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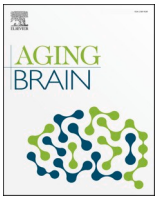

Estrogen's sex-specific effects on ischemic cell death and estrogen receptor mRNA expression in rat cortical organotypic explants

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ABSTRACT

Estrogens, such as the biologically active 17-β estradiol (E2), regulate not only reproductive behaviors in adults, but also influence neurodevelopment and neuroprotection in both females and males. E2, contingent upon the timing and concentration of the therapy, is neuroprotective in female and male rodent models of stroke. *In Vivo* studies suggest that E2 may partially mediate this neuroprotection, particularly in the cortex, via ERα. *In Vitro studies,* utilizing a chemically induced ischemic injury in cortical explants from both sexes, suggest that ERα or ERβ signaling is needed to mediate the E2 protection. Since we know that the timing and concentration of E2 therapy may be sex-specific, we examined if E2 (1 nM) mediates neuroprotection when female and male cortical explants are separately isolated from postnatal day (PND) 3–4 rat. Changes in basal levels ERα, ERβ, and AR mRNA expression are compared across early post-natal development in the intact cortex and the corresponding days in vitro (DIV) for cortical explants. Following ischemic injury at 7 DIV, cell death and ERα, ERβ and AR mRNA expression was compared in female and male cortical explants. We provide evidence that E2-mediated protection is maintained in isolated cortical explants from females, but not male rats. In female cortical explants, the E2-mediated protection at 24 h occurs secondarily to a blunted transient increase in ERα mRNA at 12 h. These results suggest that cortical E2-mediated protection is influenced by sex and supports data to differentially treat females and males following ischemic injury.

Introduction

Sex and age differences in stroke risk and outcome are apparent [1–4]. Premenopausal females have a lower incidence of stroke with better outcomes than postmenopausal females and males [5,6]. Estrogen (17β-estradiol, E2), one of the ovarian hormones depleted with menopause, is thought to be associated with this protection. Since the average age of menopause has remained constant at 51 years, while the average life expectancy for females exceeds 80 years of age, females are spending the last 30 years of their life in a

Abbreviations: 2DG/KCN, 2-deoxyglucose/ potassium cyanide; E2, 17β-estradiol; AR, androgen receptor; DIV, day (s) *In Vitro*; ER, estrogen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; hrs., hours; PND, post-natal day.

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vulnerable hypo-estrogenic state [7]. Early menopause (before 45 years of age) increases the risk of stroke [8]. In as much as E2 appears to be protective in premenopausal females, it can also increase the risk of stroke when given to females many years after postmenopausal [9–13]. While the neuroprotective effects of E2 is less clear in humans [9,10,14–19], E2 treatment in female and male rodent models of stroke reduces infarct volumes or improves neurological deficits [20–25]. This highlights the need to understand how sex and E2 mechanistically mediate this protection.

E2 is known to act on membrane-bound G-protein-coupled receptors (GPER), known as ER-X, rapidly [26–32], directly or indirectly on the mitochondria [33], and transcriptionally through the well-characterized nuclear receptors: estrogen receptor: alpha (ERα) and beta (ERβ) [34,35] and androgen receptors (AR) [36]. In the cortex, a complex region of the brain receptive to E2-mediated neuroprotection, estrogen receptors expression are dynamic throughout development and following injury. Specifically, ERα mRNA expression decreases across early postnatal development in rodents and remains low in the uninjured adult cortex, but increases following injury in the adult cortex [37]. Similarly, ER-X is expressed postnatally, absent in the adult, and increases following injury [32]. ERβ increases across early postnatal development and decreases with aging [38,39]. AR expression also increases across early postnatal development in the cortex [40] and in the adult cortex depending on the different phases of the estrus cycle [41]. Little is known about how the expression of these receptors change in the cortex of females and males with E2 treatment.

One way to simplify how the cortex intrinsically functions is to isolate it away from other regions of the brain and maintain explant slices in culture where a heterogeneous population of neurons and glial cells communicate. Initial studies showed that E2 was protective in cortical explants following a chemically, 2-deoxyglucose/ potassium cyanide (2DG/KCN) induced ischemic injury [42,43]. 2DG/KCN blocks glycolysis and oxidative phosphorylation, respectively [43], which are both altered during ischemic stroke. The E2-mediated ischemic protection was attenuated in cortical explants with ICI 182 780, which is a nonspecific estrogen receptor (ERα or ERβ) antagonist and an ER-X agonist. Additionally, cell death was not altered with 17α-estradiol, a preferred ligand for ER-X [44,45]. While these studies suggest an ERα or ERβ dependent cortical neuroprotective mechanism [42], AR is also known to alter ERα and ERβ signaling and mRNA expression in many cancers [46–49]. Since previous studies did not separate cortical explants based on sex, in the present study, we address whether ERα, ERβ, and AR mRNA expression change similarly to the intact cortex in female and male cortical explants and if E2 influences cell death and ERα, ERβ and AR mRNA expression following ischemic injury.

