**Kisspeptin system in ovariectomized mice: estradiol and progesterone regulation**

# Kisspeptin system in ovariectomized mice: estradiol and progesterone regulation

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**Abstract**

The kisspeptin system is clustered in two main groups of cell bodies (the periventricular region, RP3V and the arcuate nucleus, ARC) that send fibers mainly to the GnRH neurons and in a few other locations, including the paraventricular nucleus, PVN. In physiological conditions, gonadal hormones modulate the kisspeptin system with expression changes according to different phases of the estrous cycle: the highest being in estrus phase in RP3V and PVN (*positive feedback*), and in ARC during the diestrus phase (*negative feedback*). In this work we wanted to study these hormonal fluctuations during the estrous cycle, investigating the role played by progesterone (P) or estradiol ($E_2$), alone or together, on the kisspeptin system. Gonadectomized CD1 female mice were treated with P, $E_2$ or both ($E_2$+P), following a timing of administration that emulates the different phases of estrous cycle, for two cycles of 4 days. As expected, the two cell groups were differentially affected by $E_2$; the RP3V group was positively influenced by $E_2$ (alone or with the P), whereas in the ARC the administration of $E_2$ did not affect the system. However P (alone) induced a rise in the kisspeptin immunoreactivity. All the treatments significantly affected the kisspeptin innervation of the PVN, with regional differences, suggesting that these fibers arrive from both RP3V and ARC nuclei.

**Keywords**: kisspeptin, estrogen, progesterone, periventricular region, arcuate nucleus, paraventricular nucleus.

**Highlights**

- In ovarectomized CD1 female mice $E_2$ and P play a different roles in the kisspeptin system.

- The kiss cells in RP3V are positively influenced by $E_2$ (alone or with P), in ARC by P.

- $E_2$ and P (alone or together) affect the kisspeptin fibers of the PVN.

- Kiss innervation increases in medial PVN of animals treated with $E_2$, alone or with P.

## 1. Introduction

In the cycling rodents, the gonadal hormone levels change within a short period of time (4–5 days). Estrogen ($E_2$) and progesterone (P) are mediators of sex steroid feedback on the reproductive axis. $E_2$ is the major activator, while P is major inhibitory brake in the luteal phase of the ovarian/menstrual cycle: together they regulate the GnRH and LH secretion (McCartney et al., 2002). In proestrus and estrus the level of circulating estrogen ($E_2$) is higher than in diestrus. The administration of P before or concurrent with $E_2$ inhibits $E_2$ positive feedback and abolishes the preovulatory GnRH and gonadotrophin surge, this was observed in many species, including rat (Le et al., 1997), ewe (Kasa-Vubu et al., 1992), monkey (Dierschke et al., 1973) and women (Kuang et al., 2015). Moreover, alterations in progesterone inhibitory feedback have been implicated in infertility associated with enhanced GnRH/LH secretion (Molloy et al., 1984).

Some of the rodent hypothalamic circuits involved in the control of reproduction and/or of sexual behavior (for example the nitrergic or the kisspeptin systems) show changes according to the different phase of estrous cycle (Sica et al., 2009; Smith et al., 2006) and are sensitive to estrogens in experimental conditions (i.e. gonadectomy; Ceccatelli et al., 1996; Navarro et al., 2004).

The Kisspeptin system is an essential excitatory regulator of GnRH neurons; the absence or mutation of kisspeptin would result in suppressed GnRH secretion, and in alterations of normal pubertal development (Pinilla et al., 2012). Consistent with the role of a mediator of sex steroid feedback on the reproductive axis, the majority of kisspeptin neurons express estrogen receptor alpha (ERα of □90%; Franceschini et al., 2006; Smith et al., 2005) and progesterone receptor (PR □86%; Smith et al., 2007). In rodent brain kiss neurons are clustered in two different hypothalamic locations: the rostral periventricular region (RP3V) and the arcuate nucleus (ARC). As previously reported, the female kisspeptin system changes during the estrous cycle, but the kisspeptin immunoreactivity (kiss-ir) varies in opposing ways in the RP3V and in the ARC, showing the highest value in RP3V during estrus (*positive feedback*), when the immunoreactivity is lowest in ARC (*negative feedback*) (Smith et al., 2006).

In addition to the GnRH system, one of the major hypothalamic targets of the kisspeptin system is the paraventricular hypothalamic nucleus (PVN) (Marraudino et al., 2017). The PVN is one of the most important autonomic control centres in the brain, and its neurons,

organized into subdivisions, play essential roles in controlling several endocrine and autonomic functions including metabolism (for a review see Ferguson et al., 2008). The metabolic conditions and the amount of energy reserves of the organism are indispensable for the modulation of pubertal timing and fertility (Castellano and Tena-Sempere, 2016). Therefore, the presence of kisspeptin fibres in the PVN may represent a link among the control of metabolism and reproduction. In a previous study we demonstrated that the innervation of the PVN shows regional differences (medial versus lateral, dorsal versus ventral) and also it changes in estrus and diestrus suggesting that it derives predominantly from RP3V, but not excluding a component arising from the ARC (Marraudino et al., 2017).

