Majd, S., Majd, Z., Koblar, S., & Power, J. (2018). Beta estradiol and norepinephrine treatment of differentiated SH-SY5Y cells enhances tau phosphorylation at (Ser 396) and (Ser 262) via AMPK but not mTOR signaling pathway. Molecular and Cellular Neuroscience, 88, 201–211. https://doi.org/10.1016/j.mcn.2018.02.004

which has been published in final form at http://dx.doi.org/10.1016/j.mcn.2018.02.004

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Accepted Manuscript

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PII: S1044-7431(17)30239-7
DOI: https://doi.org/10.1016/j.mcn.2018.02.004
Reference: YMCNE 3280
To appear in: Molecular and Cellular Neuroscience
Received date: 3 July 2017
Revised date: 23 October 2017
Accepted date: 6 February 2018

Please cite this article as: Shohreh Majd, Zohreh Majd, Simon Koblar, John Power, Beta estradiol and norepinephrine treatment of differentiated SH-SY5Y cells enhances tau phosphorylation at (Ser396) and (Ser262) via AMPK but not mTOR signaling pathway. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Ymcne(2017), https://doi.org/10.1016/j.mcn.2018.02.004

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Beta Estradiol and Norepinephrine Treatment of Differentiated SH-SY5Y Cells Enhances Tau Phosphorylation at (Ser^{396}) and (Ser^{262}) via AMPK but not mTOR Signaling Pathway

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Abstract

Hyperphosphorylation of tau is one of the main hallmarks for Alzheimer’s disease (AD) and many other tauopathies. Norepinephrine (NE), a stress-related hormone 17-β-estradiol (E2) and thought to influence tau phosphorylation (p-tau) and AD pathology. The controversy around the impact of NE and E2 requires further clarification. Moreover, the combination effect of physiological and psychological stress and estrogen alteration during menopause, which affect p-tau, has not been addressed. Exposure to E2 is believed to reduce NE release, however, the link between these two hormones and AD at cellular level was also remained unknown. Here, we examined whether NE and E2 treatment of differentiated SH-SY5Y cells affected tau phosphorylation. The involvement of adenosine monophosphate kinase protein kinase (AMPK) and target of rapamycin (mTOR) as the possible mechanisms, underlying this effect was also investigated. Subsequent to SH-SY5Y differentiation to mature neurons, we treated the cells with NE, E2 and NE plus E2 in presence and absence of Compound C and Rapamycin. Cell viability was not affected by our treatment while our western blot and immunofluorescent findings showed that exposure to NE and E2 separately, and in combination enhanced p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262})/tau but not (Ser\textsuperscript{202}/Thr\textsuperscript{205})/tau. Blocking AMPK by Compound C reduced p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262}), while GSK-3β and PP2A activities were remained unchanged. We also found that blocking mTOR by Rapamycin did not change increased p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262}) due to NE+E2 treatment. Collectively, our results suggested that tau hyperphosphorylation due to exposure to NE/E2 was mediated by AMPK, the main energy regulator of cells during stress with no significant involvement of mTOR, GSK-3β and PP2A.

Key words
Norepinephrine, 17-β-estradiol, Compound C, Rapamycin, Adenosine Monophosphate Kinase Protein Kinase, Tau (Ser\textsuperscript{396, 262}) phosphorylation
Introduction

Hyperphosphoryalted tau (p-tau) is one of the two main signatures of Alzheimer’s disease (AD), the most common form of neurodegenerative disorders. AD prevalence in women is higher than in men (Vina and Lloret, 2010). Previously, it was thought that the natural decline of estrogen level during menopause increased the risk for AD (Manly et al., 2000), however numerous studies, failed to prove a significant effect of hormone therapy (HT) in reducing the risk of AD and tauopathy feature of it in human and animal trials (Imtiaz et al., 2017; Ohm et al., 2012). One essential but less studied factor in development of AD hallmarks in women, particularly during and after menopause is stress. The concurrent increase in norepinephrine (NE), along with the other stress-related hormones such as cortisol is usually anticipated to occur during menopause (Woods et al., 2009). It is believed that stress, both physiological and psychological, has a substantial role in generating tau phosphorylation, neuro-inflammation and altering blood brain barrier integrity (Feng et al., 2005; Mravec et al., 2014; Rissman et al., 2007). Interestingly, the ability of 17-β-estradiol (E2) to modulate NE functions has been reported previously (Herbison et al., 2000). It seems that E2-NE interaction enhances spatial memory during stress (Conrad et al., 2004) but impairs stress-induced working memory (Shansky et al., 2004). A role for cAMP in memory impairment during stress has been suggested in E2-NE interaction scenario (Shansky et al., 2009), however the exact mechanism by which these hormones, develop p-tau is not fully understood.

