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Estrogen protects optic nerve head astrocytes against oxidative stress by preventing caspase-3 activation, tau dephosphorylation at Ser^{422} and the formation of tau protein aggregates

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Abstract

Glaucoma, is a neurodegenerative disorder that leads to the slow degeneration of retinal ganglion cells, and results in damage to the optic nerve and concomitant vision loss. As in other disorders affecting the viability of central nervous system neurons, neurons affected by glaucoma do not have the ability to studies indicate regenerate after injury. Recent a critical role for optic nerve head astrocytes (ONHAs) in this process of retinal ganglion cell degeneration. Cleavage of tau, a microtubule stabilizing protein and constituent of neurofibrillary tangles (NFT), plays a major part in the mechanisms that lead to toxicity in CNS neurons and astrocytes. Here, we tested the hypothesis that estrogen, a pleiotropic neuro- and cytoprotectant with high efficacy in the CNS, prevents tau cleavage, and hence, protects ONHAs against cell damage caused by oxidative stress. Our results indicate that estrogen prevents caspase-3 mediated tau cleavage, and thereby decreases the levels of the resulting form of proteolytically cleaved tau protein, which leads to a decrease in

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Authors' contributions

John C. Means and Peter Koulen conceived and designed the experiments; John C. Means, Adam A. Lopez and Peter Koulen performed the experiments; John C. Means, Adam A. Lopez, and Peter Koulen analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Ethical Approval and Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of supporting data

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

NFT formation, which requires proteolytically cleaved tau protein. Overall, our data propose that by stopping the reduction of estrogen levels involved with aging the sensitivity of the optic nerve to glaucomatous damage might be reduced. Furthermore, our data suggests that therapeutic use of estrogen may be beneficial in slowing or preventing the onset or severity of neurodegenerative diseases such as glaucoma and potentially also other degenerative diseases of the CNS through direct control of posttranslational modifications of tau protein.

INTRODUCTION

Progressive neurodegenerative disorders, such as Alzheimer's disease (AD) often show significant sex differences with the incidence rate of AD in women being 2–3 times higher (Pike 2017). In addition, the likelihood of developing dementia over the long term rises with premature menopause (Ryan et al. 2014). A connection between the decline of estrogen during post-menopause and developing AD has been hypothesized (Geerlings et al. 2001). In addition, cognitive impairment in females seems to be higher when compared to males at the same stage of the disease, which also has been attributed to decreased levels of estrogen found in women who are postmenopausal (Laws et al. 2016).

When adequately utilizing therapeutic windows for estrogen replacement therapy, estrogen reduces the likelihood that healthy women will develop AD and improves cognitive function in women who have AD (Tang et al. 1996; Pike et al. 2009; Engler-Chiurazzi et al. 2015, 2016).

Glaucoma, a neurodegenerative disease of the CNS that damages the optic nerve by inducing cell death in retinal ganglion cells, results in the irreversible loss of vision (Munemasa and Kitaoka 2013). Similar to AD, decreased estrogen levels raise the risk for developing glaucoma while adequate estrogen replacement therapy appears to reduce women's risk for glaucoma (Dewundara et al. 2016; Newman-Casey et al. 2014), which is supported by strong preclinical evidence (Prokai-Tatrai et al. 2013; Kaja et al. 2003). An early decline in endogenous estrogen levels has been suggested to result in a higher susceptibility of the optic nerve to glaucoma-mediated damage (Vajaranant and Pasquale 2012).

Both AD and glaucoma appear to be linked and potentially share underlying neurodegenerative mechanisms. For example, AD patients are three times more likely to develop glaucoma when compared to the general population (Bayer et al. 2002). In addition, aggregated and mis-sorted tau accumulated in retinal neurons of a rat model for glaucoma, while knocking down tau protein levels protected neurons and axons from degeneration (Chiasseu et al. 2016).

Microglia and astrocytes are involved in mediating several mechanisms of protection against neurotoxicity (Ries and Sastre 2016). In addition, estrogen receptors are expressed on glia cells and are a target for estrogen's protective properties in a variety of pathologies (Barreto 2016; Arevalo et al. 2010; Dhandapani and Brann 2007). Reduced inflammation and oxidative stress were measured when glial cells were dosed with estrogen prior to exposure to neurotoxic signals (Villa et al. 2016; Acaz-Fonseca et al. 2014). Astrocytes are needed for estrogen to exert its neuroprotective properties on primary neurons after induction of Aβ-

mediated neurotoxicity (Sortino et al. 2004). Similarly, glial fibrillary acidic protein (GFAP) expression, a protein critical for the health and proper function of glia cells is regulated by estrogen in neuron-glia co-cultures and in ovariectomized mice that had been dosed with estrogen (Rozovsky et al. 2002). Conversely, increased reactive gliosis is observed when there is a reduction in estrogen levels in female mice independent of age (Struble et al. 2007).

Here, we hypothesized that estrogen acts as a protective agent for optic nerve head astrocytes exposed to chemically induced oxidative stress. Specifically, we determined if estrogen exerts its glioprotective effects by stopping the cleavage of tau. The results indicate that estrogen prevents caspase-3 activation and the subsequent production of the truncated tau form generated by caspase-3, and ultimately the formation of neurofibrillary tangles (NFT). We further determined that estrogen exerts its protective effects by preventing the dephosphorylation of tau at Ser^{422}, thereby reducing access of active caspase-3 to the caspase-3 cleavage site Asp^{421} in ONHAs. At the same time, treatment with estrogen also decreased apoptosis in ONHAs undergoing oxidative stress. Together, these results provide evidence for a novel mechanism of action underlying the clinical rationale that preventing the age-related decline in estrogen decreases the susceptibility of the optic nerve to oxidative stress, a component of glaucoma pathogenesis.