Material and methods

Animal Care and Housing

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health as well as the ARRIVE guidelines. Pregnant Sprague Dawley rat dams were purchased from Harlan Laboratories (Indianapolis, IN) and maintained in constant temperature conditions on a 12-hour light/dark cycle. Dams were provided food and water ad libitum. Pups were phenotypically sexed (~125 female and ~116 male total pups) and taken from their mother at postnatal day (PND) 3–4 for explants or PND 4, 10, and 18 for cortical tissue, with PND 0 as the day of birth. For the PND 25 time-point, pups were weaned from their mother at PND 22, sexed and placed in separate cages until PND 25.

Collection of cortical brain tissue

Animals were sacrificed by rapid decapitation and the brains removed. On ice, the cortex from the nucleus accombans to the beginning of the hippocampal formation, (Bregma 1.8 to 0 mm (PND4), 1.40 to −1.00 mm (PND10), and 0.8 to −1.20 mm (PND18-25)) [50], was dissected from the corpus callosum with care not to include the piriform cortex, striatum and the hippocampus. Tissue stored at −80 °C until processing.

Collection of cortical explants

Cortical explants are isolated from PND 3–4 rat pups, as previously described [39,51,52]. Pups were sexed (71 female and 82 male pups) and brains were isolated and sectioned, 300 μm, in cold dissection medium containing Gey's balanced salt solution (G9779, Sigma-Aldrich, Saint Louis, MO), 0.2 M MgCl2 and 37.5 % glucose on a vibratome from Bregma 1.8 to 0 mm [50]. Approximately 8–10 slices were harvested per brain. All same sex cortical slices, within a single litter, were group together in cold dissection medium plus ketamine HCl (Ketaset, NLS Animal Heath), the primary and secondary motor cortex along with the somatosensory cortex was dissected away from the corpus callosum, piriform cortex, striatum, and hippocampus. Cortical slices were collected and separated into individual hemispheres[50]. Three to four individual cortices were plated on Millicell-CM membranes (PICMO3050, Fisher, Hampton, NH) in wells containing 1X Basal Medium Eagle (B9638, Sigma-Aldrich), Hanks' Balanced Salt Solution (14025, Invitrogen), heat-inactivated horse serum (3H30074.03, Fisher), 37.5 % glucose in Gey's BSS, glutamax (35050, Invitrogen, Carlsbad, CA), and penicillin/streptomycin (15140, Invitrogen) with vehicle (0.01 % EtOH) or E2 (1 nM 17 β-estradiol (122323, Steraloids, Inc) in 0.01 % EtOH). Explants remained in culture at 34 °C with 5 % CO_2 for 1 to 22 days. Medium ± E2 was changed every three days. Healthy explants are transparent with smooth edges while overfed explants become opaque and underfed explants thin to the point that they are undetectable [52].

RNA isolation across development and time in culture

Cortical tissue was collected at PND 4 (28 female and 14 male pups), PND 10 (10 female and 6 male pups), PND 18 (8 female and 13 male pups), and PND 25 (8 female and 7 male pups) for RNA isolation. Cortical explants (dissected from 28 female and 27 male pups) at 1, 7, 15, 22 day (s) *In Vitro* (DIV) were collected and homogenized in TriZol (Invitrogen) for RNA isolation, per manufacturer's protocol. To ensure enough RNA, 3 explants were collected and combined for each n. The resulting pellet was air-dried and resuspended in RNase-free water (DEPC) (BP561) at 56 °C for 10 min. RNA was stored at −80 °C until reverse transcription.

Reverse transcription

One μg of total RNA was reverse-transcribed to cDNA. RNA (1ug, 20 μl) with 1 μl of Random Primers (58875, Invitrogen) and 1 μl of 10 mM dNTP's (U1515, Promega) was incubated at 65 °C for 5 min. 8 μl of Master Mix containing 4 μl of 5x first strand buffer (Y02321, Invitrogen), 2 μl of 0.1 M DTT (Y00147, Invitrogen), 1 μl of RNasin (N211B, Promega) and 1 μl of Superscript II RT (100004925, Invitrogen) was added to each reaction and incubated at room temperature for 10 min, 42 °C for 50 min, then 70 °C for 15 min. The cDNA was stored at −80 °C until quantitative real-time PCR.

