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Kisspeptin innervation of the hypothalamic paraventricular nucleus: sexual dimorphism and effect of estrous cycle in female mice

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Abstract

The hypothalamic Paraventricular nucleus (PVN) is the major autonomic output area of the hypothalamus and a critical regulatory center for energy homeostasis. The organism's energetic balance is very important for both the regular onset of puberty and regulation of fertility. Several studies have suggested a relationship among neural circuits controlling food intake, energy homeostasis and the kisspeptin peptide.

The kisspeptin system is clustered in two main groups of cell bodies (the Anterior Ventral Periventricular region, AVPV, and the Arcuate nucleus, ARC) projecting mainly to GnRH neurons and to a few other locations, including the PVN.

In the present study, we investigated the distribution of the kisspeptin fibers within the PVN of adult CD1 mice. We observed a significant sexual dimorphism for AVPV and ARC, as well as for the PVN innervation. Kisspeptin fibers showed a different density within the PVN, being denser in the medial part than in the lateral one; moreover, in female, the density changed, according to different phases of the estrous cycle (the highest density being in estrus phase).

The presence of a profound effect of estrous cycle on the kisspeptin immunoreactivity in AVPV (with a higher signal in estrus) and ARC, and the strong co-localization between kisspeptin and NkB only in ARC and not in PVN suggested that the majority of the kisspeptin fibers found in the PVN might arise directly from AVPV.

Key words: Kiss1, PVN, estrus, diestrus, arcuate nucleus, Anterior Ventral Periventricular region

Introduction

The kisspeptin peptide is encoded by the *KiSS1* gene, localized on human and murine chromosome 1. The mature peptide is formed by 52- or 54-amino-acids. This protein binds specifically to the kisspeptin receptor (Kiss1r), previously known as G-protein-coupled receptor-54, GPR54 (Kotani et al., 2001), whose mutations induce hypogonadotropic hypogonadism (de Roux et al., 2003; Seminara et al., 2003). From a physiological point of view, kisspeptin has been identified as the most powerful regulator of GnRH (Irwig et al., 2004; Pinilla et al., 2012). It is implicated in the timing of puberty onset (Han et al., 2005) and in the mechanism linking energetic status to the reproductive axis (Tena-Sempere, 2006; Castellano et al., 2010).

The neuroanatomical distribution of kisspeptin-synthesizing cell populations is conserved across mammalian species. A large population of kisspeptin neurons is described in the Arcuate hypothalamic nucleus (ARC) of mice (Clarkson and Herbison, 2006; Gottsch et al., 2004; Smith et al., 2005a), rats (Kauffman et al., 2007), hamsters (Greives et al., 2007), sheep (Franceschini et al., 2006; Goldman et al., 2007; Smith et al., 2007), mares (Decourt et al., 2008), primates (Shahab et al., 2005), and humans (Rometo et al., 2007). In this nucleus kisspeptin is coexpressed with neurokinin B (NkB), endogenous opioid peptide dynorphin A (Dyn) and other signaling molecules. These neurons, abbreviated as the KNDy subpopulation, are critical mediators of pulsatile GnRH neurosecretion (Grachev et al., 2014; Lehman et al., 2010). A second population of kisspeptin-positive cells is located in the rostral periventricular area of the third ventricle (RP3V, which includes the anteroventral periventricular region, AVPV, and the periventricular nucleus, PeN) of mice (Clarkson and Herbison, 2006; Gottsch et al., 2004; Smith et al., 2005b), rats (Irwig et al., 2004; Kauffman et al., 2007), hamsters (Greives et al., 2007), sheep (Franceschini et al., 2006; Goldman et al., 2007; Smith et al., 2007) and humans (Rometo et al., 2007).

These two hypothalamic regions are differentially regulated by testosterone and estradiol, both during development and in adulthood (Smith et al., 2005a, 2005b). Several studies performed in RP3V of adult rodents showed a peculiar dimorphism, with females displaying the highest kisspeptin expression (Kauffman, 2009; Clarkson and Herbison, 2006). Recently, a few studies showed that rodent's kisspeptin system is also dimorphic within ARC, with the kisspeptin levels significantly higher in females (Knoll et al., 2013; Overgaard et al., 2013).

Kisspeptin fibers branch out from cell bodies in RP3V and ARC, to different hypothalamic areas (Yeo and Herbison, 2011), and, among them, the Paraventricular nucleus (PVN), seems to be one of the major targets of the system. In fact, different studies mentioned that in rodents the PVN is highly innervated by kisspeptin fibers (Brailoiu et al., 2005; Clarkson et al., 2009; Yeo and Herbison, 2011). Within the hypothalamus the PVN is the major autonomic output area, with heterogeneous neuronal populations, playing essential roles in neuroendocrine/ autonomic regulation (Ferguson et al., 2008). In fact, while the lateral part of PVN contains magnocellular neurons chiefly projecting to the posterior pituitary (where they release oxytocin and arginine vasopressin into the blood), the medial part of PVN is characterized by different types of parvocellular neurons that can be identified for the presence of several neurotransmitters, neuropeptides, and enzymes involved in the synthesis of neurotransmitters [i.e tyrosine hydroxylase (TH; Ruggiero et al., 1984), neural nitric oxide synthase (nNOS; Gotti et al., 2004, 2005); but also corticotropin-releasing hormone (CRH; Wang et al., 2011), thyrotrophin releasing hormone (TRH; Kadar et al., 2010), vasopressin (Caldwell et al., 2008), somatostatin (Tan et al., 2013)].

In the present study, we describe for the first time the sexual dimorphism of kisspeptin immunoreactive system in adult CD1 mice PVN, we analyze the variations of kisspeptin distribution during the estrous cycle, and the coexistence of kisspeptin and other cellular populations within the same nucleus.

Materials and methods

Animals

CD-1 mice (*Mus musculus domesticus*) were originally purchased from Charles River Laboratories (Calco, Lecco – Italy) and maintained as an outbreed colony at the University of Torino. The animals were housed in groups of 3 males or 3 females in 45x25x15 cm polypropylene mouse cages at 22±2°C, under a 12:12 light dark cycle (light on at 8:00 am). Food and water were provided *ad libitum* (standard mouse chow 4RF21, Mucedola srl, Settimo Milanese, Italy).

We used three different groups of animals as detailed below:

Experiment 1 (kisspeptin system distribution and male to female comparison): 6 female in diestrus and 6 male mice at postnatal day 60 (PND60);
Experiment 2 (estrous cycle observation): 10 adult female mice (PND90), 4 mice in estrus and 6 mice in diestrus phase.
Experiment 3 (interaction between kisspeptin and different neuronal populations of PVN): 4 adult female mice (PND90) in estrus phase

Animal care and handling were according to the European Union Council Directive of 22th September 2010 (2010/63/UE); the Italian Ministry of Health and the Ethical Committee of the University of Torino approved all the procedures reported in the present study.