MATERIALS AND METHODS

Cell culture

Rat optic nerve head astrocytes (ONHAs) were isolated (Brown Norway; age 3 months; male) as primary cells and cultured as described previously (Kaja et al. 2015). ONHAs were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 20% Fetal Bovine Serum (FBS) and 100 U/mL penicillin and 100 mg/mL streptomycin and passaged every 3–4 days. The identity and purity of ONHA cultures was validated with immunocytochemistry measuring immunoreactivity of the astrocyte marker GFAP.

Estrogen and tBHP treatment

Cells were treated with media containing 50–100 μM *tert*-butyl hydroperoxide (*t*BHP) overnight as described previously to induce oxidative stress (Means et al. 2017). For protection assays, cells were pretreated with 25 μM estrogen (17α-estradiol; Steraloids, Inc, Newport, RI; E0950–000; or methanol as a control (vehicle)) for 2 h or 18 h prior to *t*BHP addition with mock defined as receiving vehicle only.

Cell Viability—Trypan blue was used to determine cell viability as described previously (Means et al. 2017; Matsukawa et al. 2009). The number of viable cells were determined by counting 4 fields of view and setting the untreated as 100%. Viability was determined twenty-four hours after tBHP was added.

Measurement of caspase activity

To measure caspase activity, the fluorogenic Ac-DEVD-AFC (Ac-Asp-Glu-Val-Asp-7-Amino-4-trifluoromethylcoumarin, Santa Cruz Biotechnology, Dallas, TX, USA; product

number, sc-311274) caspase-3 substrate was used as previously described (Means et al. 2017). Eighteen hours after treatment cells were collected and centrifuged at 2000 × g. The cell pellets were re-suspended in buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1mM EGTA, 1 mM DTT with protease inhibitor cocktail (Complete ULTRA Tablets, Roche Diagnostics, Indianapolis, IN, USA) and lysed by three freeze/thaw cycles. The lysate was incubated for 1 h at 37°C with caspase-3 fluorogenic substrate (0.5 μM). Caspase activity was based on the fluorescent products generated by caspase-3 activity and determined fluorometrically (excitation 405 nm, emission 535 nm) and plotted as relative arbitrary fluorescence units.

Antibodies

To detect caspase cleaved tau a mouse Asp monoclonal anti-tau (caspase cleaved at ^{421}) antibody (MilliporeSigma, Billerica, MA; product number, MAB5430; 1:500) was used. An anti-tau phospho-Ser^{422} (GenScript, Piscataway, NJ; product number, A00900; 1:1000) rabbit antiserum was used to detect phosphorylated tau at Ser^{422}. A mouse anti-tau monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA; product number/clone, 5A6; 1:500) was used to detect full-length tau. A mouse anti-GFAP monoclonal antibody (abcam, Eugene, OR; product number ab4648; clone2A5; 1:1000) was used to detect GFAP. For a loading control a mouse anti-Actin monoclonal antibody (MilliporeSigma; product number, MAB1501R; 1:1000) was used to detect actin.

Thioflavin S Staining

Thioflavin S staining was used to detect neurofibrillary tangles in ONHAs. Cells were rinsed with distilled water followed by a 5 min fixation at room temperature using 3% PFA treatment. The cells were washed 3 × 5 min with Phosphate Buffered Saline (PBS) and permeabilized (3 min) at room temperature using 0.2% Triton-X-100 in PBS. Cells were washed with PBS (3 × 5 min) and incubated with 0.05% Thioflavin S (Sigma-Aldrich, St. Louis, MO, USA; product number, T1892) in distilled water for 5 minutes. Cells were washed for 5 min in 70% ethanol. Next, several washes with distilled water (10 × 5 min, 1 × overnight) were performed.

Microscopy—Representative images were obtained using a laser-scanning confocal microscope (Leica TCS SP5). A 63×/1.4 - oil submersion objective was used. An argon ion laser (excitation 488 nm, barrier 500–555 nm) was used to visualize Thioflavin-S positive cells. Hoechst 33258 (120 ng/μL; Enzo Life Sciences Inc., Farmingdale, NY) was used to label cell nuclei. Cells were mounted using AquaPolymount (Polysciences Inc., Washington, PA).

Statistical analysis

Prism5 software (GraphPad Inc., La Jolla, CA, USA) was used for statistical analysis. For densitometric analysis of immunoblots to quantify individual bands ImageJ software (Version 1.50i, Developer: Wayne Rasband, National Institutes of Health, Bethesda, MA, USA) was utilized. Student's *t*-test for comparisons between two groups or by one-way analysis of variance (ANOVA) was used to statistically compare means. In addition, the Bonferroni post-hoc test was used for various comparisons with treatment conditions (mock,

or estrogen) and insults (*t*BHP) as variables was used. Statistical significance was set at $p \leq 0.05$. All experiments were performed in triplicate (n=3).

RESULTS

Estrogen protects ONHAs from oxidative stress

ONHAs exposed to oxidative stress induced by *t*BHP showed a decrease in cell viability that was significant (Fig. 1). To determine whether estrogen could act as a neuroprotective agent we pretreated cells with estrogen for either 2 h or 18 h prior to inducing oxidative stress. When compared to vehicle controls, estrogen pretreatment attenuated the reduction in cell viability with the longer pretreatment (18 h) being significantly more protective (Fig. 1). There was no significant difference for the 18h estrogen pretreatment when compared to the mock treatment, indicating full protection from the oxidative stress insult (Fig. 1).