In brain tissue Adenosine Monophosphate Kinase Protein Kinase (AMPK) plays a key role in managing cellular homeostasis in response to a various range of environmental stress (Alexander and Walker, 2011). Apart from its role as the master of cell energy balance, AMPK was recently introduced as one of the main tau kinases, phosphorylating tau in many residues such as Serine$^{396}$ (Ser$^{396}$) and Serine$^{262}$ (Ser$^{262}$) (Domise et al., 2016; Majd et al., 2017; Majd et al., 2016). Along with its direct effect on tau phosphorylation, AMPK inhibits target of rapamycin (mTOR) that acts as an intracellular nutrient sensor to control metabolism (Xu et al., 2012). Upregulation mTOR signaling pathway is thought to play an important role in major pathological processes of AD (Cai et al., 2015). Interestingly, NE enhances both AMPK and mTOR activation via increasing their phosphorylation, while E2 activates AMPK activation (Chen et al., 2014; Hutchinson et al., 2005; Lipovka et al., 2015; Yin et al., 2003) with a very little known about their links to AD through activating intracellular metabolic pathway of AMPK/mTOR.
Considering the role of stress in developing AD, the present study aims to investigate the effect of NE as one of the main elevated stress hormones during menopause, along with E2 on tau phosphorylation, and to study the mechanism behind it. In order to examine this hypothesis, we investigated the impact of AMPK and mTOR inhibition on p-tau (Ser^{396}) and (Ser^{262}) in the presence of NE and E2. Here we showed that the co-treatment with both NE and E2 increased p-tau (Ser^{396}) and (Ser^{262}), with no impact on cell viability between different groups. We suggest that AMPK as the main metabolic pathway, activated under stress in neurons, builds the initial underlying mechanism for stress-induced tau phosphorylation, with no or less involvement of Glycogen Synthase Kinase-3β and Protein Phosphatase 2A.
Experimental procedure

Antibodies

Phosphorylated tau rabbit polyclonal antibody (Ser$^{396}$; sc-101815), tau mouse monoclonal antibody (A-10; sc-390476), phosphorylated Glycogen Synthase Kinase-3β (p-GSK-3β) goat polyclonal antibody (Ser$^9$; sc-11757), GSK-3β rabbit polyclonal antibody (H-76; sc-9166), phosphorylated Protein Phosphatase 2A (p-PP2A-Ca/b) mouse monoclonal antibody (F-8; sc-271903), and PP2A-Aa goat polyclonal antibody (C-20; sc-6112) were purchased from Santa Cruz. Phosphorylated tau mouse monoclonal antibody (Ser$^{202}$/Thr$^{205}$; MN1020B) was purchased from ThermoFisher Scientific. Mouse anti-microtubule associated protein (anti-MAP2; ab11267) and β actin mouse monoclonal (ab6276) antibodies were purchased from Abcam, USA. The p-AMPK rabbit polyclonal (Thr$^{172}$, #2531) and AMPK rabbit polyclonal antibody (#2532) were purchased from Cell Signalling Australia. Secondary antibodies were purchase from Jackson Immuno Research, USA (HRP donkey anti-mouse, anti-rabbit, anti-goat, Alexa Fluor_488 Donkey anti-rabbit, anti-mouse, and CY3 Donkey anti-mouse).

SH-SY5Y culture and differentiation

Neuroblastoma SH-SY5Y cells (generously provided by Dr. Marie Louis Rogers, Motor Neuron Disease and Neurotrophic Research Laboratory, Flinders University) were cultured in T75 tissue culture flasks (Sarstedt, Australia) in complete Dulbecco’s Modified Eagle’s Medium and F12 (Sigma; DMEM/F12; 1:1) supplemented with 10 percent fetal bovine serum (FBS) (Sigma), 100 U/ml penicillin and 100 U/ml streptomycin (Sigma) in a humidified atmosphere of 95% air and 5% CO$_2$ at 37°C. Cell culture medium were changed each 3 days and the cells were sub-cultured once they reached 90% confluence in 6 multi-well plates (Nuncion DELTA surface; NUNC A/S) for western blot analysis, or in 4 chamber tissue culture glass slides (BD Biosciences, USA) for immunofluorescent staining. The differentiation was started after 5-7 days when cells were about 70% confluence. To differentiate cells, the medium was replaced by complete but FBS free medium, contained all supplements plus 10 µM all-trans-retinoic acid (RA, Sigma) (Lopes et al., 2010). The cells were kept in dark and humidified atmosphere of 95% air and 5% CO$_2$ at 37°C.

Treatments of differentiated SH-SY5Y cells
All treatments were begun at day 7 of differentiation. Based on the experimental groups, the cells received the following treatments for 24 or 48 hours: $10^{-5}$ M norepinephrine (NE, dissolved in PBS) (Sigma), 10nM 17-β-estradiol (E2, dissolved in DMSO) (Sigma), NE+ E2, NE+ E2 and Compound C (40 μM, dissolved in DMSO) (Sigma), NE+ E2 and Rapamycin (20 nM, dissolved in DMSO) (Sigma). The control groups received the equal volume of the solvents only.

**Cell viability assessment via trypan blue exclusion method**

Cell viability was measured in control groups and after 24 and 48 h treatment with different reagents. 1:1 volume of cell suspension in phosphate-buffered saline (PBS) and trypan blue solution (0.4% in PBS) was mixed, and the number of stained (live) and unstained (dead) cells were counted in a Neubauer chamber. Cell viability percentage was assessed by calculating the percentage of stained cells with trypan blue to the total number of stained and unstained cells by an experimenter who was blind to the identity of experimental groups.

