AIN-93G, Bio-Serv, Frenchtown, NJ, USA) to eliminate dietary estrogen sources. Age-matched males lacking AD pathology in our breeding colony (referred to below as non-transgenic animals, NTG) were used as controls in experiments involving the DTG subjects. Accordingly, the following treatment groups were formed for the present study (with seven to nine animals assigned to each group): NTG-VEH, NTG-DHED, NTG-E2, DTG-VEH, DTG-DHED, DTG-E2, where VEH denotes propylene glycol vehicle only treatment.

2.3. Treatment schedule

As our murine model expresses behavioral deficits at 6–7 months of age correlating temporally with the appearance of amyloid plaques, the experimental design focused on evaluation at 8 months of age (Jankowsky et al., 2004; Reiserer et al., 2007). Additionally due to the early initiation of estrogen therapy required to obtain a beneficial outcome treatment, our study began at 6 months of age in APPswe/PS1dE9 male mice (Sherwin, 2005). Animals (5.5–6 months, N = 7–9) were treated with vehicle (propylene glycol), E2 (2 μg/day), or DHED (2 μg/day) *via* subcutaneously (s.c.) implanted Alzet osmotic minipumps (0.025 μL/min, Durect Corp., Cupertino, CA) over the 8-week period of treatment. The concentration of the experimental agent (E2 or DHED) was 56 μg/mL. Pumps were replaced once, at the 4-week time point analogously to our recently reported studies (Prokai et al., 2015; Tschiffely et al., 2016). Testing started 24–48 h after finishing treatment with the experimental agents.

2.4. Radial arm water maze (RAWM) behavioral testing

A radial-arm water maze (Alamed et al., 2006) was used to measure cognitive deficits of the mice post-treatment period. Experimental set up was identical to that of previously reported (Tschiffely et al., 2016). On day 1 of training, twelve trials that alternated between a hidden and visible platform followed by three trials of all hidden platforms were done. On days 2 and 3, fifteen hidden platforms were used. Each mouse was assigned to the same goal arm throughout testing. The start arm changed for each trial and if the mouse did not locate the platform within 60 s, the mouse was gently directed to the platform and allowed to rest there for 10 s before being removed from the pool. A 60-second cutoff time was chosen to ensure endurance and stamina for the animals. An error was recorded as an entry *via* all four paws into an incorrect arm or the goal arm without successful location of the platform. An error score did not require that the animal swim to the back of the arm entirely before turning around. All animals were scored by the same observer blinded to the treatment schedule from a consistent site in the testing facility.

2.5. Tissue collection

Immediately following behavioral testing, animals were euthanized by cervical dislocation. The brain was immediately removed and half of each brain was flash frozen, then stored at −80 °C until further processing *via* homogenization using 1 mL of homogenization buffer (consisting of 225 mM ultra-pure mannitol, 75 mM ultra-pure sucrose, 5 mM Hepes, 1 mM EGTA, pH to 7.4) at 4 °C for biochemical and molecular analyses.

2.6. Western blot for APP

Proteins (25 μg) as determined by the standard Lowry method from forebrain homogenates were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% precast Bis-Tris gels (Bio-Rad, Hercules, CA, USA) and transferred to an Immobilon FL polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) using a Trans-Blot Turbo transfer system (BioRad). Membranes were blocked for 60 min before exposure to primary antibodies against APP (6E10, Covance, Princeton, NJ, USA) and β-actin (Cell Signaling, Danvers, MA, USA) overnight at 4 °C. Then membranes were exposed to secondary anti-mouse (6E10) and anti-rabbit (β-actin) IRdye antibodies and imaged using an Odyssey system (LI-COR, Lincoln, NE, USA). Densitometry was performed using the Odyssey software (LI-COR) and measurements were expressed as ratio of APP to β-actin.

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