# Lack of estradiol modulation of sleep deprivation-induced c-Fos in the rat brain

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## ABSTRACT

Women recover from sleep deprivation more efficiently than men, but the mechanism for this difference is unknown. Effects of estrogen on sleep suggest that it could play a role, but the brain targets on which estrogen may act to have this effect have not been identified. Sleep deprivation increases levels of the immediate-early gene protein c-Fos in selected brain regions, but it is unknown whether estrogen modulates this response. We investigated the influence of different levels of exogenous estradiol on the c-Fos response to sleep deprivation in ovariectomized female rats. Female rats were treated with low or high levels of estradiol (mimicking diestrous and proestrous levels, respectively) delivered via subcutaneous silastic tubes. Control ovariectomized females and sham-operated males were implanted with tubes filled with cholesterol. One week after surgery, half of the rats underwent a 3 h period of sleep deprivation during the light phase in a motorized Wahmann activity wheel that rotated constantly at a slow speed, while half were confined to fixed wheels. Immediately after sleep deprivation, animals were killed and their brains processed to detect c-Fos using immunohistochemistry. Sleep deprivation increased the number of c-Fos positive cells in a number of brain areas, including the caudate putamen, medial preoptic area, perifornical hypothalamus, and anterior paraventricular thalamic nucleus. Other areas, including the suprachiasmatic nucleus, posterior paraventricular hypothalamic nucleus, posterior paraventricular thalamic nucleus, arcuate nucleus, and central amygdala, did not respond to 3 h sleep deprivation with a significant increase in c-Fos levels. Levels of c-Fos induced in the selected brain regions by sleep deprivation were not modulated by estrogen levels, nor by sex.

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## 1. Introduction

The biological importance of adequate sleep is evident from the diverse psychological and physiological impairments that result from sleep disruption [1,2]. Women are more likely than men to experience sleep disruptions and, as a consequence, suffer more from their detrimental effects [3,4]. The majority of these complaints occur in relation to the menstrual cycle [5,6]. Because women experience major hormonal fluctuations across the menstrual cycle, as well as during pregnancy and in menopause, it has been hypothesized that the mechanisms controlling normal sleep and sleep homeostasis in women may be influenced by circulating sex steroids.

Although the basic structure of sleep appears to show no significant differences between normal human males and females

[7], close inspection of EEG activity across the menstrual cycle shows that the mid-luteal phase (when progesterone and estrogen serum levels are high) is associated with enhanced power density [8], a higher peak frequency of EEG spindle waves [9], and a shorter latency to stage 3 slow-wave sleep (SWS) [10]. Rapid eye movement (REM) sleep also varies across the menstrual cycle, with a small decrease during the late luteal phase [8]. These findings suggest that levels of both estrogen and progesterone may play a role in regulating sleep.

The role of female sex steroids in sleep regulation is particularly evident in postmenopausal women, who have low levels of circulating estrogen [11], and commonly experience difficulty with insomnia [12]. Treatment with synthetic estrogen (hormone replacement therapy) increases REM sleep and improves subjective sleep quality in postmenopausal women [13,14]. Similar trends have been observed in naturally cycling women who showed decreased latency to REM sleep onset and increased total REM sleep time when taking oral contraceptives (progesterone and estrogen) [10,15]. Of particular interest, women spend more time in SWS (stages 3 and 4) than men, and show greater amplitude and power of EEG slow waves

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during recovery sleep following a 40 h period of sleep deprivation (SD) [16]. Since the amount of EEG slow waves is thought to reflect previous sleep need, these findings suggest that women recover from sleep perturbations more efficiently than men. It should be noted that most of the participants in this study were taking oral contraceptives, making it difficult to assess the role of specific gonadal steroids on recovery parameters following SD [16]. However, studies using human subjects have limitations, because the complexity of the patterns of circulating hormones at different menstrual cycle phases and the variable composition of oral contraceptives make it difficult to attribute changes in sleep to particular hormones.

Animal models using controlled levels of hormones have provided some insight into how these hormones influence sleep architecture. Ovariectomized rats show less REM sleep [17] and more SWS and total sleep than intact females [18]. When treated with synthetic estrogen and progesterone, however, they show decreases in both SWS and REM sleep [19]. Naturally cycling rats show marked reductions in SWS and REM sleep during proestrus relative to metestrus and diestrus [20,21], whereas cycling female mice show subtle differences in sleep patterns across the estrous cycle [22]. As in humans, rats show a progressive increase in SWS and REM sleep amounts during pregnancy [23]. These findings suggest that changes in hormone levels experienced by female rodents can have an immediate and direct impact on baseline sleep.

Sleep is also regulated homeostatically and loss of sleep is followed by compensatory increases in the amount of SWS and EEG slow waves. Rebound increases in sleep duration after SD have been well characterized in male rats, but this process has not been studied extensively in females. One study reported that 6 h SD in naturally cycling female rats increased the amount of SWS during recovery. There were no differences in recovery SWS between the proestrous and estrous stages of the cycle, although these rats had shown differences in baseline sleep patterns across the estrous cycle [24]. A recent study in female mice showed that, despite subtle differences in baseline sleep, the recovery sleep after 6 h SD was similar between the sexes [25]. The females in that study, however, were only tested during diestrus (when levels of estrogen and progesterone are low) and therefore the potential influence of female hormones or cycle changes were not investigated. In transgenic mice lacking the aromatase enzyme necessary for conversion of androgens to estrogen, however, the light–dark distribution of sleep differs from that of wild type mice in that the knockouts display more sleep during the dark phase. Despite this difference in baseline sleep distribution, there was no difference in the response to SD [26]. These findings indicate that female sex steroids may affect spontaneous and recovery sleep differently, but further investigation is required to clarify these differences.