**Protein quantification**

Protein quantification, followed by western blot analysis were performed at the end of the treatment periods of 24-48 h. The cells were collected in the lysis buffer (5 mM Tris–HCl, 150 mM NaCl, 1 mM ethylenediaminepentaacetic acid, 0.2 % Triton X-100, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM NaF) on ice for 30 min with a 1 min vortex every 10 min. The lysed cells were centrifuged at 100,000×g for 30 min and supernatant was collected for further analysis.

The total protein in each sample was calculated, using an EZQ assay following the approved protocol (BioRad, Hercules, CA, USA) as it was described before (Domise et al., 2016). Briefly, 10 μL of sample, 25 μL of four times sample buffer (100% glycerol, 1M Tris/HCl pH 6.8, SDS, beta-mercaptoethanol, H2O) and 65 μL H2O were mixed. A quantity of 10 μL of this solution was added to 90 μL of H2O, thereafter 1 μL of each sample and the standard solution (serial dilutions of ovalbumin) were loaded on to the assay paper in triplicate each in 96-well plates and absorbance was measured, using an Image Master VDS-CL (Amersham Biosciences) and quantified by CareStream molecular imaging software.

**Western blot analysis**
For western blot analysis, 30 μg of sample in sample buffer was loaded to each well of AnykD™ TGX Stain-free gel (Bio-Rad; 569033) followed by applying the current to the gel for 20 min (100V, 300 mA). After standard SDS-PAGE separation based on their molecular weights, the proteins were transferred onto Polyvinylidene Difluoride (PVDF) membrane at 100 V for 30 min. Subsequently the membranes were blocked in solution of 5% non-fat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (pH 7.6), thereafter were incubated overnight at 4 °C with primary antibodies of: rabbit p-tau (Ser396) (polyclonal; 1:250), rabbit p-tau (Ser262) (polyclonal; 1:250), mouse p-tau (Ser202/Thr205) (polyclonal; 1:250), mouse tau (monoclonal; 1:250), p-GSK-3β (Ser9) (polyclonal, 1:500), GSK-3β (polyclonal; 1:500), p-PP2A-Cα/β (monoclonal; 1:500), PP2A-Aα (polyclonal; 1:500) and mouse β-actin (monoclonal; 1:500). On the following day, membranes were incubated for 1 h at room temperature with the HRP secondary antibodies (Donkey anti- mouse, 1:3000; Donkey anti-rabbit, 1:3000, Donkey anti-goat, 1:10,000). The blots were subsequently developed using an ECL and the chemiluminescence signal detection was performed using Fuji LAS4000 imager, quantified by CareStream molecular imaging software, and was corrected by β-actin levels.

**Immunofluorescent staining**

Culture slides were fixed with 4% paraformaldehyde (Sigma, USA). The cells were permeabilized using 0.2% Triton X-100 in TBS, and non-specific binding was blocked with 5% non-fat dry milk in TBS containing 0.1% Tween 20 (pH 7.6) for 1 h at room temperature. Immunofluorescence staining was undertaken by incubating the slides with rabbit anti p-tau (Ser396, 1:250), or mouse anti tau (1:250) for 18 h at 4° C to detect tau and p-tau (Ser396) immunofluorescence staining. Subsequently, the slides were incubated for 1 h at room temperature with the secondary antibodies of Donkey anti-rabbit (Alexa Flour _488, 1:500) and Donkey anti-mouse (CY3, 1:500). The fluorescent staining was visualized using a Leica SP5 five-channel laser scanning confocal microscope from Flinders University Microscopy Facility.

**Statistical analysis**

All of the data in this study were analysed using IBM SPSS Statistics version of SPSS Software and are expressed as the mean ± SD. One-way ANOVA was used to assess the
differences between the means of the groups followed by post hoc Tukey's. Significance was defined as $P < 0.05$.

Results

**SH-SY5Y cell differentiation to mature neurons following 7 days of RA treatment in a FBS free DMEM/F12 culture medium**

To study the effect of norepinephrine (NE) and 17-β-estradiol (E2) on tau phosphorylation (p-tau) at Ser$^{396}$, in mature neurons, SH-SY5Y neuroblastoma cells were differentiated to mature neurons. To do so, the cells were treated with Fetal Bovine Serum (FBS) free medium supplemented with 10 µM of Retinoic Acid (RA). The results showed that the differentiation began in the second day of treatment (Fig. 1a) and continued up to seven days, by showing the elongation of neuronal-like processes extending from the cells (Fig. 1b and c). Immunofluorescent staining with MAP2 as the specific marker for mature neurons, confirmed the differentiation of SH-SY5Y cells to mature neurons at day 7 (Fig. 1d).

