Effect of the interaction between food state and the action of estrogen on oxytocinergic system activity

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Abstract

Increased plasma osmolality by food intake evokes augmentation of plasma oxytocin (OT). Ovarian steroids may also influence the balance of body fluids by acting on OT neurones. Our aim was to determine if estrogen influences the activity of OT neurones in paraventricular nucleus (PVN) and supraoptic nucleus (SON) under different osmotic situations. Ovariectomized rats (OVX) were treated with either estradiol (E₂) or vehicle and were divided into three groups: group I was fed *ad libitum*, group II underwent 48 h of fasting, and group III was refed after 48 h of fasting. On the day of the experiment, blood samples were collected to determine the plasma osmolality and OT. The animals were subsequently perfused, and OT/FOS immunofluorescence analysis was conducted on neurones in the PVN and the

SON. When compared to animals which were fasted or fed *ad libitum*, the plasma osmolality of refed animals was higher, regardless of whether they were treated with vehicle or E₂. We observed neural activation of OT cells in vehicle- or E₂-treated OVX rats refed after 48 h of fasting, but not in animals fed *ad libitum* or in animals that only underwent 48 h of fasting. Finally, the percentage of neurones that co-expressed OT and FOS was lower in both the PVN and the SON of animals treated with E₂ and refed, when compared to vehicle-treated animals. These results suggest that E₂ may have an inhibitory effect on OT neurones and may modulate the secretion of OT in response to the increase of osmolality induced by refeeding.

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Introduction

Hydroelectrolytic homeostasis is maintained by several endocrine and behavioral responses that control sodium appetite, water ingestion, and the secretion of oxytocin (OT) and vasopressin (AVP) from the neurohypophysis (Fitzsimons 1998, McKinley & Johnson 2004). An increase in plasma osmolality causes a rise in plasma OT levels that, in turn, induces the excretion of sodium and chloride (Balment *et al.* 1980, Caligioni & Franci 2002).

OT is well known for its role in female reproduction where it stimulates uterine contractions and initiates milk ejection during lactation (Van Tol *et al.* 1988, Crowley *et al.* 1993). It also appears to participate in some functions that are essential for the success of reproduction, such as penile erection (Argiolas 1992), female sexual behavior (Caldwell 1992), maternal behavior (Kendrick *et al.* 1997) and stimulation of GnRH preovulatory secretion (Selvage & Johnston 2001). This hormone is synthesized by magnocellular neurosecretory cells in the supraoptic nucleus (SON) and paraventricular (PVN) nucleus of the hypothalamus. These regions are intrinsically sensitive to changes in osmolality and are activated by hypertonicity (Bourque 1989, McKinley *et al.* 1994, Oldfield *et al.* 1994). In addition to the direct or

indirect natriuretic action of OT, there is evidence that central OT also acts to modulate sodium appetite (Stricker & Verbalis 1987). The central administration of OT induces the expression of the FOS protein in regions involved in the control of food intake (Olson *et al.* 1993). Plasma osmolality also increases in response to food intake and the ingestion of sodium (Bloom *et al.* 1975, Houpt *et al.* 1983, Burlet *et al.* 1992); this increase involves both inhibitory and excitatory control mechanisms (Cunningham & Sawchenko 1991).

Although the initial focus of OT research was on its role in reproduction, its role in hydroelectrolytic control is also under investigation now (Jones & Pickering 1969, Balment *et al.* 1980, Caligioni & Franci 2002). There are several pieces of evidence indicating that gonadal steroids regulate the function of OT neurones. Some studies have shown a variation both in the levels of OT in the PVN, the SON and the neurohypophysis and in the plasma concentration of OT during the estrous cycle, with higher oxytocinergic activity during pro-estrous (Crowley *et al.* 1978, Sarkar & Gibbs 1984, Greer *et al.* 1986, Windle & Forsling 1993). The expression of OT mRNA in the magnocellular neurones of the PVN (Van Tol *et al.* 1988) increases at the onset of puberty and decreases after castration (Miller *et al.* 1989), suggesting a possible influence of estrogen on OT expression levels.

