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17β-Estradiol attenuates p38MAPK activity but not PKCα induced by angiotensin II in the brain

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Abstract

17β-Estradiol (E2) has been shown to modulate the renin–angiotensin system in hydromineral and blood pressure homeostasis mainly by attenuating angiotensin II (ANGII) actions. However, the cellular mechanisms of the interaction between E2 and angiotensin II (ANGII) and its physiological role are largely unknown. The present experiments were performed to better understand the interaction between ANGII and E2 in body fluid control in female ovariectomized (OVX) rats. The present results are the first to demonstrate that PKC/p38 MAPK signaling is involved in ANGII-induced water and sodium intake and oxytocin (OT) secretion in OVX rats. In addition, previous data from our group revealed that the ANGII-induced vasopressin (AVP) secretion requires ERK1/2 signaling. Therefore, taken together, the present observations support a novel concept that distinct intracellular ANGII signaling gives rise to distinct neurohypophyseal hormone release. Furthermore, the results show that E2 attenuates p38 MAPK phosphorylation in response to ANGII but not PKC activity in the hypothalamus and the lamina terminalis, suggesting that E2 modulates ANGII effects through the attenuation of the MAPK pathway. In conclusion, this work contributes to the further understanding of the interaction between E2 and ANGII signaling in hydromineral homeostasis, as well as it contributes to further elucidate the physiological relevance of PKC/p38 MAPK signaling on the fluid intake and neurohypophyseal release induced by ANGII.

Key Words

- fluid intake
- neurohypophyseal release
- cell signaling
- hypothalamus
- lamina terminalis

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Introduction

The constancy of the sodium concentration and the osmolality of extracellular body fluid are essential to survival. The volume and osmolality of body fluids are maintained primarily through the regulation of the ingestion and urinary excretion of water and electrolytes, mainly sodium, and are very important for proper tissue perfusion pressure and osmotic gradient across the cellular plasma membrane. The renin–angiotensin system (RAS) plays an essential role in the maintenance of hydromineral homeostasis by eliciting sodium and water intake and by inducing sodium urinary retention through aldosterone release and hemodynamic effects via angiotensin II a key component of the RAS (Hollenberg 1984, Fitzsimons 1998). The octapeptide hormone angiotensin II (ANGII) also induces vasopressin (AVP) and oxytocin (OT) secretion when injected into the brain (Antunes-Rodrigues *et al*. 2004, Almeida-Pereira *et al*. 2016). OT and AVP are synthesized by magnocellular neurosecretory neurons of the paraventricular (PVN) and the supraoptic (SON) hypothalamic nuclei and are released into the circulation from the neurohypophysis. OT participates in body fluid control by inducing natriuresis and sodium appetite inhibition, while AVP by inducing antidiuresis (Antunes-Rodrigues *et al*. 2004).

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ANGII induces different effects by acting on its angiotensinergic receptors type 1 and 2 (AT1 and AT2), but AT1 mediates most of the well-known effects of ANGII on hydromineral and cardiovascular homeostasis (Beresford & Fitzsimons 1992, Qadri *et al*. 1998, Coble *et al*. 2015). In the brain, peripheral and central ANGII induces sodium and water intake by binding to AT1 in specific structures involved in the generation of fluid intake, e.g., the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (Antunes-Rodrigues *et al*. 2004). The subfornical organ (SFO) is a key sensory circumventricular organ involved in body fluid control and blood pressure regulation, that receives, integrates and responds to both blood-borne and central nervous system signals. The increase in the circulating and central ANGII levels enhances the neural activity of the SFO, which sends efferent axonal projections to the OVLT, the median preoptic nucleus (MnPO), the SON and the PVN (Coble *et al*. 2015). In the SON and the PVN, the SFO afferent angiotensinergic projection increases the excitability of vasopressinergic and oxytocinergic neurons, leading to AVP and OT secretion (Ferguson & Renaud 1986). Furthermore, ANGII increases AVP and OT secretion by acting on its AT1 receptor expressed in the PVN (Lenkei *et al*. 1997).

The AT1 receptor is coupled to the Gq protein (GPCR), and its stimulation leads to the activation of phospholipase C, protein kinase C (PKC) and members of the mitogen-activated protein kinase family (MAPK; extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38MAPK and c-Jun N-terminal Kinase (JNK)) (Mehta & Griendling 2007). MAPK proteins are inactivated by phosphatases, which act dephosphorylating on phosphor-tyrosine residues or on serine/threonine residues or on both of their substrates. Mitogen-activated protein kinase phosphatases (MKPs) are dual specificity protein phosphatases (also known as DUSPs) that dephosphorylate both tyrosine and threonine residues on MAPK members (Salojin & Oravecz 2007). There are several MKPs isoforms all of which dephosphorylate the MAPKs with varying degrees of efficiency. MAPK phosphatase 1 (MKP-1), the first of the MKPs to be characterized, is known for dephosphorylate all three major classes of MAPK (ERK, p38MAPK and JNK) and is expressed in many tissues including the brain (Caunt & Keyse 2012).

The activation of the members of the MAPK family, mediated by AT1, can be PKC dependent or independent based on the activated conformations that the receptor may adopt (Hines *et al*. 2003). Recently, some studies have shown the physiological relevance of AT1 intracellular signaling, elucidating the proteins involved in specific behavioral and neuroendocrine responses. For example, ANGII-induced sodium intake and AVP secretion require the PKC-independent ERK1/2 signaling pathway, but water intake requires PKC, JNK and the mechanistic target of the rapamycin complex 1 (mTORC1) signaling pathways (Daniels *et al*. 2007, Almeida-Pereira *et al*. 2016, Muta *et al*. 2016).

Several epidemiological, clinical and genetic studies in humans and animals have provided remarkable insights on the relationships between high salt consumption and cardiovascular diseases such as hypertension. Woman and females usually have a salt sensitivity as well increased blood pressure at menopause or reproductive senescence, showing a gonadal hormones influence in fluid balance and blood pressure control (Meneton *et al*. 2005). Indeed, female gonadal hormones, mainly 17β-estradiol (E2), are known to mediate hydromineral homeostasis and blood pressure mainly by attenuating RAS actions. E2 attenuates sodium and water intake, OT and AVP release induced by ANGII and AT1 expression and ANGII binding in the SFO (Fitzsimons 1998, Kisley *et al*. 1999, Almeida-Pereira *et al*. 2013, 2016).

The estrogen receptor (ER) is not only known for its classic genomic actions but also for its ability to activate nongenomic cellular signaling events by activating membrane-associated ERs (mER), generating rapid effects. The mER agonism activates members of the MAPK family, such as ERK1/2, JNK and p38MAPK, and increases PKC activity (Micevych & Kelly 2012). More recently, studies from our group provided insights on the ANGII and E2 crosstalk signaling pathways and their physiological relevance to body fluid balance, which plays a critical role in the maintenance of cardiovascular homeostasis. MAPK ERK1/2 and JNK have been shown to be involved in the anti-natriorexigenic and anti-dipsogenic effects of E2 in response to central ANGII stimulation. In addition, the results strongly suggest that E2 inhibits ANGII-induced AVP secretion by increasing MKP-1 expression in the hypothalamic nuclei (PVN and SON) (Almeida-Pereira *et al*. 2016). Nevertheless, E2 signaling involvement in OT secretion in response to central ANGII remains unclear.

Given the wide complexity of the crosstalk signaling pathways, further studies are necessary to better elucidate the mechanisms of the interaction between E2 and ANGII signaling on body fluid control. In addition, the study of the interaction between E2 and ANGII signaling on body fluid control can reveal potential pharmacological targets for the prevention of cardiovascular diseases, with uncontrolled salt consumption as a predisposing factor.

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Therefore, for a better understanding of the interaction between ANGII and E2, experiments were performed to investigate whether PKC and p38 MAPK are involved in fluid intake and neurohypophysial secretion in response to central ANGII in ovariectomized rats (OVX) pretreated with E2. Although the bilateral ovariectomy partially simulates menopause, the study of E2 replacement in ovarian insufficiency is crucial for a better understanding of the E2 mechanisms.

In this context, the goal of the present study was to test the hypotheses that PKC and p38 MAPK signaling are involved in fluid intake (mainly water intake) and neurohypophysial secretion (mainly OT release) in response to central ANGII and that E2 modulates these effects of ANGII by interfering in ANGII-induced PKC/p38 MAPK signaling.

Materials and methods

Animals and ethical approval

Female Wistar rats (~250 g, 10 weeks old) obtained from the Animal Care Facility located on the Campus of Ribeirao Preto, University of Sao Paulo, Brazil, were maintained under controlled temperature (25 ± 1°C) conditions and were exposed to a daily 12:12 h light–dark cycle (06:00 h:18:00 h) with free access to tap water and pelleted food. All experiments were performed at night between 06:00 and 21:00 h This study was conducted according to the 'Guide for the Care and Use of Laboratory Animals' (NIH Publication No. 85-23, revised 1996). The experimental protocols were approved by the Ethics Committee for Animal Use of the School of Medicine of Ribeirao Preto, University Sao Paulo (protocol # 017/2012).