Fixation and tissue sampling

From PND50, female mice were inspected by daily examination of vaginal cytology smears (for details see Becker et al., 2005; McLean et al., 2012) in order to minimize the potential variations of kisspeptin expression due to the estrous cycle's phase (Adachi et al., 2007). After exhibition of 2 or more consecutive 4-days estrous cycles, female mice were killed in diestrus for the experiment 1; instead, for the experiment 2 and 3, at PND90, a group of female mice (n = 4 for exp. 2 and n = 4 for exp. 3) was killed in estrus and the others (n=6 for exp. 2) in diestrus.

Male and female mice were deeply anesthetized with a mixture of ketamine-xylazine (respectively 100 mg/mL and 20 mg/mL*)* and perfused through the heart with saline solution (0.9%) until vessels were completely blood-free, and then with the fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3). The brains were removed and stored in a freshly prepared paraformaldehyde solution for 2 h at 4 °C, followed by several washings in 0.01 M saline phosphate buffer (PBS). Finally, they were stored in a 30% sucrose solution in PBS at 4 °C, frozen in isopentane pre-cooled in dry ice at –35 °C, and stored in a deep freezer at –80 °C until sectioning.

Brains were serially cut in the coronal plane at 25 μm thickness with a cryostat, in four series. 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 a cryoprotectant solution (Watson et al., 1986) and stored at -20°C. One series was processed for kisspeptin immunohistochemistry using the free-floating technique. Brain sections were always stained in groups containing male and females sections, so that between-

assays variance could not cause systematic group differences.

Immunohistochemistry

Single-label immunohistochemistry

For experiment 1 and 2 the sections collected in the cryoprotectant solution were washed overnight in PBS at pH 7.3. The following day, sections were first washed in PBS containing 0.2 % Triton X-100 (PBS-T) for 30 min and then treated for blocking endogenous peroxidase activity (PBS solution containing methanol/hydrogen peroxide, 1:1, 20 min, at room temperature). Sections were then incubated with normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30 min and incubated overnight at 4 °C with a polyclonal rabbit anti-kisspeptin antibody (AC#566, a generous gift of drs. A. Caraty, I. Franceschini and M. Keller, Tours, France; diluted 1:10.000 in PBS-Triton X-100 0.2%). The following day, sections were incubated for 60 min in biotinilated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:200 at room temperature. The antigen-antibody reaction was revealed by 60 min incubation with biotin–avidin system (Vectastain ABC Kit Elite, Vector Laboratories, Burlingame, CA, USA). The peroxidase activity was visualized with a solution containing 0.400 mg/ml of 3,3'-diamino-benzidine (DAB, SIGMA-Aldrich, Milan, Italy) and 0.004% hydrogen peroxide in 0.05 M Tris-HCl buffer pH 7.6. Sections were mounted on chromallum-coated slides, air-dried, cleared in xylene and cover slipped with Entellan (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).

The AC#566 antibody was raised against the ten amino acid C-terminal of murine kisspeptin (amino acid residues 43–52, kp10, YNWNSFGLRY-NH2), which are required for activation of Gpr54). Mouse kp10 was coupled to BSA using glutaraldehyde and used as an immunogen in rabbits. Radioimmunoassay analysis and pre-adsorption controls showed that this antiserum is highly specific to mouse kp10: kisspeptin binding to the antisera is not inhibited by any one of different hypothalamic peptides including other RF-amide peptides (Clarkson et al., 2009; Franceschini et al., 2006).

We performed the following additional controls in our material: (a) the primary antibody was omitted or replaced with an equivalent concentration of normal serum (negative controls); (b) the secondary antibody was omitted. In these conditions, cells and fibers were totally

unstained.

Double-label immunofluorescence

For experiment 3, the sections were incubated for 24 h at 4 °C with two primary antibodies, one was always the AC053 antibody (polyclonal sheep anti-kisspeptin antibody, a generous gift of drs. A. Caraty, I. Franceschini and M. Keller, Tours, France; Franceschini et al., 2013), the second was one of the others listed in the Table 1. The primary antibodies were dissolved in a solution of PBS, pH 7.4, and containing 0.5% Triton X-100 (Merck, Darmstadt, Germany), 1% Normal Donkey Serum (Vector Laboratories, Burlingame, CA, USA), and 1% bovine serum albumin (BSA) (Sigma–Aldrich, Milan, Italy). Sections were washed and incubated, respectively, with solutions of appropriate secondary antibodies (included in Table 1). Sections were then cover slipped with antifade mounting medium Mowiol (Sigma–Aldrich, Milan, Italy).

Sections were observed and photographed with a laser scanning Leica TCS SP5 (Leica Microsystems) confocal microscope. Images were processed using Image J (version 1.46r, Wayne Rasband, NIH, Bethesda, MD, USA) and Adobe Photoshop CS4 (Adobe Systems). Only general adjustments to color, contrast, and brightness were made.

(insert Table 1 here)

Table 1: Primary and Secondary antibodies used in the double-label immunofluorescence assays

Marker	*Code*	*Source*	*Host*	*Dilution*	*Reference*
Primary Abs					
Kisspeptin	AC053	*A. Caraty*	*Sheep, pc*	1:2000	*Polling et al (2013)*
NkB	T4450	*Peninsula*	*Rabbit, pc*	1:2000	*Taziaux et al (2012)*
Vasopressin	64717	*ICN*	*Rabbit, pc*	1:8000	*Ferris et al (1997)*
nNOS	24287	*DiaSorin*	*Rabbit, pc*	1:3000	*Gillespie et al (2005)*
Oxytocin	AB911	*Millipore*	*Rabbit, pc*	1:8000	*Bean et al (2014)*
TH	22941	*Incstar*	*Mouse, mc*	1:8000	*Daadi & Weiss (1999)*
Secondary Abs					
Anti-sheep Alexa Fluor® 555	A21436	*Invitrogen*	*Donkey, pc*	1:500	
Anti-rabbit Alexa Fluor® 488	A21206	*Invitrogen*	*Donkey, pc*	1:500	
Anti-mouse Alexa Fluor® 488	A21202	*Invitrogen*	*Donkey, pc*	1:500	

mc, monoclonal antibody; pc, polyclonal antibody

Quantitative analysis (exp. 1 and exp. 2)

Selected standardized sections of comparable levels covering the arcuate hypothalamic nucleus (ARC, bregma -1.46 -1.70 mm), the anteroventral periventricular nucleus (AVPV, bregma 0.50 - 0.02 mm), the periventricular hypothalamic nucleus (PeN, bregma 0.14 - 0.22 mm), and the paraventricular hypothalamic nucleus (PVN, bregma -0.58 – 0.94 mm) were chosen (Paxinos and Franklin, 2001). For each animal, three (ARC), four (PVN) and six (AVPV, PeN) 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). Digital images were processed and analyzed by ImageJ (version 1.46r, Wayne Rasband, NIH, Bethesda, MD, USA). Measurements were performed within predetermined fields (region of interest, ROI) as follows:

– *Experiment 1: kisspeptin system distribution and male to female comparison*

Images were digitized by using a 10x (AVPV and PeN) or a 20x (ARC and PVN) objective. The ROI was a rectangular box of fixed size and shape covering a large part of each considered nucleus (350.000 μm^2 for AVPV; 284.000 μm^2 for ARC; 310.000 μm^2 for PeN, 380.000 μm^2 for PVN).

