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Kisspeptin innervation of the Hypothalamic Paraventricular Nucleus: key for reproductive and metabolic control. Effects of postnatal exposure to Genistein.

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Sigue tus sueños,

ellos saben el camino.

CHAPTER 1

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CHAPTER 1

General Introduction

Overview of the Neuroendocrine System

The organization and coordination of the body is under the control of both endocrine and the central nervous system (CNS). While CNS is very effective in short term control, acting within milliseconds or seconds, the endocrine system, through the hormones, may exert a relatively short-term (seconds to minutes), or long-term (day to weeks) control. Primary objective of the endocrine system is the maintenance of *homeostasis* with the preservation of constant conditions in the internal environment in the body, despite huge changes in the external environment. In fact, different functions such as body temperature, respiration rate, or glucose plasmatic levels are maintained within a range of normal values despite frequently changing in the external conditions.

In addition, the endocrine system plays a central role in the proliferation of species through the control of gamete development, ovulation timing, birth, lactation, tissue growth, and postnatal development.

The activity of endocrine system is highly coordinated by CNS, chiefly by the hypothalamic area. The neuroendocrine hypothalamus extends from the optic chiasma to the mammillary

bodies and it is organized into four rostral-to-caudal regions (preoptic, supraoptic, tuberal, and mammillary region), and three medial-to-later areas (periventricular, medial and lateral area). The periventricular hypothalamus contains four distinct cell clusters: the paraventricular nucleus (PVN), the arcuate nucleus (ARN), the suprachiasmatic nucleus (SCN) and the periventricular nucleus (PeN). The medial hypothalamus includes the medial preoptic nucleus (MPN), the anterior hypothalamic nucleus (AH), the dorsomedial nucleus (DMH), the ventromedial nucleus (VMH) and the mammillary nuclei. The lateral hypothalamus consists of the lateral preoptic area (POA) and the lateral hypothalamic area (LHA) (Fig. 1).

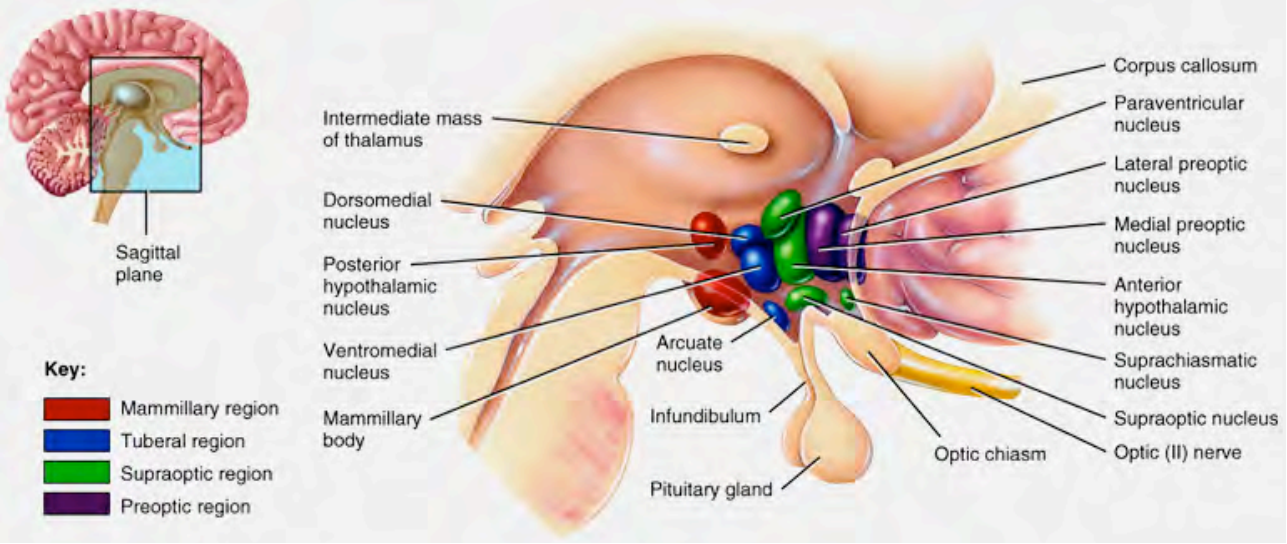

Figure 1: Organization of hypothalamic nuclei (www. humanbrainfacts.org/hypothalamus.php).