Quantitative Real-Time PCR

For real-time PCR, each reaction contained 10.125 μl DEPC H_20, 12.5 μl of Brilliant II SYBR@Green QPCR Master Mix (Agilent Technologies, 600828), 0.375 μl of Reference Dye (diluted 1:500) (Agilent Technologies), 0.5 μl of forward + 0.5 μl of reverse primer (His 3.1[53], ERα[54], ERβ[55], and AR[56]), and 1 μg of cDNA. Primer-specific concentrations were previously optimized for single PCR product with no primer-dimer formation. Each sample was run in triplicate with non-template controls. The cycling parameters were as follows: 1 cycle at 95 °C for 10 min, 40 cycles of 95 °C for 30 s, annealing temperature for 1 min, 72 °C for 30 s, and 1 cycle of 95 °C for 1 min and 55 °C for 30 s.

Injury

The deprivation of glucose and oxygen that occurs with ischemic stroke in the brain can be chemically induced with 2DG/KCN by blocking glycolysis and oxidative phosphorylation, respectively [43]. 2DG is a glucose analog that has a hydrogen in place of a hydroxyl group[57]. Hexokinase traps 2DG in the cell by adding a phosphate (2DG-P), which cannot continue in glycolysis as a substrate for phosphoglucose isomerase [58–61]. KCN binds the iron in cytochrome *c* and inhibits the mitochondrial electron transport chain from transferring electrons to oxygen and disrupts ATP production [62].

Explants at 7 DIV (isolated from 42 female pups and 60 male pups for propidium iodide (PI) analysis and 29 female and 35 male pups for RNA analysis) were treated with 1 mM 2-DG and 0.5 mM KCN (2DG/KCN) to inhibit glycolysis and oxidative phosphorylation [43] for 2 h in 1X Basal Medium Eagle (BME) or BME (control) as previously described [42,43]. Following injury, medium ± E2 was replaced and explants were treated with propidium iodide, to determine the extent of cell death, or collected for RNA isolation, as previously described in section 2.4) at 8, 12, and 24 h.

Cell death

Explants were washed with 0.1 M PBS and incubated with 5 μg/ml of PI (1 mg/mL in H2O, P4170, Sigma-Aldrich) in BME for 30 min. Explants were washed (0.1 M PBS) and visualized using a fluorescent microscope. PI entered cells that had a porous cell membrane, indicating damage, and bound to DNA. PI uptake indicated cell death and fluoresced red (emission at 630 nm) under green light (excited at 495 nm). Pictures, 20X magnification of explants, were captured using an image capture program, SPOT Advanced. Red (dead) cells per frame were then counted using an NIH program, Image J. Pictures were coded and analyzed blindly. Injured explants were compared back to the corresponding vehicle-treated non-injury for each hour and sex. As a positive control for ischemic injury, 2DG/KCN treatment had to increase PI uptake by > 20 % in the vehicle group.

Statistics

For mRNA studies, female and male cortical tissue and cortical explants were collected from at least 3 different litters at each of the time-points (PND 4, 10, 18, and 25) or days in culture (1, 7, 15, and 22 DIC), respectively. In the figure legends, n represents the number of data points. *In Vivo* n correlates to number of pups used to isolate cortex. *In Vitro* n depends on the type of experiment with RNA analysis collecting 3 explants in trizol per n to ensure enough mRNA for the analysis (i.e., 3 explants = 1n). PI analysis uses cell death measurements from individual explants (i.e., one explant = 1n). Animal numbers are estimates based on n per experiment with 8–10 cortical explant slices isolated per pup. For each gene of interest the change in threshold cycle (ΔCt) for each sample was normalized to the constitutively expressed housekeeping control gene, Histone 3.1 [53]. ΔΔCt was then calculated by comparing the ΔCt for the PND4 or 1DIC [63]. The analysis was performed in at least three independent litters. Visual inspection of the distribution of outcome variables and model residuals suggested that normality assumption of linear models was violated. Box-Cox transformations [64,65] were used to increase the tenability of this assumption. A parallel set of analyses were run on the raw and transformed data, with no substantive differences in significance. For interpretive purposes, the decision was made to present findings from the raw data.