In a previous study conducted in our lab, Martini et al. (2011) tested the sexual behavior in gonadectomized female mice subjected to a P and $E_2$ treatments, confirming that these two hormones work in synergistic fashion to facilitate female receptivity. Interestingly animals tested for lordosis behavior were only receptive after the second cycle of treatment (see Table 2 in Martini et al., 2011). They mimicked the hormonal situation typical of the late proestrus in the E2+P Group, with high levels of E2 and P, the early proestrus in the E2 Group and the diestrus in P Group. In the same study, the authors demonstrated a synergistic effect of both E2 and P on the expression of hypothalamic nNOS in ovariectomized females (Martini et al., 2011).

It is well know that $E_2$ up-regulates the expression of KiSS1 gene selectively via ERα, and virtually all kiss-ir neurons in the RP3V express this receptor isoform (Smith et al., 2005). The expression of PR on kisspeptin neurons is required for the LH surge and normal estrous cycle in mice (Gal et al., 2016; Stephens et al., 2015), and a recent study hypothesized that kiss-ir neurons in RP3V are a direct target also for P and that integration of $E_2$ and PR signaling in kiss neurons is a critical component of the LH surge (Mittelman-Smith et al., 2017).

In the present study, using sections from our previous experiment (Martini et al., 2011), we analyze the role played by $E_2$ and P (alone or together) on the kisspeptin system, including in our analysis not only the RP3V, and the ARC, but also the PVN, the major hypothalamic target of the kisspeptin system.

**2. Results**

Kiss-ir of female CD1 mice changes during the estrous cycle in different ways in the RP3V and the ARC (Smith et al., 2006), accordingly, the effect of $E_2$, P or $E_2$+P in ovariectomized females varies according to the different nuclei. In RP3V we observed the highest number of positive neurons in female mice treated with $E_2$+P (Fig. 1a). The one-way ANOVA revealed that a significant effect was present between groups analyzed for the number of Kisspeptin cells and fractional area (FA) within RP3V (see Table 2). The subsequent analysis by Tukey's HSD test reported no differences among OIL and P groups ($p=0.587$), and among $E_2$+P and $E_2$ groups ($p=0.453$), whereas there is a strongly significant differences among OIL and P groups versus $E_2$+P and $E_2$ groups ($p<0.001$) (Fig. 1b). Also for the measure of FA the one-way ANOVA revealed a significant effect. In this case the Tukey's HSD test reported similar differences among the groups: $E_2$+P vs. OIL, $p<0.001$; $E_2$+P vs. P, $p<0.001$, $E_2$+P vs. $E_2$, $p=0.072$, and OIL vs. P, $p<0.001$ (Fig. 1c).

For the ARC, the highest value for the FA covered by kiss-ir was reported in gonadectomized female treated with only Progesterone (Fig. 2a). The analysis by one-way ANOVA resulted in statistical significant (Table 1) and the two-by two comparison (Tukey's HSD test) demonstrated that the FA in P group was significantly higher than all other groups ($p<0.001$) in the ARC. No difference was observed in comparison between the control group vs. $E_2$+P ($p=1.00$) and vs. $E_2$ ($p=0.952$) (Fig. 2b).

**Kisspeptin-ir within RP3V and ARC**

| | OIL | E2+P | E2 | P | ANOVA 1 WAY | |
|---|---|---|---|---|---|---|
| | MEAN±SEM | MEAN±SEM | MEAN±SEM | MEAN±SEM | F | p |
| **RP3V** | | | | | | |
| Cell Count | 10,22±1,64 | 33,78±1,98 | 30,75±0,48 | 12,83±0,74 | 90,257 | <0,001 |
| FA | 3,65±0,35 | 16,58±0,80 | 14,11±0,57 | 8,55±0,63 | 80,496 | <0,001 |
| **ARC FA** | 5,60±0,47 | 5,45±0,55 | 4,91±0,39 | 11,54±1,07 | 17,507 | <0,001 |

**Table 1:** Quantitative data for number of Kisspeptin cells in RP3V and Fractional Area (FA) within RP3V and ARC for different groups of CD1 female mice are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

The density of Kisspeptin fibers seemed to increase in the PVN of ovariectomized female mice after all treatments (Fig. 3), the one-way ANOVA reported a significant difference (Table 2) and the Tukey's HSD test confirmed this significant increase in $E_2$+P ($p<0.001$), in

$E_2$ ($p<0.001$) and P ($p<0.001$) in comparison to OIL group (Fig. 3b). However as demonstrated in our previous study, the distribution of PVN kiss-ir fibers is not homogeneous in PVN comparing the medial with the lateral part of the nucleus (Marraudino et al., 2017). For this reason we divided the nucleus in fourteen squares (see fig.4a) to cover all the aspects of the PVN: dorso-lateral (DL), ventro-lateral (VL), dorso-medial (DM) and ventro-medial (VM). The one-way ANOVA for each part consistently revealed a significant effect (Table 2); the post-hoc analysis revealed strong differences among the different parts. In the medial subdivisions (DM and VM) the Tukey's HSD test demonstrated that all the treated groups significantly increased kiss-ir (OIL vs. E2+P, $p<0.001$; OIL vs. E2, $p<0.001$ and OIL vs. P, $p<0.001$). In contrast, in DL and VL subdivisions only OIL vs. $E_2$ was always significant ($p=0.015$ and $p=0.004$ respectively) (Fig. 4b).