In rodents, the hypothalamus originates from the neuroepithelium lining the third ventricle, due to neuronal proliferation in a lateral to medial sequence accompanying the progressive shrink of the third ventricle (Markakis 2002). These morphological changes may reflect the generation of hypothalamic nuclei (Szarek et al. 2010). The preoptic neuroephitelial patch give rise to the lateral POA between the embryonic day 11 (E11) and E14, the medial POA and the preoptic periventricular zone between E12 and E16 (Markakis 2002). The MPN is generated in two waves showing an outside-to-inside gradient between E13 and E16, with the central part created between E14 and E18. LHA nucleus develops between E12-E14, while the supraoptic nucleus (SON) is generated between E13 and E14. At last, AH, PVN and SCN nuclei arise respectively in the range E12-E15, E13 and E15, and E11-E17 (Markakis 2002).

The relay centre for CNS and endocrine system are *neurosecretory cells,* neurons able to secrete peptidic hormones (Guillemin 2005) scattered throughout hypothalamus. They can be

distinguished into two populations according to their morphology: the parvocellular system, and the magnocellular neurosecretory system. The parvocellular system consist of at least six classes of neurons regulating the anterior pituitary gland through: corticotropin-releasing hormone (CRH); gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibiting hormone (GnIH); growth hormone-releasing hormone (GHRH) and somatostatin (SS); thyrotropin-releasing hormone (TRH); and dopamine (DA). These neurotransmitters, synthesized in the neuronal cell body, located in the hypothalamus, are packaged into secretory vesicles, released directly into the portal system in the median eminence. Through this capillary system, they reach their target cells in the anterior pituitary gland, and stimulate the releases of their corresponding hormones. Besides, the magnocellular neurosecretory system consists of neuronal cells, whose axons project directly into the posterior pituitary gland, secreting two different neurotransmitters: arginine-vasopressin (AVP) and oxytocin (OT) (Calas 1985). On the other hand, the endocrine system is able to affect the CNS since the peripheral endocrine organs release hormones into the bloodstream that play a crucial homeostatic regulatory function. The target hormones are involved also in a feedback circuit to neuroendocrine cells in the hypothalamus in order to increase or decrease hormonal stimulation and maintain homeostasis. The pituitary hormones regulating endocrine glands may therefore influence target tissue to elicit specific effects.

References

Calas, A. 1985. 'Morphological correlates of chemically specified neuronal interactions in the hypothalamo-hypophyseal area', *Neurochem Int*, 7: 927-40.

Guillemin, R. 2005. 'Hypothalamic hormones a.k.a. hypothalamic releasing factors', *J Endocrinol*, 184: 11-28.

Markakis, E. A. 2002. 'Development of the neuroendocrine hypothalamus', *Front Neuroendocrinol*, 23: 257-91.

Szarek, E., P. S. Cheah, J. Schwartz, and P. Thomas. 2010. 'Molecular genetics of the developing neuroendocrine hypothalamus', *Mol Cell Endocrinol*, 323: 115-23.

Paraventricular Nucleus

The hypothalamic Paraventricular Nucleus (PVN) is a relatively small division of the vertebrate forebrain accounting for only about 1% of the brain (Swanson 1995). Despite its small size, PVN is one of the most important autonomic control centers in the brain, with neurons playing essential roles in controlling stress, metabolism, growth, reproduction, immune and other more traditional autonomic functions (gastrointestinal, renal and cardiovascular) (Ferguson, Latchford, and Samson 2008). This nucleus, in the rat, consists of approximately 100,000 neurons in a volume of about 0.5 mm^3, arranged in a wing shape structure along the dorsal portion of the third ventricle, in the anterior region of the hypothalamus.

1. Cytoarchitecture of PVN

The functional cyto- and chemo-architecture of the PVN has been extensively studied using neuroanatomic tracing and immunohistochemical techniques, particularly in the rat. Recent studies have pointed out anatomic similarities and dissimilarities between rats and mice (Biag et al. 2012).

Gurdjian (1927) described for the first time the cytoarchitectural organization of the anterior hypothalamus. He identified small cells in a medial area, medium and large cells in a dense lateral group and a dorsal group with unique Nissl staining properties in the area that was later included in the PVN. Then, densely packed neurons with large cell bodies (magnocellular neurons) with projections to the posterior pituitary gland were identified (Bargmann (1949) and Bargmann and Scharrer (1951)). Later studies, (Armstrong et al. 1980) (Swanson and Kuypers 1980) using tract tracing, Golgi impregnation, and immunocytochemical approaches clarified the cyto- and chemo-architecture of the PVN (as reviewed by (Handa and Weiser 2014)).