Caspase activation and tau cleavage are induced by oxidative stress in ONHAs and pretreatment with estrogen inhibits these markers of neurodegeneration

To determine whether oxidative stress leads to caspase activation in ONHAs we treated cells with *t*BHP and measured caspase activity. Cells undergoing oxidative stress have high levels of active caspase 3 (Fig. 2). Active caspases during oxidative stress can target tau for proteolytic cleavage, and we detected cleaved tau in ONHAs undergoing oxidative stress (Fig. 3). Next, we wanted to determine whether estrogen is able to attenuate this neurodegenerative process and could act as a neuroprotective agent during oxidative stress. ONHAs were pretreated with estrogen for either 2 h or 18 h followed by *t*BHP addition. Estrogen pretreatment significantly inhibited caspase activation at either of the two pretreatment times, but the longer pretreatment (18 h) fully inhibited caspase activation when compared to the mock treatment (Fig. 2). Consequently, the reduced caspase activity that resulted from the estrogen treatment also led to a significant reduction in cleaved tau (cTau) for both, the 2h and the 18h pretreatment times (Fig. 3) with the longer pretreatment (18h) reducing cTau production to mock treatment levels (Fig. 3B).

Tau dephosphorylation at Ser^{422} is prevented by estrogen

Under normal physiological conditions, tau is phosphorylated at Ser^{422}. This residue is important for tau proteolysis and located immediately adjacent to the caspase cleavage site, Asp^{421} (Fig. 4). During oxidative stress, Ser^{422} is dephosphorylated in ONHAs (Fig. 4A). Pretreatment of these cells with estrogen prevents this dephosphorylation at Ser^{422} with the 18h pretreatment maintaining tau phosphorylation at mock treatment levels (Fig. 4A–B).

Estrogen reverses oxidative stress induced NFT formation in ONHAs

Oxidative stress in ONHAs induced by *t*BHP treatment leads to a significant amount of Thioflavin-S positive cells, a histochemical marker that labels NFTs (Fig. 5). When ONHAs are pretreated with estrogen the number of Thioflavin-S positive cells was significantly reduced (Fig. 5A–B) with the extent of the reduction dependent on the length of the pretreatment with the longer duration (18h) being significantly more effective than the shorter period (2h). The longer pretreatment with estrogen led to a reduction in NFTs that showed no statistical difference to mock treatment (Fig. 5B). Interestingly, Thioflavin-S

staining in the 2h estrogen pretreatment condition, while significantly more intense than in the 18h estrogen pretreatment condition is more diffuse potentially indicating not only a reduction in the total amount of proteolytically cleaved cTau contributing to NFT formation, but also a concomitant change in tangle formation itself (Fig. 5A).

Estrogen attenuates increased GFAP expression, a marker of astroglial activation, in ONHAs exposed to oxidative stress

ONHAs undergoing oxidative stress display a significant increase in GFAP levels (Fig. 6). When ONHAs are pretreated with estrogen prior to *t*BHP treatment the levels of GFAP remain unchanged using the mock treatment for comparison (Fig. 6). While this prevention of astrocyte activation by pre-incubation with estrogen was highly significant, there was no significant difference with respect to the two estrogen pretreatment times tested (Fig. 6B).

DISCUSSION

The development of neurodegenerative disorders like glaucoma and AD, has been linked to decreasing levels of estrogen during aging (Dewundara et al. 2016; Newman-Casey et al. 2014; Moffat et al. 2004; Rosario et al. 2004). In addition, it has been suggested that hormone replacement therapy in women who are postmenopausal can reduce the risk of AD and glaucoma, delay the start of disease, and make cognitive or visual function better, respectively (Newman-Casey et al. 2014; Brinton 2001; Polo-Kantola and Erkkola 2001; Balderechi et al. 1998; Kawas et al. 1997; Paganini-Hill and Henderson 1996).

Mechanisms underlying estrogen's function as a neuroprotective agent have not been fully elucidated. The results show that estrogen blocks caspase-3 activation (Fig. 2) and regulates the phosphorylation state of tau at Ser^{422} (Fig. 4), thereby preventing tau cleavage (Fig. 3) and the generation of toxic tau fragments and NFTs (Figs. 3, 5, 6).

Previous work has shown that estrogen has protective effects against various noxious stimuli. For instance, estrogen can protect against apoptosis induced by staurosporine and hydrogen peroxide (Honda et al. 2001; Sur et al. 2003). Potential anti-apoptotic mechanisms of action include: Estrogen increases the anti-apoptotic protein Bcl-xL levels (Pike 1999). Estrogen attenuates caspase-3 activation, thereby preventing apoptosis (Celsi et al. 2004; Jover et al. 2002). However, neuroprotective properties of estrogen have been associated with a range of signaling pathways and networks including the attenuation of neuroinflammation and oxidative stress, affecting both Aβ and tau protein (Merlo et al. 2017).

Given that tau, a microtubule stabilizing protein, is regulated by phosphorylation at multiple sites that control its function and turnover depending on phosphorylation status, neuroprotective mechanisms involving estrogen signaling mediated effects targeting tau and specifically tau phosphorylation are potentially of high clinical relevance (Arendt et al. 2016; Johnson 2006). Under disease conditions, tau phosphorylation can become dysregulated leading to the build-up of hyperphosphorylated tau, which can aggregate into neurofibrillary tangles. This then can activate multiple signaling cascades that eventually lead to cell death contributing to the pathogenesis of neurodegenerative disorders, such as

AD (Arendt et al. 2016; Morris et al. 2011). Estrogen promotes the dephosphorylation of tau in SH-SY5Y neuronal cells and primary rat cortical neurons, where cortical neurons from females are more sensitive to estrogen when compared to the males (Zhang and Simpkins 2010; Alvarez-de-la-Rosa et al. 2005). In the present study, tau phosphorylation at Ser^{422} is necessary to prevent the proteolytic cleavage of tau. Dephosphorylation of this site allows caspases access to tau leading to the generation of tau fragments that more readily aggregate.