Immediate-early genes (IEGs), such as c-fos and junB, conventionally used as markers of neuronal activation, provide a molecular correlate of normal sleep–wake patterns and responses to perturbations such as SD. In male rats, up-regulation of c-fos mRNA or protein immediately following SD has been observed in the cerebral cortex, caudate putamen, thalamic nuclei, medial and lateral preoptic areas, arcuate nucleus, anteroventral periventricular nucleus, hippocampus, amygdala, and various brainstem areas, while decreases have been observed in other areas, such as the dorsomedial suprachiasmatic nucleus (SCN) [27–31].

In female rats, the expression of c-fos was modulated by gonadal hormones, though there are discrepancies among published findings. For instance, repeated injections of estradiol benzoate (10 μg per day) in ovariectomized rats reduced basal (unstimulated) levels of c-Fos immunoreactivity (IR) in some brain regions, including several cortical areas, and various amygdaloid, thalamic, and hypothalamic nuclei [32]. A more recent report suggests that ovariectomy itself reduces baseline c-Fos IR in several hypothalamic regions, namely the SCN and arcuate nucleus (ARC), and that this reduction is normalized by a single injection of a high dose of estradiol [30]. Several methodological differences between the two studies make comparisons difficult,

including the use of an estradiol dose almost 30 times higher in one study, the use of single versus repeated injections, and assessment of different brain regions. Since these studies did not assess circulating levels of estradiol resulting from these treatments, it is not known whether they generated physiological levels characteristic of any stage of the estrous cycle. One study that examined only cortical regions found no variation in c-fos mRNA levels across the estrous cycle [33]. There are no published reports describing the effect of estradiol on c-fos expression after a period of SD.

We studied selected brain regions thought to be involved in regulation of sleep propensity and initiation to examine whether estradiol affects the response of c-fos to 3 h SD. Estradiol was administered in some rats via subcutaneous implants to achieve physiological levels characteristic of diestrous and proestrous stages of the estrous cycle [34,35]. We compared the levels of c-Fos IR induced by SD in OVX females given estradiol treatments to those in ovariectomized females and in intact males.

## 2. Methods

### 2.1. Animals

Eight male (250–300 g at the start of the experiment) and thirty-nine female (200–225 g) Sprague–Dawley rats (Charles River Canada, St. Constant, QC) were used. Rats were housed individually under a 12:12 light:dark cycle (lights on, 07:00) and given access to food and water *ad libitum*. All animals were handled in accordance with the guidelines of the Canadian Council on Animal Care and the experimental protocol was approved by the Dalhousie University Committee on Laboratory Animals.

Female rats were divided into three treatment groups: 1) ovariectomy (OVX); 2) OVX and replacement with a low dose of estradiol (OVX+LE); 3) OVX and replacement with a high dose of estradiol (OVX+HE). Male rats with sham surgery and vehicle (cholesterol) implants constituted a fourth group. Each of these groups was further divided into sleep deprived and non sleep deprived groups for a total of 8 groups.

### 2.2. Hormonal manipulation

Low or high plasma levels of estradiol were obtained in OVX rats by subcutaneous implantation of silastic tubing containing equal amounts of estradiol. Silastic tubing segments (length = 10 mm, internal diameter = 1.60 mm, external diameter = 3.18 mm) (Cole Parmer Instrument Co., Vernon Hills, IL) were filled with 100% estradiol to achieve proestrus-like ("high") estradiol levels. Diestrous-like ("low") estradiol levels were generated by filling 4 mm of the tube segment with 10% estradiol mixed with 90% cholesterol. Control tubes were filled with 100% cholesterol for their total length. They were filled by tapping the contents into the silastic tubing and sealed with silicone adhesive (Dow Corning; Midland, MI). They were washed before use with 100% ethanol to remove any excess drug from the surface.

Surgery for OVX and drug implantation was performed under deep anesthesia using 100% isoflurane (Aerrane Baxter, Toronto, ON). An incision was made on the midline of the abdomen, blood vessels on either side of the ovary were ligated with sutures, and the ovary was excised. Tubing segments containing cholesterol or estradiol were then implanted subcutaneously in the thoracic region. Following suturing of muscle layers and skin, rats were given buprenorphine (5 mg/kg, s.c.) as an analgesic. Animals were checked daily postoperatively for any anomalies, and re-sutured under anesthesia if necessary.