Based on such parameters, the neurons of the rodent PVN were grouped into subdivisions associated with specific functions (Biag et al. 2012), in particular we can divide the neurons of the PVN into three main types (Fig. 2):

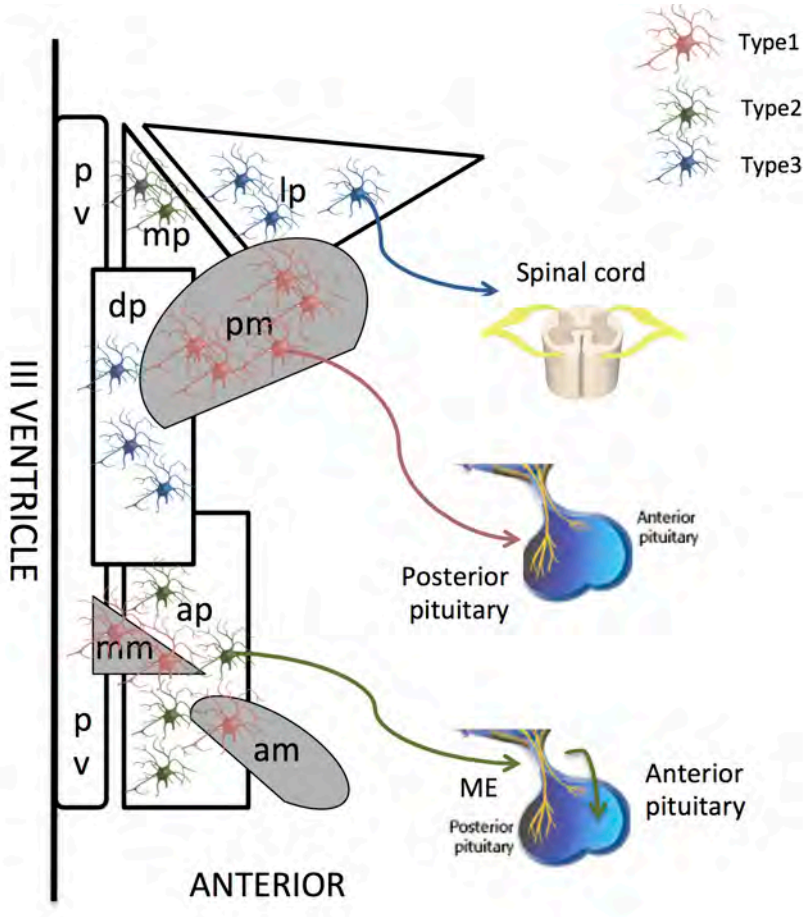

Figure 2: Schematic diagram illustrating the classical view of the organization of the paraventricular nucleus of the hypothalamus. Schematic representation of magnocellular (shaded areas) and parvocellular divisions of the PVN (modified from Swanson et al., 1981). There is a strong correlation between the areas of PVN and cellular types allocated. The magnocellular neurons (type 1) are located in anterior magnocellular (am), medial magnocellular (mm) and posterior magnocellular (pm), which synthesize and release vasopressin (AVP) or oxytocin (OXY). These neurons project to the posterior pituitary where they secrete hormones into the general circulation. The parvocellular neuroendocrine cells (type 2) synthesize corticotropin releasing hormone (CRH) and thyrotropin releasing hormone (TRH) (as well as other factors), allocated in medial parvocellular (mp) and anterior parvocellular (ap) part of PVN. These neurons project to the median eminence, where they release these hypophysiotropic factors into the pituitary portal circulation. Finally, the parvocellular preautonomic neurons (type 3), that project to autonomic control centres of the medulla and spinal cord, are present in dorsal parvocellular (dp) and lateral parvocellular (lp) part of PVN (modified from Pyner et al., 2009).

Type 1. Neurosecretory magnocellular neurons. These neurons project directly to the posterior pituitary where they release hormones (e.g. oxytocin, OXT, and vasopressin, AVP) into the general circulation.

The magnocellular neurons of the PVN are mostly distributed into two distinct but adjoining areas. Anteromedially the medial magnocellular neurons produce OXT while the lateral magnocellular part of the nucleus includes AVP-producing neurons surrounded by a ring of OXT neurons. The majority of these neurons project to the neurohypophysis (Rhodes, Morrell, and Pfaff 1981; Sawchenko and Swanson 1983; Vandesande and Dierickx 1975).

Type 2. Neurosecretory parvocellular neurons. Small to medium sized neurons, which send their axons to the median eminence where they release neuropeptides [i.e. corticotropin-releasing hormone (CRH), AVP, thyrotropin releasing hormone (TRH), somatostatin] into the hypothalamo–hypophyseal portal system to control the secretion of anterior pituitary hormones such as ACTH, TSH and growth hormone (GH).

These neurons are located in two main areas of the PVN, anterior and medial parvocellular division. The anterior parvocellular division extends from the rostral boundary of the PVN to the rostral boundary of the medial magnocellular division, lateral to the periventricular area. The medial parvocellular division lies laterally to the periventricular area and medially to the medial magnocellular division. In both divisions, parvocellular neurons are chemically very heterogeneous and express a broad list of neuropeptides including, but not limited to: OTX, AVP, TRH, CRH, angiotensin II, cholecystokinin, cocaine and amphetamine-regulated transcript (CART), enkephalin, galanin, and somatostain (Ceccatelli et al. 1989; Healy and Printz 1984; Plotsky and Sawchenko 1987; Suzuki et al. 2001).