**% Kisspeptin-ir fiber within PVN**

| | OIL | E2+P | E2 | P | ANOVA 1 WAY | |
|---|---|---|---|---|---|---|
| | MEAN±SEM | MEAN±SEM | MEAN±SEM | MEAN±SEM | F | p |
| **PVN** | 2,81±0,261 | 6,55±0,140 | 6,45±0,304 | 5,60±0,271 | 61,073 | <0,001 |
| DM | 3,55±0,447 | 8,77±0,101 | 7,73±0,478 | 5,27±0,294 | 58,792 | <0,001 |
| DL | 1,20±0,128 | 1,77±0,148 | 3,32±0,372 | 2,93±0,288 | 8,950 | 0,001 |
| VM | 3,91±0,34 | 9,68±0,291 | 8,74±0,150 | 8,76±0,396 | 106,975 | <0,001 |
| VL | 0,75±0,182 | 1,11±0,291 | 2,37±0,276 | 1,75±1,755 | 7,645 | 0,003 |

**Table 2:** Quantitative data for kisspeptin-ir fibers (FA) within PVN in total and divided in DorsoLateral (DL), DorsoMedial (DM), VentroLateral (VL) and VentroMedial (VM) for different groups of CD1 female mice are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

## 3. Discussion

Sex steroids provide feedback loops that allow the gonads to communicate with the hypothalamus to regulate GnRH release. GnRH neurons do not express androgen (AR) or estrogen (ER) receptors alpha (Herbison and Theodosis, 1992; Huang and Harlan, 1993), but they express ER-beta. However, previous studies reported the presence of ERβ in rat GnRH neurons (Hrabovszky et al., 2000; Hrabovszky et al., 2001), while in mice the data are

uncertain due to poorly specific antibodies for this receptor (Snyder et al., 2010). A recent study performed by Choeng and colleagues shows that ablation of ERβ in the forebrain of female mice results in normal oestrous cyclicity (Cheong et al., 2014). Therefore, sex steroids indirectly regulate GnRH neurons and the mediator is Kisspeptin, whose neurons express estrogen receptor alpha, ERα (□90%; Franceschini et al., 2006; Smith et al., 2005), AR (□65%; Smith et al., 2005), and the progesterone receptor (□86%; Smith et al., 2007). In rodents, sex steroids differentially regulate Kisspeptin expression in hypothalamus, which increases in the RP3V region (Kauffman et al., 2007; Smith et al., 2005) and PVN (Marraudino et al., 2017), and decreases in the ARC (Smith et al., 2005).

While PR expression in rodents is likely under ERα regulation in AVPV (Shughrue et al., 1997), progesterone down regulates ER (Simerly et al., 1996) and may counteract the induction of PR by $E_2$. Mittelman-Smith et colleague (2017) hypothesized that AVPV/PeN (or RP3V) kiss neurons are a direct target for P and that integration of $E_2$ and PR signaling in kiss neurons is a critical component of the LH surge (Mittelman-Smith et al., 2017). Effectively the block of the LH surge by progesterone might be due to PR in the AVPV, attenuating the ability of $E_2$ to induce the LH surge, but might also be due to the inactivation of Kisspeptin. In our study, in fact, the animals treated only with P present a very low activation of kisspeptin system in RP3V, for both number of cells and FA. While the situation was totally different when we administrated to the gonadectomized female mice $E_2$ alone or together with P. In these cases, that mimicked the early and the late proestrus respectively, kisspeptin-ir showed a significant increase respect to control and P animals.

In the ARC it seems that P contributes to negative feedback of the axis. Microimplants of the progesterone antagonist (RU486) in the ARC, but not in the POA, blunt the negative feedback effects of progesterone in ewes (Goodman et al., 2011). Our data showed that only females treated with progesterone (mimicking diestrus phase) presented the highest density of kisspeptin in the ARC. In fact, during the diestrus, in physiological condition the female mice express more kisspeptin in ARC than RP3V. Probably the kisspeptin cells in these two nuclei express PR and ERα in different concentration. Moreover, the progesterone down regulates ER (Simerly et al., 1996), this could explain why in animals treated with $E_2$ plus P the kisspeptin signal is comparable to females treated only with $E_2$. In addition, kisspeptin cells in the ARC co-express neuropeptides neurokinin B, NKB, and dynorphin, Dyn (KNDy neurons)

(Goodman et al., 2007) to form an interconnected and synchronized neuronal ensemble that intermittently sends a stimulatory signal to the GnRH neurons to generate pulsatile gonadotropin secretion. In fact, the presence of a dense complex of NKB fibers surrounding KiSS1 cells in the ARN would support a potential synchronizing role of NKB signaling in the coordinated control of GnRH release. In turn, Dyn would release to the same neurons, evoking a decrease in kisspeptin secretion and determining kisspeptin pulse. Therefore, $E_2$ could increase the release of Dyn and NKB in the ARC, which inhibits the kiss expression during the estrus phase; but in diestrus, when the P concentration is increased, the inhibitory effect of Dyn is weakened and consequentially there is an increase of kiss-ir in the ARC.

