Research Article:

Sex and estradiol differences in MAPK and Akt cell signalling across subregions of the hippocampus

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Abstract

Introduction: Rapid effects of estrogens within the hippocampus of rodents are dependent upon cell signaling cascades, and the activation of these cascades by estrogens varies by sex. Whether these pathways are rapidly activated within the dentate gyrus (DG) and CA1 by estrogens and across the longitudinal axis has been overlooked.

Methods: Gonadally-intact female and male rats were given either vehicle or physiological systemic low (1.1µg/kg) or high (37.3µg/kg) doses of 17β-estradiol thirty minutes prior to tissue collection. To control for the effects of circulating estrogens, an additional group of female rats were ovariectomized (OVX) and administered 17β-estradiol. Brains were extracted and tissue punches of the CA1 and DG were taken along the longitudinal hippocampal axis (dorsal and ventral) and analyzed for key MAPK and Akt cascade phosphoproteins.

Results: Intact females had higher Akt pathway phosphoproteins (pAkt, pGSK-3β, pp70S6K) than males in the DG (dorsal, ventral) and CA1 (dorsal), but lower MAPK phosphoproteins (pERK1/2, pp38) in the dorsal DG. Most of the effects of 17β-estradiol to influence cell signalling occurred in OVX animals and not in intact females or males. In OVX animals, 17β-estradiol increased cell signalling of MAPK and Akt phosphoproteins (pERK1/2, pJNK, pAkt, pGSK-3β) in both subregions but reduced MAPK phosphoproteins (pERK1/2, pp38)) in the ventral DG.

Discussion/Conclusions: Systemic 17β-estradiol treatment rapidly alters phosphoprotein levels in the hippocampus dependent on reproductive status and intact females have greater expression of Akt phosphoproteins than intact males across the hippocampus. These findings shed light on the underlying mechanisms of sex differences in hippocampal function and response to interventions that affect MAPK or Akt signaling.

Introduction

Estrogens rapidly affect object and spatial memory consolidation (1–3), short-term social memory (2,4), response learning (5), neurogenesis (6), and spine and synapse formation (Sellers et al., 2015a, 2015b; Sheppard et al., 2019). Most of these effects of estrogens occur rapidly and likely via cell signaling pathways after binding with the estrogen receptors (ERs). Estrogens exert both rapid (non-classical) and delayed (classical) actions. The classical actions of estrogens occur when they bind with ERs that dimerize, translocate to the nucleus, and act directly to affect gene expression (10). In contrast, the rapid effects of estrogens initiate a myriad of intracellular processes, including the activation of cell signaling cascades (reviewed in (2–4). Rapid (within minutes) or delayed (within hours) effects of estrogens on molecular, cellular, and behavior can vary by brain region (reviewed in (2,3,11). Rapid effects of estrogens on social, object, and spatial location recognition rely on signaling through mitogen-activated protein kinase (MAPK) and Akt (protein kinase B) pathways, whereas the delayed effects are driven predominantly by ER-estrogen response element interactions and effects on gene expression (2,3). There is, however, overlap in these effects, with the less well understood rapid effects occurring concurrently with the more established delayed effects. Thus, understanding the mechanisms, such as cell signaling cascades, through which the rapid effects act is imperative to a comprehensive view of actions by estrogens.

Two of the most studied cell signaling cascades are the MAPK and Akt pathways. These pathways are involved in a number of processes including cell proliferation, migration, and survival, apoptosis, metabolism, differentiation, immune responses, and development (12–15). There is much crosstalk between MAPK and Akt pathways (16,17), and these pathways often act in parallel to elicit similar downstream mechanisms and outputs in complementary or synergistic manners (16,18,19). However, little work has investigated both pathways in the same animals and those that do typically only measure particular phosphoproteins of interest, despite the intricacy and interconnectedness of these signaling cascades.

Sex differences have been noted in the influence of 17β-estradiol on the hippocampus. In older rats, acute 17β-estradiol increases spine density in older females but decreases spine density in males (20). Furthermore, whereas gonadectomy reduces spine density in both males and females in the CA1 (21–23), estradiol increases spine density in females (24,25) but not in males (22). Few studies have investigated sex differences in the effects of 17β-estradiol on cell signalling. Female rats have higher baseline levels of phosphorylated extracellular signal-regulate protein kinase 1/2 (pERK1/2; both p44 [ERK1] and p42 [ERK2] isoforms), pAkt, and phosphorylated glycogen synthase kinase 3β (pGSK-3β) in the prefrontal cortex (prelimbic and infralimbic cortices), nucleus accumbens, and rostral caudate putamen of rats than male rats (26). However, to our knowledge, no studies have examined sex differences in expression of both MAPK and Akt phosphoproteins in different subregions of the hippocampus thus far. Few studies have explored sex differences in these cell signalling proteins after exposure to 17β-estradiol. Intrahippocampal 17β-estradiol increases dorsal hippocampal pAkt and pERK2 in OVX females, but not in gonadally-intact or castrated males (27). Similarly, 17β-estradiol increases pERK1/2 in anteroventral periventricular nucleus and the medial preoptic area in females but not in males (28). Thus, there is a dearth of evidence exploring influence of 17β-estradiol on cell signalling across the sexes and within subregions across the hippocampus.

The hippocampus is a heterogeneous structure with distinct subfields across the longitudinal axis (dorsal, ventral), as well as dentate gyrus (DG), CA3 and CA1 subregions. Based on gene expression, receptor levels, activity, and connectivity, the dorsal hippocampus is thought to be important for learning and memory, whereas the ventral region is more important for regulation of stress and anxiety (reviewed in 29). Estradiol rapidly increases cell proliferation in both the dorsal and ventral DG (6,30). Furthermore, 17β-estradiol increases apical dendritic spines in the CA1 region but not in the DG (21,31). Given that 17β-estradiol influences both dorsal and ventral hippocampal

plasticity, it is important to examine possible subregional (i.e. DG and CA1) effects across the longitudinal axis that show rapid 17β-estradiol action in both sexes.

Most previous studies have examined the effects of estrogens on cell signaling in either the whole hippocampus (32), dorsal hippocampus (27,33–35), or dorsal CA1 (36) in OVX female rodents. Estrogens rapidly increase phosphorylated (activated) proteins in the MAPK and Akt cascades including the MAPKs pERK and pJNK and Akt pathway proteins pAkt, pGSK-3β, and p70S6K. Another MAPK protein, p38, has divergent effects from other MAPKs and is minimally affected in the hippocampus by the delayed effects of 17β-estradiol treatment in OVX female rats (37). However, even in studies investigating the rapid effects of 17β-estradiol on cell signaling cascades, methodologies (including, for example, timing and/or dose of 17β-estradiol treatment) vary and make comparison between pathways and proteins difficult.

The current study was designed to examine sex differences in cell signaling cascades, with and without 17β-estradiol, in the dorsal and ventral fields of DG and CA1 of the hippocampus. We hypothesized that sex and 17β-estradiol would differentially affect MAPK and Akt cell signaling cascades and that these differences would vary by region across the longitudinal axis. Furthermore, we hypothesized that in OVX rats 17β-estradiol would increase activation of cell signaling cascades in both the CA1 and DG, but that these effects may be more prominent in the dorsal versus ventral axis.