Surgeries

All surgeries were performed under anesthesia induced by 2,2,2-tribromoethanol (250 mg/kg, 2.5%, intraperitoneal, Sigma Aldrich), followed by prophylactic dose (0.1 mL/100 g bw, intramuscular) of veterinary pentabiotic (Fort Dodge, Campinas, SP, Brazil) (Almeida-Pereira *et al.* 2013, 2016).

The surgery for implanting a cannula (12-mm length, 0.4-mm i.d. and 0.6-mm o.d.) into the right lateral ventricle was performed aseptically, using the following stereotaxic coordinates: 0.5 mm (caudal to bregma); 1.5 mm from the midline; 3.7 mm ventral to the dura mater (Paxinos & Watson 1997). Subsequently, the rats were subjected to bilateral ovariectomy surgery under the same anesthesia. The rats were randomly separated into the OVX rats treated with vehicle (corn oil, 0.1 mL per rat, subcutaneous) or the OVX rats treated with 17β-estradiol cypionate (OVX E2; Pfizer) at a subcutaneous dose of 10 µg/rat (OVX E2) (Kisley *et al.* 1999, Almeida-Pereira *et al.* 2016). The administration of the vehicle or E2 began 24 h after OVX surgery and was conducted once a day for 8 days between 07:00 and 10:00 h. Eight days after the surgeries, the last dose of 17β-estradiol or vehicle was administered in the morning and the experiments were performed at night. The efficiency of the surgical procedure and E2 therapy were confirmed by the body weight gain and uterine index. OVX rats treated with E2 had a lower weight gain than the OVX rats treated with oil (3.50 ± 1.29 vs 16.40 ± 2.07 g, $t_{(18)} = 5.27$, $P < 0.001$, $n = 10$) and a larger uterus weight (282.4 ± 15.41 vs 73.54 ± 3.16 mg/100 g body weight, $t_{(21)} = 12.73$, $P < 0.001$, $n = 12/11$). Therefore, these data validate the OVX procedure and E2 therapy.

Water and sodium intake measures

Three days before the experiment day, the animals were placed in individual metabolic cages for appropriate adaptation, and each animal was provided with two bottles filled with hypertonic saline (1.8% NaCl) or tap water and food *ad libitum*. On the experiment day, fluid intake was evaluated. Because the body weight gain varied significantly in response to the E2 treatment, the measures were adjusted by body weight (Almeida-Pereira *et al.* 2013). Therefore, the values are expressed as mL/100 g of body weight.

Blood collection, hormone extraction and immunoassays

After decapitation, trunk blood was collected in chilled plastic tubes containing heparin (10 µL of heparin per mL of blood). Plasma was obtained, and AVP and OT hormones were extracted as previously described (Almeida-Pereira *et al.* 2013, 2016). The hormone measurements were performed using the specific radioimmunoassay techniques as previously described by Haanwinckel *et al.* (1995) and Elias *et al.* (1998). All measurements were performed in duplicate. The sensitivity of the assay was 0.12 pg/mL and the intra-assay coefficient of variation was 11.4% for AVP and 9.2% for OT in the PKC inhibitor experiment. The intra-assay coefficient of variation was 0.8% for AVP and 9.7% for OT in the p38 MAPK inhibitor experiment.

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Microdissection and Western blot

On the experiment day, the animals were decapitated and their brains were collected as previously described (Almeida-Pereira *et al.* 2016). Tissue samples of the OVLT, the MnPO, the SFO, the PVN and the SON were obtained by microdissection in a cryostat according to the coordinates of the Paxinos and Watson atlas (Paxinos & Watson 1997), exactly as described by Almeida-Pereira *et al.* (2016). The samples of the lamina terminalis contained the OVLT, the MnPO and the SFO and the samples of the hypothalamus contained the bilateral PVN and the SON from each animal.

The brain tissues were homogenized in extraction buffer (pH = 7.4) containing (in mM) 20 Tris–HCl, 150 NaCl, 2 EDTA, 1 EGTA, 1 PMSF and protease inhibitors (Thermo Scientific). Extraction of membrane and cytosolic proteins was performed as previously described by Fleegal and Sumners (2003) to quantify PKC alpha (#2056) and Paxillin (#2542). Paxillin is a cytoplasmic protein and was used as a control for the extraction of membrane vs cytosolic fractions. In addition, others brain tissue samples were homogenized in extraction buffer (100 mM Tris–HCl, 2 mM EDTA, 1% Triton X-100 and 1 M PMSF, pH = 7.4) containing protease and phosphatases inhibitors (Thermo Scientific) to determine the total p38 MAPK (#9212), phosphorylated-p38 MAPK T180/Y102 (phospho-p38, #9211), MAPK phosphatase 1 (MKP-1, #ab138265) and β-actin (#4970) as previously described (Almeida-Pereira *et al.* 2016). All antibodies were purchased from Cell Signaling Technology, except for MKP-1, which was from Abcam. The protein concentrations of membrane and cytosolic fractions (10 µg) and the total fraction (40 µg) were determined by the BCA method (Thermo Scientific). After the proteins were transferred to a nitrocellulose membrane by electrophoresis (Trans-Blot, BioRad), nonspecific binding was blocked with 10% bovine serum albumin (BSA) or with 10% skim milk specifically for Paxillin and MKP-1. Briefly, the membranes were incubated overnight with primary antibody rabbit anti-PKCα (1:500)/anti-Paxillin (1:500)/anti-p38 MAPK (1:1000)/anti-phospho-p38 MAPK (1:1000)/anti-β-actin (1:40,000)/anti-MKP-1 (1:500) at 4°C. After washing, the membranes were incubated with secondary antibody anti-rabbit peroxidase-conjugated (1:5000 or 1:2500 for PKCα, Paxillin and MKP-1; #7074, Cell Signaling Technology) at room temperature. Bands were visualized by chemiluminescence (ECL plus Kit, Amersham Biosciences, Sweden) and were quantified using imaging software and a Chemidoc XRS system (Bio-Rad). To verify the effects of E2 and ANGII, the oil/vehicle treatment was considered the control (OVX–Veh). The data are expressed as a percentage relative to the control.

Immunofluorescence

The immunofluorescence technique (including the perfusion method) was performed as previously described (Almeida-Pereira *et al.* 2016). In brief, after sections were blocked in PBS buffer containing 10% normal horse serum, they were incubated overnight with primary antibodies rabbit anti-phospho-p38 MAPK (1:1000; #9211, Cell Signaling Technology) or anti-PKCα (1:500; #2056, Cell Signaling Technology) and guinea pig anti-AVP (1:40,000; T-5048, Peninsula Laboratories, San Carlos, CA, USA) or anti-OT (1:40,000; T-5021, Peninsula Laboratories) at 4°C. After washing, the sections were incubated with secondary antibody donkey anti-guinea pig CY5 (1:200; Jackson ImmunoResearch Laboratories), biotinylated secondary antibody donkey anti-rabbit (1:200; Vector Laboratories Inc., Burlingame, CA, USA) and secondary antibody Alexa 488-conjugatedstreptavidin(1:200;JacksonImmunoResearch Laboratories) for PKCα immunofluorescence and incubated with secondary antibody goat anti-rabbit Alexa Fluor 488 (1:200; Cell Signaling Technology) for phospho-p38 MAPK at room temperature. Images were collected on a Leica TCS SP5 confocal microscope system equipped with 488 nm (argon-krypton) and 633 nm (helium-neon) laser lines (#2004/08868-0, Sao Paulo Research Foundation-FAPESP). For each nucleus, all images were detected at identical acquisition settings.

Experiment 1: Inhibitory effect of E2 on ANGII-induced fluid intake: role of the PKC inhibitor Chelerythrine

A subset of animals from both oil-treated OVX and E2-treated OVX groups received an intracerebroventricular (i.c.v.) administration of the PKC inhibitor (Chelerythrine, 100 µM/2 µL/rat, EMD Chemicals) or vehicle (0.9% saline sterile in 10% of DMSO, 2 µL/rat). Fifteen minutes later, the rats received ANGII (25 ng/2 µL/rat, i.c.v., Sigma) or vehicle (0.9% saline sterile, 2 µL/rat, i.c.v.), and the water and hypertonic saline (1.8% NaCl) intakes were measured at 5, 10, 15, 30 and 60 min after ANGII administration. The dose and timing of the Chelerythrine application were chosen based on the experiments reported by Daniels *et al.* (2009). Food was not available to the animals after the initial injection (inhibitor).

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Experiment 2: Role of the PKC inhibitor Chelerythrine on ANGII-induced OT and AVP release and inhibitory effect of E2 on ANGII-induced hormone release

Another subset of animals from both oil-treated OVX and E2-treated OVX groups received the administration of the PKC inhibitor or vehicle (same dose described above in experiment 1). Fifteen minutes later, the rats received ANGII or vehicle (same dose described above for exp. 1). After five minutes, the animals were decapitated, and trunk blood was collected for plasma OT and AVP measurements. The dose of ANGII and the timing of the blood collection were chosen based on the experiments previously reported by Almeida-Pereira *et al.* (2016). Food and fluids were not available to the animals after the initial injection (inhibitor).