– *Experiment 2: estrous cycle observation*

Images were digitized by using a 40x (PVN and ARC) or a 20x (AVPV) objective. The PVN, in each selected section, was divided into four squares (each of 25.000 μm^2) to cover its full extension. 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 PVN by dividing it into four regions: dorso-medial, dorso-lateral, ventro-medial and ventro-lateral (Fig.4A). The ROI for ARC (49.000 μm^2) as well as that for AVPV (80.000 μm^2) was placed within the boundaries of the considered nuclei to fully cover the immunopositive region, using as reference the third ventricle to position the ROI always in the same orientation.

Kisspeptin immureactivity (cell bodies and processes) was measured by calculating in binary transformations (threshold function of the software) the fractional area (percentage of pixels) covered by immunoreactive elements of the images (as previously performed in our laboratory, Pierman et al., 2008; Plumari et al., 2002; Viglietti-Panzica et al., 1994). Due to differences in the immunostaining, the range of the threshold was individually adjusted for

each section, up to cover always the immunoreactivity of smallest fibers. The results obtained from each nucleus were grouped to provide mean (± S.E.M.) values. The statistical analysis was performed, using the SPSS 22.0 statistic software (SPSS Inc, Chicago, USA), and was undertaken using ANOVA with Student's *t*-test to analyze the Experiment 1 and with *post hoc* Bonferroni test for Experiment 2; values of $p \leq 0.05$ were considered significant.

Results

Experiment 1: kisspeptin system distribution and male to female comparison

– *Qualitative results*

In the brain of CD1 adult mice the distribution of kisspeptin immunoreactive cell bodies and fibers was similar to that of other previously described strains (Clarkson et al., 2009). As detailed in previous studies, AVPV and PeN (defined together as rostral periventricular area of the 3rd ventricle, RP3V; Herbison, 2008), and ARC nuclei show the larger clusters of kisspeptin-expressing cell bodies in females than in males. Accordingly, in CD1 mouse the two most consistent populations of kisspeptin neurons identified across serial brain sections were located in these two regions.

The first group of kisspeptin-positive neurons was present within the total extension of the RP3V and clustered near the ventricular wall, making difficult to precisely discern the limits of AVPV and PeN nuclei. Kisspeptin immunoreactive (kiss-ir) cells were strongly labeled and exhibited an oval or circular cell body provided with one or two dendritic processes. The RP3V included also a large number of kiss-ir fibers. They covered the whole region and extended both dorsally and laterally from the ventricle wall into the adjacent brain regions where kisspeptin cell bodies were not present. A large number of kiss-ir fibers were located in the ventral aspect of the lateral septum and, in particular, in the anterior portion of the BNST; on the contrary only few kisspeptin fibers were present within the medial septum (MS).

A second large population of kisspeptin positive cell bodies was observed caudally, within the ARC. The kisspeptin-ir positive neurons in ARC had round or oval cell bodies, whereas their processes were difficult to distinguish due to the high density of surrounding immunoreactive processes. In fact, the densest immunostaining of the kisspeptin system was observed within the ARC, where the plexus of immunoreactive fibers clearly outlined each

level of the nucleus.

A dense innervation of kiss-ir fibers was observed within the paraventricular nucleus (PVN). These fibers outlined the entire rostro-caudal extension of the PVN (Fig.1).

– *Quantitative results*

There was a visible difference in the extension of immunoreactivity (including both positive cell bodies and processes) with females (in diestrus) displaying a higher immunoreactivity than male CD1 mice (Fig.2). The qualitative differences were confirmed by the quantitative analysis showing significant sex differences for each examined nucleus (Fig.3). This difference was particularly evident for the AVPV and PeN ($p<0.001$; Fig.2A, B and Fig.3), but also for the ARC ($p<0.05$; Fig.2E, F and Fig.3) and for the amount of immunoreactive fibers in the PVN ($p<0.001$; Fig.2C, D and Fig.3).

Experiment 2: estrous cycle observation

Whereas at low magnification (Fig.1) no differences in immunoreactivity for kisspeptin was evident within the female PVN, at higher magnification (see Fig.4B, C) the distribution of PVN kiss-ir fibers appeared not homogeneous in particular when comparing the medial (corresponding to the parvocellular region) to the lateral (corresponding to the magnocellular one) PVN. The quantitative analysis performed at higher magnification (40x objective) subdividing the nucleus into four regions (see methods and Fig.4A) was subjected to a two-way-analysis of variance for repeated measures, being the position (lateral vs medial) and the phase of the estrous cycle (estrus vs diestrus) the two independent variables, and the ventral vs dorsal position the repeated measure. This analysis reported the following F values:

Position, $F(5,48)= 196.384$, $p<0.0001$

Cycle phase, $F(1,48)= 185.551$, $p<0.0001$

Interaction Position/Cycle phase, $F(5,1,48)=23.479$, $p<0.0001$

In both estrus and diestrus the two-by two comparison (Bonferroni test) revealed a significant difference for the medial vs lateral PVN innervation (estrus, $p<0.001$; diestrus, $p<0.001$); no significant differences were observed comparing dorso-lateral vs ventro-lateral PVN (estrus $p=0.412$; diestrus $p=0.633$), whereas the comparison of dorso-medial vs ventro-medial PVN reported significant differences (estrus, $p=0.01$; diestrus, $p<0.001$).

The comparison of estrus vs diestrus females revealed a significantly higher immunoreactive fractional area in estrus in comparison to diestrus in all the considered regions: medial PVN ($p<0.001$), in particular dorso-medial PVN ($p<0.001$) and ventro-medial PVN ($p<0.001$; Fig.4D), but also in the lateral PVN ($p=0.021$).

The results for ARC and AVPV in the same animals, showed a profound effect of estrous cycle on the kisspeptin immunoreactivity. The signal was higher in AVPV ($p<0.001$) and lower in ARC ($p=0.026$) in estrus in comparison to diestrus (Fig.4D).