Caspase-3 tau cleavage was deemed an early event that occurs before tau hyperphosphorylation was being detected (Gamblin et al. 2003). In addition, caspase-3 cleaved tau has been detected prior to the onset of apoptosis and NFT formation (Rissman et al. 2004; Ugolini et al. 1997). Tau proteolysis is also mediated by calpain and can be detected before tau hyperphosphorylation occurs (Park and Ferreira 2005).

Estrogen and testosterone can both function individually as neuroprotective signaling molecules through the differential regulation of proteases targeting tau: While testosterone inhibits the activation of calpain, which generates a 17kDa tau fragment, it does not affect caspase-3 activity resulting in tau truncation at Asp^{421}, the protease signaling pathway attenuated by estrogen (Park et al. 2007). Our results indicate that neuroprotective properties of estrogen can also be found in glia, specifically astrocytes: Estrogen protects ONHAs from oxidative stress as measured by attenuation of GFAP levels as a surrogate marker of astroglial acivation and inhibits caspase-3 activation resulting in reduced tau cleavage by caspase-3, thereby preventing NFT formation. This is in line with previous work indicating estrogen-mediated control of GFAP expression as a protective mechanism (Rozovsky et al. 2002). While both 2-hour and 18-hour preincubation with estrogen effectively reduced caspase-3 activation and tau proteolytic cleavage, the longer preincubation time showed greater effects. This parallels findings in hippocampal neurons where estrogen-mediated protection from Aβ-induced neurotoxicity was effective not only after 24 h, but also after 2 h preincubation with estrogen prior to insult (Park et al. 2007).

Oxidative stress leads to the buildup of free radicals, which has been connected with numerous neurodegenerative diseases, including AD and glaucoma (Munemasa and Kitaoka 2013; Behl et al. 1995). Antioxidants can be used to safeguard cells against oxidative stress-induced cell damage and death by inhibiting oxidation and thereby preventing free radical formation (Niki and Nakano 1990). Here, we induced oxidative stress using tBHP, thereby modeling the cellular environment encountered during neurodegeneration. Estrogen acting directly as an antioxidant has been shown to protect mouse hippocampal HT22 cells and embryonic rat hippocampal cells from free radical damage and subsequent apoptosis (Behl et al. 1995; Behl 1997; Mooradian 1993), providing an alternate, potentially parallel pathway for the protection of ONHAs identified here.

Conclusions

Our data support the notion that proteolytic cleavage of tau contributes to the degeneration of the neural retina, specifically in ONHAs, during oxidative stress. In addition, we show that this degenerative signaling pathway can be attenuated by estrogen indicating that estrogen-mediated pharmacological control of neurodegeneration in the retina and optic

nerve is feasible. At the same time, specific components of these signaling pathways such as caspase-3 and specific tau phosphorylation and cleavage sites represent additional potential therapeutic targets.

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List of abbreviations

Aβ	amyloid β–peptide
AD	Alzheimer's disease
ANOVA	analysis of variance
CNS	central nervous system
GFAP	glial fibrillary acidic protein
NFT	neurofibrillary tangle
ONH	optic nerve head
ONHA	optic nerve head astrocyte
PBS	phosphate buffered saline
PFA	paraformaldehyde
tBHP	tert-butyl hydroperoxide

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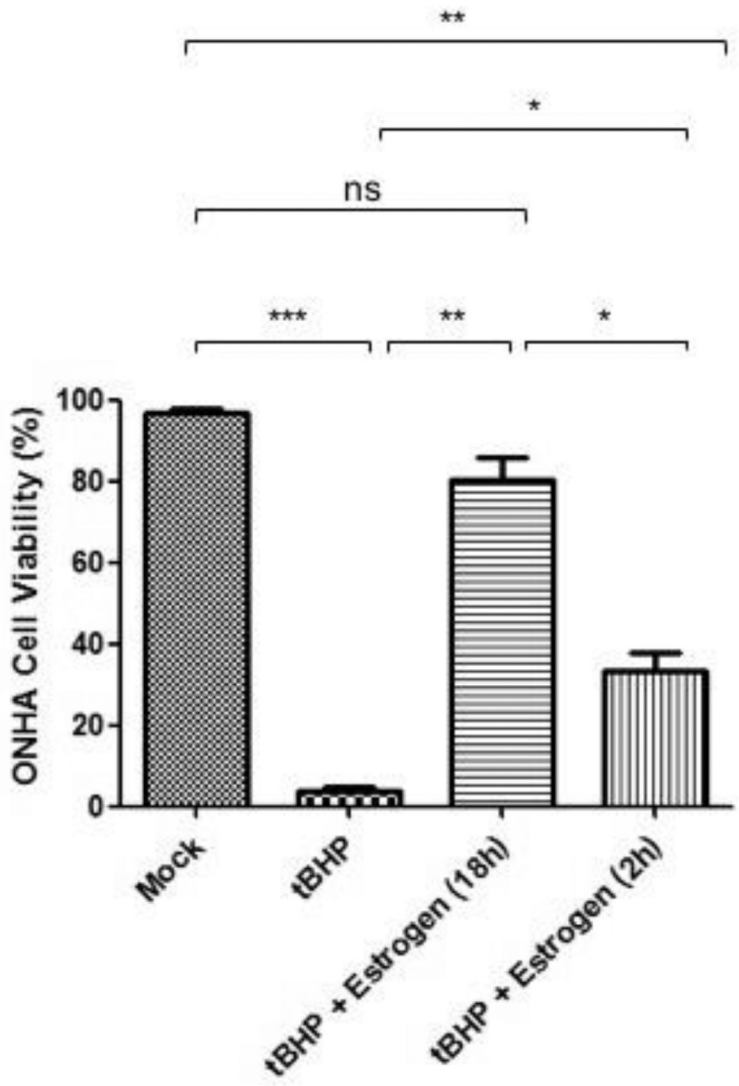

Figure 1. Estrogen prevents *t*BHP induced cell death in optic nerve head astrocytes (ONHAs).