Data are mean ± SEM and differences were considered significant at $p < 0.05$. Statistics are reported by p-value and F(DFn, DFd) for main effects and interactions. Post hoc significance are indicated with p-value. Graphing and statistics were performed with GraphPad Prism 8.4.2.

Availability of data and material. The data that support the findings of this study are available from the corresponding author upon reasonable request.

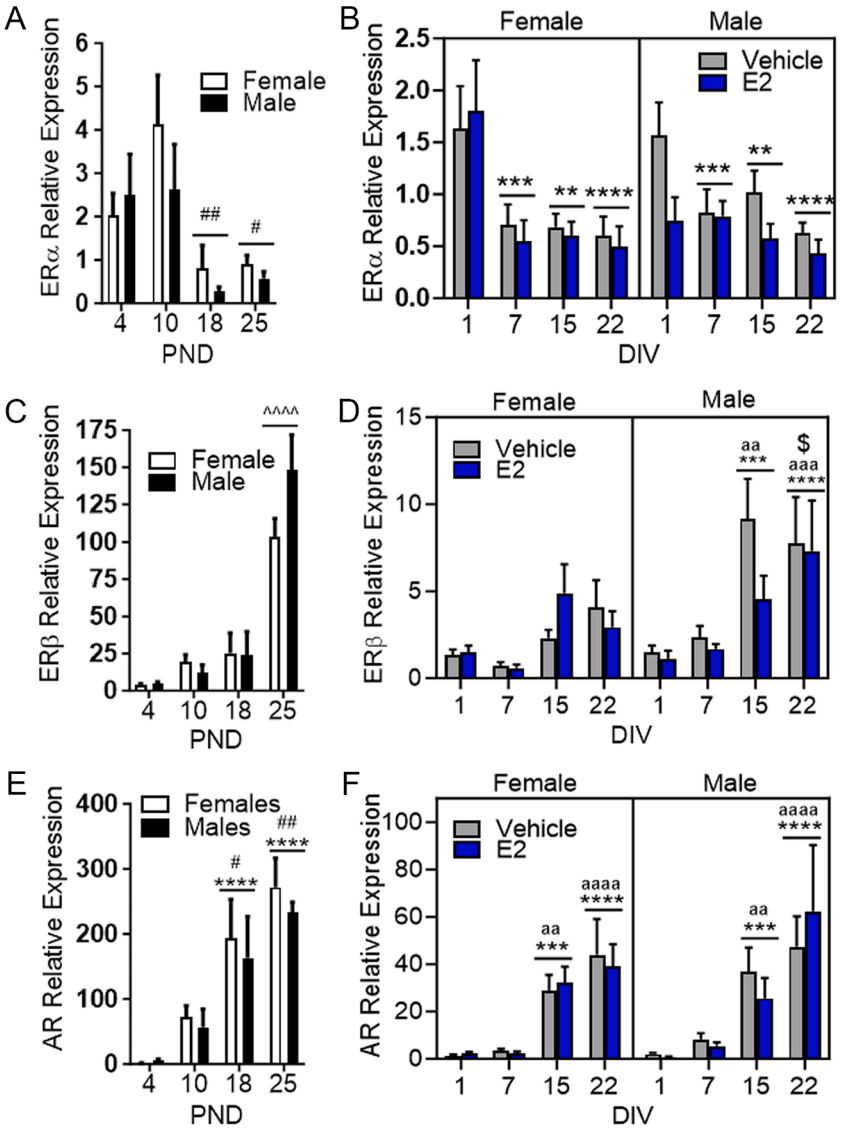

Fig. 1. Cortical and cortical explant mRNA expression across development. Changes in ERα (A, B), ERβ (C, D), and AR (E, F) mRNA expression are similar in the female and male cortex (*In Vivo; A, C, E*) and cortical explants (*In Vitro; B, D, F*). Data are mean ± SEM and relative expression normalized to housekeeping gene histone 3.1. Ct values are compared to PND4 or 1DIV sex-specific vehicle control. P values were assessed by 2- or 3-way ANOVA followed by a Tukey's post-hoc test. The analysis was performed in at least three independent litters, *In Vivo* n = 5–28/group and *In Vitro* n = 6–11/group. # ($p < 0.01$) and ## ($p < 0.0001$) indicates significance from PND10. ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$) indicates significance from PND4 or 1DIV. aa ($p < 0.01$), aaa ($p < 0.001$), and aaaa ($p < 0.0001$ indicates significance from 7DIV.^^^^($p < 0.0001$) indicates significance from PND4, PND10 and PND18. $ ($p < 0.05$) indicates significance from female 22 DIV. There was no effect of E2 treatment on ERα, ERβ, and AR in cortical tissue across development and only an effect of sex on ERβ mRNA expression.