Experiment 3: interaction between kisspeptin and different neuronal populations of the PVN

In adult CD1 estrus female mice the distribution of kisspeptin fluorescence immunoreactivity in PVN and ARC nuclei was similar to that described in experiment 1 and 2. In fact, within the PVN the immunofluorescence appeared not homogeneous with a higher concentration of kiss-ir fibers in the medial than to the lateral PVN (Fig.5A; Fig.6, left panels). In the caudal ARC we observed a large population of kisspeptin positive cell bodies and fibers (Fig.5B).

Coexistence NkB and kisspeptin: the immunoreaction for NkB revealed that there was a high expression of NkB immunoreactivity within ARC; while, in the PVN, the NkB signal was very low (Fig.5, middle panels). Merging the immunofluorescence for kisspeptin and NkB, we saw that kisspeptin strongly co-localize with NkB in the ARC but not in the PVN (Fig.5, right panels).

Kisspeptin and AVP, nNOS, OT and TH cell bodies: the four populations that we investigated were differently distributed within the PVN. The vasopressin (AVP)-containing neurons were clustered in the lateral part of the nucleus (magnocellular population) where kisspeptin fibers were very low. Only a few, scattered, small AVP cells were observed in the medial part of the PVN which was rich in kisspeptin fibers (Fig.6A). Interactions with kisspeptin fibers were very scarce in both regions. The neuronal nitric oxide synthase (nNOS)-ir cell bodies, even if present in all parts of the PVN, were mainly distributed in the lateral region of the nucleus (Fig.6B). Interactions with kisspeptin fibers were very limited. Contrary to the AVP system, the oxytocin (OT) cells were observed both in the lateral and in the medial PVN, including the ventro-medial part (Fig.6C). Interactions with kisspeptin fibers were possible both in the medial and in the lateral part. The tyrosine hydroxylase (TH)-ir cell

bodies were also scattered within the PVN, however they were particularly clustered in ventro-medial part, overlapping part of the denser innervation by kisspeptin fibers (Fig.6D).

Discussion

This study, performed in CD1 mice, confirms previous data indicating that the kisspeptin system in rodents is mainly clustered in a rostral (RP3V) and in a caudal (ARC) group of neurons. We confirm also the presence of a strong dimorphism (more cells and fibers in females than in males) in the RP3V as well as in the ARC. Previous studies (Clarkson and Herbison, 2006; Clarkson et al., 2009; Lehman et al., 2013) described the presence of kisspeptin fibers in several nuclei, including the PVN, whereas anterograde and retrograde tracing in normal adult female mice or in transgenic female mice showed that these fibers arise from ARC and AVPV (Yeo and Herbison, 2011; Yip et al., 2015). In the present study we demonstrate, for the first time, that the kisspeptin innervation is covering the entire extension of the PVN (suggesting that this nucleus is a major target for the peptide action in addition to the GnRH system), is sexually dimorphic (with females having a denser innervation than males), is not homogenously distributed within the nucleus, and, in females, it changes according to the phases of the estrous cycle.

As described in previous studies in female rat (Smith et al., 2006), kisspeptin immunoreactivity of female CD1 mice changes during the estrous cycle in a different way in the RP3V and in the ARC, showing highest value in RP3V during estrus, when the immunoreactivity is lowest in ARC. Similar changes were observed in the PVN, showing a higher density of positive fibers during the estrus. This suggests that the RP3V group could be the major source of the PVN kisspeptin fibers.

Kisspeptin neurons in RP3V and ARC have been directly related to the control of reproduction via the control of GnRH system (d'Anglemont de Tassigny and Colledge, 2010; Navarro and Tena-Sempere, 2011; Roseweir and Millar, 2009; Tsutsui et al., 2010). In addition, both KISS1 and gonadotropin inhibitory hormone (GnIH) positive cells were described in the ARC, DMH and PVN nuclei of non-human female primate suggesting a possible role for kisspeptin in the regulation also of the GnIH system (Smith et al., 2010). It is known that other neuropeptides like Dyn and NkB have been implicated in the regulation of pulsatile GnRH neurosecretion (Grachev et al., 2014; Lehman et al., 2010); moreover, a

higher co-expression of kisspeptin and NkB within ARC nucleus was previously described in mice both with ISH (Navarro et al., 2009) and immunofluorescence (Pineda et al., 2016). On the contrary, ISH showed that very few *Kiss1* neurons in the AVPV co-expressed NkB (Navarro et al., 2009). Our double-label immunofluorescence for kisspeptin and NkB confirmed the co-expression NkB/Kiss1 in ARC nucleus but showed a complete lack of NkB signal associated to Kisspeptin immunoreactivity in PVN. This confirms the hypothesis that the kisspeptin fibers observed in PVN should arrive chiefly from AVPV and PeN.

Recent tract-tracing studies in adult female mice demonstrated that only a subset of kisspeptin neurons are contacting the GnRH system (only ~36% of AVPV kisspeptin neurons are connected with GnRH neurons; (Kumar et al., 2015; Yip et al., 2015), thus suggesting that a large part of the kisspeptin neuronal population could have other targets. The large, sexually dimorphic innervation of PVN is probably one of the major targets, even if it is not directly related to reproduction, sexual behavior or puberty control. In fact, PVN plays a major role in other neuro-endocrine functions controlled by distinct neuronal subpopulations (Swanson and Sawchenko, 1980; Maniam and Morris, 2012; Bosch, 2013; Kovács, 2013; Handa and Weiser, 2014; Pyner, 2014; van Swieten et al., 2014; Sladek et al., 2015).

Therefore, the presence of positive fibers along the entire extension of the PVN suggests that kisspeptin could be implicated in the regulation of many of the physiological activities controlled by the PVN.

Our quantitative analysis performed at high enlargement magnification showed that, even if covering the entire nucleus, the innervation of mouse PVN by kisspeptin fibers was heterogeneous. In fact, the density of kisspeptin fibers was higher in the medial than in lateral PVN. While the lateral part of PVN contains magnocellular neurons chiefly projecting to the posterior pituitary (where they release oxytocin and arginine vasopressin into the blood), the medial part of PVN is characterized by the presence of different types of parvocellular neurons that can be identified for the presence of several neurotransmitters, neuropeptides, and enzymes involved in the synthesis of neurotransmitters [i.e corticotropin-releasing hormone (CRH; Wang et al., 2011), thyrotrophin releasing hormone (TRH; Kadar et al., 2010), tyrosine hydroxylase (TH; Ruggiero et al., 1984), neural nitric oxide synthase (nNOS; Gotti et al., 2004, 2005), vasopressin (Caldwell et al., 2008), somatostatin (Tan et al., 2013)].