The administration of estrogen stimulates the peripheral release of OT, increases the electrical activity in OT neurones and alters the structural organization of both the cell bodies and the dendrites of oxytocinergic neurones (Negoro *et al.* 1973, Amico *et al.* 1981, Caldwell *et al.* 1988). Taken together, these studies indicate that the action of estrogen on magnocellular neurones contributes to the regulation of OT release (Jirikowski *et al.* 1988, Van Tol *et al.* 1988, Miller *et al.* 1989, Amico *et al.* 1995, Salonia *et al.* 2005, Somponpun 2007). In contrast, some studies have found that estrogen has no significant effect on OT expression in the PVN (Van Tol *et al.* 1988, Crowley *et al.* 1993), while yet another group has shown that treatment with estrogen decreases the expression of OT mRNA in the PVN of ovariectomized rats (OVX; Shughrue *et al.* 2002). Thus, the exact relationship between estrogen and the oxytocinergic system remains under debate and the reason for these discrepancies is unclear. One possibility is that the oxytocinergic system responds differently depending on the concentration of estrogen present.

Despite mounting evidence that estrogen acts on OT neurones, it is still unclear exactly how this regulation occurs, although it may involve both direct and indirect effects. Several studies have shown that ER- β and its mRNA are both present in OT and AVP neurones. However, ER- β expression is higher in AVP neurones (Shughrue *et al.* 1996, Simonian & Herbison 1997, Hrabovszky *et al.* 1998, 2004, Somponpun & Sladek 2003, Suzuki & Handa 2005, Somponpun 2007). There is strong expression of ER- β in areas that lack or have low levels of ER- α , including both parvocellular and magnocellular neurones (Shughrue *et al.* 2002) in the SON and the PVN (Shughrue *et al.* 1996, Osterlund *et al.* 2000, Shughrue & Merchenthaler 2001, Sladek & Somponpun 2007). Some researchers have shown that AVP neurones, but not OT neurones, respond to refeeding (Timofeeva *et al.* 2005), but they used males. Therefore, they did not analyze the correlation of estrogen with oxytocinergic system in response to the increase of osmolality induced by refeeding.

In this study, we aimed to determine if the food state associated with estrogen activity alters the activity of the oxytocinergic system in the PVN and the SON.

Materials and Methods

Animals

Adult female Wistar rats weighing 160–180 g were housed in individual cages under controlled conditions of temperature ($22 \pm 1^\circ\text{C}$) and light (12 h light:12 h darkness cycle) and were allowed free access to food and water. Bilateral OVX was performed under 2.5% tribromoethanol (1 ml/100 g of body weight, i.p.). All animal experimental protocols and manipulations were approved by the ethics committee of the Medical School of Ribeirão Preto, University of São Paulo.

Experimental protocol

Fourteen days after surgery (OVX), the animals weighing 250–280 g were divided into three groups. Animals in group I were fed standard food *ad libitum*, those in group II were fasted for 48 h with access to water, and the rats in group III were refed during 2 h with standard food after 48 h of fasting. During the 3 days before the experiment began, the animals were treated with estradiol (E_2) cypionate (10 $\mu\text{g}/0.1$ ml per animal) or vehicle (oil). On the 4th day (at 1500 h), after an *ad libitum* meal (group I), 48 h of fasting (group II) or refeeding (group III), the animals were anesthetized with 2.5% tribromoethanol (1 ml/100 g of body weight, i.p.) so that blood could be withdrawn from the heart ventricle. Then, the rats were transcardially perfused through the ascending aorta with ~ 80 ml of 0.01 M PBS (pH 7.4) followed by 320 ml cold 4% paraformaldehyde (PFA).