Experiment 3: Role of the p38 MAPK inhibitor SB203580 on ANGII-induced fluid intake and E2 inhibitory effect on ANGII-induced fluid intake

Another subset of oil-treated OVX and E2-treated OVX rats were subjected to the central administration of the p38 MAPK inhibitor (SB203580, 50 µM/2 µL/rat, i.c.v., Sigma) or vehicle (0.9% saline sterile in 10% of DMSO, 2 µL/rat, i.c.v.). Twenty minutes later, the rats received ANGII (25 ng/2 µL/rat, i.c.v., Sigma) or vehicle (0.9% saline sterile, 2 µL/rat, i.c.v.) and the water and hypertonic saline solution intakes were measured at 5, 10, 15, 30 and 60 min after ANGII administration. The SB203580 dose and the time between the drugs employed were based on prior tests. Food was not available to the animals after the initial injection (inhibitor).

Experiment 4: Role of the p38 MAPK inhibitor SB203580 on ANGII-induced OT and AVP release and E2 inhibitory effect on ANGII-induced hormone release

Another subset of E2- and oil-pretreated OVX rats were centrally injected with a p38 MAPK inhibitor or vehicle (same dose described above for experiment 3). Twenty minutes later, the rats received ANGII or vehicle (same dose described above for exp. 3). After 5 min, the animals were decapitated and trunk blood was collected for plasma OT and AVP measurements. Food and fluids were not available to the animals after the initial injection (inhibitor).

Experiment 5: Role of E2 on ANGII-induced PKCα and p38 MAPK activation in the lamina terminalis structures and the hypothalamus (PVN and SON)

A subset of animals from both oil- and E2-pretreated OVX groups received ANGII (25 ng/2 µL/rat, i.c.v., Sigma) or vehicle (0.9% saline sterile, 2 µL/rat, i.c.v.). Five minutes post injection, the animals were decapitated and the brains were collected for PKC expression analysis from cytosolic and membrane fractions. While another subset of animals was

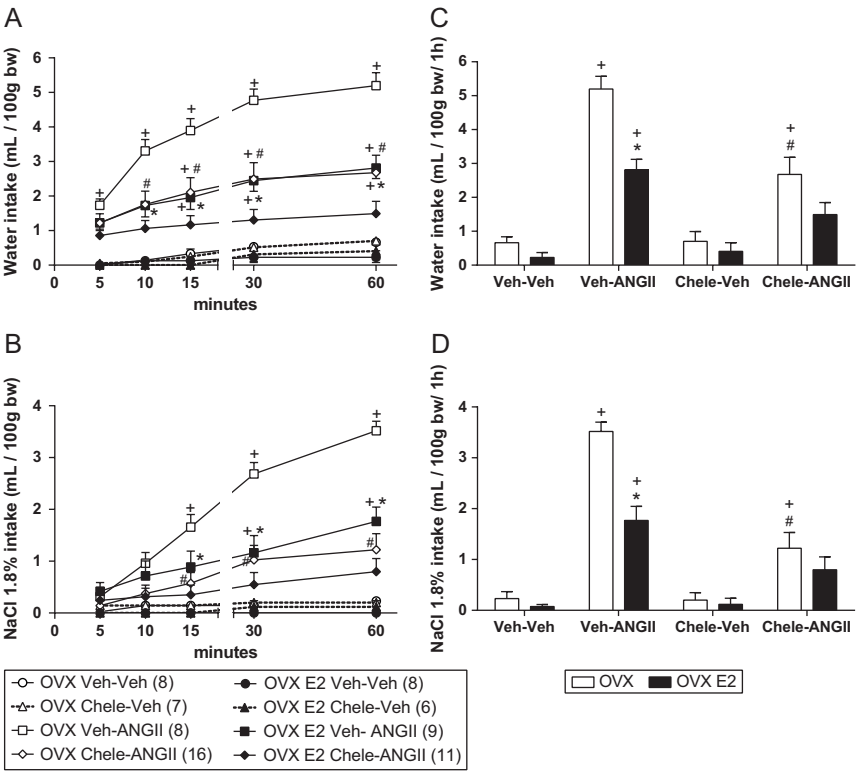

Figure 1
E2 and ANGII interaction in water and sodium intake: role of PKC inhibitor Chelerythrine. Cumulative water intake (A) and water intake at the end of the 60 min test (C), cumulative sodium intake (B) and sodium intake at the end of the 60-min test (D). A subset of animals from both oil- and E2-treated OVX groups received central injection of PKC inhibitor Chelerythrine or vehicle before ANGII or vehicle injection. After ANGII injection the fluids intake were evaluated. Values are adjusted per 100 g of body weight (bw) and expressed as means ± S.E.M. $^{+}P < 0.05$ vs respective Veh–Veh, $^{\#}P < 0.05$ vs respective Veh–ANGII, $^{*}P < 0.05$ vs respective oil-treated OVX group. The sample size (*n*) is indicated in parenthesis.

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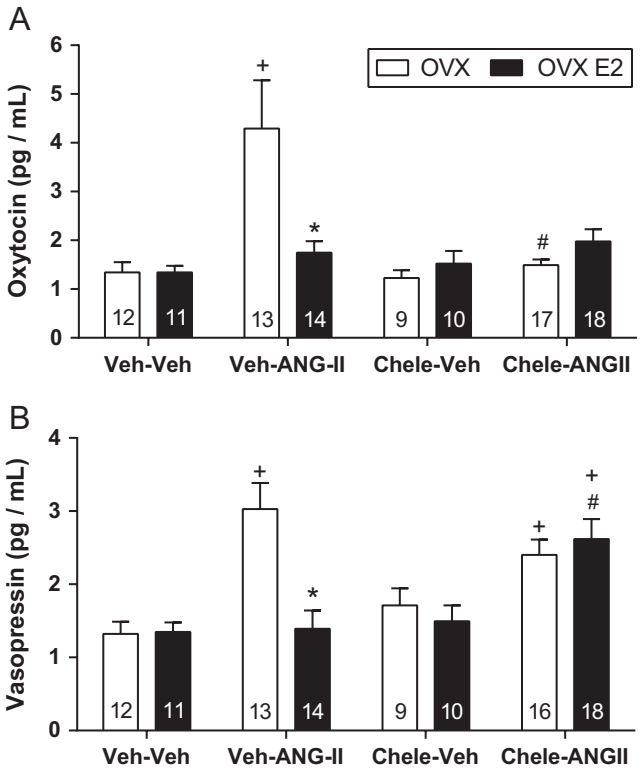

Figure 2
E2 and ANGII interaction in neurohypophysial hormones release: role of PKC inhibitor Chelerythrine. OT release (A) and AVP release (B) analyses. A subset of animals from both oil- and E2-treated OVX groups received central injection of PKC inhibitor Chelerythrine or vehicle before ANGII or vehicle injection. The blood samples were collected 5 min after ANGII injection. Values are expressed as means ± S.E.M. $^{+}P < 0.05$ vs respective Veh–Veh, $^{\#}P < 0.05$ vs respective Veh–ANGII, $^{*}P < 0.05$ vs respective oil-treated OVX group. The sample size (*n*) is indicated inside the columns.

used for total and phosphorylated p38 MAPK expression analysis. All analyses were performed by Western blot. Food and fluids were not available to the animals after ANGII injection.

Furthermore, to elucidate the role of E2 on MKP-1 expression in the lamina terminalis, another subset of oil- and E2-pretreated OVX rats received ANGII (or vehicle), and they were decapitated 10 min post injection. The brains were collected for MKP-1 expression analysis by Western blot. Food and fluids were not available to the animals after ANGII injection.

Experiment 6: Role of E2 on PKCα and phospho-p38 MAPK expression in the SFO and oxytocinergic and vasopressinergic magnocellular neurons of the SON in response to ANGII

A subset of animals from oil- and E2-treated OVX groups was deeply anesthetized with TBE 2.5% 5 min post injection of ANGII or vehicle (same dose as describe above) and the brains were collected after transcardiac perfusion. PKCα and p38 MAPK phosphorylated in the SFO and co-localization between these proteins and OT or AVP in the SON were evaluated by immunofluorescence technique. Food and fluids were not available to the animals after ANGII injection.

Statistical analysis

The data are presented as the means ± standard errors (S.E.M.) and were analyzed using Statistica (StatSoft, USA). Drinking responses were analyzed by repeated-measures ANOVA, using the hormonal profile (E2), the inhibitor drug, ANGII stimulation and time as independent variables. Hormonal release and fluid intake at 60 min were analyzed by three-way ANOVA, taking the hormonal profile (E2), the inhibitor drug and ANGII stimulation as independent variables. Protein expression was analyzed by two-way ANOVA, taking the hormonal profile (E2) and ANGII stimulation as independent variables. If the ANOVA yielded statistically significant values (*F* value significant), *post hoc* comparisons were performed using the Student–Newman–Keuls. For the analysis of the protein expression, the uterine index and the weight gain with the hormonal profile (E2) were used as the independent variables and Student's unpaired *t*-test was performed. The significance level was set at 5% ($\alpha = 5\%$).