In the PVN, both $E_2$ and P induce an increase of the kisspeptin fibers in comparison to the ovariectomized females. We know that the kiss innervation in mice PVN is heterogeneous, with higher density of fibers in the medial than in the lateral part of the nucleus (Marraudino et al., 2017). Moreover, during estrus the kiss-ir within PVN strongly increases in the medial part, suggesting that kiss innervation arrive mostly from RP3V (Marraudino et al., 2017). Present data showed that also the ARC cells may have a role in the innervation of the PVN; in fact in general no differences were present when we analyzed the total nucleus in all experimental groups, indicating that the fibers to PVN arrive from RP3V and ARC.

Analyzing the nucleus in more detail, we showed that the innervation significantly increases in the medial part of the nucleus in females treated with $E_2$, alone or together with P (a situation that mimics the early and late proestrus). In proestrus, the more rostral kisspeptin neurons (RP3V) are strongly activated by $E_2$, while more caudal neurons (ARC) are inactive. In diestrus (situation mimicked by treatment with only P), there is an opposite situation. This suggests that there is a constitutive (estrogen-insensitive) kisspeptin innervation of the PVN that arrive from both rostral and kisspeptin caudal cells. When $E_2$ increases, there is a consequent activation of kisspeptin system in RP3V that will project more fibers within the medial part of PVN.

A recent study demonstrated that in the PVN Kiss1r-expressing neurons were sparsely distributed, mainly in the medial part of the nucleus (Higo et al., 2016). This could confirm that the projection from RP3V, in late and early proestrus phases, innervates the medial area of PVN to regulate the different parvocellular neurons located in this region of nucleus. In our previous study, we already demonstrated that oxytocin and tyrosine hydroxylase strongly

related to kisspeptin fibers in PVN, but it is possible that other neurons located in the medial part of the nucleus not expressing estrogen receptors and with an important role in the regulation of energy homeostasis [as TRH, (Kadar et al., 2010)] may have some close relation with kisspeptin, witch expression is altered in conditions of reproductive impairment linked to metabolic stress (Castellano and Tena-Sempere, 2016).

In conclusion, we demonstrated that in gonadectomized CD1 female mice $E_2$ and P play different roles on the kisspeptin system. $E_2$ increases the kiss-ir in RP3V if administrated alone or together with P, while P has a direct control of activation on kiss-ir in the ARC. All the treatments significantly affected the kisspeptin innervation of the PVN, with regional differences, suggesting that these fibers arrive from both nuclei, the RP3V and the ARC.

## 4. Experimental Procedure

This study has been performed on sections stored from our previously published study on the effect of E2 and P on the hypothalamic nitrergic system (Martini et al., 2011), therefore animals, treatments and sectioning procedures are the same of the previous paper.

*Animals and treatments*

In this study we used 32 CD1 female mice (Harlan, Italy), which were housed and handled according to the EEC guidelines for European Communities Council Directives of 24th November 1986 (86/609/EEC) and with the permission of the Ethic Committee of the University of Torino and of the Italian Minister for Health.

Female mice at the age of 5 weeks were bilaterally ovariectomized under gaseous anesthesia. Two weeks after surgery, female mice were randomly assigned to four experimental groups (n=8 each) that received different hormonal treatments (Table 3, see Martini et al., 2011). From 8:00 am to 10:00 am we administered daily sexual steroids by intraperitoneal injections (Downer et al., 2001). Since it has been shown that female mice are highly sexually responsive by the second week of priming and testing, two 4-day cycles of hormonal administration have been performed (Fig. 5).

| | Cycle of steroid hormone administration | | | |
|---|---|---|---|---|
| | **Day 1** | **Day 2** | **Day 3** | **Day 4** |
| **Group OIL** | Oil | Oil | Oil | Oil |
| **Group E2+P** | EB | EB | EB | EB+P |
| **Group E2** | EB | EB | EB | EB |
| **Group P** | Oil | Oil | Oil | P |

**Table 1:** Experimental design and schedule of steroid hormone treatments for each group in a cycle of administration.

Hormones were dissolved in 0.1 ml of sesame oil. The amounts of estradiol benzoate (EB) and progesterone (P) administered in each injection have been shown to reliably induce high levels of receptivity in ovariectomized female mice (Marraudino et al., 2017). On two consecutive cycles of 4 days each, animals of the group $E_2$ received four injections of 10 µg of EB (SIGMA-Aldrich, Milan, Italy) and those of the group P received one injection of 500 µg of P (SIGMA-Aldrich, Milan, Italy). While mice of the group $E_2$+P received four injections of 10 µg of E2 and, 2 h after the last injection, 500 µg of P. Finally, the group Oil received four vehicle (sesame oil) injections (0.1 ml). Mice belonging to the same experimental group were housed by twos in 41x24x14 cm polypropylene mouse cages, under a reversed light-dark cycle (dark 07:30 am-07:30 pm). Food and water were available ad libitum.

*Fixation and tissue sampling*

In the morning, after being verified for sexual receptivity, animals were anesthetized with intraperitoneal injection of tribromoethanol (250 mg/kg) and decapitated. The brains were removed and placed into acrolein (5% in 0.01 M saline phosphate buffer, PBS) for 150 min, washed twice in PBS (30 min), placed overnight in a 30% sucrose solution in PBS at 4 °C, frozen in liquid isopentane at -35 °C, and stored in a deep freezer at -80 °C until sectioning.