Osmolality

Plasma osmolality was measured by a Fiske Mark osmometer (Fiske Associates, Norwood, MA, USA) using freezing point depression.

Hormone measurement

Plasma OT levels were measured by RIA. All samples from the same experiment were measured in the same assay to avoid interassay variation. The minimum detectable concentration was 0.8 pg/ml. The RIA was performed, as previously described (Morris & Alexander 1989), using a specific antiserum, which was a generous gift from Dr Mariana Morris from Wright State University (OH, USA), a standard from Bachem-Peninsula Labs (San Carlos, CA, USA) and a secondary antibody produced by Dr Franci's Laboratory (School of Medicine of Ribeirão Preto, University of São Paulo, Brazil). The results were reported as means $\pm$ S.E.M.

Immunofluorescence

Tissue preparation Rat brains were removed immediately after perfusion and post-fixed in 4% PFA for 2 h. Later, the brains were immersed in 30% sucrose in PBS until the tissue was saturated, frozen in cooled isopentane and stored at -70°C until sectioning. Serial coronal sections (14 μm) throughout the PVN and the SON were obtained using a cryostat and mounted onto slides.

Double-label immunofluorescence Sections of the PVN and the SON were rinsed in PBS multiple times, followed by a rinse in 0.1 M glycine in PBS to remove any excess aldehydes. After the washes in PBS, the sections were blocked in PBS containing 0.1% Triton X-100 and 1% BSA and were incubated with primary antibodies. Sections were stained for OT/FOS with an overnight incubation at room temperature with mouse monoclonal anti-OT antiserum

diluted 1:2000 (a kind gift from Dr Harold Gainer, National Institute of Health, Bethesda, MD, USA) and rabbit polyclonal anti-FOS (SC-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500 in PBS containing 0.1% Triton X-100 and 1% BSA. After the overnight incubation, the sections were rinsed and incubated for 1 h in the appropriate secondary antibodies. To visualize OT, we used a goat anti-mouse IgG labeled with Alexa Fluor 488; and to visualize FOS, we used a goat anti-rabbit IgG labeled with Alexa Fluor 594 (Molecular Probes, Grand Islands, NY, USA). Next, the slides were rinsed in PBS and coverslipped with Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA, USA). Tissue sections with immunoreactive neurones expressing OT and/or FOS were examined using an Axioskope 2 Plus microscope, and the images were captured with a Zeiss AxioCam digital camera (Carl Zeiss, Hallbergmoos, Germany). Subdivisions of the PVN and the SON were identified according to the Swanson brain atlas (Swanson 1992). Anterior (PaMA) and medial (PaMM) magnocellular subdivisions of the PVN were defined as -0.83 and -1.53 mm, respectively, from bregma and the SON was located at -0.83 mm from bregma. The immunoreactive neurones were quantified with the aid of Image J software (National Institutes of Health). In each photomicrograph, the number of neurones that expressed FOS, OT and OT/FOS in the PVN and the SON was counted. Next, we calculated the mean number of labeled neurones in two to three brain sections from similar anatomic levels of PVN and SON for each animal.

Immunocytochemical controls The omission of either the primary or the secondary antibody in single-label experiments resulted in no labeling (data not shown). For each double-label combination, primary antibodies were titrated to establish the maximum dilutions that provided robust signals with minimum nonspecific staining.

Statistical analysis

Data were analyzed by two-way ANOVA followed by a Bonferroni post-hoc test. The data were presented as means $\pm$ S.E.M. Differences were considered significant when $P < 0.05$.