### 2.3. Sleep deprivation

Acclimatization of the rats to the SD apparatus began 2 days after surgery and one week prior to SD. Each rat was transferred to a
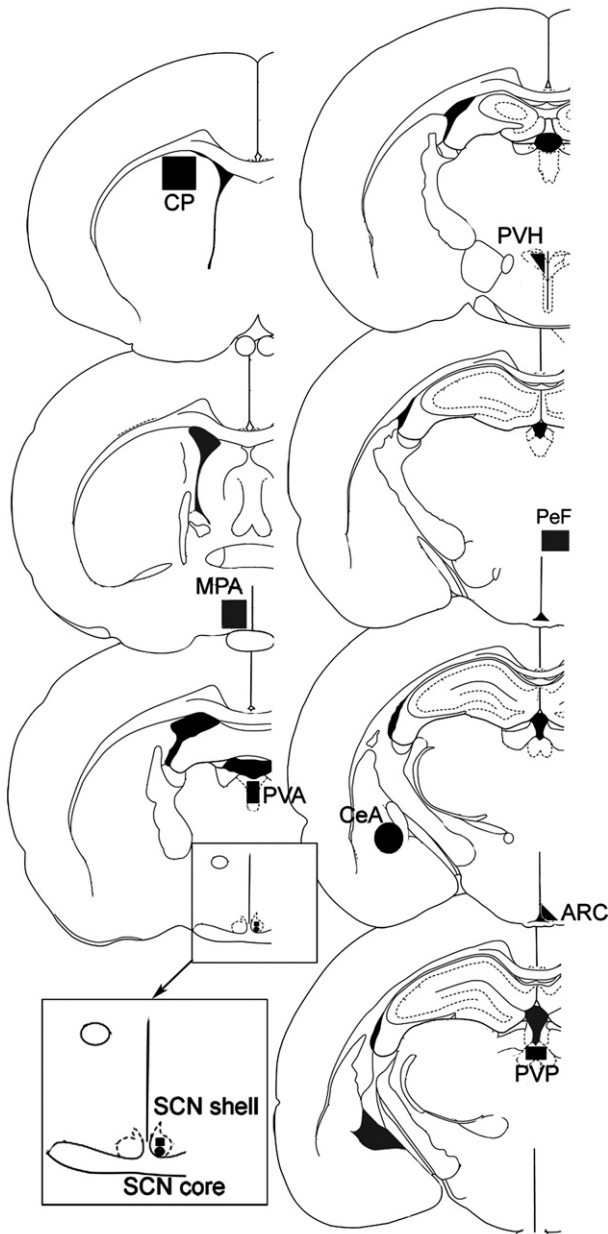
**Fig. 1.** Counting boxes for brain regions assessed for c-Fos immunoreactivity.

modified Wahmann activity wheel with an attached housing cage, in a room that was used solely for this experiment. The wheel was made of hardware cloth and was 35 cm in diameter and 12 cm in width; the attached cage was a standard polypropylene rat housing cage (47×24×20.5 cm), modified at one end with a short piece of PVC pipe connecting the cage to the running wheel, thereby allowing entrance into and exploration of the wheel. During this one week period, the wheel was fixed so that it could not be rotated by the rat. Food (Purina rat chow) and water were available *ad libitum*.

On the day of SD, 4 of 8 rats in each hormone treatment group were sleep deprived for a period of 3 h. Sleep deprivation began 3 h into the light phase (10:00 AM). Rats were confined to the wheel portion of the apparatus and the housing cages were replaced with a modified feeder. The feeder allowed access to food and water while the rat was in the wheel to ensure that the animal was not deprived of water or food. To sleep deprive the rats, the wheel was driven at a rate of approximately 1 rpm by an electric motor. In a previous study, this speed was determined to be optimal for sleep deprivation, as it is incompatible with the postural adjustments of the body required for

sleep [29]. The control animals were also confined to their wheels, but these remained fixed, thereby limiting imposed and voluntary activity.

### 2.4. Perfusion

At the end of the 3 h SD period, animals were anesthetized with a 60% ketamine hydrochloride (final does 60 mg/kg, Ketalean, Bimedia-MTC, Cambridge, ON), 16% xylazine (3.2 mg/kg, Rompun Bayer, Etobicoke, ON), 6% acepromazine maleate (0.6 mg/kg, Atravet, Ayerst, Montreal, PQ) and 18% saline solution at a dose of 1 ml/kg, i.p. and perfused intracardially with a 50 ml 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by approximately 150 ml of fixative containing 4% paraformeldahyde in 0.1 M phosphate buffer (PB; pH 7.4). Each perfusion was initiated and completed within 30 min of the SD period. Brains were removed, post-fixed in the same fixative overnight and then transferred into 30% sucrose in 0.1 M PB (pH 7.4) at 4 °C until they sank.

### 2.5. Blood collection and radioimmunoassay

Blood samples were collected into heparinized tubes via cardiac puncture in anesthetized animals just prior to perfusion, and centrifuged at 800 ×g to separate blood plasma. Samples (2 ml) were stored at −20 °C until assayed. Plasma levels of estradiol were measured using a commercially available radioimmunoassay kit (Catalog # TKE22; Inter Medico, Markham, ON, Canada).

