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Estradiol stimulates an anti-translocation expression pattern of glucocorticoid co-regulators in a hippocampal cell model

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Abstract

A consistent clinical finding in patients with major depressive disorder (MDD) is hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis, the system in the body that facilitates the response to stress. It has been suggested that alterations in glucocorticoid receptor (GR)-mediated feedback prolong activation of the HPA axis, leading to the dysfunction observed in MDD. Additionally, the risk for developing MDD is heightened by several risk factors, namely gender, genetics and early life stress. Previous studies have demonstrated that GR translocation is sexually dimorphic and this difference may be facilitated by differential expression of GR co-regulators. The purpose of this study was to determine the extent to which ovarian hormones alter expression of GR and its co-regulators, *Fkbp5* and *Ppid*, in HT-22 hippocampal neurons. The impact of corticosterone (cort), estradiol (E2), and progesterone (P4) treatments on the expression of the genes *Nr3c1*, *Ppid*, and *Fkbp5* was assessed in HT-22 hippocampal neurons. Treatment of cells with increasing doses of cort increased the expression of *Fkbp5*, an effect that was potentiated by E2. Exposure of HT-22 cells to E2 decreased the expression of *Ppid* and simultaneous exposure to E2 and P4 had combinatory effects on *Ppid* expression. The effects of E2 on *Ppid* extend previous work which demonstrated that serum E2 concentrations correlate with hippocampal *Ppid* expression in female rats. The results presented here illustrate that E2 generates an anti-translocation pattern of GR co-regulators in hippocampal cells.

Keywords

Corticosterone; HT-22; Estradiol; Progesterone ; *Fkbp5*; *Ppid*; *Nr3c1*; Glucocorticoid receptor

1. Introduction

Chronic stress exposure has been linked to the pathophysiology of multiple diseases and disorders [1]. The hypothalamic–pituitary–adrenal (HPA) axis mediates the stress response and response to stress is terminated by a negative feedback mechanism. In the acute state, the response to stress is adaptive and provides the necessary energy for an organism to cope with the stressor. However, when stress exposure becomes chronic, negative feedback on the HPA axis can become insensitive, leading to prolonged exposure to glucocorticoids, the hormones that mediate the stress response [2].

Negative feedback on the HPA axis is stimulated when circulating glucocorticoids bind to cytosolic glucocorticoid receptors (GR) in the hippocampus, prefrontal cortex, and pituitary gland. Ligand binding to the GR leads to nuclear translocation and subsequent interaction with DNA response elements, ultimately leading to gene transcription and termination of the stress response. Upon binding to glucocorticoids, the GR dimerizes and subsequently translocates into the nucleus, a process that is modulated by several immunophilin co-chaperones. These proteins are postulated to regulate both GR sensitivity to circulating glucocorticoids and its translocation upon ligand binding [3]. FK506 protein 51, also known as *Fkbp5*, is a normal component of 55% of GR complexes that lowers GR's affinity for glucocorticoids. *Ppid*, an immunophilin peptidyl-prolyl cis trans isomerase also known as Cyp40, competes with *Fkbp5* and facilitates GR translocation by increasing GR's affinity for the motor protein dynein [3]. Once in the nucleus, GR activates a multitude of genes, including *Fkbp5* in an ultra-short feedback loop that negatively regulates further GR translocation [4]. Given that *Fkbp5* suppresses GR translocation, increased expression of *Fkbp5* is hypothesized to reduce GR negative feedback on the HPA axis. Furthermore, functional polymorphisms of *Fkbp5* lead to higher expression and blunted negative feedback on the HPA axis and have repeatedly been associated with a higher risk for developing mood disorders [5].

The manifestation of stress related disorders such as depression is greater in women than in men [6], and the susceptibility of women is heightened during times of fluctuation in ovarian hormones [7–9]. Rodent studies suggest that these differences first appear during adolescence, with females displaying more depressive behavior after exposure to chronic stress compared to males [10–16]. Furthermore, the differences in stress-induced changes in behavior may be a reflection of sex differences in the stress-induced changes in the HPA axis. Recently, our group documented that both GR translocation and expression of GR co-chaperones differed between male and female rats exposed to chronic adolescent stress. Females with a history of chronic adolescent stress displayed increased depressive-like behavior [10], impaired negative feedback of the HPA axis, and a reduction in GR sensitivity; results that were not observed in males [12].