Results

Osmolality

Figure 1A shows that the plasma osmolality (mOsm/kg H₂O) of OVX animals treated with the vehicle was significantly higher in the group that was refed with standard food after 48 h of fasting (318.1 ± 1.4) than in rats fed *ad libitum* (305.4 ± 1.6 , $P < 0.001$) or in rats that were fasted for 48 h (301.6 ± 1.4 , $P < 0.001$). Similarly, the plasma osmolality of OVX animals treated with estrogen was significantly higher

Figure 1 (A) Plasma osmolality, expressed in mOsm/kg H₂O, in each experimental group. (B) Plasma OT concentration of ovariectomized (OVX) rats treated with vehicle or estrogen. Differences: * $P < 0.05$ compared to groups fed *ad libitum* and those that were fasted for 48 h when given the same treatment and # $P < 0.05$ compared to animals refed with standard food and treated with estrogen as opposed to vehicle. Results are expressed as the mean $\pm$ S.E.M. Number between parentheses indicates the group size.

in the group refed with standard food after 48 h of fasting (320 ± 3.8) compared to the rats that were fed *ad libitum* (309.7 ± 1.9 , $P < 0.001$) and animals that were fasted for 48 h (302 ± 3.2 , $P < 0.05$). However, there was no significant difference between the treatments (vehicle or estrogen) in any of the three groups.

Hormone measurement

Figure 1B shows plasma OT (pg/ml) levels in OVX animals treated with either vehicle or estrogen. The plasma OT levels of the group that was fasted for 48 h (16.5 ± 5) were similar to the levels of the group fed *ad libitum* (22.7 ± 3), when both were treated with the vehicle. However, these levels increased in the group that was refed with standard food after 48 h of fasting (50.7 ± 9) when compared either to the group that was fasted for 48 h (16.5 ± 5 , $P < 0.001$) or to the group that was fed *ad libitum* (22.7 ± 3 , $P < 0.002$). In OVX animals treated with estrogen, no significant differences were observed

among any of the groups. Therefore, estrogen treatment seems to have hindered the increase in plasma OT levels that was induced by the osmotic stimulus caused by refeeding after 48 h of fasting. This finding was confirmed by the observation that plasma OT levels were significantly higher in the vehicle-treated group (50.7 ± 9) than in the estrogen-treated group (21.6 ± 2 , $P < 0.001$).

Immunofluorescence

The data show (Fig. 2A) that there was no significant difference in the number of OT neurons in the PaMA between vehicle- or estrogen-treated OVX animals among any of the three food states (fed *ad libitum*, 48 h of fasting, or refed with standard food after 48 h of fasting). The same result was found for OT neurons in the PaMM (Fig. 2B) with the exception of animals that underwent 48 h of fasting. In this study, the number of OT neurons was lower in animals treated with estrogen (60 ± 6.6 , $P < 0.03$), as opposed to vehicle (80 ± 9.1). In the SON (Fig. 2C), there were no differences among any of the groups in the number of OT neurons between vehicle- or estrogen-treated animals. However, for the three groups of vehicle-treated animals, the number of OT neurons was significantly lower in those fed *ad libitum* (43 ± 1.7 , $P < 0.05$) compared to those that were refed after 48 h of fasting (70 ± 5.4).

As shown in Fig. 3A, in OVX rats treated with vehicle, the number of FOS-immunoreactivity (-ir) neurons in the PaMA was significantly higher in animals refed after fasting (28.8 ± 2.1 , $P < 0.001$) than in those fed either *ad libitum* (0.3 ± 0.2) or after 48 h of fasting (0.4 ± 0.4). The number of FOS-ir neurons in the PaMA was also significantly higher in refed vehicle-treated animals (28.8 ± 2.1 , $P < 0.001$) than in refed animals treated with estrogen (10.6 ± 3.7). In the animals treated with estrogen, the number of FOS-ir neurons in the PaMA was significantly higher in the group refed after fasting (10.6 ± 3.7 , $P < 0.01$) than in the group fed *ad libitum* (0 ± 0.3).