**Viability of differentiated SH-SY5Y cells following 24 and 48 hours of treatment with NE and E2**

To evaluate the effect of NE and E2 individual treatment and in combination in the presence and absence of Compound C and Rapamycin on cell survival, trypan blue exclusion assay was performed to calculate the percentage of cell survival. Our results showed no effect of our treatment on cell survival. That was presented by the percentage of trypan blue positive cells (stained) in the total population of cells (stained and unstained) after 24 and 48 h of treatment with NE and E2, the combination of both and with and without Compound C and Rapamycin compare to control group (Fig. 2).

**Effect of NE and E2 individually and in combination on p-tau (Ser$^{396}$), p-tau (Ser$^{202}$ Thr$^{205}$) and p-tau (Ser$^{202}$ Thr$^{205}$) and.**

Considering the demonstrated role of stress-related hormones on p-tau and the effect of E2 on regulating stress response, we examined the effect of NE/ E2 treatment separately and in combination on p-tau level at Ser$^{396}$, Ser$^{262}$, and p-tau (Ser$^{202}$/Thr$^{205}$) after 24 and 48 h. These
four residues are some of the major phosphorylated sites in AD with different characteristics. Ser\textsuperscript{396} and Ser\textsuperscript{262} are both sensitive to kinase activity of AMPK, while Ser\textsuperscript{202} and Thr\textsuperscript{205} are affected by the other tau kinases but not AMPK. Compare to control groups, western blotting revealed a significant time-dependent increase in p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262}), but not p-tau (Ser\textsuperscript{202}, Thr\textsuperscript{205}), by showing the increase in the p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262}) to tau ratio, in groups that separately treated with NE or E2 (One Way ANOVA, \( *p < 0.01 \)) (Fig. 3). Also Co-treatment with NE and E2 significantly increased p-tau (Ser\textsuperscript{396}) and p-tau (Ser\textsuperscript{262}) (One Way ANOVA, vs control group, \( *p < 0.01 \)) but not p-tau (Ser\textsuperscript{202}/Thr\textsuperscript{205}) compared with control group, however the level of increase was less than treatment with E2 or NE individually (the difference was not significant) (Fig. 3). Beta-actin levels remained the same across all lanes, confirming the equal loading of proteins in all wells.

Effect of Compound C and Rapamycin individually and in combination on p-tau (Ser\textsuperscript{396}) and p-tau (Ser\textsuperscript{262}) following NE and E2 co-treatment

Previous studies suggested a role for AMPK/mTOR axis in tau pathology and AD development (Caccamo et al., 2013; Domise et al., 2016). In view of these results, along with the reports on the positive impact of E2 and NE on AMPK and mTOR activation, we examined the effect of inhibition of AMPK and mTOR pathways separately and together, on p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262}) in the presence of NE and E2. Blocking AMPK by Compound C reduced p-tau at Ser\textsuperscript{396} and Ser\textsuperscript{262} (One Way ANOVA, vs control group, \( *p < 0.01 \)) (Fig. 4), by showing the significant decrease in the ratio of p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262}) to tau, while the level of \( \beta \)-actin remained the same across all lanes. Rapamycin treatment did not affect the elevated p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262}) in presence of NE and E2, however, co-treatment with Rapamycin and Compound C significantly reduced p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262}) after 24 (Fig. 4A) and 48 h of treatment (Fig. 4B) (One Way ANOVA, vs NE+E2 group **\( p < 0.01 \)). Immunofluorescent (IF) staining results showed an increase in p-tau (Ser\textsuperscript{396}) (Fig. 5, e, f, g, h) in NE+E2 treated compared with control group (Fig. 5 a, b, c, d), while the staining of tau (Ser\textsuperscript{396}) decreased subsequent to exposing the cells to Compound C (40 \( \mu \)M) (Fig. 5, i, j, k, l), supporting our western blot findings.

Effect of NE and E2 individually and in combination on p-AMPK (Thr\textsuperscript{172}) in the presence and absence of Compound C and Rapamycin

With the respect to the suggested kinase role for p-AMPK (Domise et al., 2016) and to address the involvement of activated AMPK in mediating the effect of NE and E2 on tau
phosphorylation, the activity level of AMPK was assessed in NE and E2 treated with and without Compound C and Rapamycin. Our results revealed a significant AMPK activity (p-AMPK/AMPK ratio) in the presence of NE and E2 (One Way ANOVA, vs control group, *p < 0.01) compared with control group. Our finding showed that compound C blocked this activation (Fig. 6) while treatment of the cells with Rapamycin had no effect on the enhanced AMPK activity in the presence of NE and E2 (Fig. 6). Our results from immunofluorescent staining confirmed the higher level of p-AMPK in the presence of NE and E2 after 24 h (Fig. 7 d, e, f) than control group (Fig. 7 a, b, c). Compound C treatment reduced p-AMPK (Fig 7 g, h, i), however AMPK remained highly activated in the presence of Rapamycin in NE+ E2 treated groups (Fig 7 j, k, l).