Results

ERα, ERβ, and AR mRNA expression in the female and male cortical explants compared to in vivo

To establish basal expression patterns and understand hormone receptor expression changes when E2 is present, we compared ERα, ERβ, or AR mRNA expression changes in the intact rat cortex to cortical explants grown in culture. Female and male cortical explants, isolated from PND3 pups and grown in culture for 1, 7, 15, and 22 Days *In Vitro* (DIV), were compared to the intact cortex at PND4, 10, 18, and 25. ERα mRNA expression was not significantly different between females and males, but did decrease over time ($F(3,72) = 5.79$; $p < 0.01$, Fig. 1A) in the cortex by PND18 and in cultured cortical explants ($F(3,119) = 10.10$; $p < 0.0001$, Fig. 1B) by 7DIV. An increase ($p < 0.0001$, $F(3,81) = 54.56$, Fig. 1C) in ERβ mRNA expression in the cortex occurred significantly by PND25, with no effect of sex. However, effects of sex ($F(1,130) = 11.30$; $p = 0.001$) and DIV ($F(3,130) = 13.60$; $p < 0.0001$, Fig. 1D) were noted due to the male cortical explants having a significant increase in ERβ mRNA expression by 15DIV, compared to 1DIV, and male cortical explants having significantly higher ERβ mRNA, than female cortical explants, by 22DIV. An increase in AR mRNA expression occurred, regardless of sex, in the cortex across PND ($F(3,66) = 15.54$, $p < 0.0001$, Fig. 1E) by PND18 and PND25. In cortical explants AR mRNA expression significantly increased across DIV ($F(3,123) = 22.67$, $p < 0.0001$, Fig. 1F), regardless of sex, by 15DIV and 22DIV. While all of the gene expression changes are time-dependent, either PND or DIV, an effect of sex was only seen in cortical explants with ERβ mRNA expression and none of the observed genes were affected by E2 treatment. Therefore, cortical explants are an in vitro model that may add insight into innate signaling specific to the cortex.

E2 reduces ischemic cell death in female, but not male cortical explants

The deprivation of glucose and oxygen that occurs with ischemic stroke in the brain can be chemically induced with 2DG/KCN by blocking glycolysis and oxidative phosphorylation, respectively [43]. Cell death, measured by propidium iodide uptake, was analyzed at 8, 12, and 24 hrs. following a two-hr. injury with 2DG/KCN in female and male cortical explants. Propidium iodide is a fluorescent compound that enters cells when plasma membranes are damaged and binds to DNA. Female cortical explants had an effect of injury with 2DG/KCN ($F(1,96) = 18.41$, $p < 0.0001$, Fig. 2 A,B), an effect of time following injury ($F(2,96) = 3.604$. $p < 0.05$), and an interaction of injury and treatment ($F(1,96) = 9.332$, $p < 0.01$). A significantly increase of cell death at 24 hrs. was attenuated with E2 treatment. Significant cell death ($F(1,146) = 23.06$, $p < 0.0001$, Fig. 2 C,D) was also noted in male cortical explants, with no effect of E2. Therefore, cortical explants may provide a platform for studying sex-specific E2-mediated protection following ischemia that is innate to the cortex.