The number of FOS-ir neurons in the PaMM (Fig. 3B) was not significantly different among any of the animals treated with estrogen and subjected to the three different food states. However, in vehicle-treated animals, the number was significantly higher in the group refed after fasting (53.6 ± 1.1 , $P < 0.001$) when compared to the group fed *ad libitum* (0.5 ± 0.5) and that underwent a 48-h fasting period (0.8 ± 0.5). The number of FOS-ir neurons in the PaMM from the vehicle-treated group that was refed after fasting was also higher (53.6 ± 1.1 , $P < 0.001$) than the animals treated with estrogen and refed after fasting (12 ± 6.3).

In the SON of animals refed after fasting for 48 h (Fig. 3C), the number of FOS-ir neurons was significantly higher in vehicle-treated animals (80.8 ± 8.6 , $P < 0.001$) than in animals treated with estrogen (47.3 ± 6.6). However, both groups showed a higher number of FOS-ir neurons when compared to the animals fed *ad libitum* (0.1 ± 0.1 ; 0 ± 0) and animals that underwent 48 h of fasting (0 ± 0 ; 4.4 ± 1.2) for both treatments.

We also looked for FOS-ir co-expression in OT neurons from the PVN and the SON (Figs 4 and 5). Little or no co-expression was observed in the PVN (both PaMA and PaMM) and the SON from animals that were fed *ad libitum* or fasted for 48 h and were treated with either estrogen or vehicle. However, co-expression was found in these areas in

Figure 2 The number of OT-ir neurons in (A) the anterior magnocellular part (PaMA), (B) the medial magnocellular part (PaMM) of paraventricular nucleus and (C) the supraoptic nucleus (SON) of vehicle- or estrogen-treated ovariectomized (OVX) rats. Differences: # $P < 0.05$ compared to estrogen-treated animals that underwent 48 h of fasting and * $P < 0.05$ compared to animals fed *ad libitum* and given the same treatment. Results are expressed as the mean $\pm$ S.E.M. Number between parentheses indicates the group size.

Figure 3 The number of FOS-ir neurones in (A) the anterior magnocellular part (PaMA), (B) the medial magnocellular part (PaMM) of the paraventricular nucleus and (C) the supraoptic nucleus (SON) of vehicle- or estrogen-treated OVX rats. Differences: * $P < 0.05$ compared to animals fed *ad libitum* and those that were fasted for 48 h when given the same treatment; # $P < 0.05$ compared to animals refed with standard food and treated with estrogen; α represents statistical significance compared to animals fed *ad libitum* and treated with estrogen. Results are expressed as the mean $\pm$ S.E.M. Number between parentheses indicates the group size.

animals refed after fasting and treated with vehicle (PaMA = 42.9 ± 6.1 ; PaMM = 48 ± 5.2 ; SON = 52.5 ± 7.8 FOS-OT neurones/section) or with estrogen (PaMA = 11.9 ± 5.3 ; PaMM = 2.9 ± 1.8 ; SON = 24.5 ± 6.2 FOS-OT neurones/section). Statistically significant differences were demonstrated when these values were compared to the groups fed *ad libitum* ($P < 0.001$) and those that were fasted for 48 h ($P < 0.01$). Moreover, the FOS/OT co-expression in the PaMA, the PaMM and the SON from animals refed after fasting was significantly higher in animals treated with vehicle than in animals treated with estrogen.

Discussion

It is well established in the literature that OT magnocellular neurones from the PVN and the SON project to the neurohypophysis. From there, the hormone OT is released to affect reproductive function (Russell & Leng 1998, Fisher *et al.* 2006) and maintain hydroelectrolytic homeostasis, which is regulated by changes in plasma osmolality (Balment *et al.* 1980, McKinley & Johnson 2004). We found a significant increase in plasma osmolality in E_2 -primed and unprimed OVX animals that were refed with standard food after fasting when compared to animals fed *ad libitum* or animals that were fasted for 48 h. Our results are in keeping with the results of a previous study which showed that refeeding may increase plasma osmolality (Gill *et al.* 1985, Burlet *et al.* 1992). The estrogen treatment did not change this response. Previous reports had also shown a similar response of estrogen (Caligioni & Franci 2002, Hartley *et al.* 2004).