Methods

Animals

Ten- to eleven-week-old, gonadally intact female (n=65) and male (n=19) Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada) were received at the University of British Columbia and pair-housed (with the exception of one triple-housed cage of males) upon arrival. Rats were housed in transparent polyurethane bins (48×27×20cm) with aspen chip bedding and were given Purina rat chow and tap water *ad libitum*. Rats were maintained under a 12/12h light/dark cycle (lights on 07:00h) in standard housing conditions (21 ± 1ºC; 50 ± 10% humidity). Animals were handled every other day prior to the experiment. Animals were 12-13 weeks old when administered 17β-estradiol and brains were collected. Females weighed 255-360g, whereas males weighed 425-600g. To determine estrous phase during testing, intact females underwent vaginal lavage immediately following sacrifice. Lavage samples were stained with cresyl violet and estrous phase was evaluated (38). One week following arrival, 36 female rats were bilaterally ovariectomized using aseptic techniques under isoflurane anesthesia, with ketamine (30 mg/kg, Bimeda-MTC, Cambridge, ON), xylazine (2 mg/kg, Bayer HealthCare, Toronto, ON), and bupivacaine (applied locally; 4 mg/kg, Hospira Healthcare Corporation, Montreal, QC). Following surgery, rats were single-housed and allowed to recover for at least one week before testing. All experiments were conducted in accordance with the ethical guidelines set by the Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. All efforts were made to reduce the number and the suffering of animals.

Treatment

Rats were subcutaneously administered 1.1µg/kg 17β-estradiol in sesame oil (low 17β-estradiol; Females n=10, Males n=6, OVX n=13), 37.3µg/kg 17β-estradiol in sesame oil (high 17β-estradiol; Females n=10, Males n=7, OVX n=13), or sesame oil vehicle (Females n=9, Males n=6, OVX n=10) 30 minutes prior to sacrifice. These doses were chosen because they result in circulating levels of 17β-estradiol observed on the morning of proestrus (high 17β-estradiol) or during diestrus (low 17β-estradiol)(39) and both doses enhance cell proliferation 30 minutes after injection in OVX females (6,30). Effort was made to ensure that within sex groups did not differ in body weight. All rats

were injected between 09:00h and 11:15h. Rats were sacrificed by decapitation, and brains were excised, hemisected, and flash-frozen on dry ice.

Brain tissue processing

Brains were sliced into 300µm sections at -10°C using a Leica CM3050 S cryostat (Nußloch, Germany). Punches were taken from the DG and CA1 of both the dorsal and ventral horns of the hippocampus using modified 18G needles with an internal punch diameter of 0.838mm (see Figure 1). Tissue was collected and homogenized using an Omni Bead Ruptor (Omni international, Kennesaw, GA) with 40µl of cold lysis buffer. Homogenates were centrifuged at 4°C and 1000×g for 15min then stored at -20°C. Total protein concentrations in homogenates were quantified using the Pierce Micro BCA Protein Assay Kit (ThermoFisher Scientific) and used according to manufacturer instructions, with samples run in triplicates.

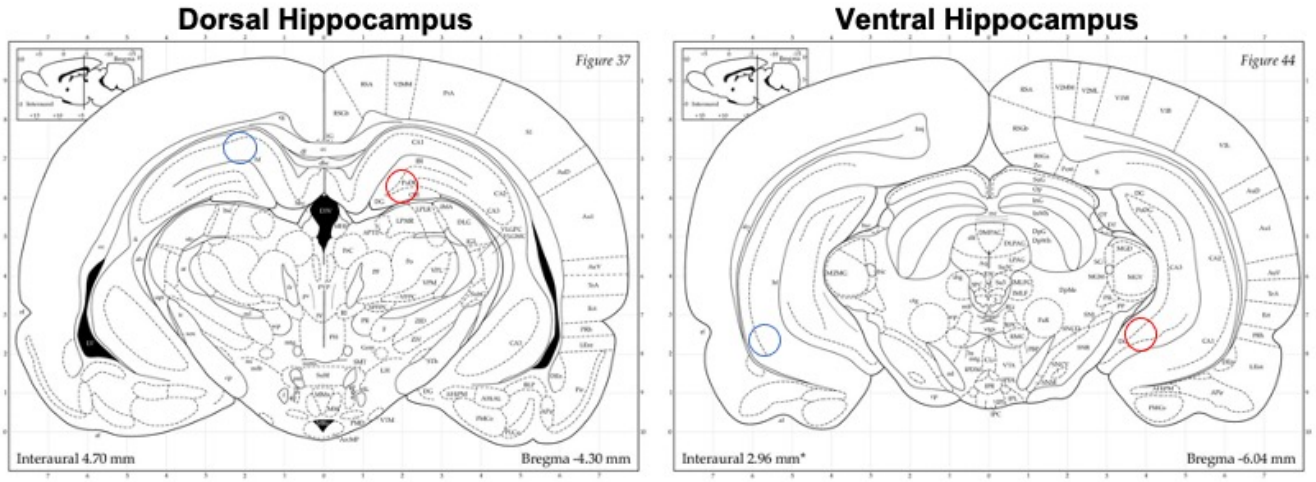

Figure 1: Example of CA1 and dentate gyrus punch location
Blue circles indicate approximate CA1 tissue punch location. Red circles indicate approximate dentate gyrus tissue punch location. Punches were 0.838mm in diameter.

Phosphoprotein quantification

Multiplex electrochemiluminescence immunoassay kits (MAPK Kinase Phosphoprotein and Akt Signaling Panel Assay Whole Cell Lysate Kits) were purchased from Meso Scale Discovery (Rockville, MD) and used according to manufacturer instructions – with the exception of incubating the samples at 4°C overnight – to measure phosphoprotein levels. The antibody precoated plates allowed for the simultaneous quantification of the following phosphoproteins: pERK1/2 (combined p42 and p44 isoforms), pJNK, and pp38 (MAPK kit) and pAkt, pGSK-3β, and pp70S6K (Akt kit). Samples were run in duplicates, plates were read with a Sector Imager 2400 (Meso Scale Discovery) using the Discovery Workbench 4.0 (Meso Scale Discovery). Phosphoprotein values were normalized to total protein concentrations for statistical analyses. Inter- and intra-assay coefficients of variation (CV) were below 10%. Phosphoprotein signal data were excluded when the CV between duplicates exceeded this threshold.

Statistical analyses

Statistical analyses were run using Statistica v.8.0 (StatSoft Inc, Tulsa, OK) software. Two-way ANOVAs were conducted for each analyte and region with treatment (oil, low 17β-estradiol, high 17β-estradiol) and sex (female, male) as between groups factors. Phosphoprotein levels in intact vehicle

treated rats were compared across the estrous cycle (proestrus vs non-proestrus, corresponding to high and low 17β-estradiol phases, respectively) using individual t-tests. One-way ANOVAs were conducted for each analyte and region in the OVX animals, with treatment (vehicle, low 17β-estradiol, high 17β-estradiol) as the between-subjects factor. *Post hoc* comparisons used Newman-Keuls. Any *a priori* analyses were subjected to Bonferroni correction. Any statistical outliers (± 2 standard deviations from the group mean) were removed. Statistical significance was set at $p<0.05$. Eta squared (η^2) and Cohen's *d* effect sizes were calculated where appropriate.