Sex differences in the effects of stress on GR may be facilitated by gonadal hormones. On a molecular level, gonadal steroids influence the activity of both the GR itself and its co-regulators [11,12]. For example, estradiol (E2) has been shown to reduce GR action, and progesterone (P4) competes with cort for GR binding [17,18]. Additionally, gonadal hormones influence GR co-regulators. In breast cancer cells, E2 has been shown to regulate the expression of *Ppid*, whose gene-product is believed to facilitate GR translocation [19]. Additionally, *Fkbp5* has hormone response elements for androgens and progestins, as well as glucocorticoids [20,21]. Crosstalk in regulation of co-regulators by ovarian steroids suggests a molecular mechanism by which ovarian steroids can modulate GR sensitivity. Taken together, these studies suggest a role of ovarian steroids in the modulation of the GR system. Several studies have examined GR regulation *in vitro* but they have focused on cells of peripheral origin and cell type has been demonstrated to impact the influence of ovarian steroids on co-regulators [22–25]. Further study is therefore necessary to determine if ovarian steroids modulate expression of GR co-regulators in neurons.

Previous work suggests that ovarian steroids influence hippocampal GR regulation more than testosterone [12], therefore, the current study focused on the effects of E2 and P4 on the expression of *Nr3c1*, the gene that encodes GR, and expression of two of GR's co-regulators, *Fkbp5* and *Ppid*. To determine whether E2 and P4 could alter the expression of GR co-regulators in hippocampal cells, we assessed the impact of multiple E2 and P4 concentrations in combination with cort on the expression of *Nr3c1*, *Fkbp5*, and *Ppid* *in vitro* in HT-22 mouse hippocampal neurons. HT-22 cells represent an ideal model system

for these hormone studies, as they express functional GR, PR and both isoforms of the ER [26–28].

2. Materials and methods

2.1. Cell line

HT-22 cells, which are immortalized cell line derived from mouse hippocampi were obtained from Salk Institute (La Jolla, CA). Cells were cultured in media made of Dulbecco's modified eagle medium (DMEM) (Invitrogen, Grand Island, NY) and 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY) with PenStrep (Life Technologies) under standard conditions (37 °C, 5% CO₂).

2.2. Hormone preparations

Hormone doses were based on previous *in vivo* observations of serum hormone level in rats during following stress exposure [12]. Prior to each hormone exposure, cells were seeded at a density of 6.2×10^4 into six-well plates using 0.05% Trypsin–EDTA, and allowed to grow for 24 h. The medium was then replaced with a stripped medium containing a phenol red free DMEM/F12 (Invitrogen, Grand Island, NY) and 10% charcoal-stripped fetal bovine serum (Sigma Aldrich, St. Louis, MO) for 24 h. This medium has minimal hormones, and thus will not confound the effects of exposing cells to hormones. After this period, cells were exposed to various hormone concentrations for a 24 h period. In total, eight wells (n = 8) were used for each hormone condition, taken from four separate passages of HT-22 cells. Hormone concentrations used in this study were determined based on peak serum hormone levels observed previously in our lab [12].

The cort solution was prepared by dissolving cort (Sigma Aldrich, St. Louis, MO) in EtOH to make a 29 mM solution (10 mg/mL). Cort was further diluted 1:200 in sterile PBS to make 0.145 mM (50,000 ng/mL) stock solution. The E2 solution was prepared by dissolving -estradiol (Sigma Aldrich, St. Louis, MO) in EtOH to make 3.7 mM (1 mg/mL) solution. The E2 solution was diluted 1:100,000 in sterile PBS to make a 37 nM stock solution (10 ng/mL). The P4 solution was prepared by dissolving P4 (Sigma Aldrich, St. Louis, MO) in EtOH to make a 3.2 mM (1 mg/mL) solution. The P4 solution was diluted 1:200 in sterile PBS to make 0.016 mM (5000 ng/mL) stock solution. For baseline conditions, EtOH was diluted 1:200 in sterile PBS to make vehicle solution, and control samples were treated with the same amount of this vehicle solution. These stock solutions were added to stripped media to make the appropriate concentrations of hormones.