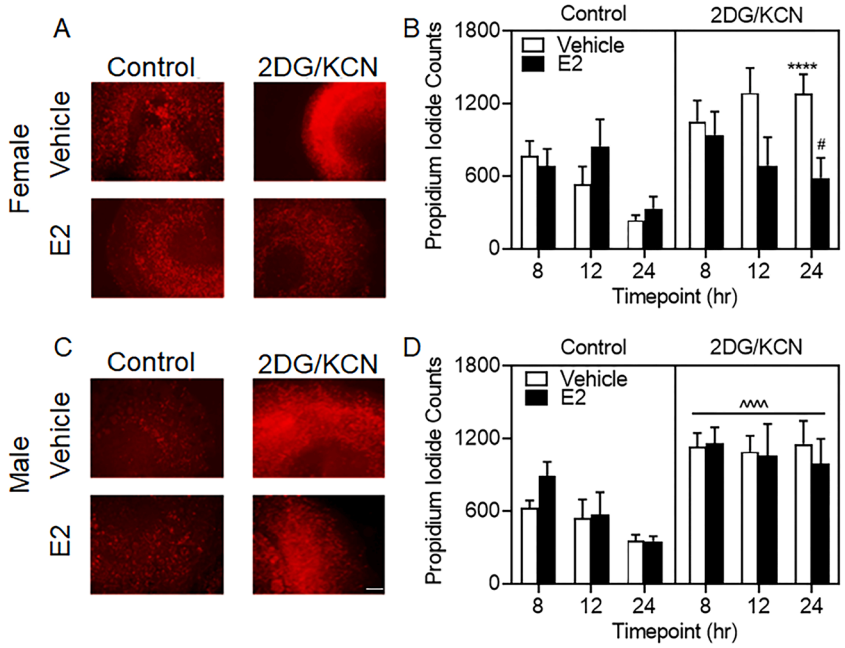

Fig. 2. Cell death time-line following injury in cortical explants. Representative 10X image at 24 hrs. (A, C), and PI uptake (red dots) quantification at 8, 12, and 24 hrs. in female (B) and male (D) cortical explants. Scale bar = 50 μm. Data are mean + SEM. PI uptake was quantified by image J. The analysis was performed in at least three independent litters, n = 5–25/group. P values were assessed by 3-way ANOVA followed by a Tukey's post-hoc test. **** ($p < 0.0001$) indicates significance from 24 hr. vehicle and E2-treated controls. # ($p < 0.05$) indicates significance from 24 hr. Vehicle-treated 2DG/KCN.^^^^($p < 0.0001$) indicates significance from control.

Changes in ERα, ERβ, and AR mRNA expression following 2DG/KCN

Expression of ERα, ERβ, and AR mRNA was measured at 8, 12, and 24 hrs. following injury with 2DG/KCN in female and male cortical explants treated ± E2. In the female cortical explants, effect of injury with 2DG/KCN ($F(1,63) = 18.80, p < 0.0001$, Fig. 3A), time following injury ($F(2,63) = 51.27$, $p < 0.0001$), and treatment ($F(1,63) = 12.10$, $p < 0.01$) were observed with ERα mRNA expression. Following injury, ERα mRNA expression was significantly increased at 12 hrs. in the vehicle, while E2 treatment significantly lowered ERα mRNA expression. By 24 h, ERα mRNA expression, in female cortical explants, had returned to baseline. No significant changes (Fig. 3B) in ERα mRNA expression were observed in male cortical explants ± E2 at 8, 12, or 24 hrs. following injury. ERβ mRNA expression significantly decreased in the female ($F(2,45) = 14.14$, $p < 0.0001$, Fig. 2C) and male ($F(2,46) = 3.654$, $p < 0.05$, Fig. 3D) cortical explants by 12 hrs. and by 24 hrs., respectively, regardless of injury or E2 treatment. In female cortical explants, an effect of injury ($F(1,46) = 12.04$, $p < 0.01$, Fig. 3E) and time following injury ($F(2,46) = 10.14$, $p < 0.001$) were observed with a significant increase in AR mRNA expression observed at 8 hrs. that significant decreased by 24 hrs. AR mRNA expression did not significantly differ with injury, time after injury, or E2 treatment in male cortical explants (Fig. 3F). Therefore, E2 treatment only significantly attenuated the increase in ERα mRNA that was associated with injury.