Results

First, we analysed the effects of estrous cycle on phosphoproteins across the dorsal/ventral axis and in all regions. There were no significant effects (all ps>0.206; Supplementary Figures 1 and 2) and, as such, these were not considered further.

Females have higher levels of Akt pathway phosphoproteins in the dorsal and ventral dentate gyrus

Females had greater phosphoprotein signal than males for each analyte in the Akt pathway in both the dorsal and ventral DG (main effects of sex: dorsal DG: pAkt – $F_{(1,39)}=39.91$, $p<0.0001$, $\eta^2=0.482$; pGSK-3β – $F_{(1,36)}=26.33$, $p<0.0001$, $\eta^2=0.394$; pp70S6K – $F_{(1,39)}=20.91$, $p<0.0001$, $\eta^2=0.327$; ventral DG: pAkt – $F_{(1,36)}=24.95$, $p<0.0001$, $\eta^2=0.394$; pGSK-3β – $F_{(1,35)}=12.07$, $p=0.0014$, $\eta^2=0.246$; pp70S6K – $F_{(1,37)}=39.91$, $p=0.0005$, $\eta^2=0.227$; Figure 2A). There were no other main or interaction effects in this pathway (all ps>0.094).

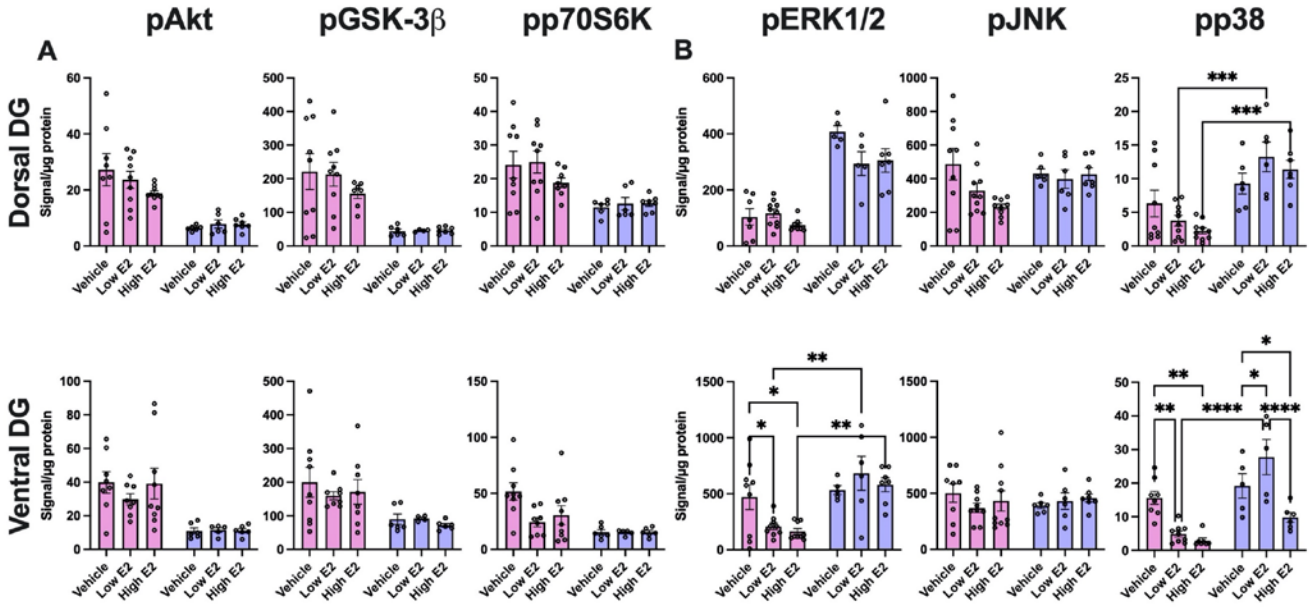

Figure 2: Dentate gyrus Akt and MAPK phosphoprotein levels in female and male rats

Phosphoprotein signal normalized by amount of total protein in sample. A) Phosphoprotein levels were higher in females in all analytes in the dorsal (pAkt, pGSK-3b, and pp70S6K: p<0.0001) and ventral DG (pAkt: p<0.0001; pGSK-3b: p=0.0014; pp70S6K: p=0.0005). B) Males had higher pERK1/2 and pp38 (ps<0.0001) in the dorsal DG than females. Whereas no differences in dorsal DG pp38 were seen in male and female rats given vehicle or within either sex with estradiol treatment, low and high estradiol produced significant differences between male and female rats (ps<0.0006). In the ventral DG, males had higher pERK1/2 and pp38 (ps<0.0001) than females. Estradiol treatment produced significant reductions in pERK1/2 in females (ps<0.032), but not in males, resulting in lower pERK1/2 levels in females than males when given low or high estradiol (ps<0.008). Low and high estradiol reduced ventral DG pp38 in females (ps<0.007). In males, low estradiol increased pp38 (p=0.013), whereas high estradiol decreased pp38 (compared to vehicle [p=0.02] and low estradiol [p<0.0001]) in the ventral DG. Divergent effects of estradiol treatment produced a significant difference in pp38 between

male and female rats given low estradiol ($p<0.0001$).

Females have higher levels of Akt pathway phosphoproteins in the dorsal, but not ventral, CA1

Females had greater pGSK and pp70S6K than males in the dorsal CA1 (main effect of sex: pGSK-3β – $F_{(1,38)}=7.608$, $p=0.0089$, $\eta^2=0.143$; pp70S6K – $F_{(1,39)}=6.031$, $p=0.0186$, $\eta^2=0.118$; Figure 3A) but not in the ventral CA1 (all ps>0.082). High 17β-estradiol increased pAkt in females ($p=0.05$, Cohen's d=0.929) in the dorsal CA1 but not in males ($p=0.355$; sex by 17β-estradiol treatment interaction: $F_{(2,37)}=3.731$, $p=0.0334$, $\eta^2=0.143$; Figure 3A) compared to vehicle treatment. There was also a main effect of sex for pAkt in the dorsal CA1 ($F_{(1,37)}=7.725$, $p=0.0085$, $\eta^2=0.148$) but no other main or interaction effects of Akt pathway phosphoproteins in the ventral CA1 (all ps>0.05).

Males had greater pERK1/2 in the dorsal DG than females. 17β-estradiol reduced MAPK phosphoproteins in the ventral DG in intact females but not in intact males.