Plasma OT levels in all groups were higher than previously reported values because we did collect the blood under anesthesia. However, we did not observe a significant difference in plasma OT levels in E_2 -primed and unprimed OVX animals after 48 h of fasting when compared to animals fed *ad libitum*, regardless of estrogen treatment. In this study, we also analyzed photomicrographs from portions of the anterior and medial magnocellular PVN and the SON of OVX animals that were fasted for 48 h. There was no expression of FOS in magnocellular OT neurones of these nuclei in vehicle- or estrogen-treated animals. Previous studies showed no change in OT levels in the PVN and the SON from rats that were fasted for 48 h (Burlet *et al.* 1992). Thus, it appears that the OT magnocellular neurones in these nuclei do not participate in the responses to 48 h of fasting.

In contrast, our results have shown that refeeding after 48 h of fasting induced OT secretion and FOS expression in the PVN (anterior and medial magnocellular portion) and the SON of unprimed OVX animals to a greater extent than in E_2 -primed OVX animals. Moreover, approximately half of the neurones that expressed FOS protein in the PVN and the SON were OT-ir. FOS expression has been reliably used to assess brain activity in response to food intake (Naimi *et al.* 1997, Timofeeva *et al.* 2002). It is also known that OT and AVP neurones in the PVN and the SON express FOS in

response to an increase in osmolality (Somponpun & Sladek 2003, Ruginsk *et al.* 2007, Vilhena-Franco *et al.* 2011).

Timofeeva *et al.* (2005) have shown that AVP neurones, but not OT neurones, respond to refeeding. The difference

Figure 4 The percentage of OT-ir neurones that co-expressed FOS-ir in the (A) anterior magnocellular part (PaMA), (B) medial magnocellular part (PaMM) of the paraventricular nucleus and (C) supraoptic nucleus (SON) of vehicle- or estrogen-treated OVX rats. Differences: * $P < 0.05$ compared to animals fed *ad libitum* and those that were fasted for 48 h when given the same treatment and # $P < 0.05$ compared to animals refed with standard food and treated with estrogen. Results are expressed as the mean $\pm$ S.E.M. Number between parentheses indicates the group size.

between this report and our results may be due to the sexual difference, as they used males. Björkstrand & Uvnäs-Moberg (1996) studied the effect of OT injection on food intake and daily weight gain in males and females from two substrains of Sprague–Dawley rats. They demonstrated that central, but not peripheral OT injection, caused lower food intake and daily weight gain in females than in males.

The potential involvement of circumventricular organs, such as the subfornical organ (SFO), in monitoring plasma osmolality after hyperosmotic challenge or after feeding has been suggested previously (Starbuck & Fitts 2002, Hiyama *et al.* 2004). The hypertonicity of the extracellular fluid activates the SFO and the AV3V regions, which in turn stimulate magnocellular neurones in the PVN and the SON to release OT from the neurohypophysis (Cunningham & Sawchenko 1991, Oliet & Bourque 1993, Somponpun *et al.* 2004). Therefore, our results from this study indicate that refeeding after fasting increases plasma osmolality, which in turn activates OT neurones in the PVN and the SON to release OT to the plasma.

E₂-primed OVX animals that were refed after 48 h of fasting had a lower co-expression of FOS protein in OT neurones of the PVN and the SON when compared to unprimed OVX animals. Thus, in our experimental model, estrogen inhibited the activation of oxytocinergic neurones induced by refeeding.