Results

Experiment 1: Inhibitory effect of E2 on ANGII-induced fluid intake: role of the PKC inhibitor Chelerythrine

E2 therapy attenuated the ANGII-stimulated water (E2 × ANGII interaction: $F_{1,65} = 6.1$, $P < 0.05$, Fig. 1A; E2 × ANGII interaction: $F_{1,65} = 5.6$, $P < 0.05$, Fig. 1C) and sodium intake (E2 × ANGII interaction: $F_{1,65} = 6.0$, $P < 0.05$, Fig. 1B; E2 × ANGII interaction: $F_{1,65} = 5.9$, $P < 0.05$, Fig. 1D). Likewise, the central administration of Chelerythrine (PKC inhibitor) attenuated both water (Chele × ANGII interaction: $F_{1,65} = 8.0$, $P < 0.01$, Fig. 1A; E2 × ANGII interaction: $F_{1,65} = 11.5$, $P < 0.01$, Fig. 1C) and sodium intake (Chele × ANGII interaction: $F_{1,65} = 11.9$, $P < 0.001$, Fig. 1B; E2 × ANGII interaction: $F_{1,65} = 19.4$, $P < 0.001$, Fig. 1D) induced by ANGII in oil- and E2-treated OVX rats. Therefore, PKC inhibition did not change the E2 inhibitory effect on the ANGII-induced fluid intake.

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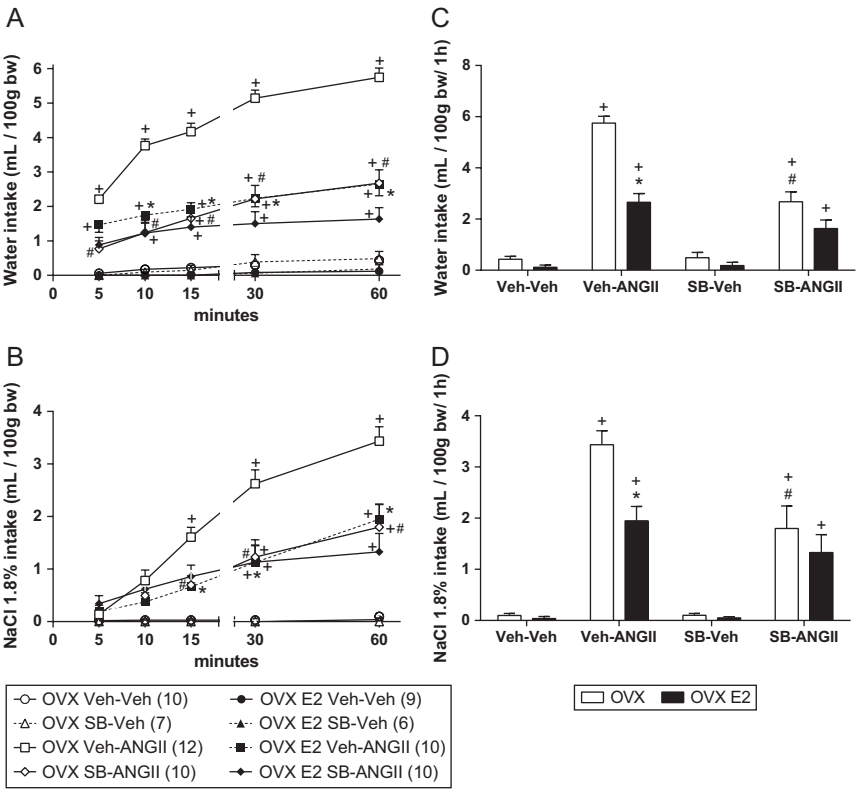

Figure 3
E2 and ANGII interaction in water and sodium intake: role of p38 MAPK inhibitor SB203580. Cumulative water intake (A) and water intake at the end of the 60 min test (C), cumulative sodium intake (B) and sodium intake at the end of the 60 min test (D). A subset of animals from both oil- and E2-treated OVX groups received central injection of p38 MAPK inhibitor SB203580 or vehicle before ANGII or vehicle injection. After ANGII injection the fluids intake were evaluated. Values are adjusted per 100 g of body weight (bw) and expressed as means ± S.E.M. $^{+}P < 0.05$ vs respective Veh–Veh, $^{\#}P < 0.05$ vs respective Veh–ANGII, $^{*}P < 0.05$ vs respective oil-treated OVX group. The sample size (*n*) is indicated in parenthesis.

Experiment 2: Role of the PKC inhibitor Chelerythrine on ANGII-induced OT and AVP release and inhibitory effect of E2 on ANGII-induced hormone release

The pretreatment with E2 attenuated the ANGII-stimulated OT secretion (E2 × ANGII interaction: $F_{1,96} = 4.0$, $P < 0.05$, Fig. 2A) and AVP secretion (E2: $F_{1,95} = 4.8$, $P < 0.05$, Fig. 2B). The central administration of the PKC inhibitor attenuated the OT secretion induced by ANGII (Chele × ANGII interaction: $F_{1,96} = 5.2$, $P < 0.05$), but did not change the ANGII-induced AVP secretion in oil-treated OVX rats. Interestingly, PKC inhibition in the E2-treated rats reversed the E2 inhibitory effect on ANGII-stimulated AVP release (Chele × E2 × ANGII interaction: $F_{1,95} = 8.1$, $P < 0.01$).

Experiment 3: Role of the p38 MAPK inhibitor SB203580 on ANGII-induced fluid intake and E2 inhibitory effect on ANGII-induced fluid intake

As shown in Fig. 3, E2 therapy attenuated the ANGII-stimulated water (E2 × ANGII interaction: $F_{1,66} = 14.8$, $P < 0.001$, Fig. 3A; E2 × ANGII interaction: $F_{(1,66)} = 19.2$, $P < 0.001$, Fig. 3C) and sodium intake (E2 × ANGII interaction: $F_{1,66} = 5.8$, $P < 0.05$, Fig. 3B; E2 × ANGII interaction: $F_{(1,66)} = 5.7$, $P < 0.05$, Fig. 3D). Similarly, the central administration of SB203580 (p38 MAPK inhibitor) attenuated both water (SB × ANGII interaction: $F_{1,66} = 29.9$, $P < 0.001$, Fig. 3A; ANGII × SB interaction: $F_{(1,66)} = 27.4$, $P < 0.001$, Fig. 3C) and sodium intake (SB × ANGII interaction: $F_{1,66} = 7.2$, $P < 0.01$, Fig. 3B; ANGII × SB interaction: $F_{(1,66)} = 7.2$, $P < 0.01$, Fig. 3D) induced by ANGII in oil- and E2-treated OVX rats. Therefore, p38 MAPK inhibition did not change the E2 inhibitory effect on the ANGII-induced fluid intake.

Experiment 4: Role of the p38 MAPK inhibitor SB203580 on ANGII-induced OT and AVP release and E2 inhibitory effect on ANGII-induced hormone release

The pretreatment with E2 attenuated the ANGII-stimulated OT secretion (E2 × ANGII interaction: $F_{1,71} = 16.0$, $P < 0.001$, Fig. 4A) and AVP secretion (E2 × ANGII interaction: $F_{1,69} = 12.1$, $P < 0.001$, Fig. 4B). The p38 MAPK inhibition attenuated OT (SB × ANGII interaction: $F_{1,71} = 8.4$, $P < 0.01$) and AVP (SB × ANGII interaction: $F_{1,69} = 10.1$, $P < 0.01$) secretion induced by ANGII, but did not change the E2 inhibitory effect on ANGII-induced hormone release.

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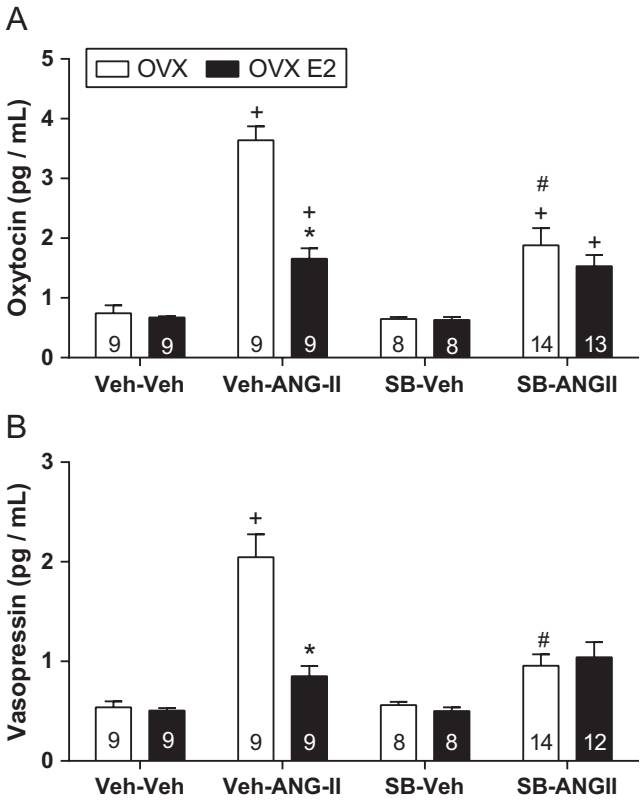

Figure 4
E2 and ANGII interaction in neurohypophysial hormones release: role of p38 MAPK inhibitor SB203580. OT release (A) and AVP release (B) analyses. A subset of animals from both oil- and E2-treated OVX groups received central injection of p38 MAPK inhibitor SB203580 or vehicle before ANGII or vehicle injection. The blood samples were collected five minutes after ANGII injection. Values are expressed as means ± S.E.M. $^{+}P < 0.05$ vs respective Veh–Veh, $^{\#}P < 0.05$ vs respective Veh–ANGII, $^{*}P < 0.05$ vs respective oil-treated OVX group. The sample size (*n*) is indicated inside the columns.