Male rats had significantly greater pERK1/2 than females, regardless of dose, in dorsal DG (main effects of sex: $F_{(1,37)}=117.4$, $p<0.0001$, $\eta^2=0.717$; Figure 2B). Inspection of the figure suggests both doses of 17β-estradiol decreased pERK1/2 in males but not females in the dorsal DG, but these effects just fail to reach statistical significance with Bonferroni corrections (ps>0.207, Cohen ds>1.12; interaction: $F_{(2,37)}=2.569$, $p=0.09$, $\eta^2=0.031$; main effect of dose: $F_{(2,37)}=3.184$, $p=0.053$, $\eta^2=0.039$). Despite a significant interaction, there were no significant effects of estradiol on pp38 in the dorsal DG within either sex, although there were sex differences seen after treatment with 17β-estradiol but not in the vehicle groups (males > females [low estradiol: $p=0.0004$, Cohen's d=2.257; high estradiol: $p=0.0006$, Cohen's d=3.350] but not in the vehicle groups [$p=0.151$, Cohen's d=0.592; interaction: $F_{(2,42)}=3.242$, $p=0.049$, $\eta^2=0.134$]; Figure 2B). There was also a main effect of sex on pp38 ($F_{(1,42)}=38.26$, $p<0.0001$, $\eta^2=0.427$; Figure 2B), but no main effect of 17β-estradiol ($p=0.486$, $\eta^2=0.0164$). There were no significant effects found in pJNK in the dorsal DG (all ps >0.075).

In the ventral DG, 17β-estradiol reduced pERK1/2 in females regardless of dose (ps<0.032, low 17β-estradiol Cohen's d=1.070, high 17β-estradiol Cohen's d=1.261), an effect that was not seen in males (ps >0.41, Cohen's ds<0.525; interaction $F_{(2,39)}=3.436$, $p=0.0422$, $\eta^2=0.094$; Figure 2B). There was a significant main effect of sex ($F_{(1,39)}=22.14$, $p<0.0001$, $\eta^2=0.302$), but no significant effect of 17β-estradiol ($p=0.295$, $\eta^2=0.0344$).

Both doses of 17β-estradiol decreased pp38 in the ventral DG of females (low 17β-estradiol: $p=0.007$, Cohen's d=2.48; high 17β-estradiol: $p=0.003$, Cohen's d=3.07; Figure 2B; interaction: $F_{(2,34)}=9.873$, $p=0.0004$, $\eta^2=0.177$). However, in males high 17β-estradiol decreased pp38 ($p=0.020$, Cohen's d=2.08), whereas low 17β-estradiol increased pp38 ($p=0.013$, Cohen's d=0.85). There were also main effects of sex ($F_{(1,34)}=34.24$, $p<0.0001$, $\eta^2=0.307$) and 17β-estradiol ($F_{(2,34)}=13.78$, $\eta^2=0.247$, $p<0.0001$). There were no significant effects of pJNK (all ps>0.461).

MAPK protein phosphorylation in the CA1 does not differ between sexes or with 17β-estradiol in intact rats

There were no sex (all ps>0.145) or 17β-estradiol (all ps>0.134) effects observed for any analyte in either the dorsal or ventral CA1. Although a significant sex by 17β-estradiol treatment interaction was observed in pERK1/2 in the dorsal CA1 ($F_{(2,36)}=3.371$, $p=0.0455$, $\eta^2=0.156$; Figure 3B), *post hoc* analyses found no significant differences between groups (all ps >0.30).

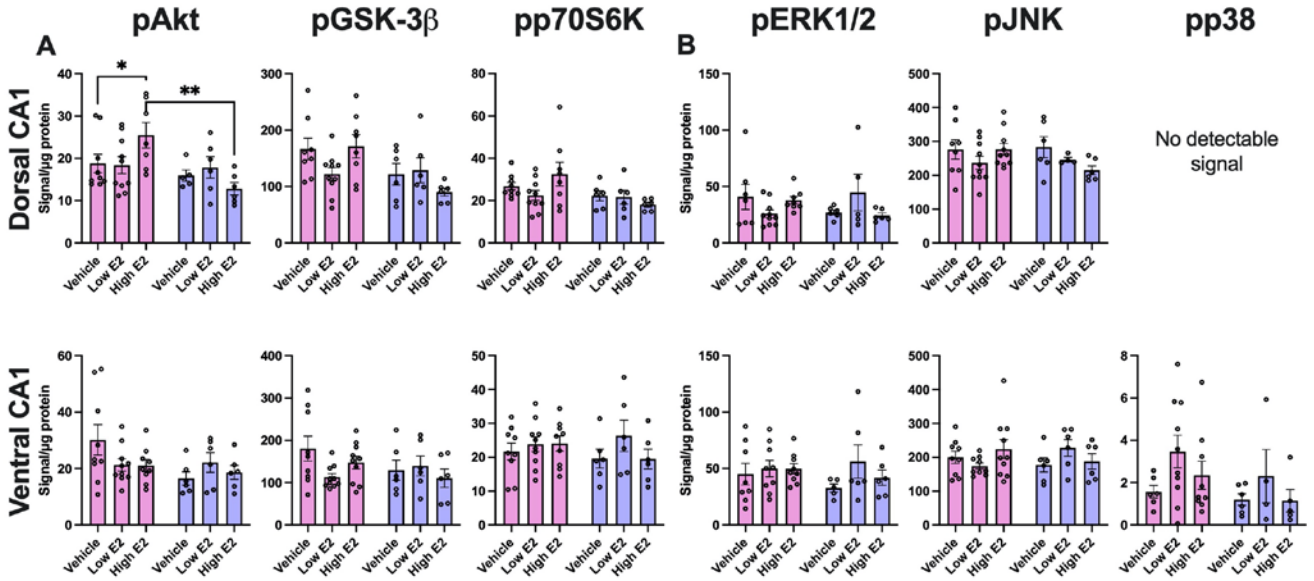

Figure 3: Hippocampal CA1 Akt and MAPK phosphoprotein levels in female and male rats
Phosphoprotein signal normalized by amount of total protein in sample. A) Phosphoprotein levels were higher in females in all analytes in the dorsal CA1 (pAkt: p=0.0085, pGSK-3b: p=0.0089, and pp70S6K: p=0.0186) High estradiol increased dorsal CA1 pAkt in female rats (p=0.05), leading to a difference between males and females with this treatment (p<0.01). B) MAPK phosphoproteins did not differ by sex or treatment in the dorsal or ventral CA1. pp38 did not produce sufficient signal for analysis in this region.

Low 17β-estradiol increases pERK1/2 and pJNK in the dorsal dentate gyrus of OVX rats

A priori, we expected 17β-estradiol to increase pERK1/2 and pJNK activation. Low 17β-estradiol increased pERK1/2 (relative to vehicle: p=0.03, Cohen's d=1.356; relative to high 17β-estradiol: p=0.02, Cohen's d=1.249; main effect of 17β-estradiol on pERK1/2: $F_{(2,14)}$=4.254, η^2=0.378, p=0.036; Figure 4B). Low 17β-estradiol also increased pJNK in the dorsal DG (relative to vehicle: p=0.02, Cohen's d=1.402; relative to high 17β-estradiol: p=0.01, Cohen's d=1.401; main effect of 17β-estradiol: $F_{(2,14)}$=5.077, p=0.022, η^2=0.420; Figure 4B). There was no significant effect of 17β-estradiol on pp38 in the dorsal DG of OVX rats (p=0.505, η^2=0.0931). All analytes for the MAPK cascade failed to reach significance for 17β-estradiol effects in the ventral DG (all p>0.303). Similarly, all analytes for the Akt cascade failed to reach significance for 17β-estradiol effects in both the dorsal and ventral DG of OVX rats (all ps>0.270).