The hormone concentrations are summarized as follows: In the primary study, cort doses of 0, 50, 400 and 800 ng/mL were applied in conjunction with 0, 20, or 40 pg/mL of E2. Further, cort doses of 0, 50, 400, or 800 ng/mL were applied in conjunction with 0, 10, or 30 ng/mL of P4. In the third study, cort doses of 0, 50, or 400 ng/mL were applied in conjunction with both E2 (0, 20, 40 pg/mL) and P4 (0, 10, 30 ng/mL).

2.3. RT-PCR

After 24 h of hormone exposure, cells were pelleted using 0.05% Trypsin–EDTA. RNA was extracted and RT-PCR was performed as previously described [12]. Briefly, Taqman primer/probe chemistry was used along with Taqman gene expression Master Mix. cDNA was synthesized via AB High Capacity CDNA RT-Kit (Applied Biosystems). Gene expression was quantified for *Nr3c1*, *Ppid*, and *Fkbp5*. *Tfrc* was used as the housekeeping reference gene, and C_t values were calculated in the same manner. Additionally, C_t were normalized to baseline conditions. Changes in gene expression are reported as fold change (2^{-C_t}).

2.4. Statistical analyses

GraphPad Prism 4.0 and SPSS were used for all statistical analyses in this study, and an alpha value was set to 0.05 for all tests. Sample sizes were as follows: baseline condition (hormone-free), $n = 12$, and for all other conditions, $n = 7$ or 8 . In rare cases, wells were removed due to the fact that they did not amplify during PCR which lowered the sample size from 8 to 7 . Three-way ANOVAs were conducted to analyze whether P4 and Cort or E2 and Cort had combinatory effects on gene expression. In the cases of the combined effects of E2 and P4, two way ANOVAS were conducted for each of the Cort concentrations followed by Tukey posthoc tests if a main effect was found. If an interaction effect was found, a main effect test was conducted in order to determine the level at which the hormones interacted.

3. Results

3.1. Expression of *Nr3c1* was not altered by hormone exposure

Exposure to 24 h of any hormone tested did not alter *Nr3c1* expression, the gene which encodes GR, as compared to the hormonefree condition. There was no effect of cort exposure ($F_{3,88} = 0.37$, $p > 0.05$; Fig. 1A, B), E2 exposure ($F_{2,88} = 0.48$, $p > 0.05$; Fig. 1A), or P4 exposure ($F_{2,88} = 0.28$, $p > 0.05$; Fig. 1B) on the expression of *Nr3c1*. In addition, no combination of these hormones resulted in altered expression of *Nr3c1* ($p > 0.05$).

3.2. Expression of *Fkbp5* increased in response to cort and E2, but not P4

Exposure to cort was independently sufficient to increase *Fkbp5* expression ($F_{3,87} = 87.34$, $p < 0.001$; Fig. 2A, B) at concentrations of 400 ng/mL and 800 ng/mL ($p < 0.001$). E2 did not independently alter expression of *Fkbp5* ($F_{2,87} = 0.60$, $p > 0.05$; Fig. 2A); however, cort and E2 interacted to alter *Fkbp5* expression ($F_{6,87} = 2.27$, $p < 0.05$; Fig. 2A). Specifically, E2 increased *Fkbp5* expression when cort was held at 400 ng/mL ($p < 0.01$), but not when cort was at 0 ng/mL ($p > 0.05$), 50 ng/mL ($p > 0.05$), or 800 ng/mL ($p > 0.05$). P4 did not have a significant effect on *Fkbp5* expression, and there were no interaction effects between E2, P4 and cort ($p > 0.05$; Fig. 2B).

3.3. Expression of *Ppid* decreased in response to E2

Twenty-four hours of exposure to E2 decreased *Ppid* expression ($F_{2,85} = 4.90$, $p < 0.01$; Fig. 3A) as compared to hormone-free conditions. Exposure to cort did not alter *Ppid* expression ($F_{3,85} = 1.05$, $p > 0.05$; Fig. 3A; B). The effects of E2 were specific to exposure at the 20 pg/mL concentration and resulted in decreased expression of *Ppid* compared to 0 pg/mL of E2 ($p < 0.05$; Fig. 3A). Dual exposure to cort and E2 did not interact to further alter *Ppid* expression ($p > 0.05$; Fig. 3A). Exposure to P4, whether alone or in combination with cort did not alter *Ppid* expression ($F_{2,85} = 0.081$, $p > 0.05$; Fig. 3B).