Experiment 5: Role of E2 on ANGII-induced PKCα and p38 MAPK activation in the lamina terminalis structures and the hypothalamus (PVN and SON)

Because the alpha isoform of PKC is involved in ANGII-stimulated drinking responses (Coble *et al.* 2014) and PKC inhibition did not change the E2 inhibitory effect on ANGII-induced fluid intake, the role of E2 on PKCα activation in response to ANGII was evaluated. Both ANGII and E2 were found to induce PKCα activation (ANGII: $F_{1,21}=9.2$, $P<0.01$; E2: $F_{1,21}=7.9$, $P<0.05$), but E2 pretreatment did not change the PKCα activation induced by ANGII in the lamina terminalis (Fig. 5A). On the other hand, no PKCα activation was observed in any experimental condition in the SON or the PVN (Fig. 5B). Therefore, this result suggests that the alpha isoform of PKC is not involved in ANGII-stimulated OT release and E2 inhibitory effect on ANGII-induced AVP release.

Likewise the role of E2 on ANGII-induced p38 MAPK phosphorylation in the lamina terminalis and the hypothalamus was evaluated since p38 MAPK inhibition did not alter the E2 inhibitory effect on ANGII-induced fluid intake and hormone release. Central administration of ANGII increased p38 MAPK phosphorylation in the lamina terminalis (ANGII: $F_{1,29}=6.5$, $P<0.05$, Fig. 6C) and the hypothalamus (ANGII: $F_{1,26}=9.0$, $P<0.01$, Fig. 6A), but E2 pretreatment did not. Furthermore, E2 pretreatment prevented ANGII-induced p38 MAPK phosphorylation in the lamina terminalis (E2 × ANGII interaction: $F_{1,29}=6.1$, $P<0.05$) and the hypothalamus (E2 × ANGII interaction: $F_{1,26}=4.1$, $P<0.05$). Because E2 increases MKP-1 expression in the hypothalamus (Almeida-Pereira *et al.* 2016), it was investigated whether E2 attenuates p38 MAPK phosphorylation by increasing MKP-1 expression in the lamina terminalis. However, E2 pretreatment and ANGII administration did not alter MKP-1 expression in the lamina terminalis (Fig. 7).

Experiment 6: Role of E2 on PKCα and phospho-p38 MAPK expression in the SFO and oxytocinergic and vasopressinergic magnocellular neurons of the SON in response to ANGII

As shown in the Figs 8 and 9, there is co-expression between phospho-p38 MAPK and AVP/OT in magnocellular neurons of the SON. This observation corroborates the hypothesis that both AVP and OT release induced by ANGII require p38 MAPK pathway signaling. In addition, E2 pretreatment attenuated phospho-p38 MAPK immunostaining induced by ANGII in the SON, which emphasizes the hypothesis that E2 prevents ANGII-induced p38 MAPK phosphorylation. Conversely, PKC alpha isoform immunostaining in the SON was not observed, showing that this isoform is not expressed in the magnocellular neurons in the SON.

Additionally, immunofluorescence was performed to further investigate the role of E2 on p38 MAPK and PKCα expression induced by ANGII specifically in the SFO, a crucial brain structure involved in hydromineral and cardiovascular homeostasis. It was observed that E2 pretreatment attenuated phospho-p38 MAPK immunostaining induced by ANGII (Fig. 10), but not PKCα immunostaining in the SFO (Fig. 11) according to the data obtained from Western blot.

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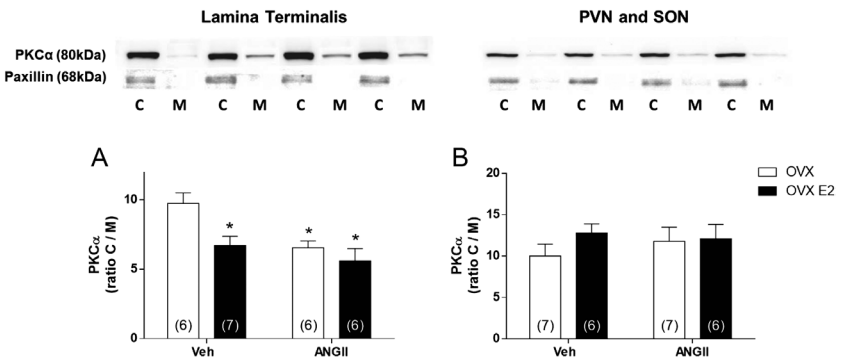

Figure 5
Effect of E2 on ANGII-induced PKCα translocation in the lamina terminalis and hypothalamus. PKCα translocation in the lamina terminalis structures (A) and in the PVN and SON (B) analyses. A subset of animals from both oil- and E2-treated OVX groups received central injection of ANGII or vehicle. The brain samples were collected five minutes post injection. Above A: representative blots and corresponding to the groups in the graph below, showing PKCα and paxillin protein levels in the lamina terminalis (MnPO, OVLT and SFO). A: bar graph shows means ± S.E.M. (n = 6–7) of the ratio of C/M PKCα protein expression in the lamina terminalis structures. C refers to the cytosolic fractions and M to the membrane fractions of the lamina terminalis extracts. Above B: representative blots and corresponding to the groups in the graph below, showing PKCα and paxillin protein levels in the hypothalamus (PVN and SON). B: bar graph shows means ± S.E.M. (n = 6–7) of the ratio of C/M PKCα protein expression in the hypothalamus. C refers to the cytosolic fractions and M to the membrane fractions of the hypothalamic extracts. $^{+}P < 0.05$ vs oil-treated respective group, $^{*}P < 0.05$ vs OVX–Veh group. The sample size (n) is indicated inside the columns.

Discussion

A substantial amount of work has been performed to investigate the cellular mechanism of AT1 and estrogen receptor (ER) activity on the cardiovascular system. However, the interaction between AT1 and ER receptors and its physiological relevance in the maintenance of body fluid control remain unclear. Recently, our group contributed to the elucidation of the physiological relevance of ERK1/2 and JNK signaling on the ANGII-induced drinking response and neurohypophysial secretion in OVX rats treated with E2 (Almeida-Pereira *et al.* 2016). Now, the present study investigated the role of E2 and PKC/p38 MAPK signaling on fluid intake and neurohypophysial secretion in response to central ANGII in OVX rats.

The central inhibition of the PKC and p38 MAPK activity decreased both water and sodium intake induced by ANGII in OVX rats, suggesting that PKC/p38 MAPK signaling is involved in fluid intake induced by ANGII. Consistent with these results, Coble *et al.* (2014) also showed that PKC, specifically the alpha isoform, is involved in water and sodium intake within the SFO in response to increased brain ANGII in mice. In addition, similar to PKC, p38 MAPK was also demonstrated to be involved in ANGII-induced fluid intake, suggesting that the p38 MAPK pathway can be PKC dependent. Conversely, Daniels *et al.* (2007) showed that PKC signaling is specifically involved in ANGII-induced water intake in male rats. These divergent results suggest a sexually dimorphic aspect to the AT1 signaling pathway involved in ANGII-induced fluid intake. Indeed, several studies have reported that the RAS is differentially regulated in males and females (Fischer *et al.* 2002, Krause *et al.* 2003, Sandberg & Ji 2012). The OVX rats have a pronounced sodium appetite compared to intact female and male rats (Fitzsimons 1998), suggesting that the lack of ovary steroids leads to a higher sensitivity to the effects of ANGII. E2 attenuates sodium and water intake in female rats during proestrus and OVX in response to ANGII (Antunes-Rodrigues & Covian 1963, Almeida-Pereira *et al.* 2016) and reduces ANGII binding (Kisley *et al.* 1999) and AT1 receptor expression in the SFO (Krause *et al.* 2006, Almeida-Pereira *et al.* 2013). Therefore, the sexually dimorphic aspect of the intake behavior can be attributed to differences in gonadal and steroid profiles (Stricker *et al.* 1991, Fitzsimons 1998) that affect RAS functioning. However, although gonadal sex is a major difference, it is not the only difference. The sex chromosomes, regardless of the gonadal hormone milieu, and the cell-autonomous actions of the sex chromosomes, for example, can also contribute to the sexually dimorphic aspect as shown recently by Ji *et al.* (2010) and Dadam *et al.* (2014) in cardiovascular and hydromineral homeostasis.