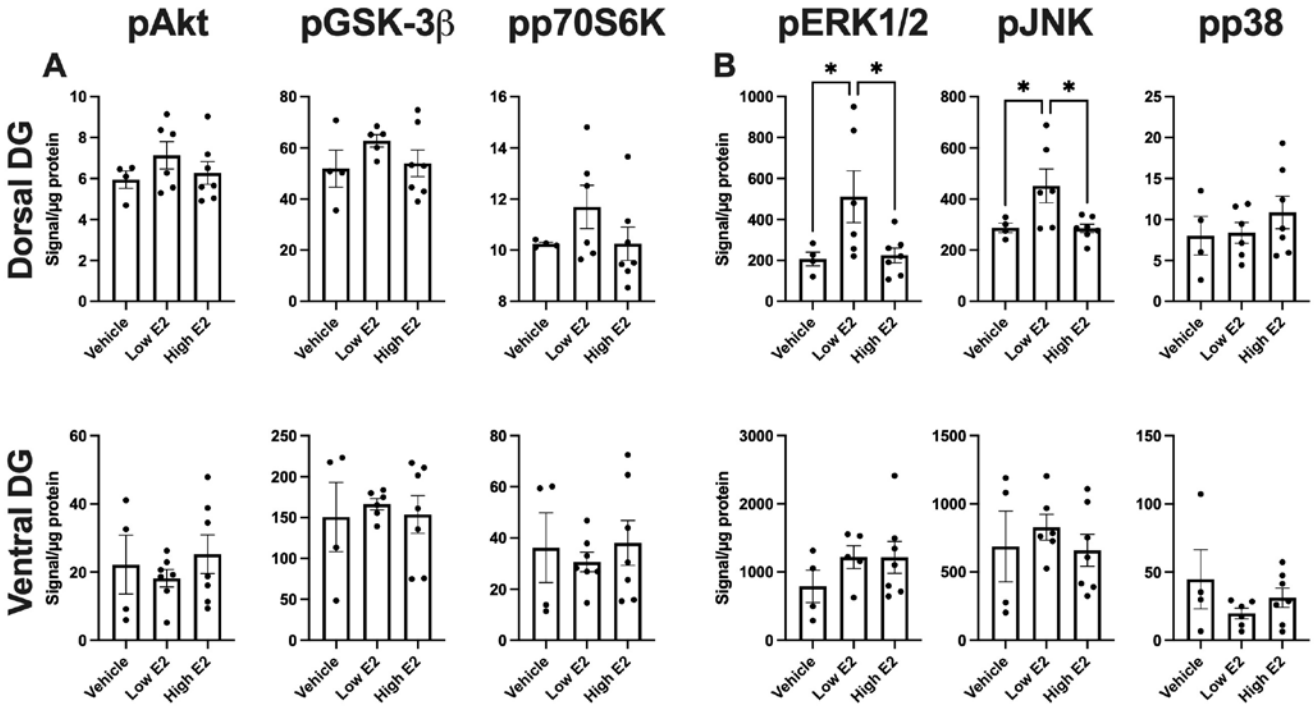

Figure 4: Dentate gyrus Akt and MAPK phosphoprotein levels in ovariectomized female rats
Phosphoprotein signal normalized by amount of total protein in sample. A) Estradiol did not affect Akt pathway phosphoprotein levels in the dorsal or ventral DG of OVX rats. B) Low estradiol increases pERK1/2 (relative to vehicle [p=0.03] or high estradiol [p=0.02]) and pJNK (relative to vehicle [p=0.02] or high estradiol [p=0.01]) in OVX rats. Estradiol did not affect MAPK pathway phosphoprotein levels in the ventral DG of OVX rats.

Both doses of 17β-estradiol increase pJNK and pGSK-3β in the dorsal CA1 of OVX rats.

Both 17β-estradiol doses increased pJNK (low 17β-estradiol: p=0.017, Cohen's d=1.56; high 17β-estradiol: p=0.009, d=1.89; Figure 5B; main effect of 17β-estradiol: $F_{(2,15)}$=6.288, p=0.0104, η^2=0.456) in the dorsal CA1. Both 17β-estradiol doses increased pGSK-3β (low 17β-estradiol: p=0.024, Cohen's d=2.37; high 17β-estradiol: p=0.032, Cohen's d=1.32; main effect of 17β-estradiol: $F_{(2,13)}$=4.889, p=0.026, η^2=0.4293; Figure 5A) in the dorsal CA1. There were no 17β-estradiol effects in pERK, pAkt, or pp70S6K in the dorsal CA1 (all ps>0.189).

Low 17β-estradiol increases MAPK and Akt pathway phosphoproteins in the ventral CA1 of OVX rats

Low 17β-estradiol increased pERK1/2, pJNK, pAkt, and pGSK-3β in the ventral CA1 of OVX rats. Low 17β-estradiol increased pERK1/2 (p=0.003, Cohen's d=2.434; Figure 5B), pJNK (p=0.027, Cohen's d=1.537; Figure 5B), pAkt (p=0.042, Cohen's d=1.397; Figure 5A) and pGSK-3β (p=0.008, Cohen's d=1.859; Figure 5A) compared to vehicle, but high 17β-estradiol did not (all ps>0.15). Main effects of 17β-estradiol were significant for pERK1/2 ($F_{(2,15)}$=8.731, p=0.0031, η^2=0.538; Figure 5B), pJNK ($F_{(2,14)}$=5.64, p=0.0160, η^2=0.446; Figure 5B), pAkt ($F_{(2,14)}$=4.373, p=0.0335, η^2=0.385; Figure 5A), and pGSK-3β ($F_{(2,14)}$=6.81, p=0.0086, η^2=0.493; Figure 5A; all other ps>0.205).