3.4. Exposure to E2 and P4 had combinatory effects on *Ppid* expression

When cells were exposed to P4 and E2 simultaneously, *Ppid* expression was altered in a manner distinct from E2 exposure alone. In the absence of cort, E2 (40 pg/mL) in combination with P4 (30 ng/mL) stimulated a 20% increase in *Ppid* expression compared to baseline ($F_{2,63} = 6.27$, $p < 0.01$; Fig. 4A). This increase in *Ppid* expression in the absence of cort is in contrast to no effect of P4 alone on *Ppid* expression and a decrease in *Ppid* expression stimulated by exposure to E2 alone. When cort (50 ng/mL) was included in the culture media, the combination of the high concentration of E2 (40 pg/mL) and either concentration of P4 (10 ng/mL or 30 ng/mL) reduced *Ppid* expression ($F_{2,62} = 4.46$, $p < .05$; Fig. 4B). This is in contrast to no independent effect of P4 exposure and the demonstration of E2 only precipitating an effect on *Ppid* at the lower concentration (20 pg/mL) when cort was included in the media (50 ng/mL). Finally, in the presence of high cort (400 ng/mL), the

combination of E2 (40 pg/mL) and P4 (10 ng/mL) had no effect on *Ppid* expression; which coincided with the fact that neither E2 nor P4 independently altered *Ppid* expression.

4. Discussion

The goal of the current study was to determine if either E2 or P4, independently or in combination with one another, could alter the expression of GR co-regulators. The data presented here demonstrate that E2 modified the effects of cort on *Fkbp5* leading to augmented expression of *Fkbp5*. Conversely, E2 independently decreased expression of *Ppid*. In contrast to the effects of E2, P4 did not alter expression of either *Fkbp5* or *Ppid*, alone or in combination with cort. Combination of P4 with E2 appeared to antagonize the effects of E2 on *Ppid* expression and the combination of P4 and E2 had no effect on *Fkbp5* expression. Collectively, these data demonstrate that E2 has the capacity to influence the expression of GR co-regulators in hippocampal cells. The influence of E2 on GR co-regulators may explain previously documented sex differences in the effects of chronic stress on expression of GR co-regulators in the hippocampus. The profile of altered expression documented, that is, increased *Fkbp5* and decreased *Ppid*, may lead to inhibited translocation of the GR as previously described in females following chronic stress [12].

4.1. Expression of GR in HT-22 cells does not respond to Cort, E2, or P4

In contrast to previous reports, the current data set demonstrates that neither cort nor ovarian steroids regulate the expression of GR. Multiple studies have presented evidence of down-regulation of GR in the presence of cort in multiple peripheral cell lines and tissue [23–25], but the cell line in the current study was of hippocampal origin and this contrasting data may represent a difference between peripheral cells and neurons. Previous work demonstrates the necessity of activation of the mineralocorticoid receptor (MR) in the down-regulation of GR in the hippocampus [29]. MR is the type I glucocorticoid receptor that has a higher affinity for circulating cort than GR, and MR is occupied at basal levels of cort. Some studies suggest that HT-22 cells do not express MR [30]. If this is the case, this may explain why treatments with cort did not change expression of GR. In fact, previous *in vivo* studies showed that rats exposed to stress had levels of GR expression in the hippocampus no different than non-stressed controls [12]. Data from our HT-22 cells support the observation that cort does not always alter GR expression in hippocampal neurons.