Brains were serially cut in the coronal plane at 25µm thickness with a cryostat. The plane of sectioning was oriented to match the drawings corresponding to the coronal sections of the mouse brain atlas (Paxinos and Franklin, 2001). Sections were collected in four series in a cryoprotectant solution (Watson et al., 1986) and stored at -20 °C until used for immunohistochemistry.

In a previous study (Martini et al., 2011), we stained two series for nNOS immunohistochemistry and toluidine blue (Nissl staining). For the present study we used a subgroup of animals (n=4) for each experimental group and we utilized the third series for Kisspeptin immunohistochemistry.

*Kisspeptin immunohistochemistry*

After washing the cryoprotectant solution, the sections were incubated in sodium borohydride (0.1% diluted in PBS) for 15 min, followed by several washing in PBS, exposed to Triton X-100 (0.2% in PBS) and then treated for blocking endogenous peroxidase activity with methanol/hydrogen peroxide solution for 20 minutes. Sections were then incubated for 30 minutes with normal goat serum (Vector Laboratories, Burlingame, CA, USA) and incubated overnight at room temperature with the polyclonal antibody AC#566 (1:10000 in PBS-Triton X-100 0,2%, pH 7,3-7,4) directed against a 10 amino acid peptide corresponding to 43–52 residues of mouse kisspeptin (kp-10). A biotinilated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) was then used at a dilution of 1:200 for 60 min at room temperature. The antigen-antibody reaction was revealed by incubation with avidin-peroxidase complex (Vectastain ABC Kit Elite, Vector Laboratories, Burlingame, CA, USA) for 60 min. The peroxidase activity was visualized with a solution containing 0.400 mg/mL of 3,3'- diamino-benzidine (SIGMA-Aldrich, Milan, Italy) and 0.004% hydrogen peroxide in 0.05M Tris–HCl buffer pH 7.6. Sections were collected on slides pre-treated with chrome alum, air-dried, washed in xylene and coverslipped with Entellan mounting medium (Merck, Milano, Italy).

The production and characterization of this polyclonal kisspeptin antibody has been described in previous studies (Clarkson et al., 2009; Franceschini et al., 2006).

In addiction, we performed other controls in our material: (i) the primary antibody was omitted or replaced with an equivalent concentration of normal serum (negative controls); (ii) the secondary antibody was omitted. In these conditions, cells and fibers were totally unstained.

*Quantitative analysis*

For quantitative analysis, selected standardized sections of comparable levels covering the Arcuate nucleus (ARC; Bregma -1.58 mm; -1.70 mm; -1.82 mm), the Paraventricular nucleus

(PVN, Bregma -0,58 mm; -0,82 mm; -0,94 mm) and rostral periventricular region of the third ventricle (RP3V; Bregma 0,26 mm; 0,02 mm; -0,22 mm) were chosen in according to the mouse brain atlas (Paxinos & Franklin, 2001). The sections were acquired with a NIKON Digital Sight DS-Fi1 video camera connected to a NIKON Eclipse 80i microscope (Nikon Italia S.p.S., Firenze, Italy). Images were digitized by using a 40x (PVN) or a 20x (ARC and RP3V) objective. Digital images were processed and analyzed by IMAGEJ (version 1.47v; Wayne Rasband, NIH, Bethesda, MD, USA). Measurements were performed within predetermined fields (region of interest, ROI), i.e. rectangular boxes of fixed size and shape covering a large part of each considered nucleus (63.366 $\mu m^2$ for ARC; 104.048 $\mu m^2$ for RP3V). While the PVN, in each selected section, was divided into fourteen squares (40,150 $\mu m^2$ each) to cover its full extension. As in our previous study (Marraudino et al., 2017), these squares did not match with the sub-nuclei of the PVN, but were chosen in order to have a topographical reference to analyze in more detail the density of immunoreactivity within the PVN by dividing it into four regions: dorso-medial, dorso-lateral, ventro-medial and ventro-lateral. The measure of total PVN was a mean of signal measured in each four subdivisions of nucleus. Positive Kisspeptin neurons were identified for the presence of a clearly labeled cell body only within RP3V; while in ARC and in PVN we quantified the presence of immunoreactive material with the method of the fractional area (FA). Briefly, immunoreactive structures were separated from the background by using the threshold function of the software. In this way it is possible to measure the FA covered by immunoreactive structures within the ROI. The results obtained were grouped to provide mean (± S.E.M.) values.

*Statistical analysis*

Quantitative data were examined with SPSS 24 statistic software (SPSS inc., Chicago, USA) by one-way analysis of variance (ANOVA), followed, when appropriate, by a post-hoc Tukey's HSD test. Differences were considered statistically significant for values of $p<0.05$.