PKC and p38 MAPK inhibition also decreased both water and sodium intake induced by ANGII in OVX rats treated with E2 similar to the OVX oil group. These results suggest that E2 can change the PKC activity and p38 MAPK phosphorylation induced by ANGII, maintaining its inhibitory effect on fluid intake. However, E2 did not

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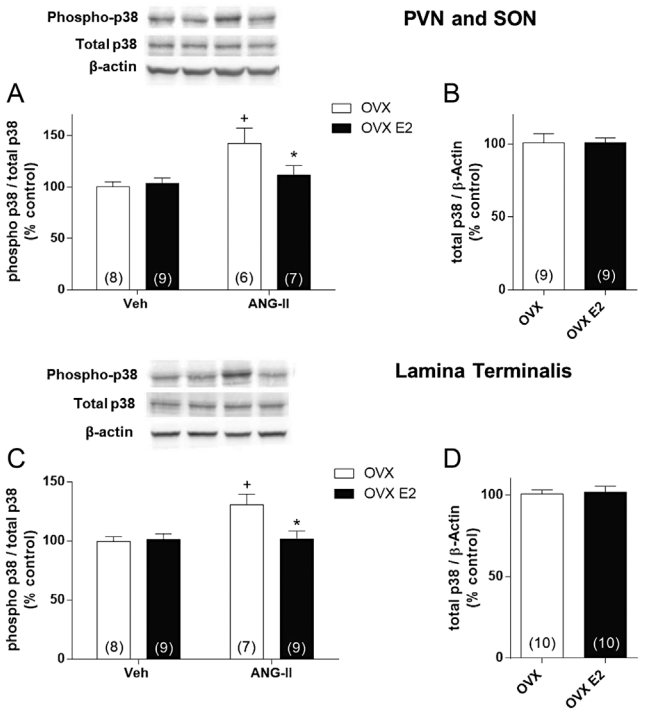

Figure 6
Effect of E2 on ANGII-induced p38 MAPK phosphorylation in the lamina terminalis and hypothalamus. p38 MAPK phosphorylation in the PVN and SON (A and B) and in the lamina terminalis structures (C and D) analyses. A subset of animals from both oil- and E2-treated OVX groups received central injection of ANGII or vehicle. The brain samples were collected five minutes post injection. Above A: representative blots and corresponding to the groups in the graph below, showing phosphorylated p38 MAPK, total p38 MAPK and β-actin protein levels in the hypothalamus (PVN and SON). A: bar graph shows means ± S.E.M. of phospho-p38/total p38 protein expression in the hypothalamus. B: bar graph shows means ± S.E.M. of total p38/β-actin protein expression in the hypothalamus. Above C: representative blots and corresponding to the groups in the graph below, showing phosphorylated p38 MAPK, total p38 MAPK and β-actin protein levels in the lamina terminalis (MnPO, OVLT and SFO). C: bar graph shows means ± S.E.M. of phospho-p38/total p38 protein expression in the lamina terminalis. D: bar graph shows means ± S.E.M. of total p38/β-actin protein expression in the lamina terminalis. $^{+}P < 0.05$ vs oil-treated respective group, $^{*}P < 0.05$ vs OVX–Veh group. The sample size (*n*) is indicated inside the columns.

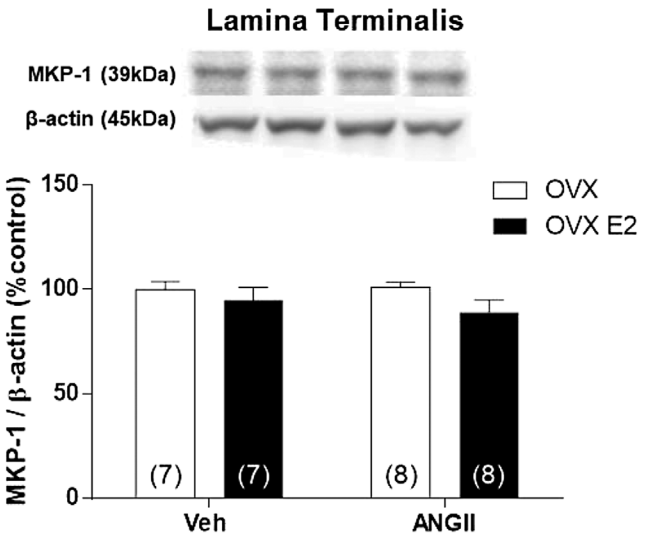

Figure 7
Role of E2 on MKP-1 expression in the lamina terminalis. A subset of animals from both oil- and E2-treated OVX groups received central injection of ANGII or vehicle. The brain samples were collected ten minutes post injection. In the top: representative blots and corresponding to the groups in the graph below, showing MKP-1 and β-actin protein levels in the lamina terminalis (MnPO, OVLT and SFO). At the bottom: bar graph shows means ± S.E.M. of MKP-1/β-actin protein expression in the lamina terminalis. The sample size (*n*) is indicated inside the columns.

alter PKCα activity in the lamina terminalis induced by ANGII, suggesting that PKCα does not mediate the inhibitory effect of E2 on fluid intake induced by ANGII. PKC is known to be a key enzyme in the signal transduction of GPCR and is involved in the activation of several other proteins and the consequent activation of several intracellular cascades that are responsible for numerous cellular functions (Tanaka & Nishizuka 1994). Then, it is suggested that the E2 could alter the activation of other proteins in the PKC signaling cascade. In fact, the E2 treatment inhibited p38 MAPK phosphorylation in the lamina terminalis induced by ANGII and this mechanism can explain, at least in part, the E2 inhibitory effect on the fluid intake induced by ANGII. Moreover, the attenuation of ANGII-induced water and sodium intake via E2 treatment and p38 MAPK inhibition are not additive supporting the hypothesis that the E2-induced p38 MAPK dephosphorylation in response to ANGII can contribute to its inhibitory effect on the fluid intake. Recently, Almeida-Pereira *et al.* (2016) showed that JNK signaling is also involved in ANGII-induced water and sodium intake in OVX rats and that E2 treatment prevented ANGII-induced JNK phosphorylation in the lamina terminalis. These results suggest that the JNK signaling that is involved in ANGII-induced intake behavior can also be PKC-dependent and that E2-induced JNK inhibition can contribute to its inhibitory effect on ANGII-induced fluid intake.

Phosphatases are important regulators of intracellular signaling events and are responsible for the dephosphorylation of the residues of their substrates. MAP kinase phosphatases act as negative feedback regulators of MAPK activity (Caunt & Keyse 2012). Because E2 increases MKP-1 expression in vascular smooth muscle cell cultures and the hypothalamus (Takeda-Matsubara *et al.* 2002, Almeida-Pereira *et al.* 2016), we investigated whether MKP-1 is involved in E2-induced p38 MAPK dephosphorylation in the lamina terminalis.

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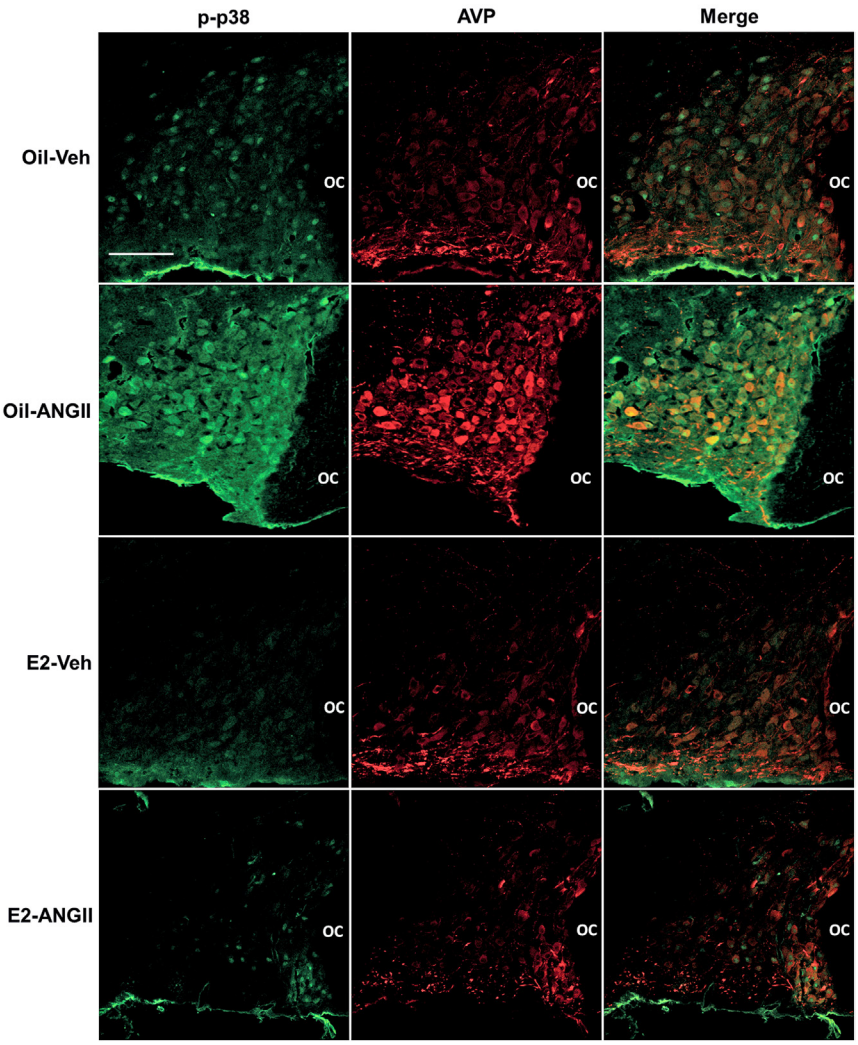

Figure 8
Role of E2 on phospho-p38 MAPK expression in the vasopressinergic magnocellular neurons of the SON in response to ANGII. Representative immunofluorescence photomicrographs of coronal sections showing the AVP and phospho-p38 MAPK staining and their co-localization (merge) in the supraoptic nucleus in response to ANGII stimulation in oil- and E2-treated OVX rats. OC, optic chiasm. Objective is ×40. The scale bar represents 100 µm. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0095.