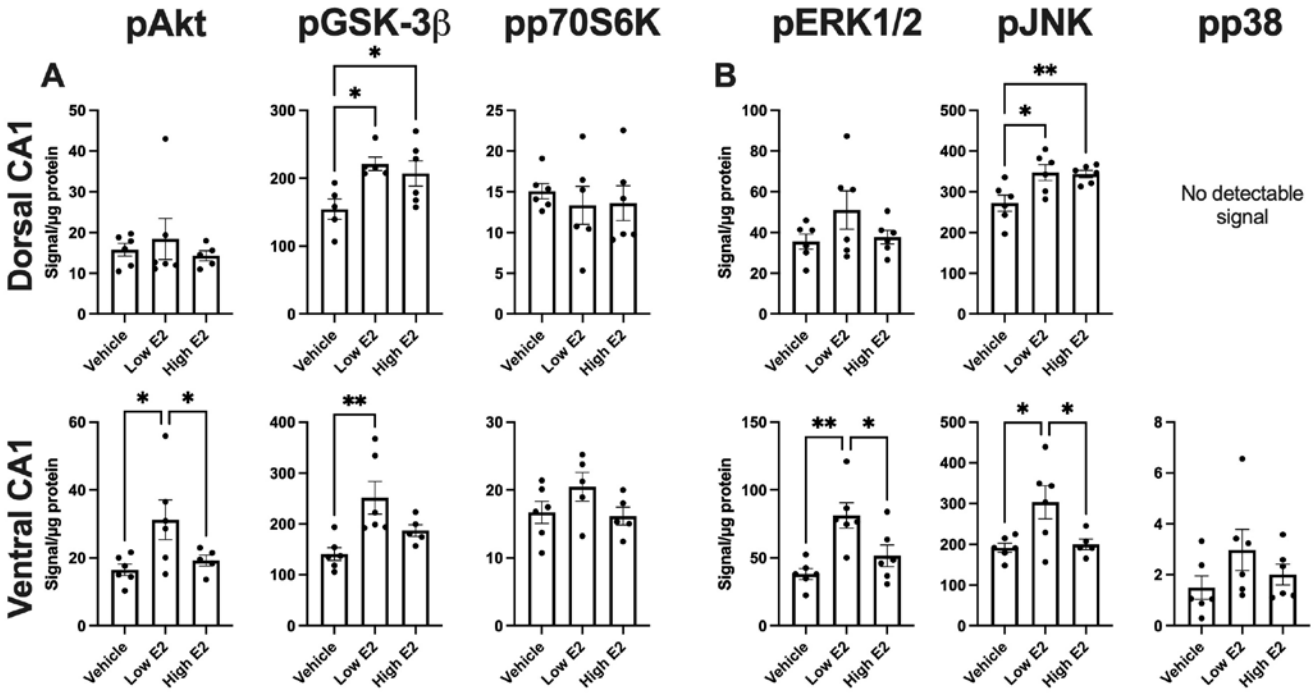

Figure 5: Hippocampal CA1 Akt and MAPK phosphoprotein levels in ovariectomized female rats
Phosphoprotein signal normalized by amount of total protein in sample. A) Low (p=0.024) and high (p=0.032) estradiol increased pGSK-3b in the dorsal CA1 of OVX rats. In the ventral CA1, low estradiol increased pAkt (relative to vehicle and high estradiol, ps<0.042) and pGSK-3b (relative to vehicle, p=0.008). Signal from pp38 was too low to detect in this region. B) Low (p=0.017) and high (p=0.009) estradiol increased pJNK in the dorsal CA1 of OVX rats. In the ventral CA1, low estradiol increased pERK1/2 (relative to vehicle [p=0.003] and high estradiol [p<0.01]) and pGSK-3b (relative to vehicle and high estradiol, ps<0.05).

Discussion

Past characterization of the rapid effects of estrogens on cell signaling cascades has predominantly focused on the CA1 of the dorsal hippocampus or the dorsal hippocampus as a whole and has rarely investigated sex differences. Here, we found sex differences in phosphoprotein levels in the dorsal and ventral DG and dorsal CA1 in MAPK and Akt cascades. 17β-estradiol had more dramatic effects in OVX females compared to intact females and males on MAPK and Akt pathways. In intact rats, 17β-estradiol significantly decreased MAPK phosphoproteins in females but increased pp38 in males in the ventral DG. In contrast, dose-dependent effects of 17β-estradiol were observed mainly in the CA1 in OVX female mice in both Akt and MAPK pathways, although low 17β-estradiol increased MAPK phosphoproteins (pERK and pJNK) in the dorsal DG. Collectively, these findings are the first characterization of sex differences in MAPK and Akt phosphoprotein levels across the longitudinal axis of the hippocampus in the DG and CA1. We also saw rapid MAPK and Akt phosphoprotein responses to 17β-estradiol in both sexes, with greater influence of 17β-estradiol in OVX compared to intact females. These findings illustrate that both sex and 17β-estradiol differentially influence phosphoprotein levels in region-specific ways in the hippocampus.

Sex differences in MAPK and Akt cell signalling pathways in the DG and CA1

Consistent with investigations of other brain regions, we observed sex differences in cell signaling pathways in the DG and CA1 of the dorsal and ventral hippocampus, mostly in the Akt pathway (see Table 1 for summary). Females had higher Akt pathway phosphoproteins across the longitudinal axis and between subregions of the hippocampus than males. On the other hand, males only had higher pERK1/2 than females in the dorsal DG. Females showed higher levels of all Akt

phosphoproteins tested (pAkt, pGSK-3β, pp70S6K) in the dorsal and ventral DG and the dorsal CA1. In contrast, males had higher pERK1/2 in the dorsal DG than females. These findings are partially consistent to findings in the frontal cortex, caudate and nucleus accumbens (26). Female rats had higher baseline levels of pERK1/2, pAkt, and pGSK-3β in the frontal cortex (prelimbic and infralimbic cortices), nucleus accumbens, and rostral caudate putamen than males (26), that for at least the Akt pathway proteins mirrors our findings in the hippocampus. In male mice, pERK1/2 expression in the anteroventral periventricular nucleus (AVPV) was higher, irrespective of gonad status, compared to females (28), similar to our findings in the dorsal DG. Regrettably, while much has been made of sex differences in MAPK and Akt pathway proteins in skeletal muscle, heart, and other peripheral tissues (e.g. (40,41)), these are among the only investigations into sex differences in MAPK or Akt signaling in the brain. Our findings suggest more sex differences, favouring females, in the Akt signalling pathways across the dorsal and ventral axis and subregions (DG, CA1) of the hippocampus and with males only showing greater activation of ERK1/2 in the dorsal DG.

Table 1: Summary of sex differences in Akt and MAPK phosphoproteins

	pAkt	pGSK-3β	pp70S6K	pERK1/2	pJNK	pp38
Dorsal DG	Female****	Female****	Female****	Male****	–	Male****
Ventral DG	Female****	Female**	Female***	Male****	–	Male****
Dorsal CA1	Female**	Female**	Female*	–	–	No data
Ventral CA1	–	–	–	–	–	–

*Significant sex differences phosphoprotein levels with the sex indicated having higher phosphoprotein levels. * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.*

Effects of 17β-estradiol on hippocampal cell signalling pathways

We found more dramatic effects of 17β-estradiol on cell signalling activation in the hippocampus of OVX rats compared to intact rats (see Table 2 for summary). Estradiol rapidly increased MAPK and Akt pathway phosphoproteins dependent on dose and longitudinal axis mainly within the CA1 region of OVX rats. It is perhaps not surprising that the CA1 region was particularly sensitive to activation of cell signalling proteins in response to 17β-estradiol as others have found that the dorsal CA1 region shows rapid upregulation of spine density and memory consolidation (27,33,34,36,42–45). Our results are consistent with Koss et al. (2018) who found that whole dorsal hippocampi of OVX female mice, but not gonadally-intact males had increased pAkt and pERK2 (p42 isoform) following acute 17β-estradiol treatment. Although we did not see statistically significant effects in the dorsal CA1 or ventral DG, the direction of the means favours an increase in pERK1/2 with 17β-estradiol. It is important to note here that Koss and colleagues (2018) saw differences in pERK only in pERK2 and not pERK1 whereas our analysis did not discriminate between isoforms. In addition to the hippocampus, estradiol rapidly increases pERK1/2 in the medial preoptic area and AVPV (28). Further, effects of 17β-estradiol on pAkt were present only in the ventral CA1 and not in the dorsal CA1, which is interesting given the functional implications of these regions. Future research should consider the longitudinal axis, as findings can differ based on this and may be important for therapeutic outcomes.