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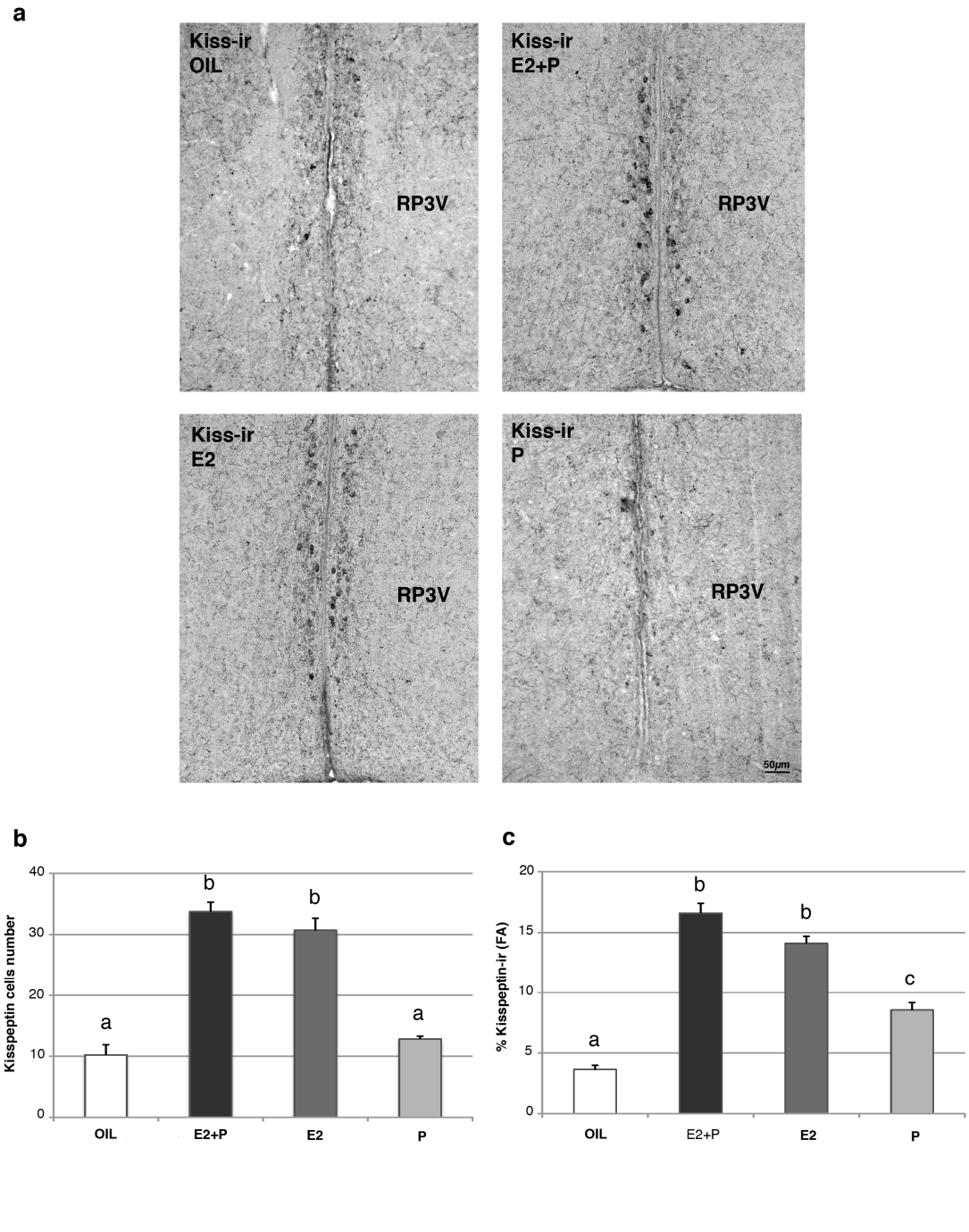
**Figure 1. (a)** Effects of estradiol+progesterone ($E_2$+P), estradiol ($E_2$) and progesterone (P) administration on Kisspeptin system in rostral periventricular region of the third ventricle (RP3V) of female mice. **(b and c)** Histograms representing the number of cells and the fractional area (FA) (expressed as mean±SEM) covered by kisspeptin-ir structures in RP3V in ovariectomized mice treated with $E_2$, P and $E_2$+P. Significant differences (ANOVA followed by the Tukey's HSD test; $p \leq 0.05$) are denoted by a, b or c. Scale bar = 50µm.

**Figure 2. (a)** Photomicrographs representing the effects of estradiol+progesterone ($E_2$+P), estradiol ($E_2$) and progesterone (P) administration on Kisspeptin system in arcuate nucleus (ARC) in comparison with control group (OIL). **(b)** Histogram representing the fractional area (FA) (expressed as mean±SEM) covered by kisspeptin-immunoreactive structures in ARC in ovariectomized mice treated with estradiol ($E_2$), progesterone (P) and estradiol+progesterone ($E_2$+P). Significant differences (ANOVA followed by the Tukey's HSD test; $p \leq 0.05$) are denoted by a or b. * = third ventricle. Scale bar = 100µm.

**Figure 3. (a)** Photomicrographs representing the comparison between control group (OIL) and the effect of the estradiol ($E_2$) administration on Kisspeptin system in Paraventricular Nucleus (PVN) of female mice. **(b)** Histogram representing the fractional area (FA) (mean±SEM) covered by kisspeptin-immunoreactive fibers in paraventricular nucleus (PVN) in ovariectomized mice treated with estradiol ($E_2$), progesterone (P) and estradiol+progesterone ($E_2$+P). Significant differences (ANOVA followed by the Tukey's HSD test; $p \leq 0.05$) are denoted by a or b. Scale bar = 50µm.