**PP2A activity in NE and E2 treated groups in the presence and absence of Compound C and Rapamycin**

Recent findings indicate a major role for protein phosphatase 2A (PP2A) down-regulation in tau hyperphosphorylation in AD (Qian et al., 2010; Sontag and Sontag, 2014). There is also a suggested role for NE and mTOR in modulating PP2A activity (Peterson et al., 1999; Pullar et al., 2003). To investigate the role of PP2A in tau hyperphosphorylation under NE and E2 treatment, PP2A activity (p-PP2A/PP2A ratio, normalized by β-actin intensities) has been evaluated. The results showed that PP2A activity remained at same levels in all treated and control groups with NE and E2 (β-actin levels remained the same across all lanes). The level of PP2A activity also did not show any significant change after blocking the AMPK and mTOR pathways with Compound C and Rapamycin, respectively (Fig 8A).

**GSK-3β activity in NE and E2 treated groups in the presence and absence of Compound C and Rapamycin**

As reported previously, AMPK activation can increase Glycogen Synthase Kinase-3β (GSK-3β) activation through reducing its phosphorylation at Ser9 (Horike et al., 2008). Because GSK-3β is one of the main kinases responsible for tau phosphorylation in AD brains (Hanger and Noble, 2011), we investigated its GSK-3β in tau hyperphosphorylation that we observed in differentiated SH-SY5Y cells after 24 and 4h h treatment with NE/E2. The results showed no significant difference between treated groups compared with control in GSK-3β activity (p- GSK-3β/ GSK-3β). The β-actin levels remained the same across all lanes (Fig. 8B) and p-GSK-3β to GSK-3β ratio were normalized by β-actin intensities.
Discussion

We previously demonstrated a specific role for adenosine monophosphate kinase protein kinase (AMPK) in phosphorylating tau, a main hallmark for Alzheimer’s disease (AD) and other tauopathies in neurons under hypoxic stress (Majd et al., 2017). It is believed that stimulation of hypothalamic-pituitary-adrenal (HPA) stress axis, leading to overproduction of cortisol and norepinephrine (NE), may participate in tau phosphorylation (p-tau) and beta amyloid production (Feng et al., 2005; Mravec et al., 2014; Rissman et al., 2007; Sotiropoulos and Sousa, 2016). From two main hallmarks of AD, p-tau seems to be an essential element in developing the pathology under stress (Lopes et al., 2016). The cellular mechanisms, linking NE release to p-tau, are not fully understood. In current study, we used an in vitro model of differentiated human neuroblastoma SH-SY5Y cells. The full differentiation of cell was proven by their positive staining with microtubule associated protein 2 (MAP2) in our study. For these cells, the presence of adrenergic receptors have been documented previously (Kazmi and Mishra, 1989). Specifically, it was demonstrated that differentiated SH-SY5Y cells to mature neurons under retinoic acid treatment (the same as our protocol), express NE receptors, NE transporter (NET) and the vesicular monoamine transporter (VMAT), some of the characteristic of adrenergic neurons (Kovalevich and Langford, 2013).

Our results in particular revealed a timely increase of p-tau (Ser^{396}) and (Ser^{262}), two AMPK sensitive residues of tau following exposure to NE. Ser^{396} and Ser^{262}, have been proven as two residues of tau that are sensitive to kinase activity of AMPK, the main energy regulator of cells under stress (Gu et al., 2013; Thornton et al., 2011). This finding is consistence with other studies, including ours, reporting long-term tau hyperphosphorylation at some residues including Ser^{396} and Ser^{262} under stressful conditions such as hypoxia, hypothermia, and hibernation (Arendt et al., 2003; Majd et al., 2017; Planel et al., 2004).
It was suggested that stress stimulated release of central NE (Nisenbaum et al., 1991). Our finding showed the positive effect of NE on tau phosphorylation. This finding suggests that NE, an important component of stress response could play a critical role in tauopathy. In accordance with our results, Braak et al. demonstrated an extensive level of tau neuropathy, which happened at the very first stages of AD in locus coeruleus (LC), the main NE source of brain (Braak et al., 2011). Many other experimental evidence proposed a substantial role for NE receptors, especially β1 and β2 in tau hyperphosphorylation, through activating protein kinase A (PKA) and c-Jun N-terminal kinase (JNK) cascade (Wang et al., 2013). Norepinephrine also increases the expression of serine-threonine kinases, some of them with tau kinase activity (Chenal and Pellerin, 2007; Hutchinson et al., 2005). The role of stress in tauopathy was further confirmed when a substantial elevation in p-tau was reported through exposing animals to long period of restraining stress, a stimulatory model to anxiety and social stress in humans (Rissman et al., 2012).