4.2. E2 increases *Fkbp5* expression in HT-22 cells

Increasing concentrations of cort applied to HT-22 cells increased the expression of *Fkbp5* (Fig. 2A; B). A positive regulation of *Fkbp5* transcription by cort is consistent with previous reports of an up-regulation of *Fkbp5* expression in hippocampus and hypothalamus following glucocorticoid exposure both *in vitro* in hippocampal cell lines and *in vivo* as a result of stress [31,32]. This up-regulation of *Fkbp5* after exposure to glucocorticoids is believed to be part of an ultra-short feedback loop whereby GR regulates its own sensitivity [33]. Specifically, an increase in circulating cort results in increased *Fkbp5* expression, which prevents prolonged GR translocation. Mechanistically, cort has been shown to exert epigenetic effects on the expression of *Fkbp5*. Specifically, prolonged exposure to cort reduced DNA methylation of the *Fkbp5* gene in the hippocampus both *in vivo* and *in vitro* [31,34]. DNA methylation can be associated with gene silencing, and thus the cort-induced decrease in DNA methylation results in higher expression of *Fkbp5*. Thus cort could affect *Fkbp5* expression by an epigenetic mechanism instead of direct binding to the DNA.

Exposure to E2 augmented the effects of cort on *Fkbp5* expression. At a cort concentration of 400 ng/mL, there is an interaction effect in which E2 concentrations of 20 pg/mL and 40 pg/mL augment the cort-induced increase in expression of *Fkbp5* (Fig. 2A). *Fkbp5* does not

have an estrogen response element [21], but E2 may modulate gene expression through indirect interaction with transcription factors that regulate *Fkbp5* expression. Indeed, human studies show that pregnant women with a history of mood disorders have an increase in the expression of *Fkbp5* [35]. The prolonged elevations of gonadal steroids during pregnancy suggest an influence of these steroids in regulation of those genes [35]. Additionally, estrous cycle stage can predict stress reactivity [36]. The present study suggests that estradiol sensitizes the *Fkbp5* response to cort. Taken together, these studies suggest an important role of estradiol in modulation of the GR system.

Conversely, the administration of P4 did not have an effect on the expression of *Fkbp5*. This finding is somewhat perplexing given the fact that *Fkbp5* has a response element where the glucocorticoid receptor, androgen receptor (AR) and P4 receptor (PR) all bind. However, induction of *Fkbp5* by progestins has only been shown in human carcinoma cell lines and human breast cancer cells [21,37]. Some studies in the uterus and hypothalamus show that PRs require E2 “priming” in order for P4 to successfully bind to its own receptor. Accordingly, the present study also determined whether the treatment of E2 and P4 simultaneously would influence co-regulator gene expression. These results suggest that E2 independently influences *Fkbp5* expression and P4 does not. Various combinations of E2, P4 and cort produced no larger effect on *Fkbp5* expression than any one hormone alone.

4.3. E2 decreases expression of *Ppid* in HT-22 cells

E2 treatment was sufficient to reduce expression of *Ppid* in hippocampal cell culture. Treatment of HT-22 cells with 20 pg/mL E2 reduces the expression of *Ppid* (Fig. 3A), but neither cort nor P4 independently affected expression of *Ppid* (Fig. 3). The simultaneous administration of E2 and P4 produced treatment-induced expression patterns different from those of either hormone individually (Fig. 4). These data suggest that E2 and P4 may uniquely interact to regulate expression of *Ppid*. This is significant given the vast hormone fluctuation in females as a function of the reproductive state and estrous cycle, which may change GR activity at different points. The lack of interaction at higher cort concentrations could be explained by the fact that cort and P4 have been shown to have cross-talk at the receptor level with progesterone binding at an allosteric site on the GR [38]. *Ppid*, also known as Cyp40, is a major immunophilin present in approximately 10% of all GR complexes, and the most common in estrogen receptor complexes. It is believed to facilitate the nuclear translocation of glucocorticoid receptors through recruitment of the motor protein dynein to the steroid receptor complex. For estrogen receptors, which are already localized in the nucleus, *Ppid* is thought to facilitate the conformational change of the estrogen-bound receptor [39]. As mentioned, *Ppid* is a positive regulator of GR translocation to the nucleus. *Ppid* occupies the same binding site on the GR receptor as *Fkbp5*, a negative regulator of translocation. Therefore, E2-induced decreases in *Ppid* expression would, in theory, facilitate additional binding of *Fkbp5* and sequester GR in the cytoplasm leading to glucocorticoid resistance.