The influence of estrogen on the balance of body fluids has been frequently investigated, although its effects are not fully understood (Curtis 2009). Therefore, the mechanism used by estrogen to control the secretion of OT is complex and poorly understood. The variation in food and water intake has been demonstrated during the estrous cycle, due to increased estrogen secretion. Food and water intake decrease during pro-estrous and estrous. Also, urinary output and urinary sodium excretion are decreased in estrous compared to diestrus (Forsling & Peysner 1988). It is known that physiological concentrations of estrogen stimulate the release of OT by the dendrites of magnocellular neurones in the SON (Wang *et al.* 1995), and studies in our laboratory have shown that the secretion of OT varies during the estrous cycle, increasing during pro-estrous and the morning of estrous (Caligioni & Franci 2002). In addition, several studies have shown that estrogen may regulate many aspects of the function of OT neurones, including the expression of OT mRNA (Shughrue *et al.* 2002), peptide synthesis (Levin & Sawchenko 1993), electrical activity (Negoro *et al.* 1973) and the release of OT (Skowsky *et al.* 1979). In turn, OT inhibits salt appetite (Stricker & Verbalis 1987), reduces food consumption (Arletti *et al.* 1989), induces the expression of the FOS protein in regions involved in the control of food intake (Olson *et al.* 1993) and causes natriuresis directly mediated by kidney OT receptors (Verbalis *et al.* 1991) or indirectly through stimulation of the atrial natriuretic peptide release from heart (McCann *et al.* 1994, Gutkowska *et al.* 1997).

Figure 5 Photomicrographs (20 $\times$) showing colocalization of OT-ir (green) and FOS-ir (red) in coronal sections (14 μ m) from the PaMM and PaML portions of the PVN and the supraoptic nucleus (SON) of ovariectomized (OVX) rats treated with vehicle (A–C) or estrogen (D–F) and refed with standard food after fasting for 48 h. In each panel (A–F), the magnification of a neurone OT-FOS-ir is shown in square on the left bottom corn. Scale bar: 100 μ m.

Our study was the first to analyze the interaction of the effect of osmotic stimulus induced by refeeding and estrogen influence on OT system activity. We have shown that estrogen hindered or reduced the activation of OT system normally induced by the increase of osmolality, in the refeeding condition. Taken together, our results and literature reports indicate that estrogen and OT could induce similar responses in some situations or some estrogen actions could be mediated by OT. How does estrogen modify the response of the OT system to the hyperosmolality induced by refeeding after fasting? Is it a direct action on OT neurones or an indirect action through circumventricular organs or both?

The idea that estrogen may directly regulate OT neurones is highly controversial. Previous studies suggest that estrogen directly regulates the gene expression of OT in the PVN and the SON through ER- β (Shughrue *et al.* 1996, Laflamme *et al.* 1998, Somponpun & Sladek 2003, Hrabovszky *et al.* 2004). Thus, estrogen increases the level of OT mRNA in neurones of the PVN and SON (Chung *et al.* 1991, Amico *et al.* 1995) and increases plasma OT levels (Amico *et al.* 1981, Wang *et al.* 1995, Caligioni & Franci 2002). The expression of ER- β in OT neurones is well established in the literature for the PVN, but has not been observed in the SON (Shughrue *et al.* 1996, Simonian & Herbison 1997, Alves *et al.* 1998, Hrabovszky *et al.* 1998, Laflamme *et al.* 1998, Somponpun & Sladek 2003, Suzuki & Handa 2005). There are conflicting data regarding the SON because some researchers have demonstrated the expression of ER- β mRNA and protein in the SON (Laflamme *et al.* 1998, Hrabovszky *et al.* 2004).

It has been reported that treatment with estrogen in OVX rats promotes a decrease in ER- β expression in the PVN (Suzuki & Handa 2004). Moreover, variation in the expression of ER- β was not found to be dependent on the concentration of a circulating gonadal steroid (Greco *et al.* 2001). Somponpun & Sladek (2003) found that the

expression of ER- β is negatively correlated with changes in plasma osmolality in males. This regulation only occurred in magnocellular neurones of the PVN and the SON that were activated in response to an osmotic stimulus. ER- β protein is located in the nucleus of magnocellular and parvocellular neurones and the attenuation of ER- β expression occurs in both AVP and OT neurones hyperosmotically stimulated. This alteration of ER- β expression could suppress the inhibitory effect to facilitate a greater increase in the secretion of AVP and OT.