As eluded to above, 17β-estradiol treatment increased pERK1/2 in the DG and CA1 of OVX rats but not in gonadally intact male and female rats. Consistent with our findings Koss et al (27) found that 17β-estradiol increased pERK2 in OVX female rats, and not in intact males. Much of the previous work on the rapid effects of 17β-estradiol has focused on comparing to an OVX group. However, with the exception of pERK1/2 and pp38 in the ventral DG, the effects of 17β-estradiol treatment in this study were only present in OVX rats and not intact rats. One possible reason for this is that the

intracellular response to estrogens is already at ceiling in gonadally-intact female rats. While not directly compared, phosphoprotein levels (e.g. Akt analytes in the dorsal DG) in OVX rats were lower than gonadally-intact females. Further supporting this is the inverted U-shaped dose response in OVX rats in which low 17β-estradiol increased phosphoproteins whereas high 17β-estradiol did not. This is frequently seen in sex steroid research (9,46) and likely results from higher doses activating off target effects. As such, treatment of gonadally-intact female mice with 17β-estradiol may have resulted in fewer effects on MAPK and Akt analytes due to the ongoing presence of gonad-derived hormones. This has substantial implications for the conclusions and translatability of preclinical 17β-estradiol research. For example, if 17β-estradiol treatment leads to an improvement in a cognitive task in OVX rodents in a MAPK- or Akt-dependent manner, it should not be assumed that the same improvements would be seen in gonadally-intact rodents.

Table 2: Summary of effects of 17β-estradiol on Akt and MAPK phosphoproteins in OVX female rats

	pAkt	**pGSK-3β**	**pp70S6K**	**pERK1/2**	**pJNK**	**pp38**
Dorsal DG	–	–	–	Low E2*	Low E2*	–
Ventral DG	–	–	–	–	–	–
Dorsal CA1	–	Both E2 */*	–	–	Both E2 */**	No data
Ventral CA1	Low E2*	Low E2**	–	Low E2**	Low E2*	–

*Significant increases phosphoprotein levels following E2 treatment in OVX females. In cases where both doses of E2 increased phosphoprotein levels, significance is indicated as low dose / high dose. * p<0.05, ** p<0.01.*

Differences across hippocampal subregions and along the longitudinal axis

Estrogens, in particular 17β-estradiol, affect neuroplasticity in a subregion-dependant manner in the hippocampus. For example, 17β-estradiol increases apical dendritic spine density in the dorsal CA1 (21,25,31,42–45) but not in the DG (21,31). However, 17β-estradiol rapidly increases cell proliferation in the dorsal and ventral DG of OVX rats (6,30), with the effects on males or gonadally-intact females to be determined. Inhibition of ERK signaling mediates the increased dendritic spine densities in dorsal CA1 and medial prefrontal cortex following 17β-estradiol treatment (45). Given our findings that low 17β-estradiol increases pERK and pJNK in dorsal DG, perhaps the rapid effects of 17β-estradiol on cell proliferation in the DG (6) involves activation of these phosphoproteins. As such, investigations into the intracellular mechanisms driving rapid increases in cell proliferation in the DG are required.

It remains possible that the other subregions of the hippocampus drive effects seen in previous studies that were absent in ours. Here, we focused on the CA1 and DG as many of the established rapid effects of estrogens occur within these regions (e.g. (6,30,42–45); for recent reviews, see (3,9)). As many previous studies had less regional specificity, perhaps the effects, such as increases in pERK in the dorsal hippocampus response to 17β-estradiol, are driven by hippocampal regions other than the CA1 and DG, such as the CA3 region.

Estrous cycle

In the present study, we did not observe any statistical effects of estrous cycle, although our study was not designed to specifically examine estrous cycle changes. There is a paucity of research making comparisons across the estrous cycle with regards to cell signaling cascades in the brain. One study found that transcription of MAPK and, potentially, Akt cascade phosphoprotein mRNA in the medial prefrontal cortex of rats was upregulated in proestrus (47). In the ventral hippocampus, chromatin dynamics and resulting MAPK pathway protein gene expression vary across the estrous cycle and between male and female mice (48). In addition to changes in dendritic spine densities in the rat hippocampus across the estrous cycle (49,50), changes in total hippocampal volume across the mouse estrous cycle have been observed, with high estrogen phases associated with greater

hippocampal volume (51). Similar changes have been found across the human menstrual cycle (52,53). With so many neuroplastic changes occurring with natural fluctuations of estrogens, further investigations into the effects of the estrous cycle on estrogen-responsive kinase pathways are needed.

Conclusions

Sex differences in MAPK and Akt cell signaling phosphoproteins exist in both dorsal and ventral DG and in dorsal CA1 in rats. Estradiol treatment increased MAPK and Akt signaling in the CA1 region in OVX rats with far fewer effects in intact females or males. These results shed light on the possible underlying mechanisms of sex differences in hippocampal function in response to 17β-estradiol that affect MAPK and Akt kinase pathways. They further show the heterogeneity of estrogen and sex effects between hippocampal subregions and along the longitudinal axis. With these subregions serving often distinct purposes, and the functional dissociation between the dorsal and ventral hippocampus, the present results provide possible mechanisms through which these differences may arise. Furthermore, with many neurological disorders having profound effects on the hippocampus, these sex, hormonal status, and regional differences provide valuable insights not only into the potential underlying mechanisms, but also into therapeutic possibilities.

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Statement of Ethics

All experiments were conducted in accordance with the ethical guidelines set by the Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee. All efforts were made to reduce the number and the suffering of animals.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Author Contributions

PASS – conception and design of the work; data acquisition, analysis, and interpretation; writing and revising

TP - conception and design of the work; data acquisition, analysis, and interpretation; writing and revising

LAMG - conception and design of the work; data acquisition, analysis, and interpretation; writing and revising

Data Availability

All data is available upon request to corresponding author.