In this study we compared by double-immunofluorescence the distribution of some of these PVN neuronal populations and that of kisspeptin fibers. On the basis of our results we can assume that AVP- and nNOS-containing neurons were not strongly related to kisspeptin

system. Instead, the presence of several OT and TH positive neurons in the medial PVN, where the concentration of kiss-ir fibers is massive, is suggestive of a possible interrelation between these systems.

On the other hand, kisspeptin could play a role in the regulation of both AVP and OT neurons. In fact, in rat, Rao et al. (2011) showed that kisspeptin significantly increased AVP and OT mRNA expression, and, very recently, an in situ hybridization study revealed that in rat diestrus female Kiss1r is co-expressed in subpopulations of oxytocin neurons of the medial part of the PVN (Higo et al., 2016). Moreover, in AVPV more than half of kisspeptin immunoreactive neurons express also TH immunoreactivity (Clarkson and Herbison, 2011).

A question about the putative functions of the kisspeptin innervation in the PVN arises from the literature on the Kiss1r distribution within the mammalian brain. At our knowledge only three studies detailed the neuroanatomical distribution of Kiss1r. One was performed with low-resolution autoradiography for GPR54 mRNA (Lee et al., 1999): the figures are showing the presence of the mRNA in the periventricular hypothalamus, but no details are provided to eventually identify the signal within the PVN. A second study (Herbison et al., 2010) used a transgenic GPR54 LacZ knock-in mouse model to detail a map of GPR54-expressing elements within the mouse brain. In this study, in addition to regions where are located GnRH neurons, the authors reported the existence of several nuclei expressing GPR54 LacZ where no kisspeptin fibers have been detected (i.e. hippocampus, supramamillary and pontine nuclei), and regions (as the PVN or the supraoptic nucleus) where the transgene was not expressed even if kisspeptin fibers have been described. Finally, a recent in situ hybridization study detailed the presence of Kiss1r in several hypothalamic and extrahypothalamic regions including the PVN (Higo et al., 2016). Therefore, there is some disagreement on the presence of Kiss1r especially in the PVN that may depend on technical issues, but could also indicate the existence of other ligands for Kiss1r and other receptors for kisspeptin (Herbison et al., 2010).

Kisspeptin belongs to the family of RF-amide peptides and it shows high binding activity to neuropeptide FF receptors (FF1 and FF2, also known as GPR74 and 147; Oishi et al., 2011). The distribution of these two receptors has been studied by autoradiography for mRNA in the rat brain (Liu et al., 2001); in particular, FF1 is widely distributed within the hypothalamus, including the PVN. Thus, it is possible that a subpopulation of kisspeptin neurons (mainly from the rostral hypothalamus) project to the PVN to activate the FF1

receptor. This could also be one possible explanation for the anorexigenic effect of centrally injected kisspeptin (Stengel et al., 2011).

In conclusion, we demonstrated that, in CD1 mice, the kisspeptin fibers cover the entire extension of the PVN and that this innervation is sexually dimorphic (with females having a denser innervation than males). Moreover, we confirmed that kisspeptin system in ARC was sexually dimorphic also in CD1 mice. In addition, our data show a heterogeneity in the innervation of the PVN by the kisspeptin, with changes during the estrous cycle (higher density of positive fibers during the estrus), and, finally, they suggest that the source of this innervation may be located in the rostral group of Kisspeptin neurons.

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Author contributions

MM performed experiments, analyzed data and wrote the paper. AF, DM, and GP performed experiments and analyzed data. GCP and SG designed experiments, wrote and supervised the paper.

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660 **Figure legends**

661

662 **Fig.1. Distribution of kisspeptin immunoreactive fibers within PVN of CD1 (diestrus)**
663 **female mice, from rostral to caudal sections.**
664 Kisspeptin fibers outline the boundaries of the paraventricular nucleus (PVN) and run along
665 the wall of the third ventricle towards the optic chiasm (ox). A moderate innervation is
666 present also within the supraoptic nucleus (SON), while the suprachiasmatic nucleus (SCN) is
667 almost totally empty of immunoreactivity, as well as the ventromedial hypothalamic nucleus
668 (VMH).
669 f = fornix; * = third ventricle; mt = mammillothalamic tract. Scale bar = 100 μm.
670
671
672 **Fig.2. Dimorphic kisspeptin system in the hypothalamic nuclei**
673 Comparison of the distribution of kisspeptin fibers in diestrus female (left panels) and male
674 (right panels) CD1 mice. The expression of kisspeptin is higher in females than in males. **A,**
675 **B)** rostral periventricular area of the third ventricle (RP3V). **C, D)** paraventricular nucleus
676 (PVN). **E, F)** arcuate nucleus (ARC).
677 ox = optic chiasm; aca = anterior commissure; f = fornix; * = third ventricle; SCN =
678 suprachiasmatic nucleus; VMH = ventromedial hypothalamic nucleus; DMH = dorsomedial
679 hypothalamic nucleus; ME = median eminence. Scale bar = 100 μm.

680

681

682 **Fig.3. Quantitative study of kisspeptin system in the hypothalamic nuclei**
683 Histograms representing the fractional area covered by kisspeptin-immunoreactive structures
684 (mean ± SEM) in arcuate nucleus (ARC), anteroventral periventricular nucleus (AVPV),
685 periventricular nucleus (PeN) and paraventricular nucleus (PVN) of male (black bars) and
686 female (white bars) CD1 mice. Male showed a lower immunoreactivity in comparison with
687 female group.
688 ** $p<0.01$, *** $p<0.001$ different from males. ($p<0.05$; Student's *t*-test).
689
690

Fig.4. Effect of estrous cycle on the kisspeptin immunoreactivity in adult mice

A) The representative subdivision of PVN in four quadrants (DM, dorso-medial; DL, dorso-lateral; VM, ventro-medial; VL, ventro-lateral). **B, C)** The comparison of kisspeptin immunoreactive fibers within paraventricular nucleus (PVN) of female CD1 mice in estrus (**B**) and in diestrus (**C**) phases respectively. **D)** Histograms representing the fractional area covered by kisspeptin-immunoreactive structures (mean ± SEM) in PVN (dorso-medial, dorso-lateral, ventro-medial, ventro-lateral), anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC) of female adult mice in estrus (dark grey) and in diestrus (light grey) phases.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ different from diestrus.

($p<0.05$, Bonferroni test). Scale bar = 50 μm.