### 2.6. Immunohistochemistry for c-Fos

The brain was sliced into 40 μm-thick coronal sections using a freezing microtome. Sections were collected in 4 sets of 1:4 series in 0.05 M Tris-buffered saline (TBS, pH 7.4). To visualize c-Fos IR, one series of sections was first incubated for 5 min in 10% methanol, 3% hydrogen peroxide in 0.05 M TBS (pH 7.4) to inhibit any endogenous peroxidases, followed by three 10 min rinses in TBS. Sections were then incubated with a rabbit anti-c-Fos antibody (1:20,000; Ab-5, Oncogene Research Products, Uniondale, NY) in a solution of 0.01% sodium azide, 2% normal donkey serum (Sigma, St. Louis, MO) and 0.3% Triton X-100 in 0.05 M TBS (pH 7.4) on a shaker, either overnight at room temperature or for 2 days at 4 °C. Once the primary antibody incubation was complete, sections were rinsed (as described earlier) and incubated in a biotinylated donkey anti-rabbit IgG (1:1000; Jackson Laboratories, West Grove PA) for 90 min. Sections were then rinsed and incubated in avidin–biotin peroxidase complex (1:200; ABC Elite Kit from Vector Laboratories, Burlingame, CA) for 60 min at room temperature. The peroxidase-conjugated proteins were visualized by incubating sections in 0.02% diaminobenzidine and 0.6% nickel ammonium sulfate in 0.05 M TB to which 0.006% hydrogen
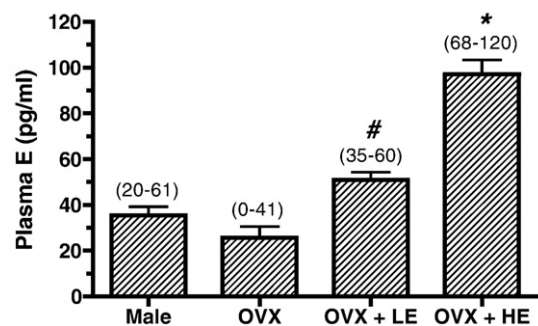


**Fig. 2.** Mean plasma estradiol levels (±SEM) for ovariectomized (OVX; $n=9$), low estradiol (OVX+LE; $n=10$), high estradiol (OVX+HE; $n=10$) and male ($n=12$) groups. Ranges are indicated within the parentheses for each group, *indicates significantly different from all other groups; #indicates significantly different from OVX.

peroxide was added to produce a dark-purple reaction product. After the reaction was complete, sections were mounted on subbed slides, dehydrated in an ascending alcohol series, and coverslipped.

The anti-c-Fos antibody was raised against the N-terminal sequence (residues 4–17) of c-Fos protein that is conserved among human, mouse and rat. It recognized a ~55 kDa protein on Western blot; this corresponds to the molecular weight of c-Fos (manufacturer's technical information). Omission of the primary antibody abolished staining, confirming the specificity of the secondary antibody used.

## 2.7. Image analysis

To assess patterns of c-Fos immunoreactivity, eight brain regions were analyzed (coordinates are reported relative to bregma according to Paxinos and Watson, 1998; Fig. 1): caudate putamen (CP: dorsal region adjacent to the corpus callosum; 0.2–1.2 mm anterior), medial preoptic area (MPA: 0.26–0.4 mm anterior), suprachiasmatic nucleus (SCN: 1.3–1.4 mm posterior), the paraventricular nucleus of the hypothalamus (PVH: 1.8 mm posterior), perifornical hypothalamic area (PeF: 2.8 mm posterior), anterior paraventricular thalamic
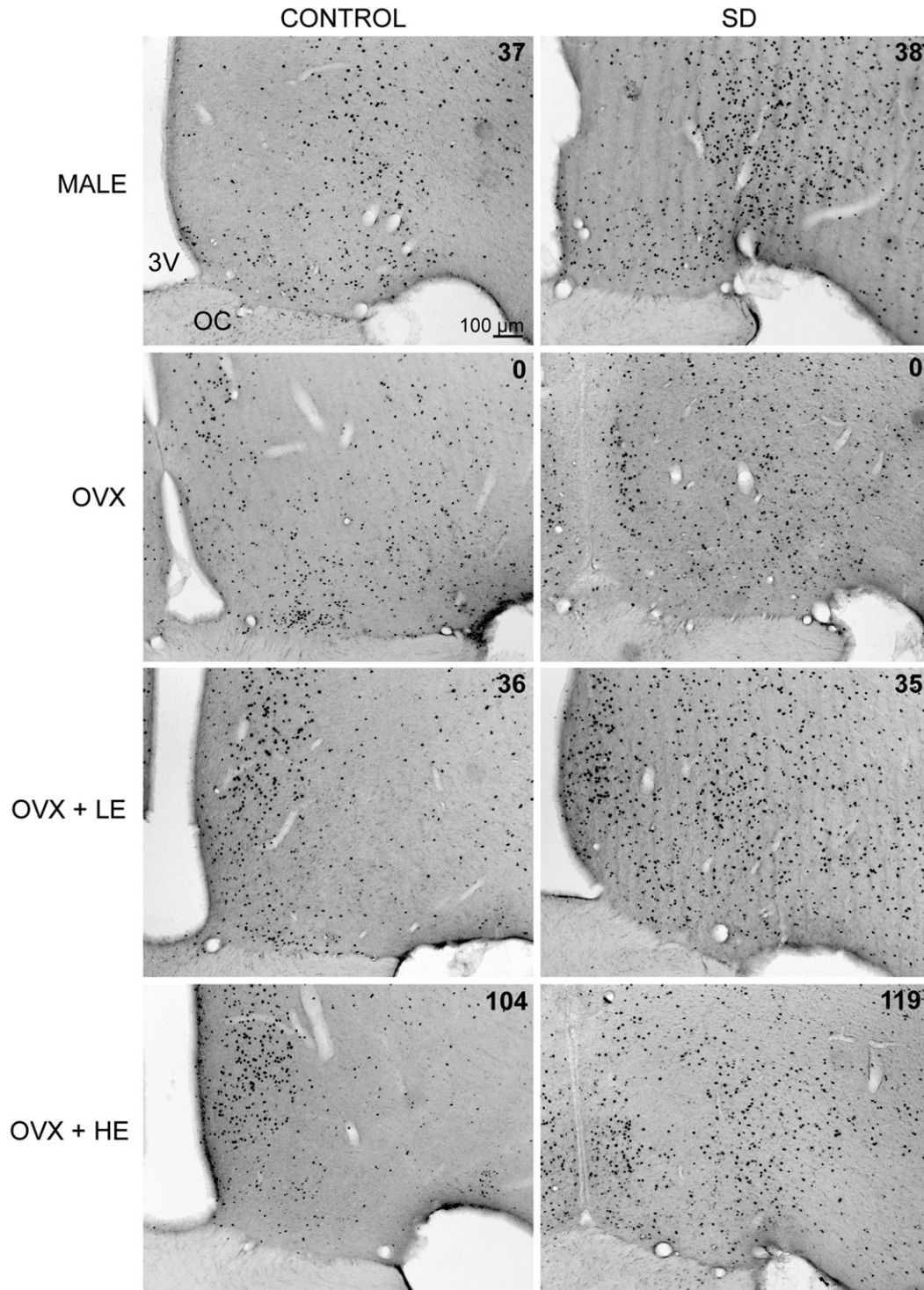