ONHAs were pretreated with 25 μM estrogen for 2 h or 18 h followed by treatment with *t*BHP. Twenty-four hours later viability was determined using Trypan Blue. In *t*BHP treated compared to cells there was a significant reduction in cell viability cells that were mock treated (***, p=0.0002). ONHAs pretreated with estrogen for 2 h (*, p=0.0236) or 18 h (**, p=0.0053) prior to *t*BHP treatment had a significant increase in viable cells compared to *t*BHP treated cells. When compared to mock treated ONHAs, pretreatment with estrogen for 2 h still showed significantly reduced cell viability (**, p=0.0052), while pretreatment with estrogen for 18 h had no significant difference in viable cells compared to mock treated (ns, p=0.0982). Estrogen pretreatment for 18 h was significantly more protective than pretreatment for 2 h (*, p=0.0221). Experiments were performed in triplicate (n=3) and values were depicted as mean +/− SEM and analyzed using ANOVA (p=0.0002). For statistical comparison, the Bonferroni post-hoc test and Student t-test were used. Statistical significance was set at $p \leq 0.05$.

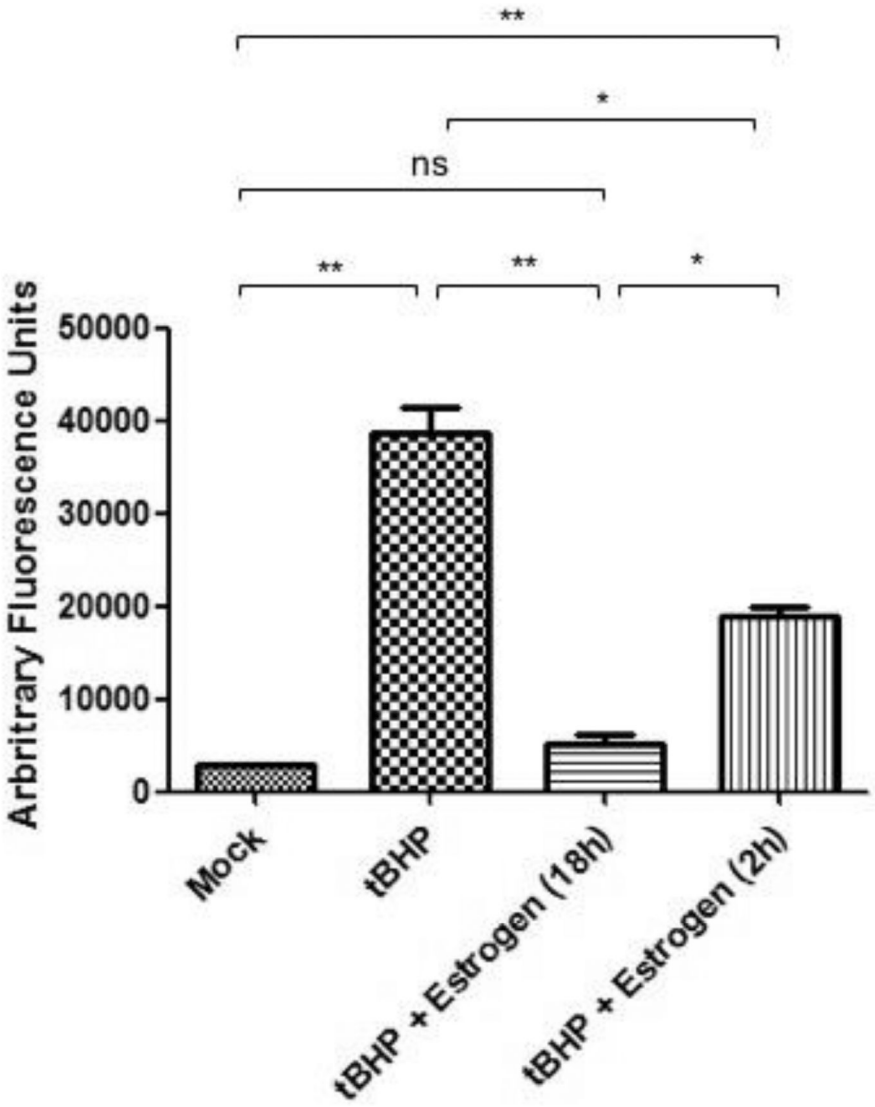

Figure 2. Estrogen inhibits oxidative stress induced caspase activation in ONHAs.

Oxidative stress in ONHAs were induced using *t*BHP and caspase activity was measured using Ac-DEVD-AFC. Caspase activity in ONHAs that were treated with *t*BHP was significantly higher compared to mock treated (**, p=0.0058). ONHAs pretreated with 25 μM estrogen for 2 h (*, p=0.0208) or 18 h (**, p=0.0077) before tBHP addition had a significant decrease in caspase activity compared to *t*BHP treated cells. Estrogen pretreatment for 18 h was significantly more effective at inhibiting caspase activity compared to a 2 h pretreatment (*, p=0.0144). ONHAs pretreated for 2 h with estrogen had a significant level of active caspases versus mock treated (**, p=0.0035) while pretreatment with estrogen for 18 h showed no significant difference in caspase activity versus mock treated (ns, p=0.1974). Experiments were done in triplicate (n=3) and values were depicted as mean +/− SEM and analyzed using ANOVA (p=0.0003). For statistical comparison, the Bonferroni post-hoc test and Student t-test were used. Statistical significance was set at $p \leq 0.05$.