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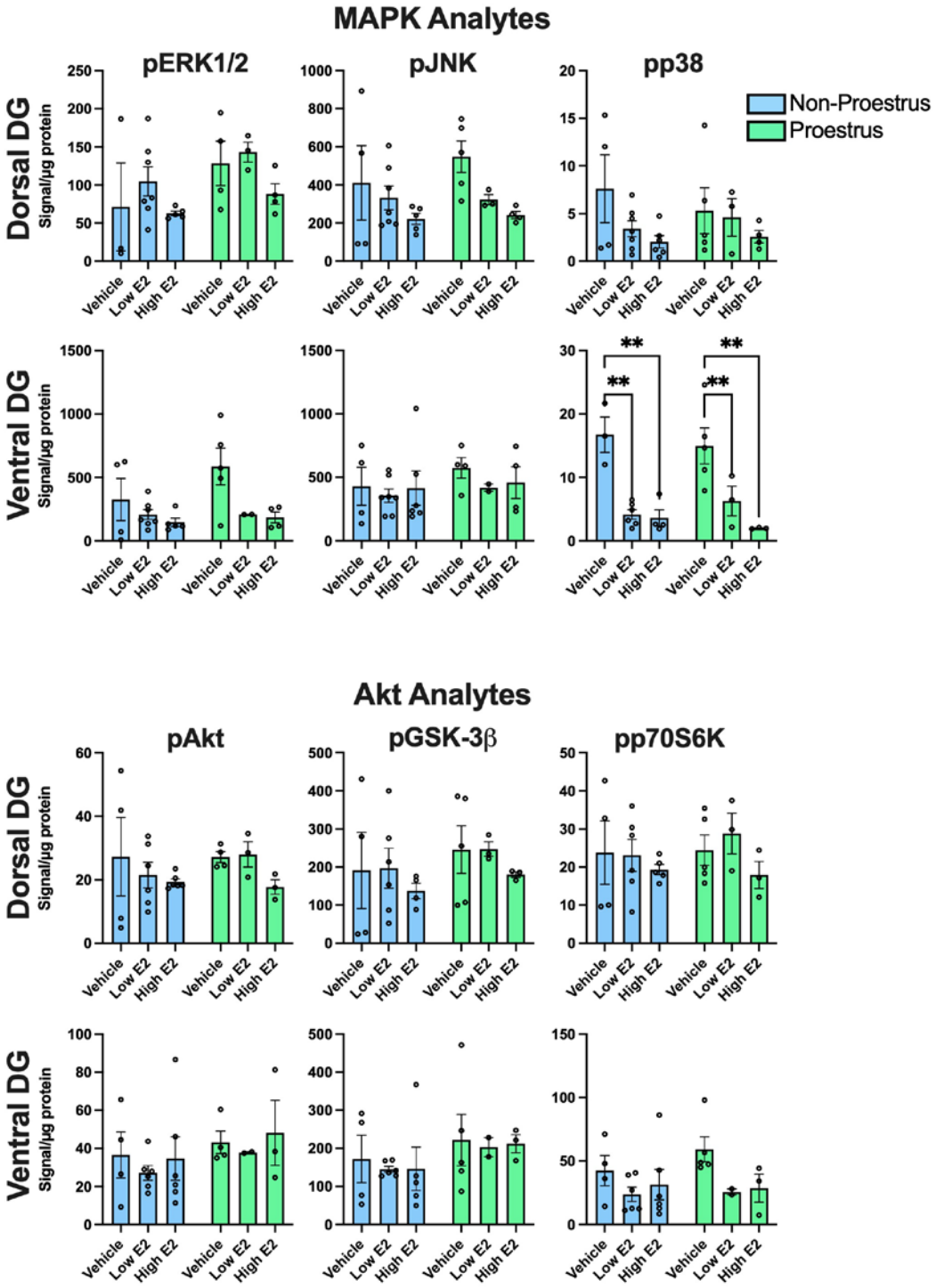
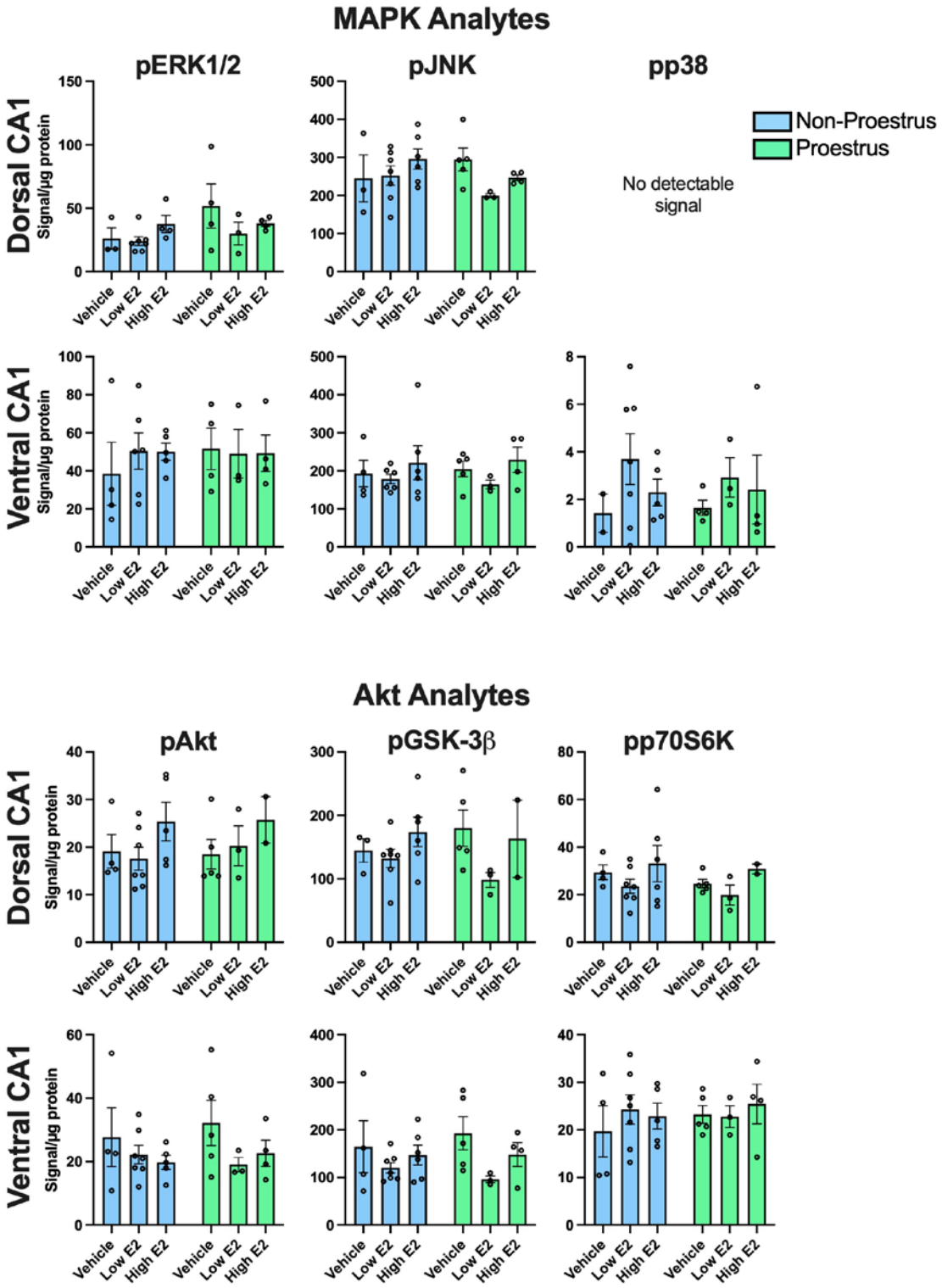
Supplementary Figures:



Supplementary Figure 1: Estrous phase evaluation of MAPK and Akt phosphoprotein levels in the dentate gyrus

Phosphoprotein signal normalized by amount of total protein in sample. Vehicle treated rats did not differ by estrous phase. Treatment effects were observed in dorsal DG pJNK ($p=0.0366$), ventral DG pERK1/2 ($p=0.0148$), and ventral DG pp38 ($p<0.0001$). Estradiol decreased pp38 in proestrous and non-proestrous groups ($p<0.01$). No other effects were observed.



Supplementary Figure 2: Estrous phase evaluation of MAPK and Akt phosphoprotein levels in the hippocampal CA1

Phosphoprotein signal normalized by amount of total protein in sample. Vehicle treated rats did not differ by estrous phase. No significant effects of estrous phase or estradiol were observed.