Adult rats release AVP and OT in response to osmotic stimuli even in the presence of circulating estrogen (Caligioni & Franci 2002, Vilhena-Franco *et al.* 2011). The down-regulation of ER- β during hyperosmolality may remove this inhibitory influence, allowing the release of AVP and OT in situations that require maximum secretion. Changes in receptor expression may alter the responsiveness of target cells and determine the physiological conditions in which gonadal steroids act to regulate cellular function (Somponpun & Sladek 2003). On the other hand, our data show that estrogen hindered or reduced the activation of OT system normally induced by the increase of osmolality in the refed condition after fasting. This condition could characterize another mechanism that allows the action of estrogen to take advantage of the osmotic stimulation. Maybe estrogen could be changing the sensitivity of the osmoreceptor mechanisms related to the control of activity of OT neurones.

In our study, approximately half of the neurones that expressed FOS protein in the PVN and the SON were OT-ir. Thus, other non-oxytocinergic neurones in the PVN and the SON are also activated by refeeding. These neurones may, at least in part, be AVP neurones that also participate in the control of hydroelectrolytic balance (Skott 2004, Timofeeva *et al.* 2005, Curtis 2009). However, as the expression of FOS was lower in OVX estrogen-treated animals that were refed

after fasting, and some of the vasopressinergic neurones may be involved, we cannot rule out a role for estrogen in inhibiting the release of AVP through ER- β (Skowsky *et al.* 1979, Skott 2004). The presence of gonadal steroids certainly does not eliminate the control of AVP release, but it could modulate the response.

Additionally, we cannot rule out a possible indirect regulation of estrogen on OT and AVP neurones via ER- α , which is expressed in neurones located in the organum vasculosum of lamina terminalis and the medial preoptic area, where projections to the SON originate (Voisin *et al.* 1997, Sladek & Somponpun 2007, Grassi *et al.* 2010). However, these brain areas were not a focus of this work.

Other point to consider should be a possible activation of oxytocinergic neurons by gastric distension induced by refeeding. Gastric distension may stimulate satiety mechanisms and cause a slight increase in OT secretion in rats (McCann *et al.* 1988). This distension may activate the area postrema (AP) and the nucleus of the solitary tract (NTS) in rats through vagal excitatory signals. These areas send projections to specific structures of the brain involved in control of eating behavior, such as the dorsomedial hypothalamus, PVN and central amygdala (Ricardo & Koh 1978, Rinaman *et al.* 1998). It has been shown that refeeding increases the expression of FOS in the PVN, SON, NTS and AP (Timofeeva *et al.* 2002). NTS neurons activated by gastric distension project primarily to the parvocellular portion in the PVN, while the magnocellular division is activated by stimulation from oropharyngeal region, osmoreceptors or circumventricular organs such as the SFO (Starbuck & Fitts 2002, Timofeeva *et al.* 2005). Our work has analyzed the magnocellular portion in the PVN, so our results are probably not due to gastric distension.

When the previously cited literature and the data from this study are taken together, we can make the following conclusions: first, in OVX animals, treatment with estrogen reduced the activity of OT neurones due to the increase in osmolality induced by refeeding after fasting. This reduced activity occurred despite a probable downregulation of ER- β caused by the osmotic stimulus induced by refeeding after fasting. Also, in unprimed OVX animals, the absence of estrogen allowed for the highest activity of OT neurones due to the increase in osmolality induced by refeeding after fasting.

Conclusion

The present study aimed to investigate the effect of estrogen on OT in response to increased osmolality, due to refeeding. We found that OVX rats treated with estrogen had a lower activation of OT neurones induced by refeeding when compared to OVX rats treated with a vehicle control. Thus, our data show that estrogen exerts an inhibitory effect on the activity of oxytocinergic neurones, which is increased by refeeding-induced osmolality after fasting.

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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