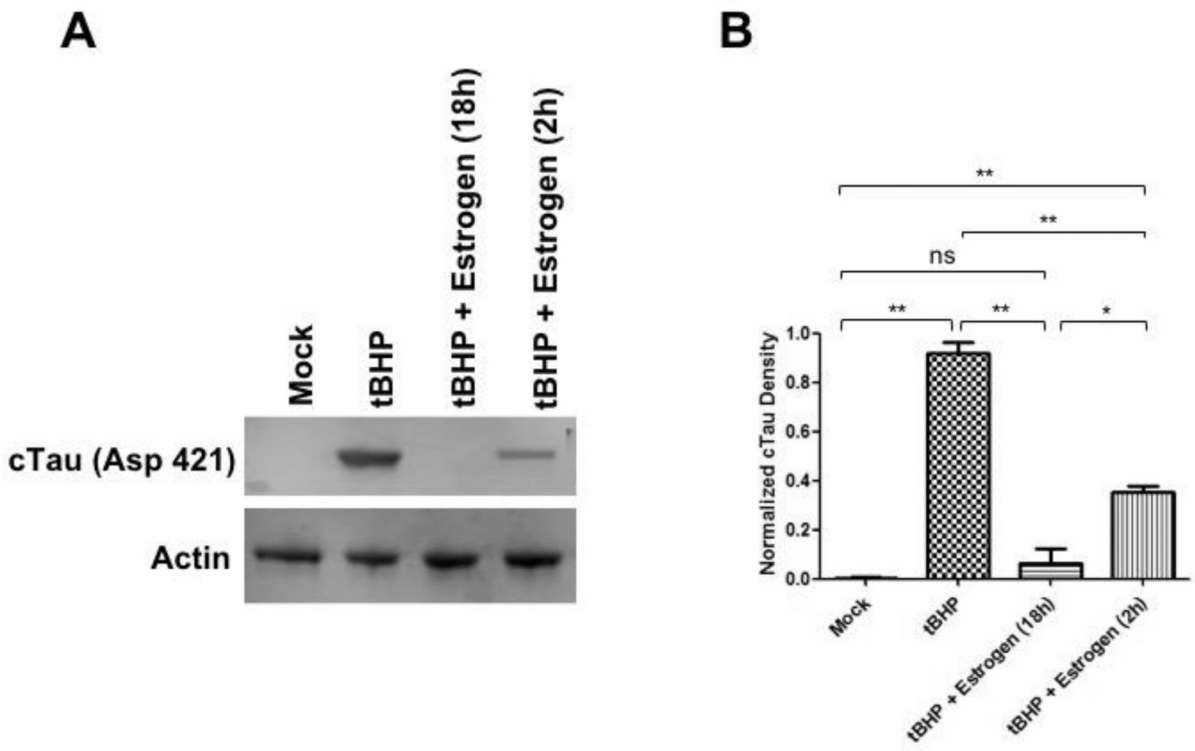

Figure 3. Estrogen inhibits oxidative stress induced tau cleavage in ONHAs.

ONHAs were pretreated with 25 μM estrogen followed by induction of oxidative stress with tBHP. (A) tBHP treated ONHAs were used in immunoblotting assays to measure cleaved tau (cTau) levels. For a loading control actin was used. (B) tBHP treated ONHAs had a significant amount of cTau compared to mock treated (**, p=0.0025). Estrogen pretreatment for 2 h led to a significant decrease in detectable cTau (**, p=0.0083). Estrogen pretreatment for 18 h further decreased cTau levels significantly (**, p=0.0078). Pretreatment with estrogen for 18 h was more effective than a 2 h pretreatment in attenuating cTau levels (*, p=0.0486). ONHAs pretreated with estrogen for 2 h still showed a significant amount of cTau compared to mock (**, p=0.0055). Pretreatment of ONHAs for 18 h had no significant difference from mock (ns, p=0.4565). Experiments were done in triplicate (n-=3) and values were depicted as mean +/− SEM and analyzed using ANOVA (p=0.0003). For statistical comparison, the Bonferroni post-hoc test and Student t-test were used. Statistical significance was set at $p \leq 0.05$.