Type 3. Long-projecting neurons. These neurons send their axons to the brainstem and the spinal cord (regions involved in controlling autonomic and somatosensory functions). Moreover, they can be further subdivided by: phenotype, afferent inputs, cell size, density, and dendritic morphology (Armstrong et al. 1980; Kiss, Martos, and Palkovits 1991; Rho and Swanson 1989).

These neurons of the PVN are allocated in three main regions: the dorsomedial cap, the ventral PVN and the posterior PVN (Blair et al. 1996; Ferguson, Latchford, and Samson 2008; Swanson 1977; Swanson and Kuypers 1980). The dorsomedial cap projects to the lateral gray horn of the

spinal column (intermediolateral cell columns). The ventral and posterior regions project to a wide range of spinal cord and brainstem regions, as the dorsal vagal motor nucleus, nucleus of the solitary tract, periaqueductal gray, dorsal raphe, locus coeruleus, parabrachial nucleus and ventrolateral reticular nucleus (Hosoya et al. 1991; Luiten et al. 1985; Pyner and Coote 2000; Saper, Swanson, and Cowan 1976; Shafton, Ryan, and Badoer 1998; Shapiro and Miselis 1985). These descending connections utilize glutamate, gamma-aminobutyric acid (GABA) or various neuropeptides as CRF, AVP and OXT (Jansen, Wessendorf, and Loewy 1995; Milner et al. 1993; Sawchenko and Swanson 1983; Sofroniew and Schrell 1982).

The PVN regulation of the sympathetic outflow depends by the integration of inhibitory and excitatory neurotransmitter activation of more than 30 neurotransmitters identified within the PVN (Sawchenko and Swanson 1983). Some of them are briefly descripted here.

GABA

Only two neuronal populations of PVN are inhibitory neurons: gamma-aminobutyric acid (GABA) and neuronal nitric oxide synthase (nNOS) neurons. While nNOS plays an important role in the tonic inhibitory regulation of sympathetic outflow, GABA, provides a substantial synaptic innervation of the PVN (Decavel and Van den Pol 1990), and together influence the tonic regulation of sympathetic outflow (Zhang and Patel 1998).

The peri-PVN area contains numerous GABAergic neurons projecting to the PVN (Roland and Sawchenko 1993). This architecture suggests that inputs to the PVN are first processed, or filtered, locally before contacting the PVN neurons. Glutamate injections in this region near PVN result in GABA-dependent inhibition of PVN neurons (Boudaba, Szabo, and Tasker 1996). This effect is mediated mainly by $GABA_A$ receptors, although GABAergic presynaptic neurotransmission, as well as glutamatergic one, could regulate the $GABA_B$ receptors (Benarroch 2005). GABAergic neurones are a target for nitric oxide, whose release from alternate neuronal sources modulates sympathetic outflow by enhancing synaptic GABAergic function to depress PVN-sympathetic activity (Pyner 2009).

nNOS

nNOS neurons reside within PVN, mainly in the magnocellular compared to parvocellular part

(Stern 2004) (Watkins, Cork, and Pyner 2009). Nitric oxide (NO) has a major role in regulating PVN neurons; it inhibits both magnocellular AVP (Kadekaro 2004) and sympathoexcitatory PVN neurons (Li et al. 2004; Qadri et al. 2003). NO is a gas, so it readily diffuses across cell membranes to activate cGMP mechanisms to exert its biological effect (Kennedy 2000). NO is produced by the enzyme nitric oxide synthase, which has three isoforms: neuronal (nNOS), endothelial and inducible; only nNOS is present in the PVN. Studies about nNOS neurons outputs showed that they accounts for 6–10% of PVN-spinally projecting neurones (Watkins, Cork, and Pyner 2009; Weiss et al. 2001) and 12–25% of the PVN– rostral ventrolateral medulla projecting neurones (Kantzides and Badoer 2005; Li, Zhang, and Stern 2003). Moreover, it seems that nNOS potentiates local GABAergic synaptic inputs by acting to enhance release of GABA from presynaptic terminals (Chen and Pan 2006; Li et al. 2004; Zhang, Esenturk, and Walker 2001).

TH

Tyrosine hydroxylase (TH) is a key enzyme in catecholamine biosynthesis. Although it is also present in noradrenergic and adrenergic fibers, the only labelled cell bodies in the hypothalamus belong to dopaminergic cells (Sawchenko and Swanson 1981). Immunohistological studies demonstrated that, in rats, approximately 500 TH–ir cells are present in PVN, located within the periventricular the anterior, medial and lateral parvocellular regions of the PVN (Sawchenko and Swanson 1981). Moreover, their distribution is similar to that of somatostatin-stained cells (Sawchenko and Swanson 1983).