One of the examples of the stressful situation in humans is the transition phase to menopause, and the menopause itself in women. The controversy around the impact of this transition on tau phosphorylation and AD development is remained to be clear. While our findings showed the positive impact of each 17-β-estradiol (E2) and NE on p-tau, combination of both generated a milder effect. It was previously reported that NE effect could be modulated by estradiol (Conrad et al., 2004; Herbison et al., 2000; Shansky et al., 2004). The effect of estradiol on memory by itself, as the main female sex hormone is still controversial. A suggested role for estradiol in memory improvement through binding to neurotrophin receptors, activation of phosphatidylinositol3-kinase (PI3K) and mTOR pathway followed by protein synthesis (Luine, 2014) was suggested before. In opposite, a large human study showed that giving estradiol to women at late menopause actually increased the risk of dementia (Luine, 2008). Some studies reported that estradiol improved cognitive performance in adult and middle-aged ovariectomized rats and mice via activation of estrogen alpha (ERα) and beta (ERβ) receptors (Rodgers et al., 2010; Talboom et al., 2008), while some others showed no significant effect of hormone therapy in reducing the risk of AD (Imtiaz et al., 2017; Ohm et al., 2012). While experimental situation could be the reason for this conflict, the level of estrogen receptors’ involvement during different periods of time and in different conditions could be an explanation.

A recent study demonstrated that activation of ERα by estradiol positively regulated the p-tau (Xiong et al., 2015). Differentiated SH-SY5Y cells contains a substantial level of estradiol receptors (Barbati et al., 2012; Grassi et al., 2013). Our findings showed that exposure to NE
and E2, enhanced p-tau \( \text{Ser}^{396} \) and \( \text{Ser}^{262} \) after 24 and 48 h of treatment. These results could be explained by the previous finding, showing the existence of two ER\( \alpha \) and ER\( \beta \) receptors for estradiol, while ER\( \alpha \) positively, whereas ER\( \beta \) negatively, regulated p-tau (Xiong et al., 2015). Other evidence suggest the preference of E2 in acting though ER\( \alpha \) but not ER\( \beta \) in a variety of situations (Wang et al., 2004). Interestingly, the proposed neuroprotection effect of estradiol through its anti-inflammatory effect is believed to be mediated by ER\( \alpha \) (Vegeto et al., 2003) while the impact of this activation on p-tau at the same time could limit its positive effect. This could clarify the contradiction of the findings on protective effects of estradiol in different studies. Our findings showed that tau hyperphosphorylation was less prominent when we combined E2 with NE. That could be explained by the modulatory role of E2 on NE function (Manly et al., 2000), or more probably, the effect of NE in reducing the nuclear estrogen receptors which has previously reported (Montemayor et al., 1990).

Tau phosphorylation in our study remained significantly higher in NE+E2 treated groups compared with control group. Apart from modulatory effect of E2 on NE (Conrad et al., 2004; Herbison et al., 2000; Shansky et al., 2004), the observed significant increase of p-tau \( \text{Ser}^{396} \) and \( \text{Ser}^{262} \) in presence of NE and E2, could be explained by the others’ reports. Previous evidence showed a common stimulatory effect of both NE and E2 on AMPK pathway (Chen et al., 2014; Hutchinson et al., 2005; Lipovka et al., 2015; Yin et al., 2003). Interestingly, p-tau at Ser\(^{202}/\text{Thr}^{205} \) two AMPK insensitive sites of tau (Domise et al., 2016) was not affected by NE and E2 in the current study. This supports our hypothesis, suggesting that tau hyperphosphorylation effect of NE and E2 is mediated through AMPK. Our current findings showed blocking AMPK by Compound C significantly reduced p-tau \( \text{Ser}^{396} \) and \( \text{Ser}^{396} \) in differentiated SH-SY5Y cells following NE and E2 co-exposure. AMPK is one of the main intracellular pathways that activates under different stress conditions (Batandier et al., 2014). AMPK family of proteins act as one of the main tau kinases phosphorylating tau at many residues including \( \text{Ser}^{396} \) and \( \text{Ser}^{262} \) (Gu et al., 2013; Thornton et al., 2011). Demonstrating AMPK activation by E2 and NE in this study is in compliance with previous reports, proposing that activation of AMPK by E2 enhances AMPK \( \alpha \)-catalytic subunit phosphorylation (Rogers et al., 2009). Further studies revealed that the AMPK activation via E2 is mediated through estrogen receptor \( \alpha/\beta \) (Lipovka et al., 2015). Adrenergic signaling pathway also leads to increase in AMPK activation through AMPK \( \alpha 1 \) subunit phosphorylation (Mulligan et al., 2007), with a specific role for \( \beta \)-adrenergic receptor activation by NE (Hutchinson et al., 2005). It is not still clear whether AMPK activation plays any role in mediating NE and E2 effect on tau phosphorylation. Using the specific
inhibitor of AMPK, Compound C, in the presence of NE and E2, we observed a significant reduction in p-tau that was occurred before applying Compound C. This finding suggests that the hyperphosphorylation of tau in our study was mainly mediated through AMPK activation. Along with AMPK, mTOR is the other element in regulating cellular response to stress as downstream target of AMPK. It was shown that upregulation of mTOR signaling pathway plays an important role in major pathological processes of AD, including beta Amyloid accumulation and tau phosphorylation (Cai et al., 2015; Xu et al., 2012). mTOR activation itself is modulated by other factors including NE and E2. It is reported that both NE and E2 increase activation of mTOR (Luine, 2014; Yu and Henske, 2006), which raises the possibility of mTOR involvement in their effects on tau phosphorylation. Our results showed that mTOR inhibition by Rapamycin did not affect p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262}) elevation due to NE/ E2 treatment. Co-treatment of CC and Rapamycin, in order to block the possible activated mTOR due to AMPK inhibition, did not make any change in the effect of AMPK blockage on reducing p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262}). This finding suggests that NE and E2 effect on tau hyperphosphorylation is mediated through AMPK activation in this study. Serin\textsuperscript{396} could also be phosphorylated by Glycogen Synthase Kinase-3β (GSK-3β), and dephosphorylated by Protein Phosphatase 2A (PP2A), two main tau kinase and phosphatase. Human studies showed the enhanced activation of GSK-3β and declined activity of PP2A, in AD brains (Ferrer et al., 2002; Rudrabhatla and Pant, 2011). To determine the involvement of these two enzymes in p-tau (Ser\textsuperscript{396}) and (Ser\textsuperscript{262}) increase due to NE/E2 treatment, the activity of GSK-3β and PP2A were examined in our study. The current findings showed no significant change in activity levels of these enzymes, suggesting the AMPK-mediated effect of NE/E2 co-treatment as the possible mechanism for tau hyperphosphorylation. It is also important to note that in parallel with this cascade, the interaction between NE and E2 could be considered as the other element to modulate the mediatory role of AMPK, which requires further investigation.