Fig. 3. Representative photomicrographs of c-Fos immunoreactivity in the medial preoptic area in the four different hormonal conditions (ovariectomized (OVX), low estradiol (OVX+ LE), high estradiol (OVX+HE) and male) groups with or without SD. Each micrograph is matched for estradiol level which is indicated in the upper right corner (in pg/ml).

nucleus (PVA: 1.3–1.4 mm posterior); posterior paraventricular thalamic nucleus (PVP: 3.3–3.6 mm posterior); the central nucleus of the amygdala (CeA: 3.6 mm posterior) and arcuate nucleus (ARC: 2.56–3.30 anterior). These areas are involved in the regulation of sleep (MPA, PVA, PVP, and SCN), wakefulness (PeF), emotional arousal (CeA), locomotion (CP), and neuroendocrine function (PVH and ARC). Three sections were selected in each animal that were centered on the levels indicated above, except that only one section through the PVH, SCN, and PeF was analyzed. All sections were analyzed bilaterally.

The black and white images of each brain region in the selected sections were captured using an AxioCam HRc camera mounted on a Zeiss Axiovert 200 microscope. The white–black reference levels of the captured images were adjusted using the Adobe Photoshop Adjust Levels command. The "white" level of the intensity histogram (gray value of 0) was set to the whitest point on the image which was restricted to a blood vessel or a blank area of the slide. The "black" level of the intensity histogram (gray value of 245) was set at the darkest immunohistochemical staining visible in each image. An optimal threshold setting for counting was determined by comparing the counts for different threshold values to subjective eye counts in a representative section. This threshold value was then used for all images analyzed.

For each brain region, a counting box was used to delineate an area of quantification (Fig. 1). The size and placement of this box were determined on the basis of the boundaries of the nucleus of interest determined according to Paxinos and Watson [36]. For the PVA a box of 400×600 μm (length×width) dimension was placed 50 μm below the ventral surface of the roof of the third ventricle (D3V) so that the middle of the box was aligned with the midline of the brain section. Cell counts in the MPA were made using a rectangle measuring 650×700 μm, which was placed 100 μm from the third ventricle (3 V) while being anchored to the dorsal border of the optic chiasm. For the SCN, a circle with a radius of 150 μm was anchored to the dorsal border of the optic chiasm and a rectangle (150×100 μm) was then centered at the top of this circle to delineate between the core and shell, respectively. To distinguish between the magnocellular and parvocellular regions of the PVH, a triangle of 250×400 μm was placed 30 μm ventral and 75 μm lateral to the roof of the 3 V. For the CP a box of 1×1 mm was used at the most dorsal part of the CP section. For the arcuate nucleus, a trapezoid with a height of 250 μm, a width of 400 μm at the top, and 600 μm at the bottom was used. The top of the trapezoid was aligned with the ventromedial edge of the 3 V where it widens just dorsal to the median eminence. The perifornical area was counted using a box measuring 800×650 μm placed 250 μm dorsal to the centre of the fornix. For the central nucleus of the amygdala, a 650 μm circle was placed 50 μm lateral to the commissural stria terminalis. The number of c-Fos-immunoreactive cells within each brain region of interest was calculated with Scion Image (version 4.02) using the threshold described above.