TRH

Thyrotropin-releasing hormone (TRH) regulates many physiological functions, but it is best known as the central regulator of the hypothalamic-pituitary-thyroid (HPT) axis (Reichlin 1989). In rats, the cell bodies of TRH neurons lie in the periventricular and medial parvocellular subdivisions while their axons reach the median eminence (Fekete et al. 2000; Ishikawa et al. 1988; Merchenthaler and Liposits 1994). There, they release TRH into the portal capillary system, which is conveyed to the anterior pituitary gland (Fekete and Lechan 2007), where regulates the TSH release. Interestingly, there are important differences in distribution of TRH neurons in rodents. In fact, while in mice, these neurons are located in the compact part and in the neighboring region in the mid-level of the PVN and are absent from the anterior and posterior parts and in periventricular zone of the PVN (Kadar et al. 2010), in rats only a few

TRH neurons can be identified in the magnocellular division (Lechan and Jackson 1982). Although TRH neurons in mice have been found intermingled with vasopressin- and oxytocin-IR neurons, in the compact part, these are no co-localization between TRH and AVP or OXT (Kadar et al. 2010). In rats (Fekete and Lechan 2007) as in mice (Kadar et al. 2010), TRH neurons co-express CART, both the perikarya in the mid-level of the PVN neurons and in their axon terminals innervating the median eminence. The co-localization TRH-CART, in mice was only rarely observed also in the anterior and posterior parts of the PVN (Kadar et al. 2010). TRH secretion is continuously regulated by neurons located in the ARC (POMC and NPY neurons) belonging to the circuit modulating food intake and by, the hormone leptin, released by the fat tissue. During fasting, thyroid activity decreases to reduce energy expenditure in humans and mice, and this is associated to low leptin levels with a reduction of TRH gene expression and TRH formation (Perello, Stuart, and Nillni 2006).

Moreover, THR release is stimulated by α-MSH (the major product of POMC cleavage in this system) predominantly via melanocortin 4 receptor (MC4R), whereas the aguti-related peptide (AgRP, co expressed in NPY neurons) inhibits TRH release mainly by acting on MC3R (Kim et al. 2002). NPY projections from ARC to PVN (Elias et al. 1999) promote suppression of the activity of TRH neurons with a positive energy balance trough via NPY Y1 and Y5 receptors (Fekete et al. 2002) and NPY reduces also α-MSH-induced increase in TRH production by decreasing the amount of α-MSH and α-MSH-induced CREB phosphorylation in the PVN (Cyr et al. 2013).