Fig.5. Kisspeptin and NkB expression within PVN and ARC in adult CD1 female mice

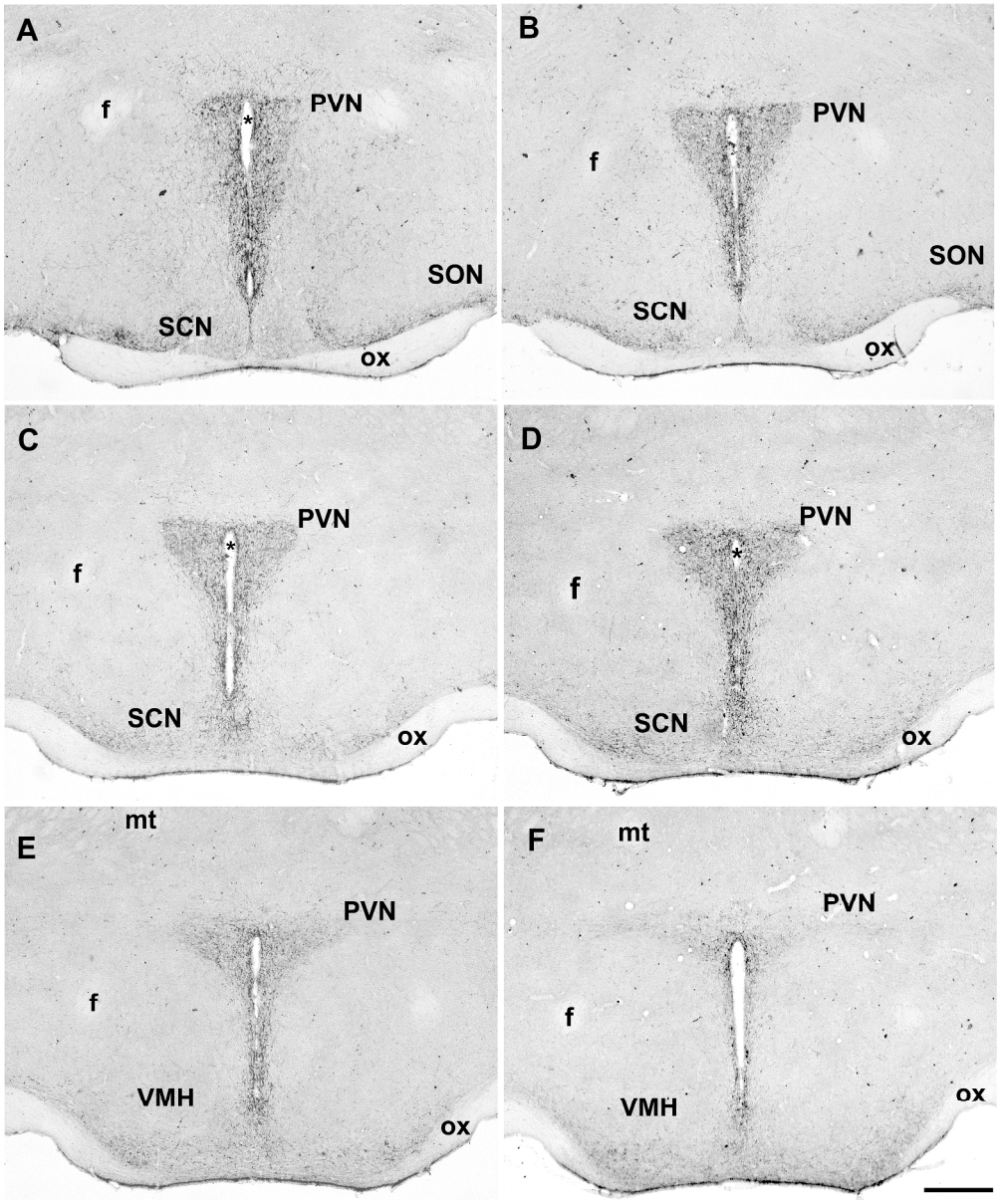
Kisspeptin (Kiss; red) and Neurokinin B (NkB; green) immunoreactivity in a coronal section of paraventricular nucleus (PVN) **(A)** and arcuate nucleus (ARC) **(B)** of the adult CD1 female mice in estrus phase. A dense kisspetin expression is evident in both nuclei, respect to NkB signal: is clearly present only in ARC; in PVN the NkB signal is very low. The merge indicates that the kisspeptin and Nkb immunoreactivity co-localise with the ARC nucleus.

Fig.6. Kisspeptin and PVN cellular populations (AVP, nNOS, OT, TH)

Coronal section of the adult CD1 female mice paraventricular nucleus (PVN) in estrus phase. It is possible to observe the relations with kisspeptin (Kiss; red) and different PVN neuronal populations: **A)** vasopressin (AVP; green); **B)** neuronal nitric oxide synthase (nNOS, green); **C)** oxytocin (OT, green); **D)** (TH, green). Note that the totally AVP and the majority of nNOS neuronal cell bodies were distributed in lateral PVN, where the concentration of Kisspeptin

718 fibers was lower; in the medial PVN instead were presents a conspicuous number of OT and
719 TH cell bodies.

720
721
722 **Table 1. Primary and Secondary antibodies used in the double-label**
723 **immunofluorescence assays**
724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PVN
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Fig.1_new

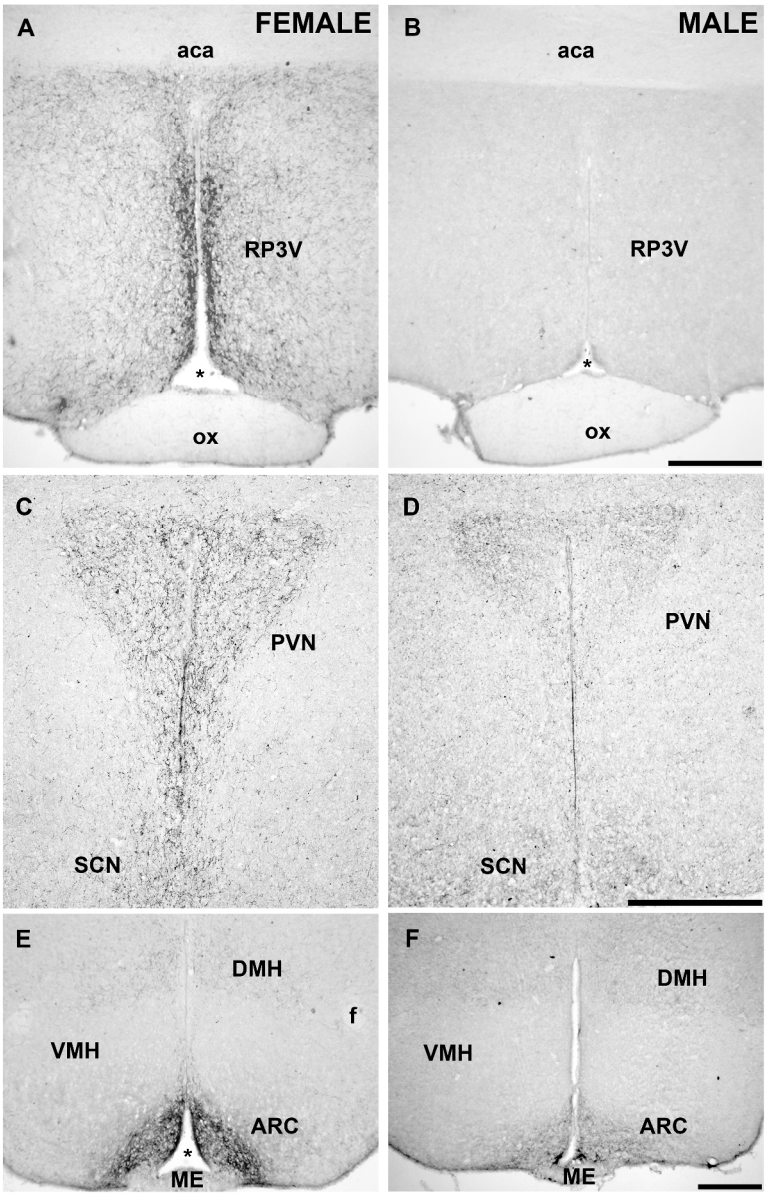
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Fig.2_new