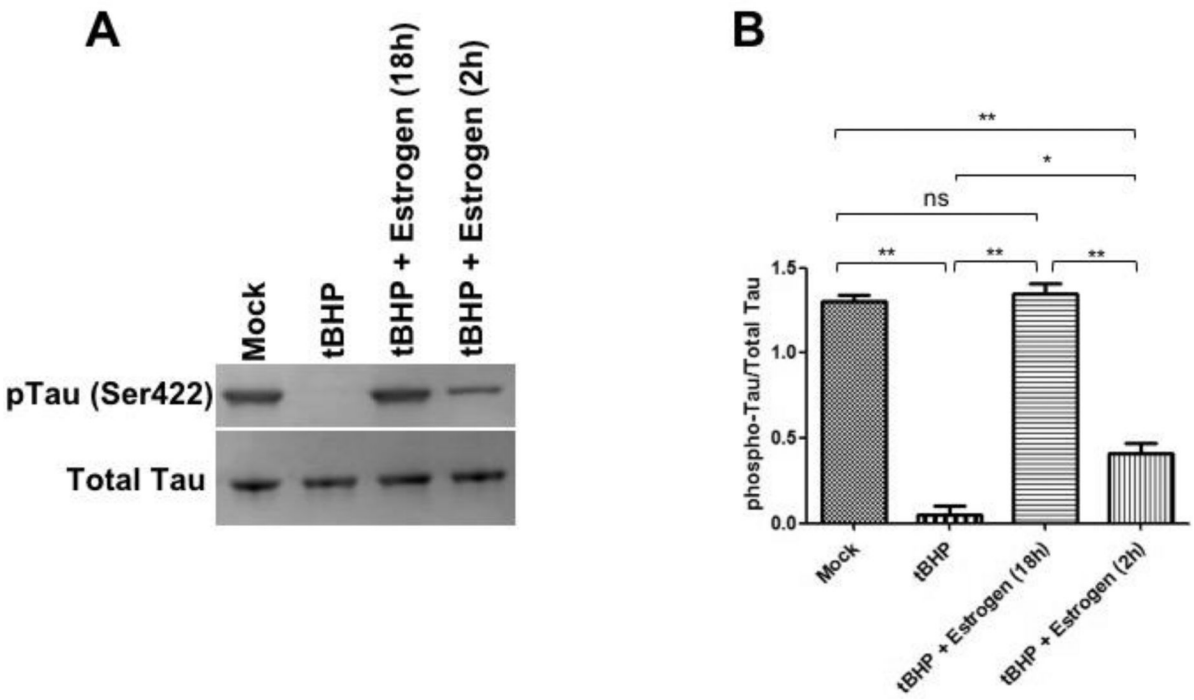

Figure 4. Dephosphorylation of tau at Ser^{422} is blocked by estrogen in ONHAs.

ONHAs pretreated with 25 μM estrogen were exposed to *t*BHP to induce oxidative stress. (A) Phosphorylation of Tau at Ser^{422} was examined by immunoblotting. ONHAs showed Ser^{422} tau phosphorylation under control conditions (see mock). (B) tBHP treated ONHAs had a significant reduction in tau phosphorylation versus mock (**, p=0.0027). ONHAs pretreated with estrogen for 2 h followed by *t*BHP treatment had a significant increase in phosphorylated tau versus ONHAs treated with *t*BHP alone (*, p=0.0482). When estrogen pretreatment was increased to 18 h tau phosphorylation was increased significantly when compared to *t*BHP treatment (**, p=0.0037). Estrogen pretreatment for 18 h was more effective than a 2 h pretreatment (**, p=0.0085) in restoring phosphorylation of tau at Ser^{422}. ONHAs pretreated with estrogen for 18 h (ns, p=0.5598) followed by *t*BHP treatment showed no significant difference from mock treated ONHAs, while the 2h pretreatment still showed a significant difference compared to mock treated (**, p=0.0070). Experiments were done in triplicate (n=3) and values were depicted as mean +/− SEM and analyzed using ANOVA (p=0.0002). For statistical comparison, the Bonferroni post-hoc test and Student t-test were used. Statistical significance was set at $p \leq 0.05$.

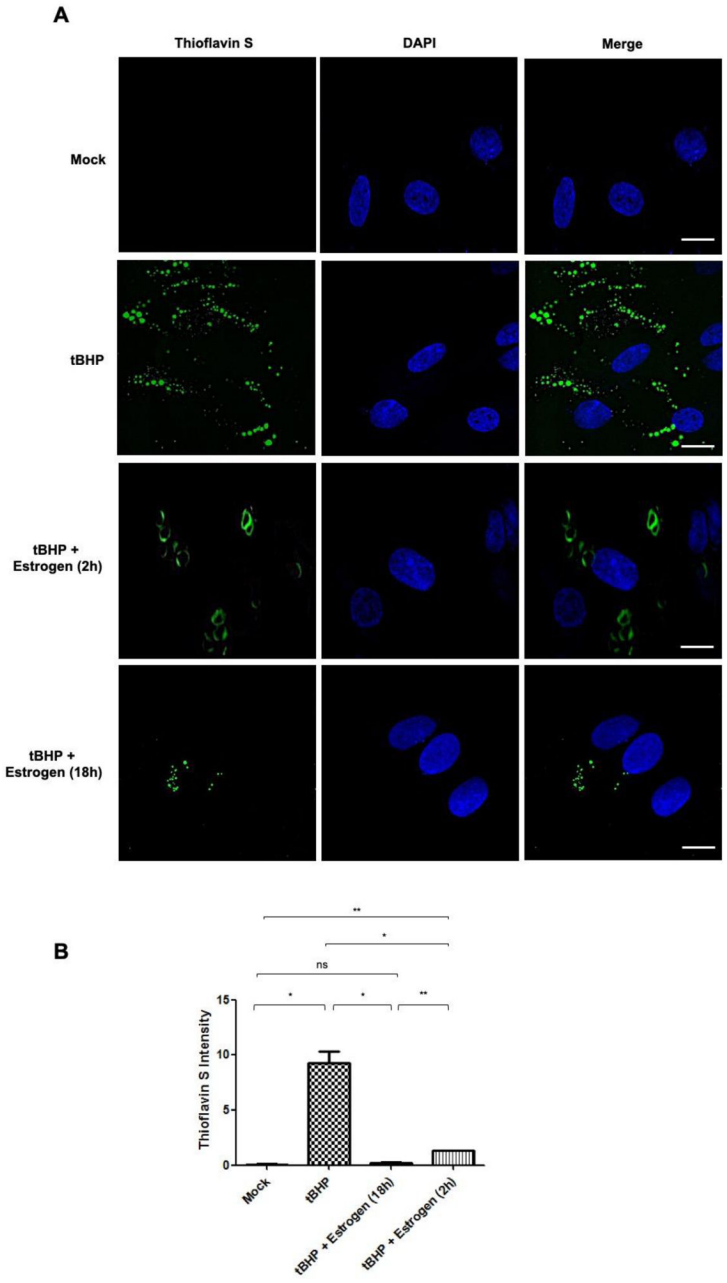

Figure 5. Estrogen prevents NFT formation induced by *t*BHP treatment in optic nerve head astrocytes (ONHAs).