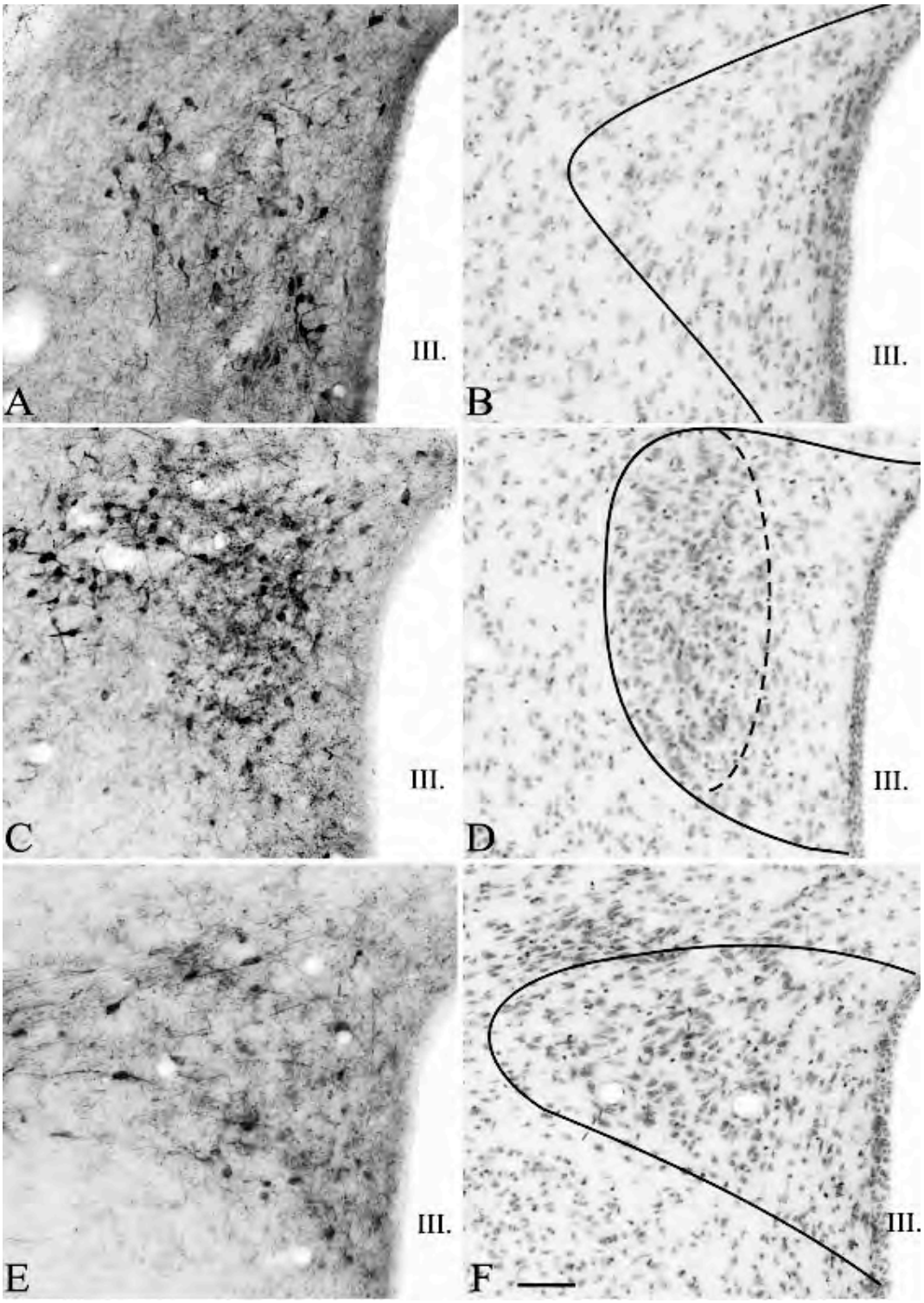

Figure 3: Distribution of TRH neurons in the PVN of mice. A,C,E: Low-magnification images illustrate TRH-IR neurons at three anteroposterior levels of the PVN. B,D,F (Kadar et al., 2010).

CRH

One population of PVN parvocellular neurons express CRH. This population is located laterally to TRH neurons, which are in turn found lateral to the periventricular somatostatin neurons. Beside being expressed in many brain regions where it is implicated in the modulation of a wide variety of behavioral and neurobiological functions (Bale and Vale 2004), CRH is the regulator of hypothalamic–pituitary–adrenal axis through direct projections to the median eminence via the tuberoinfundibular system. In fact it is the main regulator of ACTH secretion in the anterior pituitary (Antoni et al. 1983).

CRH can be released together with AVP and OXT (Sawchenko, Swanson, and Vale 1984; Whitnall and Gainer 1988), because AVP and OXT enhance the secretagogue properties of CRH at the anterior pituitary gland (Bilezikjian and Vale 1987; Gillies, Linton, and Lowry 1982; Rivier and Vale 1983). Interesting, AVP and OXT can stimulate ACTH secretion even in the absence of CRH (Gillies, Linton, and Lowry 1982) (Schlosser et al. 1994); but their direct injection in PVN inhibits HPA responses (Landgraf and Neumann 2004; Windle et al. 1997). This indicates that these neuropeptides may have regulatory effects within the PVN through their local release (paracrine action), that are very different from their actions on the anterior pituitary (Handa and Weiser 2014). Moreover, it was shown that hypothalamic CRH neurons in the mouse do not possess detectable levels of OXT or AVP receptors (Chen et al. 2017), but a very few parvocellular CRH neurons express mRNA transcript for the oxytocin receptor (OXTR) postsynaptically (Dabrowska et al. 2011; Dabrowska et al. 2013). In fact, it seems that CRH and OXT neurons have processes that intermingle within the PVN enabling a possible cross-talk between these neural populations. Recently, a study showed that OXT could inhibit spontaneous excitatory synaptic transmission onto CRH neurons providing a possible mechanism by which OT suppresses CRH neuronal activity (Jamieson, Nair, and Iremonger 2017).

OXT

Oxytocin (OXT) is a nonapeptide that plays a very important role in sociability, parturition, lactation and food intake (as described later). It is produced exclusively in the PVN, supraoptic (SON), and intermediate accessory nuclei (Sawchenko and Swanson 1983). Within PVN, OXT neurons are classified in magnocellular and parvocellular neurons, that differ by size and shape, subnuclear location, amount of OXT production, and involvement in distinct circuitries and functions (Armstrong et al. 1980; Sawchenko and Swanson 1983; Swanson and Kuypers 1980).