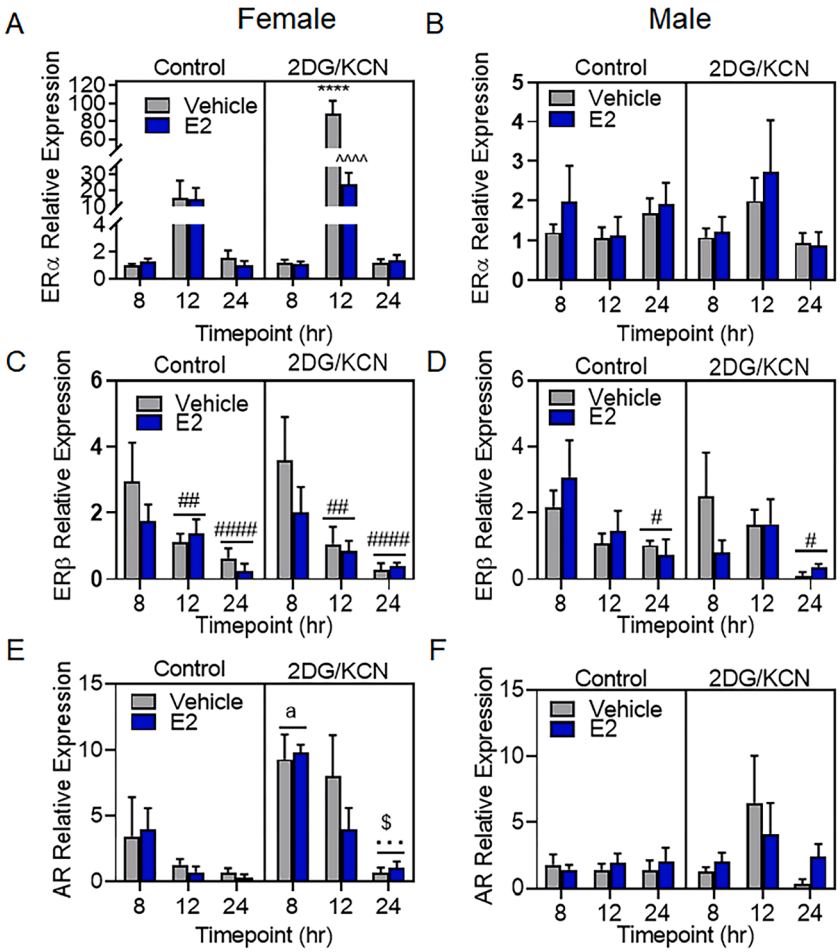

Fig. 3. Changes in mRNA expression following injury in cortical explants. Changes in ERα (A, B), ERβ (C, D), and AR (E, F) mRNA expression at 8, 12, and 24 hrs. following 2DG/KCN injury in female (A, C, E) and male (B, D, F) cortical explants ± E2. Data are mean + SEM and relative expression normalized to housekeeping gene histone 3.1. Ct values are compared back to vehicle-treated controls for each time point. The analysis was performed in at least three independent litters, n = 6–11/group. P values were assessed by 2- or 3-way ANOVA followed by a Tukey's post-hoc test. **** ($p < 0.0001$) indicates a significant increase at 12 hrs. from the uninjured controls and a significant increase at 12 hrs., compared to 8 and 24 hrs., within vehicle-2DG/KCN.^^^^($p < 0.0001$) indicates a significant decrease with E2-2DG/KCN from the vehicle-2DG/KCN at 12 h. ## ($p < 0.01$) and #### ($p < 0.001$) indicates significance from 8 hrs. "a" ($p < 0.05$) indicates significance from 8 hr. control. $ ($p < 0.01$) and··· ($p < 0.0001$) indicates significance from 8 and 12 hr. 2DG/KCN, respectively.

Discussion

Premenopausal females have a lower incidence of stroke with better outcomes, presumable due to E2-mediated protective effects. Therefore, understanding E2-mediated mechanisms of protection is essential to improving outcomes and long-term care following stroke. The regulation and expression patterns of ERα, ERβ, or AR, during early postnatal development can potentially help us understand how E2 and these receptors are regulated at other times during an animal's lifetime, i.e. following a brain injury. One theory is that the brain reverts back to its developmental state following injury to aid in repair [66].

Steroid hormones and their receptors influence the cytoarchitecture of the developing cortex by influencing synaptogenesis, cell differentiation, apoptosis, neurite outgrowth, connectivity, and migration [67–70]. As expected, based on previous studies, ERα mRNA expression decreases in both the intact cortex [71,72] and cortical explants across development [71]. While ERβ mRNA does not significantly increase across time in cortical explants, it does follow similar trends to the intact cortex. As previously reported, AR mRNA was also not sexually dimorphic in the cortex across development [73,74]. However, to our knowledge, we are the first to report that increases in AR mRNA also occur in cortical explants, regardless of sex. Increasing AR expression is correlated to neuronal circuit organization [74,75], suggesting higher cognitive function. Interestingly, we did not see an effect of E2 treatment on ERα, ERβ, or AR mRNA expression in female or male cortical explants across time. Previous reports in cortical explants indicate cell death and the ratio of glial and neuronal cells are stable across DIV. This suggests these dynamic changes are innate to cortical development rather than through cell death or input from other brain regions. These changes in the cortex correlate to cellular organization and further establish that the cortex can "age" in culture without signaling cues from the vasculature, infiltrating immune cells, and projections from other parts of the brain.

An important factor in the extent of brain damage following ischemic injury in animal models is the level and expression pattern of estrogen receptors (ER). Generalized pharmacologic blockade of ERs exacerbates ischemic injury in mice [76] and blocks estrogen-induced neuroprotection in cultured neurons [42,77] and cortical explant cultures [42]. Studies using ERα knockout female mice demonstrate that E2-mediated neuroprotection following stroke is dependent on the presence of ERα in the cortex [78] and that ERβ alone is not sufficient for neuroprotection. In ovariectomized rodents, a model of menopause, ERα mRNA and protein increase in the cortex by 24 hrs. following stroke [79,80], but is seen earlier with E2 pre-treatment [80]. Similarly, to these previously published studies, our data suggest that E2 neuroprotection in the cortex of female rodents may partially be mediated transiently with ERα mRNA.