(A) ONHAs were pretreated with 25 μM estrogen followed by induction of oxidative stress using tBHP. Production of NFTs was determined by staining with Thioflavin S (green). (B) tBHP treated cells showed significant Thioflavin S staining, indicative of NFT formation, compared to mock (*, p=0.0129). Cells pretreated with estrogen for 2 h showed a significant reduction of Thioflavin S staining compared to cells treated with *t*BHP treated (*, p=0.0171). In addition, NFTs appeared hallo like and more diffuse for the 2h pretreatment. Increasing pretreatment time to 18 h with estrogen reduced NFT formation significantly (*, p=0.0134) compared to *t*BHP treated cells. Pretreatment with estrogen for 18 h was

significantly more effective than the 2 h pretreatment (**, p=0.0024). ONHAs pretreated with estrogen for 2 h still had significant NFT formation compared to mock treated (**, p=0.0024). Estrogen pretreatment for 18 h was the most effective showing no significant difference in the level of NFT formation compared with mock treated ONHAs (ns, p=0.1326). Experiments were done in triplicate (n=3) and values were depicted as mean +/− SEM and analyzed using ANOVA (p=0.0007). For statistical comparison, the Bonferroni post-hoc test and Student t-test were used. Statistical significance was set at $p \leq 0.05$.

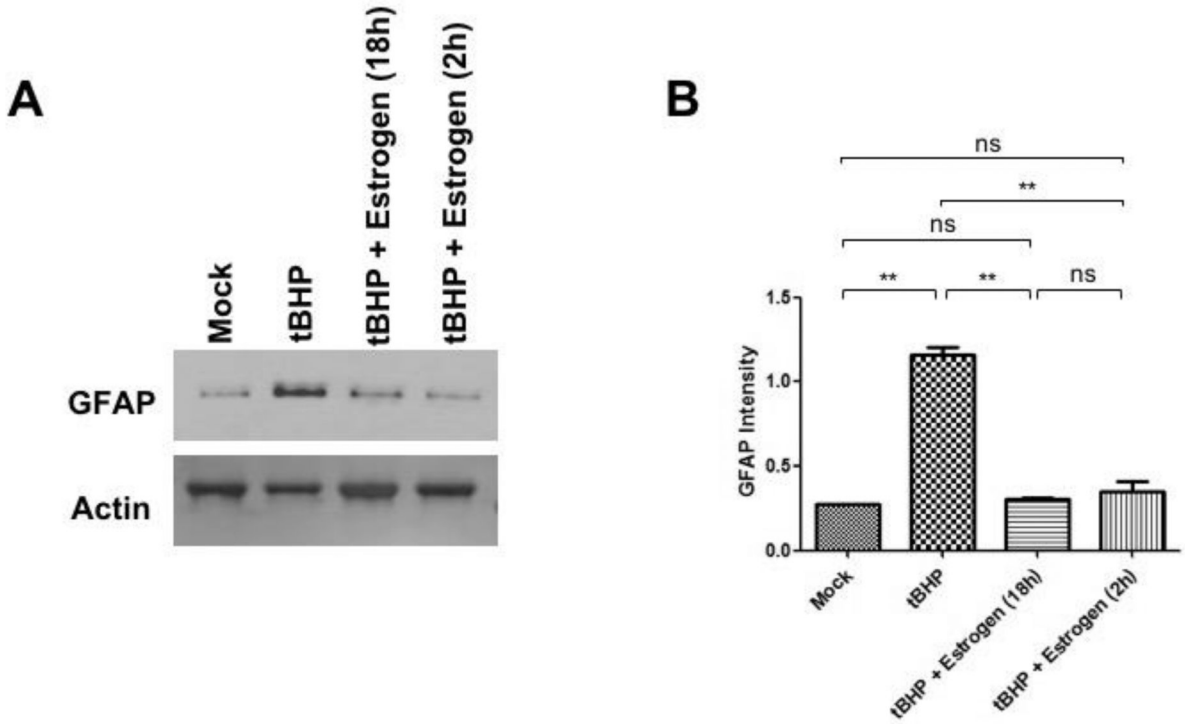

Figure 6. During oxidative stress induced by tBHP estrogen stabilizes GFAP levels in optic nerve head astrocytes (ONHAs).

(A) GFAP levels are elevated during oxidative stress as determined by immunoblot assay (B) During oxidative stress GFAP levels are significantly upregulated versus mock (**, p=0.0020). 25 μM estrogen pretreatment for 18 h (**, p=0.0022) or 2 h (**, p= 0.0074) significantly reduced GFAP levels compared to *t*BHP treated ONHAs. When compared to mock treated ONHAs both 2 h (ns, p=0.3054) and 18 h (ns, p=0.0954) estrogen pretreatment had no significant changes to the levels of GFAP. GFAP levels showed no significant difference when comparing estrogen pretreatment for 2 h or 18 h (ns, p=0.4926). Experiments were done in triplicate (n=3) and values were depicted as mean +/− SEM and analyzed using ANOVA (p=0.0002). For statistical comparison, the Bonferroni post-hoc test and Student t-test were used. Statistical significance was set at $p \leq 0.05$.