Magnocellular OXT neurons provide systemic OT supply by release into the blood via the posterior pituitary. They project also to the forebrain, including the nucleus accumbens (Dolen et al. 2013; Knobloch et al. 2012; Ross et al. 2009) and the central nucleus of amygdala (Knobloch et al. 2012). On the other hand, parvocellular OXT neurons project to distinct brainstem nuclei and different regions of the spinal cord (SC) (Sawchenko and Swanson 1983) modulating: cardiovascular functions, breathing, feeding behavior, and nociception (Atasoy et al. 2012; Mack et al. 2002; Petersson 2002). Moreover, it is still unknown how parvocellular OXT neurons are incorporated into the entire OXT system and functionally interact with magnocellular OXT neurons (Eliava et al. 2016). Notably, a very recent study identified a small group of parvocellular OXT neurons (~30) that simultaneously project collaterals to magnocellular OXT neurons in the SON and to deep layers of the SC. It has been proposed that that they represent a new type of OXT neurons, which coordinate central and peripheral inhibition of nociception and pain perception, and hence, play a role in promoting analgesia (Eliava et al. 2016).

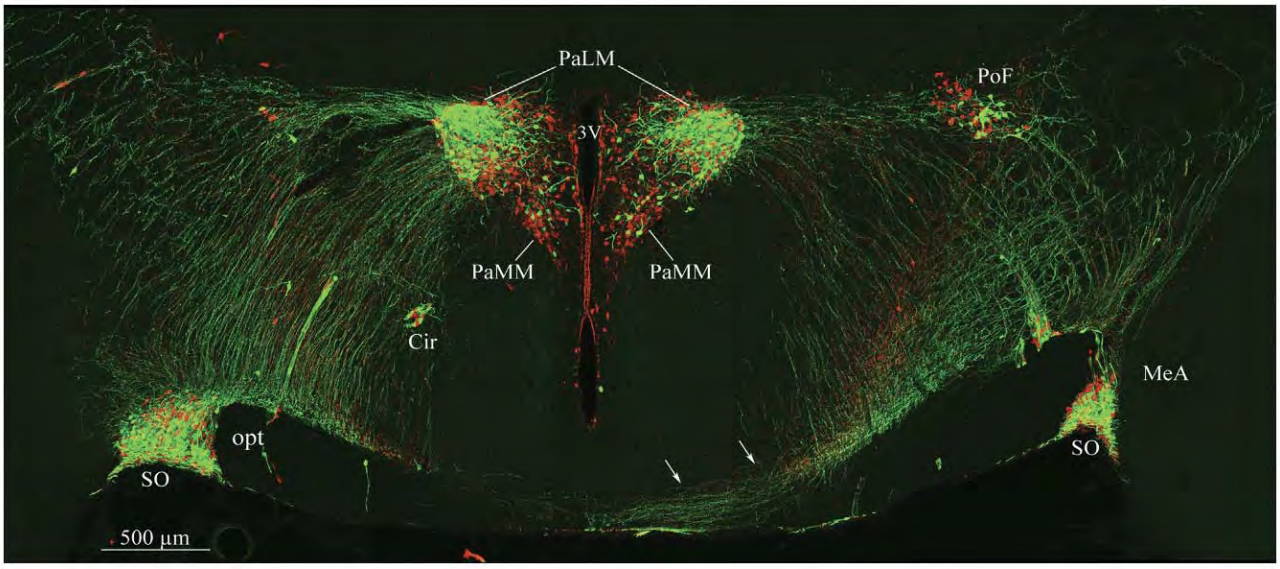

Figure 4: Immunocytochemically stained OXT (red) and AVP (green) neurons in coronal section. Both the medial (PaMM) and lateral (PaLM) divisions of the PVN are show, with PaLM containing a majority of AVP neurons (but with some surrounding OXT neurons, and the PaMM a majority of OXT neurons (with some scattered AVP neurons).opt, optic tract; Cir, nucleus circularis; PoF, posterior fornical nucleus; MeA, medial amygdaloid; 3V, third ventricle; SO, supraoptic (Armostrong, 2014).