**Figure 4. (a)** The representative subdivision in the Paraventricular Nucleus (PVN) of female mice treated with estradiol+progesterone ($E_2$+P) in fourteen quadrants to identify the four parts of nucleus (DorsoMedial; DorsoLateral; VentroMedial; VentroLateral). **(b)** Histograms representing the fractional area (FA) covered by kiss-ir fibers (mean±SEM) in the PVN (DorsoMedial; DorsoLateral; VentroMedial; VentroLateral) in ovariectomized mice treated with estradiol ($E_2$), progesterone (P) and estradiol+progesterone ($E_2$+P). Significant differences (ANOVA followed by the Tukey's HSD test; $p \leq 0.05$) are denoted by a, b or c.

**Figure 5.** Experimental time line. Female mice at the age of 5 weeks were bilaterally ovariectomized; two weeks after surgery, they were randomly assigned to four experimental groups that received two cycles (each of 4-days) of hormonal administration.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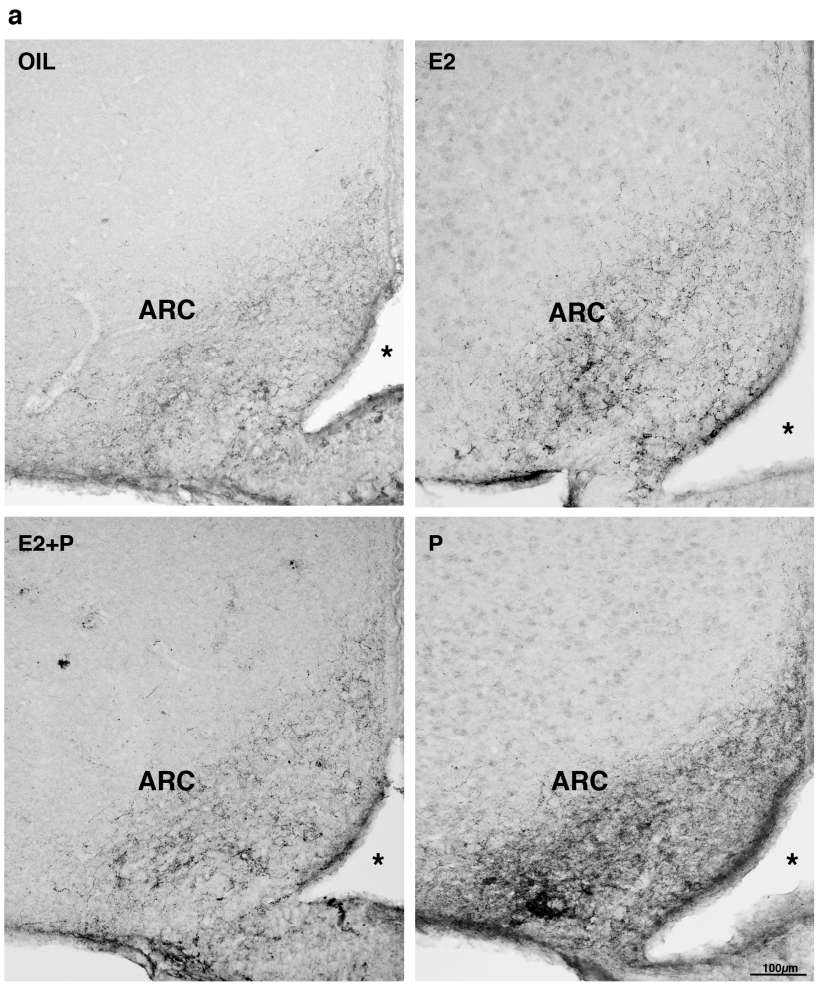

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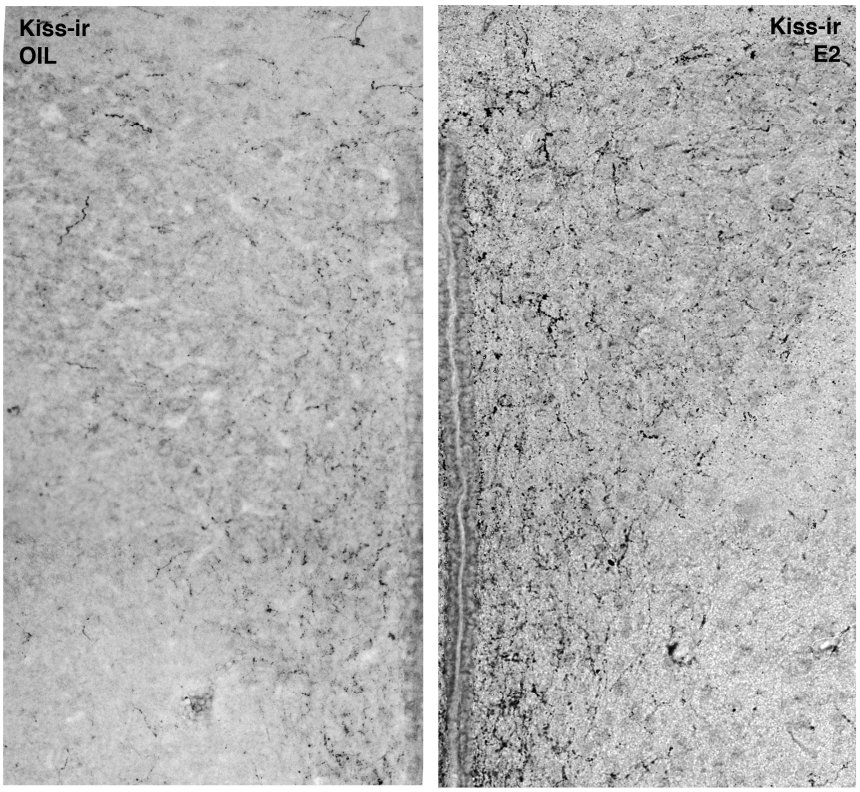