Conclusions

In conclusion, our study suggests that exposure to NE and E2 might develop tau hyperphosphorylation at Ser\textsuperscript{396} and Ser\textsuperscript{262}, one of the main hallmarks of AD. This hyperphosphorylation in mainly mediated via AMPK activation, with no significant involvement of mTOR, GSK-3β and PP2A pathways. Combining these findings, with the view of stressful condition of menopause, brings this point to attention that, trail therapeutic...
strategies such as hormone therapy to prevent AD, must be taken in action with extra cautious along with stress management.

**Acknowledgments**

This study was supported by the Establishment grant from Flinders University (39468). The authors would like to thank the Motor Neuron Disease (MND) laboratory of Centre for Neuroscience, and the Microscopic and Proteomics facilities of Flinders University for their enthusiastic support. All authors were involved in the study design, development of the model and completing the experiments. The initial draft was written by SM and reviewed by ZM, SK and JP.

**Conflict of interests**

There are no conflicts of interest to disclose in this study.
Figure 1. SH-SY5Y differentiation to mature neurons. Differentiation of SH-SY5Y cells began at the second day of treatment with 10 µM of Retinoic acid (RA) in Fetal Bovine Serum (FBS) free DMEM/F12 culture medium (Fig. 1a). The elongation of the cellular processes continued for 7 days (Fig. 1b and c), and the immuno-fluorescent staining with specific marker for the mature neurons, MAP2, confirmed the fully differentiation of the cells to mature neurons after 7 days of differentiation (Fig. 1d). Scale bars, 25 µm.

Figure 2. The effect of 24 and 48 h treatment with norepinephrine (NE) (10⁻⁵ M) and 17-β-estradiol (E2) (10 nM) in the presence and absence of Compound C (CC; 40 µM), Rapamycin (Rap; 20 nM), and a combination of both on cell viability using trypan blue exclusion method. The percentage of cell death did not show any significant difference between groups. Error bars depict the SD.

Figure 3. Norepinephrine and 17-β-estradiol (E2) individually and in combination increase tau phosphorylation at Ser³⁹⁶ and Ser²⁶². A) Western blot analysis of p-tau (Ser³⁹⁶), (Ser²⁰²/Thr²⁰⁵), (Ser²⁶²) and tau protein in control, norepinephrine (NE), 17-β-estradiol (E2) and NE+E2 groups. (B) Quantification of the p-tau (Ser³⁹⁶) and (Ser²⁶²) to tau ratio after normalizing data with β-actin intensities showed significant increase in tau phosphorylation at Ser³⁹⁶ in treated groups with 10⁻⁵ M of NE, 10 nM of E2, and NE+ E2 compared with control group (One Way ANOVA, *p < 0.01) (Fig. 3) while p-tau (Ser²⁰²/Thr²⁰⁵) showed the same intensities in all bands. The level of p-tau (Ser³⁹⁶) and (Ser²⁶²) increase in NE+ E2 group was less than individual treatment with each NE or E2, in both 24 and 48 h of treatment, however the difference between them was not significant. Error bars depict the SD. All values are expressed as percent change relative to control group and were corrected by the β-actin level.
Figure 4. Compound C (CC), but not Rapamycin (Rap) treatment decreased p-tau (Ser^{396}) and p-tau (Ser^{262}) elevation in NE+E2 treated groups after 24 and 48 h. Western blot analysis of p-tau (Ser^{396}), p-tau (Ser^{262}) and tau protein along with the quantification assay of p-tau (Ser^{396})/tau and p-tau (Ser^{262})/tau after 24 h (A) and 48 h (B) treatment of NE+E2 groups with Compound C (CC; 40 μM), Rapamycin (Rap; 20 nM), and the combination of both. Significant increase in p-tau (Ser^{396})/tau and p-tau (Ser^{262})/tau were observed in NE+E2 group compared with control group (One Way ANOVA, vs control group, *p < 0.01). The results showed the significant decrease in p-tau (Ser^{396})/tau and p-tau (Ser^{262})/tau following 24 and 48 h treatment with Compound C, and the combination of Compound C and Rapamycin (One Way ANOVA, vs NE+E2 treated group, **p < 0.01) but not Rapamycin alone, compared with NE+E2 treated group (Fig. 4). Error bars depict the SD. All values are expressed as percent change relative to control group and were corrected by the β-actin level.