However, neither E2 pretreatment nor ANGII administration altered MKP-1 expression, suggesting that this isoform is not involved in the ER and ANGII receptor activity, specifically in the lamina terminalis.

Some studies have reported the involvement of PKC and protein kinase A (PKA) in the phosphorylation process of the AT1 receptor, inducing its desensitization (Beltman *et al*. 1996, Iglesias *et al*. 2001, Kohout & Lefkowitz 2003). PKC phosphorylates residues of the C-terminal portion of GPCRs exposed or not to agonists (Ferguson 2001). Since E2 was able to induce PKCα activation in the structures of the lamina terminalis, E2-mediated PKCα activation could be reasonably assumed to induce AT1 desensitization, contributing to its inhibitory effect on the ANGII-induced intake behavior. Although the PKC isoforms involved in the AT1 phosphorylation process have not been fully elucidated in the literature, the inhibition of classical PKC isoforms is known to reduce AT1 phosphorylation (Beltman *et al*. 1996), taking the PKCα isoform a potential candidate.

The present study reports for the first time that PKC signaling is involved in ANGII-induced OT release but not in AVP release. Vasopressin release from the neurohypophysis requires ERK1/2 signaling as shown previously by Almeida-Pereira *et al*. (2016) in OVX rats. Taken together, these data provide interesting insights that neurohypophysial secretion in response to ANGII involves distinct signal transduction pathways. In addition, we observed that PKC inhibition reverses the E2 inhibitory effect on the ANGII-induced AVP release, suggesting that E2 requires PKC signaling to inhibit AVP release induced by ANGII. E2 increases MKP-1 expression in the PVN and the SON, which was implicated in the E2-induced dephosphorylation of ERK1/2 and the consequent attenuation of AVP release in response to ANGII (Almeida-Pereira *et al*. 2016).

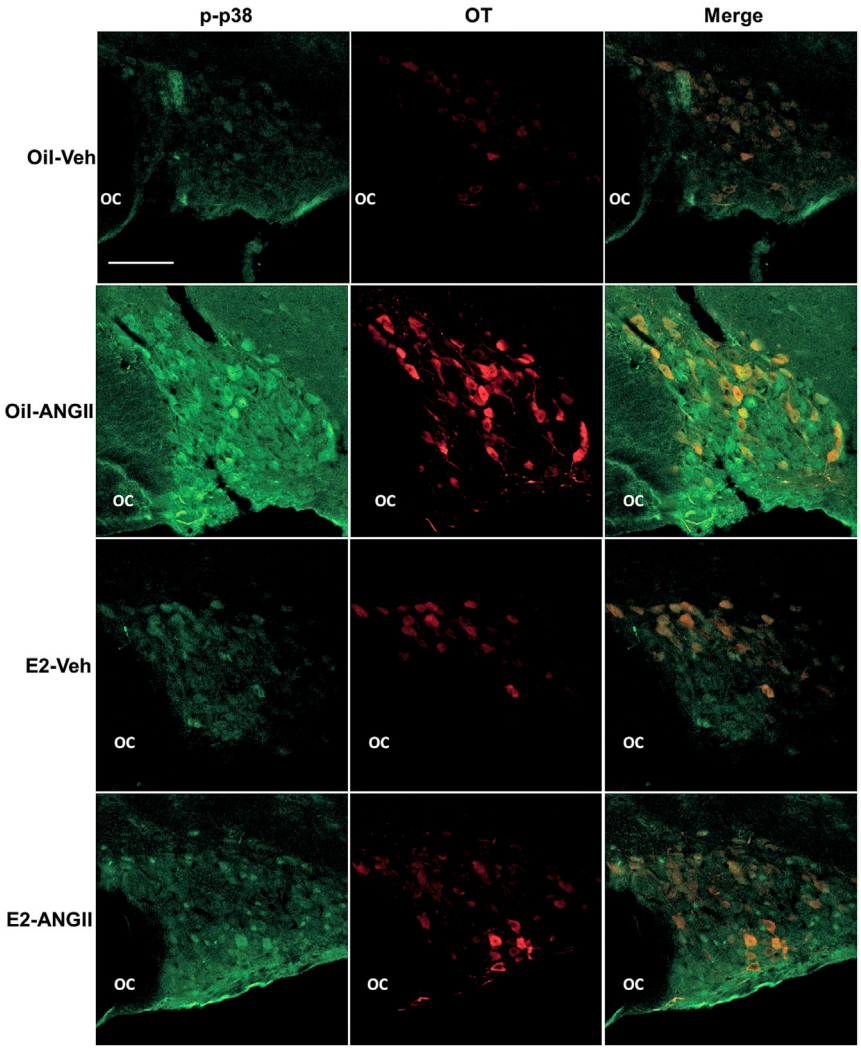

Figure 9
Role of E2 on phospho-p38 MAPK expression in the oxytocinergic magnocellular neurons on the SON in response to ANGII. Representative immunofluorescence photomicrographs of coronal sections showing the OT and phospho-p38 MAPK staining and their co-localization (merge) in the supraoptic nucleus in response to ANGII stimulation in oil- and E2-treated OVX rats. OC, optic chiasm. Objective is ×40. The scale bar represents 100 µm. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0095.

Therefore, these observations lead to the hypothesis that MKP-1 expression can be PKC dependent. Indeed, some studies showed that MKP-1 expression is mediated by PKC (Beltman *et al.* 1996, Stawowy *et al.* 2003, Short *et al.* 2006). Therefore, these data suggest that E2 inhibits ANGII-induced AVP release via PKC-mediated MKP-1 induction and consequent ERK1/2 dephosphorylation.

Because PKCα is involved in fluid intake in response to ANGII, we evaluated whether PKCα is also be involved in neurohypophysial secretion induced by ANGII. However, no differences were observed between the experimental groups in the PKCα activation in the hypothalamus or in PKCα immunostaining in the magnocellular neurons in the SON, showing that this isoform is not expressed in the SON. Therefore, these results suggest that the alpha isoform of PKC is not involved in ANGII-stimulated OT release and in the E2 inhibitory effect on ANGII-induced AVP release. Chelerythrine has been reported to inhibit both classical and novel PKC isoforms (Herbert *et al.* 1990, Saraiva *et al.* 2003), suggesting that they are probably the PKC isoforms that are involved in ANGII-induced OT release and in the inhibitory effect of E2 on ANGII-induced AVP release. On the other hand, another important point to be considered is that ANGII injected intracerebroventricular stimulates AVP and OT release by acting mainly in the SFO, which send angiotensinergic projections to magnocellular neurons of the PVN and SON (Ferguson & Bains 1996, Coble *et al.* 2015). Thus, PKCα within the SFO induced by ANGII can be involved in OT release.

SFO neurons have been shown to express both AT1 and ER type α (ERα) whereas magnocellular neurons in the PVN and the SON express ER type β (ERβ) (Alves *et al.* 1998, Hrabovszky *et al.* 1998, Rosas-Arellano *et al.* 1999), suggesting that E2 can act directly and/or indirectly

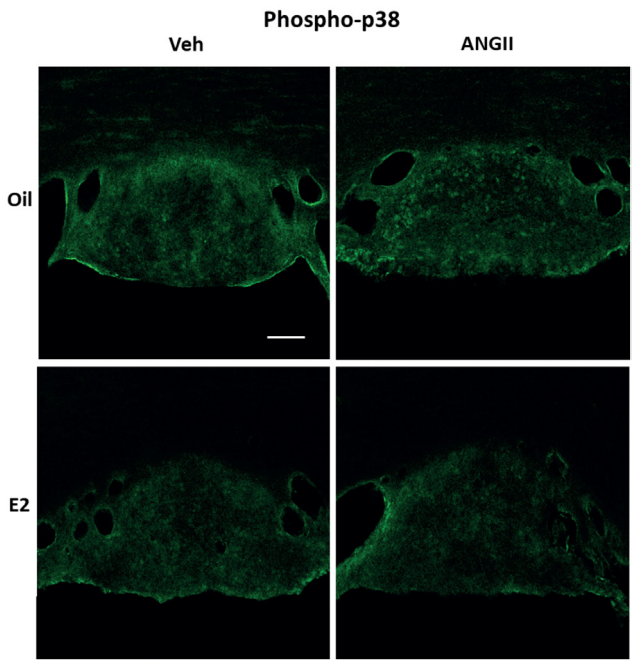

Figure 10
Role of E2 on phospho-p38 MAPK expression in the SFO in response to ANGII. Representative immunofluorescence photomicrographs of coronal sections showing the phospho-p38 MAPK protein staining in the subfornical organ in response to ANGII stimulation oil- and E2-treated OVX rats. Objective is ×20. The scale bar represents 100 µm. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0095.