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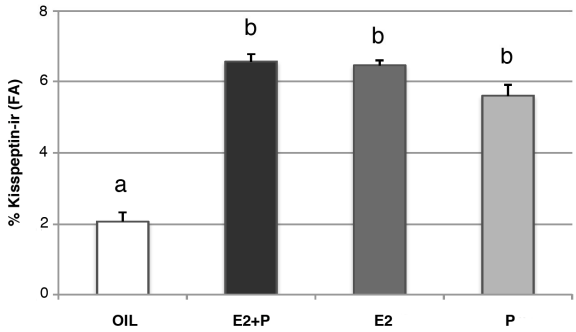

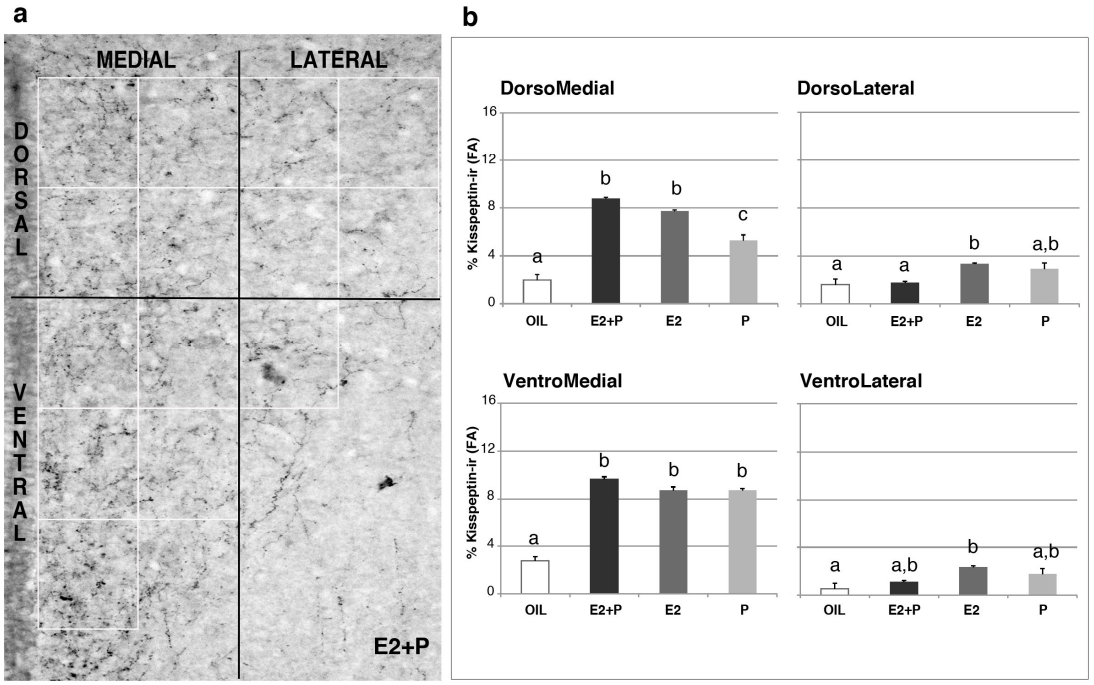

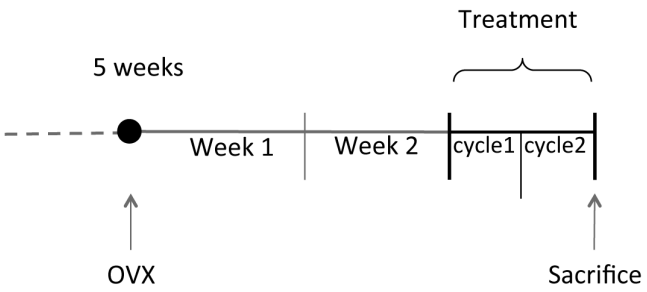

**Highlights**

- In ovarectomized CD1 female mice $E_2$ and P play a different roles in the kisspeptin system.

- The kiss cells in RP3V are positively influenced by $E_2$ (alone or with P), in ARC by P.

- $E_2$ and P (alone or together) affect the kisspeptin fibers of the PVN.

- Kiss innervation increases in medial PVN of animals treated with $E_2$, alone or with P.