### 2.8. Statistical analysis

Group differences in hormone levels were assessed using a one-way analysis of variance (ANOVA) with a post-hoc Tukey's test. Unilateral cell counts from the selected sections for a given brain region in each animal were averaged and the mean was used for statistical analysis of treatment effects. To determine whether hormonal condition affected basal c-Fos IR, a one-way ANOVA was first performed for non-sleep deprived conditions. Differences in the number of immunoreactive cells were analyzed statistically using a two-way (deprivation condition and hormonal condition) ANOVA. If significant interactions were obtained, Tukey's post-hoc tests were used to clarify differences between individual groups. Data were square-root transformed to satisfy homogeneity of variance where required. Data are presented as mean±S.E.M. $p$ values less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. Plasma estradiol levels

Four of the ovariectomized females (two High E, one Low E, and one OVX animal) were omitted from the analysis because their measured estradiol levels were well in excess of the target plasma levels for their respective groups. For the remaining animals, the mean plasma estradiol levels (±SEM) in OVX, Low E, High E and Male groups were 24.6 (±5.0), 51.1 (±3.3), 97.1 (±6.2) and 35.6 (±3.8) pg/ml, respectively (Fig. 2). There was a significant difference among the groups ($F_{3,37}$=46.23, $p<0.001$). Tukey's post-hoc analysis revealed that the high estradiol group had significantly higher plasma estradiol levels than the low E group ($p<0.001$), OVX group ($p<0.001$) and the male group ($p<0.001$). The estradiol levels of the low E group were in turn significantly greater than those of the OVX group ($p=0.002$). No significant difference in plasma estradiol levels was found between the male group and the OVX or Low E groups.

### 3.2. c-Fos immunoreactivity levels

Examples of c-Fos IR in the MPA in the eight treatment groups are shown in Fig. 3. Quantitative analyses indicated that, under the basal (no deprivation) conditions, estradiol treatments did not significantly alter c-Fos IR in any of the brain regions studied (Table 1, Fig. 4). However, baseline c-Fos IR tended to be higher in the OVX and Low E females, compared to males or High E females in sleep/wake-regulatory areas (PeF, MPA, and PVA; Fig. 4A–C), as well as in predominantly neuroendocrine nuclei (ARC and PVH, Table 1).

The effects of SD in animals were variable across the regions studied. SD significantly increased the number of c-Fos IR cells in the MPA ($F_{1,28}$=9.047, $p=0.006$, Fig. 4A), PVA ($F_{1,33}$=5.735, $p=0.022$, Fig. 4B), PeF ($F_{1,33}$=4.468, $p=0.042$, Fig. 4C), and CP ($F_{1,33}$=15.43,

**Table 1**
c-Fos immunoreactive cell counts in brain regions that did not show a significant effect of sleep deprivation (SD) or hormone condition (ovariectomized (OVX), low estradiol (OVX+LE), high estradiol (OVX+HE) and males)

| Brain Region | Male Control | SD | OVX Control | SD | OVX+Low E2 Control | SD | OVX+High E2 Control | SD |
|---|---|---|---|---|---|---|---|---|
| SCN core | 18.2±7.7 | 9.2±4.3 | 50.6±21.6 | 19.5±12.5 | 33.4±11.4 | 36.0±18.7 | 25.3±15.1 | 23.2±11.1 |
| SCN shell | 23.6±8.6 | 14.5±6.3 | 37.6±14.4 | 32.3±19.9 | 29.8±7.2 | 36.8±15.5 | 30.6±17.1 | 24.8±9.0 |
| PVH | 10.8±2.4 | 29.0±10.7 | 40.3±27.4 | 37.4±12.0 | 37.1±12.5 | 45.7±16.5 | 12.9±3.6 | 29.0±11.4 |
| ARC | 12.2±3.5 | 12.7±4.0 | 19.8±10.8 | 20.3±6.3 | 16.5±8.2 | 11.7±5.1 | 3.4±1.1 | 11.3±3.4 |
| CEA | 16.0±6.1 | 24.6±8.0 | 25.8±10.4 | 18.7±5.4 | 22.7±7.0 | 18.3±5.7 | 29.2±11.5 | 21.0±7.5 |
| PVP | 67.0±27.9 | 97.9±24.9 | 82.9±24.9 | 114.4±27.9 | 97.3±27.9 | 100.3±27.9 | 61.3±27.86 | 91.1±24.9 |

Mean (±SEM) of cell counts in the suprachiasmatic nucleus (SCN) core and shell, paraventricular nucleus of the hypothalamus (PVH), arcuate nucleus (ARC), central nucleus of the amygdala (CEA) and posterior paraventricular nucleus of the thalamus (PVP) are shown.
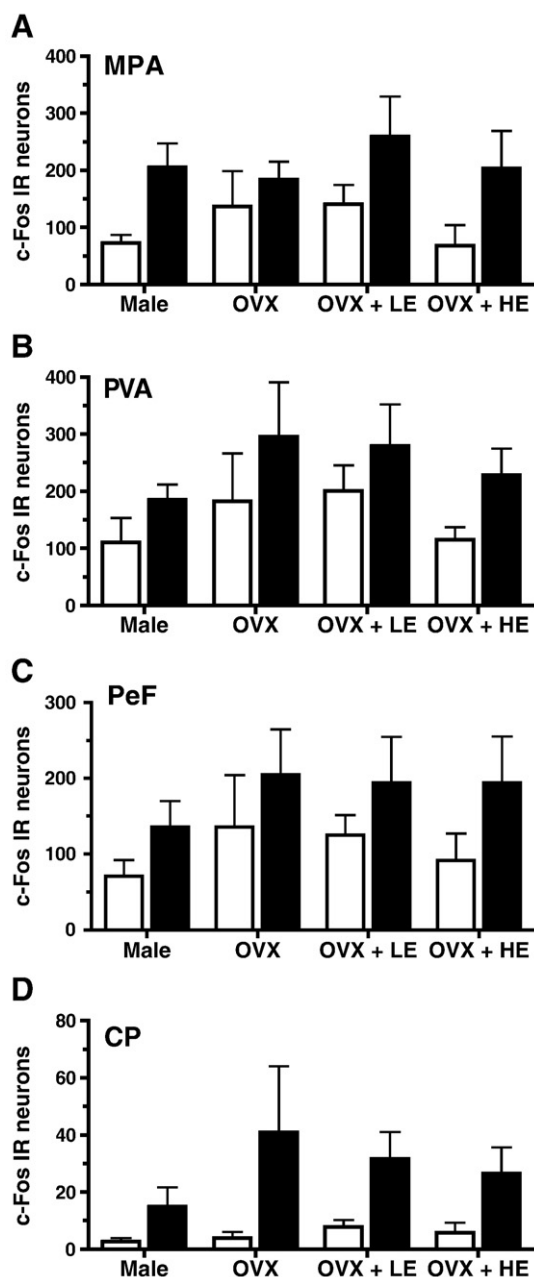
Fig. 4. Mean numbers of c-Fos-immunoreactive cells (±SEM) for ovariectomized (OVX; $n=9$), low estradiol (OVX+LE; $n=10$), high estradiol (OVX+HE; $n=10$) and male ($n=12$) groups that were either sleep deprived (dark bars) or left undisturbed (control, white bars) for the (A) MPA (medial preoptic area), (B) PVA (anterior paraventricular nucleus of the thalamus), (C) PeF (periformical region of the hypothalamus) and (D) CP (caudate putamen). A main effect of SD was found for all areas.