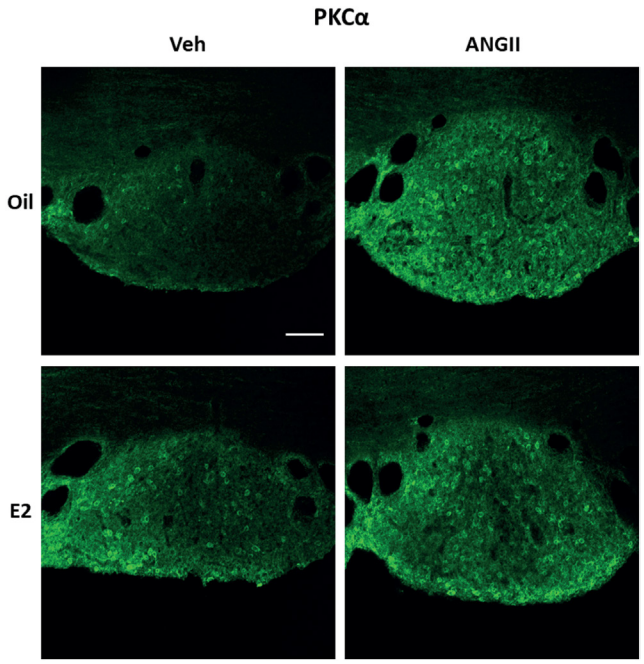

Figure 11
Role of E2 on PKCα expression in the SFO in response to ANGII. Representative immunofluorescence photomicrographs of coronal sections showing the PKCα protein staining in the subfornical organ in response to ANGII stimulation oil- and E2-treated OVX rats. Objective is ×20. The scale bar represents 100 µm. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0095.

on ANGII-induced OT and AVP release. In regarding to indirect effect of E2, Ciriello and Roder (2013) showed that E2 treatment in OVX animals via ERα decreases the spontaneous discharge rate of SFO neurons that projected to SON and are responsive to ANGII, as well as inhibits the response of these neurons to ANGII. These findings are in agreement with our results which showed that E2 prevents MAPKs phosphorylation in response to ANGII within the SFO, although induces PKCα activation. As discussed previously, E2-mediated PKCα activation within the SFO could be reasonably assumed to induce AT1 desensitization, contributing, at least in part, to its inhibitory effect on the ANGII-induced OT and AVP release. Because E2 requires PKC signaling to inhibit AVP release induced by ANGII, it is suggested that E2 can activate some phosphatase PKC-dependent within the SFO. However, more studies are needed to test these hypotheses. On the other hand, as only E2, not ANGII, was able to induce MKP-1 expression specifically in the PVN and the SON, it is suggested that there also is an important direct mechanism of E2 in the PVN and the SON. In fact, E2 has also been shown to modulate OT and AVP release directly via its ERβ or mER (mainly AVP release) in magnocellular neurons in the PVN and SON (Swenson & Sladek 1997, Somponpun & Sladek 2002, Somponpun *et al*. 2004).

Interestingly, we observed that p38 MAPK is involved in both ANGII-induced OT and AVP release, since p38 MAPK inhibition decreased neurohypophysial hormone secretion. This hypothesis was also confirmed by the presence of AVP/phospho-p38 and OT/phospho-p38 co-expression in the magnocellular neurons of the SON. Taken together, these results provide new insights that ANGII-induced OT release requires the PKC/p38 MAPK signaling pathway and ANGII-induced AVP release requires ERK1/2 and p38 MAPK signaling. Because PKC is not involved in AVP release induced by ANGII, it is reasonable to suggest that the activation of p38 MAPK can be PKC independent. Indeed, recent studies have reported PKC-independent p38 MAPK activation in some tissues (Lemonnier *et al*. 2004, Samuvel *et al*. 2005, Slone *et al*. 2016). Moreover, we observed that E2 prevented the ANGII-induced p38 MAPK phosphorylation in the PVN and the SON, which can explain the inhibitory effect of E2 on ANGII-induced OT and AVP secretion. Therefore, this result suggests that the inhibitory effect

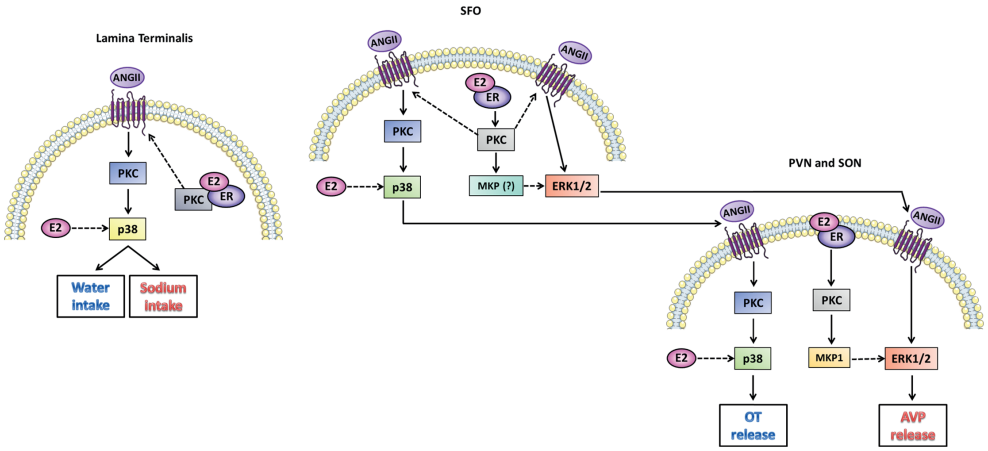

Figure 12
E2 and ANGII interaction in body fluid control. Schematic summary of the proposed interaction between E2 and ANGII in water and sodium intake (left): E2 modulates fluid intake through attenuation of p38 MAPK phosphorylation and PKC mediated by E2 can be involved in AT1 desensitization in the lamina terminalis. In the middle and right, the proposed interaction between E2 and ANGII in oxytocin (OT) and vasopressin (AVP) release: middle, effect of E2 on ANGII-induced OT release by preventing p38 MAPK phosphorylation within the SFO that represents the major site of action of circulating ANGII on the neurohypophysial hormone release through its angiotensinergic connections to PVN and SON; PKC mediated by E2 could be involved in some phosphatase activation which is responsible by dephosphorylation of ERK1/2 in the SFO; right, direct effect of E2 on ANGII-induced OT release by preventing p38 MAPK phosphorylation in the PVN and SON; E2 requires PKC signaling to modulate AVP release induced by ANGII in the PVN and SON. Legend: continuous arrow indicates stimulation and dashed arrow indicates inhibition/desensitization. A full colour version of this figure is available at https://doi.org/10.1530/JOE-18-0095.

of E2 on neurohypophysial secretion involves the dephosphorylation of MAPK family members (p38 and ERK1/2 (Almeida-Pereira *et al*. 2016)) in the PVN and the SON in response to ANGII.

A significant contribution of this work is the identification of some steps of ANGII signaling modulated by E2, which can explain its regulation on the central ANGII effects. In addition, the present report confirms previous findings that PKC is involved in the water intake induced by ANGII and notably expands upon these data with the demonstration that PKC signaling is also involved in sodium intake in female rats, showing the role of p38 MAPK in ANGII-induced fluid intake. The present observations also support the novel hypothesis that different intracellular signaling from ANGII can elicit distinct neurohypophysial hormone release.

In conclusion, this work contributes to the further understanding of E2 and ANGII interaction in the control of body fluid homeostasis and reveals potential pharmacological targets to prevent cardiovascular diseases, such as hypertension, during female reproductive senescence. The present results are the first to demonstrate that PKC/p38 MAPK signaling is involved in ANGII-induced fluid intake and OT secretion in OVX rats and suggest that E2 modulates the ANGII effects through the attenuation of MAPKs phosphorylation induced by ANGII in the brain (Fig. 12).

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
G A P and J A R designed the research; G A P, T V F, R C, S Q C and H V P S performed the experiments; G A P, L L K E and J A R analyzed the data; G A P wrote the manuscript; L L K E and J A R contributed to the preparation of the manuscript.

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