Interestingly, although male rodents can be protected by E2 (depending on the concentration and time frame of treatment), ERα expression is not known to change *in vivo* following injury in gonadally intact males [81]. E2 treatment did not protect male cortical explants from cell death at 24 hrs. or alter ERα mRNA expression. In alternate models of ischemia, Chanana et al, demonstrated that ERα expression is necessary to mediate neuroprotection with a tyrosine kinase B receptor agonist. [82] Future studies may examine E2 mediated neuroprotection and how changes in sex-steroid hormone receptors are needed to mediated this protection.

The injury did not alter ERβ mRNA expression in female or male cortical explants. Previously published studies elude to non-receptor mediated mechanisms for E2 neuroprotection, such as ion fluxes, activation of protein kinases, direct or indirect mitochondrial-mediated changes, neuroglobin upregulation, and post-translational modification (reviewed in [83–86]). In addition to the E2 mediated neuroprotection, innate sex-specific effects on sex hormones receptors may be regulated by contributions of X and Y chromosomal genes [87], tissue-specific promoter regulation (e.g., transcriptional and co-regulator factors) [88], and epigenetic modulators[89]. Histone lysine demethylase is known to escape X-inactivation resulting in increased expression in females, [89] which results in sexually dimorphic expression of epigenetic modulators that may alter receptor expression. [38,81,90,91] While utilizing cortical explants allows us to remove the systemic influence of circulating hormones produced by the gonads, we cannot rule out the *de novo* production of sexually dimorphic hormones, enzymes, or regulatory elements that are innate to the female and male cortical tissue (reviewed in [92 93]).

A transient increase in AR mRNA expression occurred at 8 hrs. following 2DG/KCN injury in both vehicle and E2-treated female cortical explants. This transient increase in AR mRNA precedes the increase in ERα mRNA at 12 hrs. In agreement with breast cancer studies, our data also supports AR transcriptionally regulating ERs [48,49].This transient increase has also been reported in other injury paradigms, such as wound healing.[94] Additionally, Wilson et al. previously reported an 2DG/KCN injury-induced activation of Akt kinase in cortical explants that occurred earlier with E2 pretreatment.[43] Kinases, such as Akt kinase and Protein kinase C,[95] are known to regulate the transcription activity of sex hormones, pro-survival signals [96–98], and the phosphorylation state of the AR. [99,100] E2 pretreatment leads to earlier activation of Akt and decreased cell death at 24 hrs. While not in the context of this manuscript, future studies may look at changes in AR phosphorylation and Akts contributions to the sex specific and E2 mediated neuroprotection.

Due to the lack of antibody specificity for ERα, ERβ, or AR, one major limitation of our study, is the lack of correlation between mRNA and protein expression [101,102]. While out of the scope of this paper, future studies should evaluate the specificity of additional antibodies and evaluate changes in sex steroid hormone protein relationship to mRNA expression, as this may not be linear [103]. Additional studies should investigate later time-points (e.g., 48 hrs.) and higher pharmacological doses of E2 in cortical explants, as well as, non-transcriptional E2-mediated mechanisms of neuroprotection are needed to understand E2-mediated neuroprotection in the cortex.

Conclusions

Here we provide evidence that E2-mediated protection in females is maintained in isolated cortical explants but not in males. E2

attenuates an injury-induced transient increase in ERα mRNA expression. These results may have implications for sex-specific treatments in neurodegenerative diseases.

Authors' contributions

ALT and MEW conceptualized and designed the studies. ALT performed the *in vitro* experiments/analyzed the data with assistance from CJM, JMW and TS. ALT, CJM, and MEW contributed to writing of the manuscript, but all authors critically reviewed the manuscript and approved it as submitted.

Ethics approval and consent to participate

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health as well as the ARRIVE guidelines.

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CRediT authorship contribution statement

Amanda L. Trout: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition. **Christopher J McLouth:** Formal analysis, Writing – review & editing. **Jenne M. Westberry:** Conceptualization, Data curation, Investigation, Writing – review & editing. **Tomoko Sengoku:** Data curation, Investigation, Writing – review & editing. **Melinda E. Wilson:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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