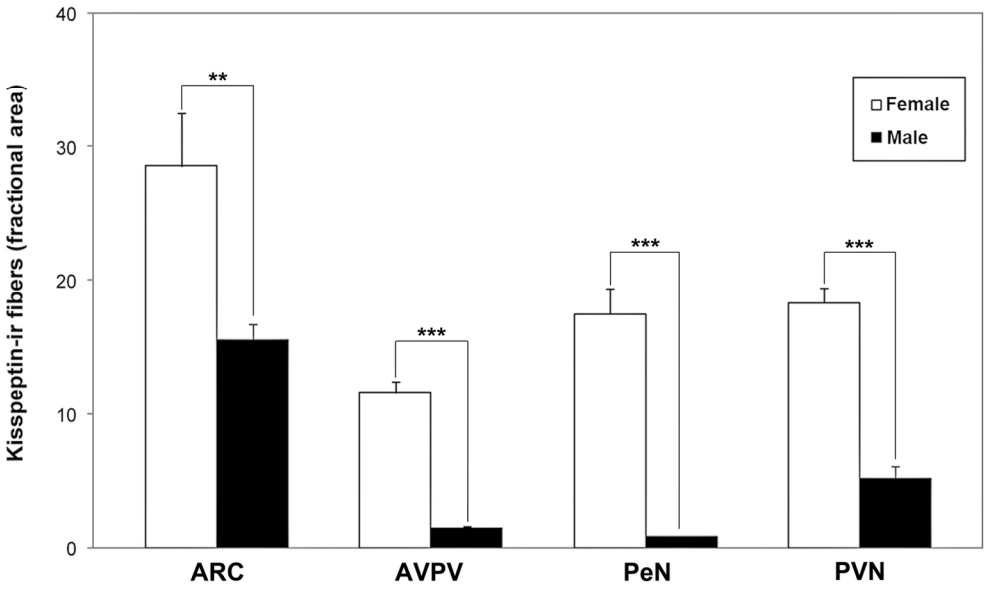

Fig.3_new

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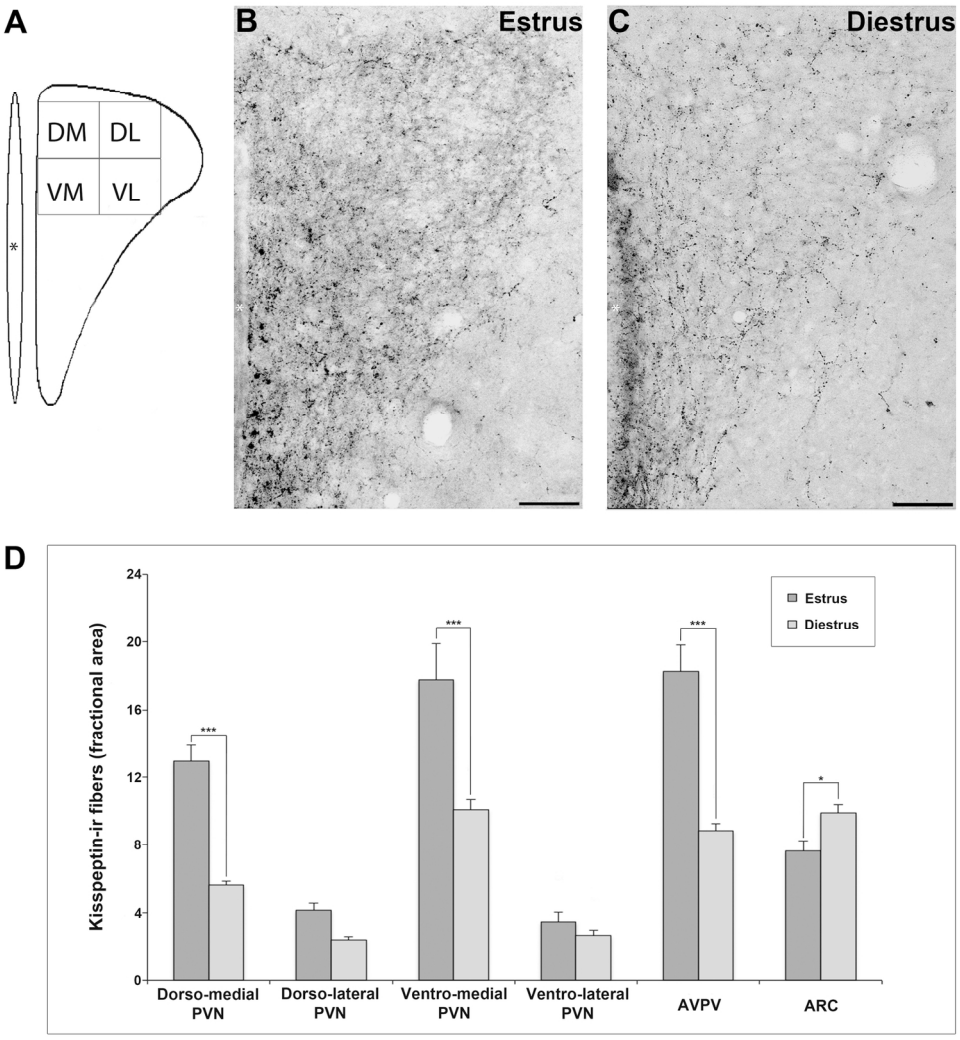