$p<0.0001$ Fig. 4D). However, SD did not significantly affect the number of labeled cells in the SCN core and shell regions (core, $F_{1,26}=0.921$, ns; shell $F_{1,26}=0.129$, ns), PVH ($F_{1,32}=1.379$, ns), ARC ($F_{1,31}=0.049$, ns), PVP ($F_{1,27}=0.876$, ns) and CeA ($F_{3,16}=0.353$, ns) (Table 1).

For rats undergoing SD, estradiol treatment did not modify c-Fos IR in the regions in which SD increased the numbers of labeled cells, nor in the regions in which SD had no effect (Fig. 3, Table 1). However, in the nuclei that showed a trend for an increase in baseline c-Fos IR (PeF, MPA, PVA, ARC and PVH), the effect of SD tended to be greater in the High E group than in the OVX and Low E groups (Fig. 4 and Table 1), but these apparent differences were not statistically reliable.

## 4. Discussion

These results confirm that 3 h of sleep deprivation up-regulates c-Fos IR in both the PVA and the MPA, two brain regions known to play a role in homeostatic regulation of sleep and sleep need [37–39]. SD also enhances c-Fos IR in the PeF, which has been proposed to play an important role in maintaining wakefulness [40], and in the CP, which has a well-known role in motor activity [41]. They also demonstrate that neither of two physiological doses of estradiol modulates either basal or SD-evoked levels of c-Fos IR in any of the brain regions studied.

The increases in c-Fos IR in the MPA and PVA in females following 3 h sleep deprivation are consistent with previously reported results in male rats [29,42]. Many neurons in the MPA increase their firing rates during NREM sleep while others increase their firing during wakefulness, and some of these neurons increase firing in proportion to the duration of time awake [43]. Lesions of the MPA in cats result in severe and persistent decreases in sleep [44]. A previous study examining c-fos expression after sleep deprivation of different durations (3 and 6 h) that began at different circadian phases suggested that the MPA might integrate both homeostatic and circadian information [29].

The increase in c-Fos IR in the PeF after SD in both males and females in this experiment is consistent with the role of this area in wake maintenance. The PeF and surrounding regions of the lateral hypothalamus house neurons containing the neuropeptide orexin, which has been implicated in promoting wakefulness [45]. The expression of c-fos in these neurons is highest during the dark period of the light–dark cycle when rats are normally awake [46], and it can be enhanced by sleep deprivation or administration of stimulant drugs such as methamphetamine or caffeine [46,47]. The enhancement of c-Fos IR in the CP following SD in male and female rats in the present study is consistent with previous findings in males [27,29]. The constant slow rotation of the wheel during the period of SD prevents sleep by imposing constant movement or postural adjustments. SD rats were, therefore, engaged in more locomotor activity than non-deprived rats, which could account for the increased c-Fos IR in this area (cf. [48]).

It is evident from these findings that the response of c-fos to a short period of sleep deprivation is similar in male and female rats, and furthermore that this response does not appear to be modulated by levels of circulating estradiol. This is somewhat surprising given that in situ hybridization studies demonstrated both estrogen receptor (ER)-α and ER-β in the SD-responsive areas examined in the present experiment. Both receptor subtypes were detected in the MPA, PVA, and lateral hypothalamic regions, with relatively more ER-β than ER-α in the MPA [49] and relatively even distributions of the receptor subtypes in the lateral hypothalamus [50]. The PVA has a lower density of ER, with relatively more ER-α than ER-β [50]. The presence of ERs in these regions suggests a potential role of estrogen in sleep regulation as ERs can alter the transcriptional milieu by their interactions with SD-regulated transcription factors such as c-Fos that act at the AP-1 site [51]. Nevertheless, in the present study estradiol treatment did not alter the effects of SD on c-Fos IR in these regions.