Figure 5. Immunofluorescent (IF) staining showed no significant p-tau (Ser^{396}) elevation in NE+E2 group treated with Compound C. Differentiated SH-SY5Y cells showed an increase in their p-tau (Ser^{396}) levels (Green) following 48 hours treatment with NE+E2 (Fig. 5. e, f, g, h) compared with control group (Fig. 5 a, b, c, d). Compound C (40 μM) treatment decreased tau phosphorylation at Ser^{396} (Fig. 5 I, j, k, l). The tau showed the same level of staining in all groups (Red). Scale bars (a-d: 10 μm; e-l: 25 μm)

Figure 6. Adenosine monophosphate protein kinase protein (AMPK) activity increased under NE and E2 treatment. A) Western blot data of p-AMPK (Thr^{172}) and total AMPK in norepinephrine (NE), 17-β-estradiol (E2), NE+ E2, NE+ E2 with 40 μM Compound C (CC), NE+ E2 with 20 nM Rapamycin (Rap) and 40 μM Compound C, and NE+ E2 with 20 nM Rapamycin only, showed the substantial increase in NE+ E2 group after 24 and 4 h. The increased activity was blocked with Compound C, but not Rapamycin. B) Quantification analysis revealed a significant increase in AMPK activity (p-AMPK/AMPK ratio) in NE+ E2 treated groups compared with control group. The enhanced activity was diapered after Compound C treatment. Error bars depict the SD. All values are expressed as percent change relative to control group and were corrected by the β-actin level.

Figure 7. Immunofluorescent (IF) imagining showed a substantial increase in Adenosine monophosphate protein kinase protein (AMPK) phosphorylation at Thr^{172} (green) following
24 h cell treatment with norepinephrine (NE) and 17-β-estradiol (E2) (d, e, f) compared with control group (a, b, c). Compound C (CC) reduced p-AMPK (g, h, i) in NE+E2 treated groups, while AMPK remained highly phosphorylated in the presence of Rapamycin (Rap) in NE+E2 group (j, k, l). Scale bars, 10 μm.

**Figure 8.** A) Protein Phosphatase 2A (PP2A) activity remained unchanged in all groups. Western blot results of p-PP2A and total PP2A in norepinephrine (NE), 17-β-estradiol (E2), NE+ E2, NE+ E2 with 40 μM Compound C (CC), NE+ E2 with 20 nM Rapamycin (Rap) and 40 μM Compound C, and NE+ E2 with 20 nM Rapamycin only, showed the same intensity in the bands in all lanes. Quantification analysis revealed no significant difference in p-PP2A/PP2A in all treated groups after 24 and 48 h compared with control group. B) Glycogen Synthase Kinase-3β (GSK-3β) activity in norepinephrine (NE) and 17-β-estradiol (E2) treated groups in the presence and absence of Compound C (CC) and Rapamycin (Rap) remained unchanged. Western blot results of p-GSK-3β and total GSK-3β in all tread groups (norepinephrine (NE), 17-β-estradiol (E2), NE+ E2, NE+ E2 with 40 μM Compound C, NE+ E2 with 20 nM Rapamycin and 40 μM Compound C, and NE+ E2 with 20 nM Rapamycin only) revealed the same intensities for p-GSK-3β and total GSK-3β bands in all groups. Quantification analysis of western blot results showed no significant difference in p-PP2A/PP2A between treated groups after 24 and 48 h compared with control group. Error bars depict the SD. All values are expressed as percent change relative to control group and were corrected by the β-actin level.
References


Highlights:

1- NE and E2 treatment separately, and in combination increase tau phosphorylation at Ser^396 and Ser^262 (AMPK-sensitive residues) but not Ser^202/Thr^205 (AMPK-insensitive residues).

2- Compound C inhibits AMPK activity and blocks p-tau (Ser^396, 262) elevation due to NE and E2 treatment.

3- Rapamycin (m-TOR inhibitor) has no effect on NE+ E2 dependent p-tau elevation at (Ser^396, 262).
Figure 3
Figure 6

A

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B

Relative p-AMPK(Thr^{172})/AMPK Density to Control (Normalized to Actin Intensities)

- Control
- NE+E2-24 h
- NE+E2+CC-24 h
- NE+E2+CC+Rap-24 h
- NE+E2+Rap-24 h
- NE+E2-48 h
- NE+E2+CC-48 h
- NE+E2+CC+Rap-48 h
- NE+E2+Rap-48 h

* indicates statistical significance.