Fig.4_new

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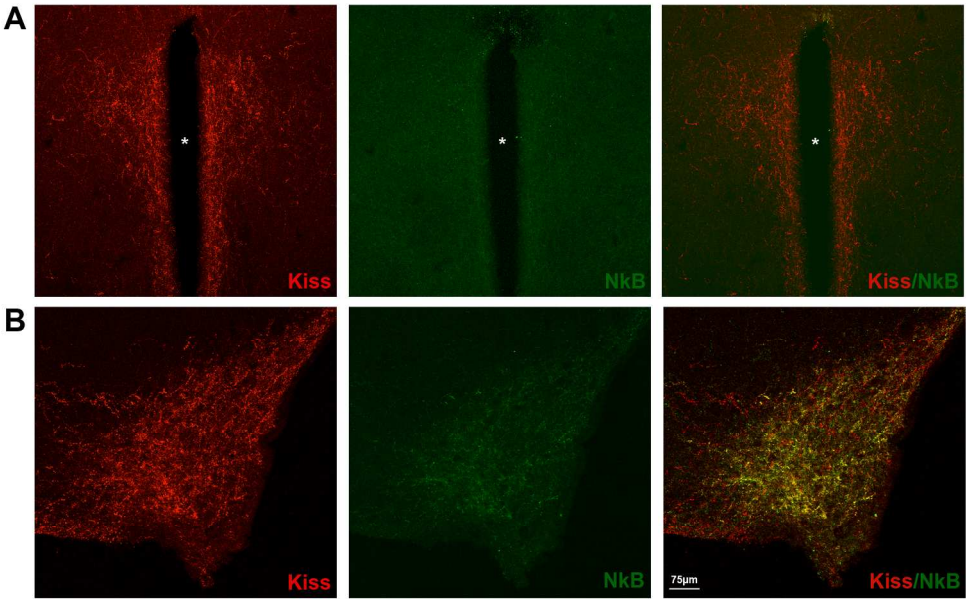

Fig.5_new

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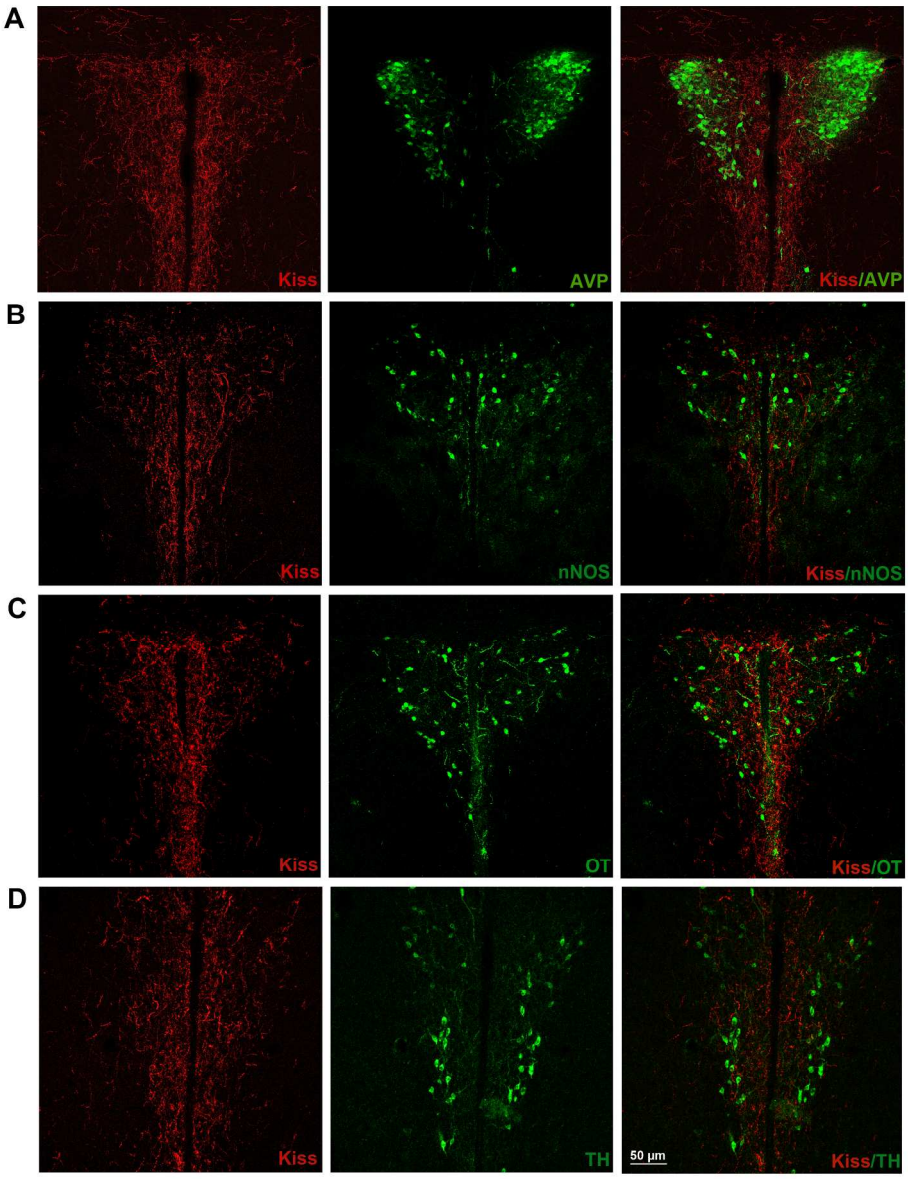

Fig.6_new

209x271mm (300 x 300 DPI)

Marker	*Code*	*Source*	*Host*	*Dilution*	*Reference*
Primary Abs					
Kisspeptin	AC053	*A. Caraty*	*Sheep, pc*	1:2000	*Polling et al (2013)*
NkB	T4450	*Peninsula*	*Rabbit, pc*	1:2000	*Taziaux et al (2012)*
Vasopressin	64717	*ICN*	*Rabbit, pc*	1:8000	*Ferris et al (1997)*
nNOS	24287	*DiaSorin*	*Rabbit, pc*	1:3000	*Gillespie et al (2005)*
Oxytocin	AB911	*Millipore*	*Rabbit, pc*	1:8000	*Bean et al (2014)*
TH	22941	*Incstar*	*Mouse, mc*	1:8000	*Daadi & Weiss (1999)*
Secondary Abs					
anti sheep Alexa Fluor® 555	A21436	*Invitrogen*	*Donkey, pc*	1:500	
anti rabbit Alexa Fluor® 488	A21206	*Invitrogen*	*Donkey, pc*	1:500	
anti mouse Alexa Fluor® 488	A21202	*Invitrogen*	*Donkey, pc*	1:500	

mc, monoclonal antibody; pc, polyclonal antibody