In some respects, the lack of effect of hormone manipulation on the c-fos response to SD is not entirely surprising, since previous studies examining the effects of estrogen treatment on basal (i.e. unstimulated) c-Fos IR found apparently discrepant results. Rachman et al. [32] reported that estrogen decreased basal c-fos expression globally in the brain, whereas Peterfi et al. [30] found increases in basal expression following estrogen treatment. Taking into account the very different dosage treatments in these studies, one interpretation of their results might be that there is a non-linear, dose-dependent effect of estrogen on c-Fos levels. Indeed, a well characterized non-linear relationship between estradiol and hippocampal plasticity has been described; low

levels of estrogen promote neurogenesis and LTP, whereas higher levels inhibit these processes [52,53]. Future studies should determine whether estrogen effects on c-Fos levels in the brain are dose-dependent, with higher doses suppressing c-fos expression, as the non-significant trend in this study suggests.

One alternative explanation for the lack of effect of estradiol on SD-induced c-Fos IR in the present experiment is that OVX rats still had low levels of circulating progesterone when studied. Progesterone can have an anxiolytic effect by acting at GABA receptors, facilitating sleep and decreasing the latency to NREM in a dose-dependent manner [54]. Given progesterone's sleep promoting effects and the role of estrogen in priming progesterone receptors [55] it may be important to monitor and manipulate progesterone levels in future studies of the role of estrogen and progesterone in neural responses to sleep deprivation. In addition, the estradiol levels in the different hormonal treatment groups in the present study showed considerable overlap, particularly between the OVX and low estrogen groups, suggesting that endogenous estrogen may not have fully cleared at the time of sleep deprivation, approximately 9 days after OVX. However, we are confident that the circulating estradiol levels in the low and high estradiol groups achieved blood levels that mimicked diestrus (ranging from 35–75 pg/ml) and proestrus (ranging from 80–140 pg/ml), respectively [35,56].

Counting immunoreactive cell nuclei to assess levels of c-Fos IR is a commonly used method for measuring cellular activation [57]. This approach is limited, however, because classifying cells as c-Fos positive or negative does not allow for detection of subtle changes that may affect levels of c-Fos without changing their classification. Quantification of c-fos expression levels using other approaches such as in situ hybridization may be more useful in detecting possible modulatory effects of hormones, especially in terms of anatomical localization of c-fos mRNA.

Another possible explanation for the lack of effect of estradiol is the period of SD used in the current study. We chose a 3 h period of SD since we previously found increased c-Fos IR in certain brain regions in males after 3 h SD, and in most regions examined, this increase was not significantly enhanced with a longer (6 h) period of SD [29]. Similar findings were reported by others using periods of SD from 3–12 h [27]. Furthermore, a 3–4 h period of SD is sufficient to induce a variety of molecular and cellular changes within the rat brain [58,59]. However, it is possible that a longer period of SD is necessary to produce large changes in c-Fos IR that are sensitive to estradiol levels, since longer periods of SD (i.e., 12 and 24 h) more strongly enhance recovery EEG slow-wave activity, while this is only mildly elevated after shorter periods of sleep deprivation [60–62].

When conducting studies involving SD, care must be taken to try to differentiate between the stress associated with SD procedures and forced wakefulness itself. Almost all forms of SD (i.e., wheel rotation, gentle handling, and platform-over-water method) involve some form of stress resulting from the novelty or unpredictability of the manipulations [63–65]. Not surprisingly, SD activates the hypothalamic pituitary adrenal (HPA) axis and a 6 h SD causes a two-fold increase in plasma corticosterone levels [64,66]. While SD may be inherently stressful, their effects on some neural processes such as aspects of LTP, neurogenesis, and EEG spectra are not identical [63,64,67].

In female mice tested during the diestrous phase of the cycle, there was little difference between recovery sleep after 6 h of SD between the sexes; however, male mice showed a much larger REM rebound after a 1 h restraint period during the subsequent dark phase [25,68]. Since the female mice were tested only in the diestrous phase, it is not possible to speculate whether estrous cycle differences in hormonal milieu mediated the distinct responses of males and females to SD and stress. High levels of estradiol enhance both endocrine and behavioural measures of stress reactivity [68] while stress-induced c-fos mRNA in cortical structures is differentially activated in female rats

depending on the phase of the estrous cycle [69]. Therefore, it is possible that female sex steroids could also influence stress-induced changes in certain aspects of sleep that were not assessed in the present study. However, given that SD in the present study did not affect c-Fos IR in the paraventricular hypothalamic nucleus, we do not have any evidence to suggest that the SD procedure was stressful at the time of sacrifice, and therefore this could help to explain why our estradiol manipulations did not result in distinct c-Fos responses to SD. Nevertheless, some stressful components of the SD procedure may influence IEG expression in different brain regions and at different time points during the SD period.

There have been few studies of SD in female rodents. We have shown effects of SD on c-Fos IR levels in hormonally manipulated female rats that resemble those in intact males. We failed to observe any effect of the level of estradiol on this response, consistent with the previous demonstration that sleep deprivation had similar EEG effects at different stages of the estrous cycle [24]. It will be necessary to address, in future studies, the effect of exogenous estradiol on EEG measures during spontaneous and recovery sleep. Future studies will also need to assess whether the mode of hormone delivery in this study (continuous levels) or a possible interaction with progesterone levels might have contributed to the lack of effect observed.

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