5. Conclusions

Collectively, these results suggest that gonadal steroids provide a causal link between concentrations of serum gonadal hormones and previously observed changes in the GR signaling system [12]. The results in this study provide a basis for the attenuated GR translocation observed in females with a history of chronic adolescent stress [12]. The present study demonstrates that E2 is capable of augmenting cort-induced increases in *Fkbp5* expression, and E2 can independently lead to a down-regulation of *Ppid*. This co-regulator expression pattern could reduce GR sensitivity to circulating glucocorticoids through a reduction in GR translocation.

These results lend support to the hypothesis that female adaptations to chronic stress are mediated by interactions among ovarian steroids and the GR system. To this end, female rats react to physical and psychological stressors with a larger endocrine response than males [40], and estrogen has been shown to increase basal levels of cort and to exaggerate the cort response to stressors [41]. Estrogen also prolongs the ACTH release in response to a stressor [41], suggesting that estrogen impairs GR negative feedback. Handa et al. (1994) propose that this estrogen-mediated inhibition of GR function could reflect an evolutionary strategy to prevent the deleterious actions of elevated glucocorticoids on reproduction [41]. Understanding the role of GR co-regulators in negative feedback and the potential for ovarian steroids to modify function of GR co-regulators is important given the clinical association of depression with various events in the female reproductive cycle. For example, pre-menstruation, the postpartum phase, and menopause are time periods associated both with significant hormone changes and depressive changes in females [42]. Importantly, the present study provides additional insights into the role of gonadal steroids in modulation of the GR system and suggests a framework for future *in vivo* studies on the role of sex in the etiology of depression.

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HIGHLIGHTS

- Estradiol is capable of augmenting corticosterone-induced increases in *Fkbp5* expression.
- Estradiol in combination with a low concentration of corticosterone caused a decrease in expression of *Ppid*.
- Estradiol in combination with progesterone can cause increased expression of *Ppid* in the absence of corticosterone.
- Estradiol in combination with progesterone decreases *Ppid* expression when corticosterone is present.

Fig. 1. HT-22 cells were exposed to varying concentrations of corticosterone (cort), estradiol (E2), and progesterone (P4) for 24 h and then expression of *Nr3c1*, the gene that encodes the glucocorticoid receptor (GR), was assessed. None of the tested combinations of cort and E2 or cort and P4 altered expression of *Nr3c1* ($p > 0.05$ in all cases). Data are presented as mean $\pm$ SEM.

Fig. 2.

Corticosterone and estradiol treatments increase *Fkbp5* expression in HT-22 cells. HT-22 cells were exposed to varying concentrations of corticosterone (cort), estradiol (E2), and progesterone (P4) for 24 h and then expression of *Fkbp5* was assessed. Treatment with increasing doses of cort significantly increased expression of *Fkbp5* (***) compared to either 0 or 50 ng/mL cort). At a cort concentration of 400 ng/mL, E2 potentiates the cort-induced increase in *Fkbp5* expression (* p < 0.05 compared to 0 ng/mL E2). Data are presented as mean $\pm$ SEM.

Fig. 3.

Estradiol decreases *Ppid* expression in HT-22 cells. HT-22 cells were exposed to the indicated hormone concentrations for 24 h and then expression of *Ppid* was assessed using quantitative RT-PCR. A) Treatment with increasing doses of estradiol (E2) at either 0, 50, or 800 ng/mL corticosterone (cort) reduced expression of *Ppid* (* $p < 0.05$ compared to control). B) Progesterone (P4) did not alter expression of *Ppid* at any concentration. Data are presented as mean $\pm$ SEM.

Fig. 4.

Progesterone and estradiol have combined effects on *Ppid* expression in HT-22 cells. Expression of *Ppid* in HT-22 cells after a 24-h exposure to steroid hormone treatments. HT-22 cells were exposed to cort in conjunction with both estradiol (E2) and progesterone (P4). A) In the absence of cort, E2 (40 pg/mL) and P4 (30 ng/mL) caused a 20% increase in *Ppid* expression compared to hormone-free conditions (** $p < 0.01$ compared to control). B) At a cort concentration of 50 ng/mL, E2 (40 pg/mL) and P4 (10 ng/mL or 30 ng/mL) reduced *Ppid* expression (* $p < 0.05$ compared to control). C) At a cort concentration of 400 ng/mL neither E2 or P4 altered *Ppid* expression at any concentration tested. Data are presented as mean $\pm$ SEM.