AVP

Vasopressin (AVP, also known as antidiuretic hormone, ADH) is a nonapeptide very close to the structure of OXT. AVP is expressed in both magnocellular and parvocellular neurons within the PVN. In the parvocellular neurons, AVP may co-localize with CRH, especially after adrenalectomy (Kiss 1985; Sawchenko, Swanson, and Vale 1984; Whitnall and Gainer 1988). These parvocellular neurons can also co-express somatostatin, or other regulatory hormones and project to the neurohemal zone of the median eminence, where they regulate the anterior pituitary secretion (Sawchenko et al. 1996). Furthermore, AVP/CRH neurons receive stress signals trough brain stem and limbic pathway. In particular, the neurons allocated in medial PVN display glucocorticoid receptors for direct feedback (de Souza and Franci 2010). Moreover, *in situ* hybridisation has revealed that 40% of the parvocellular neurones project to the spinal cord. These neurons, in physiological conditions, have been found mostly in the lateral and ventral part of the medial subdivision and, in a small number, in the dorsal parvocellular one (Hallbeck and Blomqvist 1999). In this contest, the role of AVP is to control the cardiovascular functions (Riphagen and Pittman 1989). On the other hand, magnocellular AVP neurons give rise to the hypothalamus-neurohypophyseal system (HNS) and project to the posterior pituitary where they release AVP into the blood stream. The activity of these magnocellular AVP neurons is regulated by blood osmolarity and cardiovascular reflexes (Leng, Brown, and Russell 1999), in fact hypovolemia and hypotension activate AVP secretion from magnocellular via humoral and neural mechanisms (Potts et al. 2000).

2. Afferent inputs to PVN

Parvocellular PVN neurons receive and integrate direct inputs from brainstem neurons in order to initiate responses to systemic stressors. This area of PVN is densely innervated with noradrenergic (NA) fibers (Hornby and Piekut 1989; Kitazawa, Shioda, and Nakai 1987; Swanson et al. 1981) mostly arising from the locus coeruleus, nucleus of the solitary tract (NTS) and ventrolateral medulla. NA fibers from the medullary project to AVP cells in the lateral PVN. While NA fibers from the locus coeruleus reach the medial periventricular zone (Cunningham and Sawchenko 1988) (Swanson et al., 1986; Cunningham and Sawchenko, 1988), where target both TRH (Diano et al., 1998) and CRH (Kitazawa et al, 1987) neurons. CRH neurons receive a dense NA innervation from NTS (Hornby and Piekut 1989; Kitazawa, Shioda, and Nakai 1987). Notably, stress increase NA release in the PVN, inducing an increase of CRH release in the median eminence and of ACTH plasma levels (Chen, Du, and Wang 2004; Pacak 2000).

In addition, also glucagon-like peptide-1 (GLP-1) neurons from the NTS project directly to parvocellular CRH neurons (Sarkar et al. 2003; Tauchi et al. 2008), which highly express GLP-1 receptor (Gu et al. 2013).

Also serotonin fibers have been largely observed within the PVN. They arise from the median and dorsal raphe nuclei of the brainstem (Sawchenko and Swanson 1983) to stimulate the PVN parvocellular neurons (Van de Kar and Blair 1999). Indeed, parvocellular neurons expressing serotonin (5-HT) receptors (Zhang et al. 2002) are required to induce the activation of the HPA axis (Heisler et al., 2007). For instance, 5-HT1A (Rossi et al. 2010), and 5HT-7 receptors modulate the release of corticosterone and ACTH (Garcia-Iglesias et al. 2013). Beside its excitatory effect, 5-HT fibers inhibit GABAergic synaptic transmission in the PVN (Lee et al. 2008). Interestingly, the effect of 5-HT on PVN neurons depend on where afferent fibers terminate and is regulated by gonadal steroid hormones, e.g. the 5-HT excitatory effect on PVN is desensitized by estradiol treatment (Handa and Weiser 2014).

Parvocellular neurons in the PVN receive substantial input from other limbic areas. The bed nucleus of the stria terminalis (BnST) is the main inhibitor of parvocellular neurons in the PVN. It is a complex group of several related subnuclei, extensively projecting to the PVN (Cullinan, Herman, and Watson 1993). The majority of the PVN-projecting neurons in BnST are GABAergic (Cullinan, Herman, and Watson 1993). Beside BnST, also the central and medial amygdaloid nuclei project directly to parvocellular neurons (including median eminence and spinal projecting groups (Gray, Carney, and Magnuson 1989)), as well as to AVP and OXT neurons (Oldfield et al. 1985). Although, other brain regions as the hippocampus, prefrontal cortex, medial amygdala and lateral septum inhibit PVN, they always require an intermediary synapse on GABAergic neurons in BnST and peri-PVN (Dong, Petrovich, and Swanson 2001).

The suprachiasmatic nucleus (SCN) projects to parvocellular and subparaventricular zone of the PVN: therefore, these projections give a direct access to the circadian clock for the PVN circuits. In particular, the SCN dorsomedial shell, which consists of AVP neurons, is largely responsible for the PVN inputs (Vrang, Larsen, and Mikkelsen 1995), while the SCN ventrolateral core (including mainly vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide neurons) project to the subparaventricular zone (Hermes et al. 2009).

3. An overview of the role of PVN on food-intake control

Two different neuronal populations localized in the ARC regulate the central control of food-intake. The anabolic neurons are characterized by the co-expression of agouti-related peptide (AgRP) and neuropeptide Y (NPY) while the catabolic neurons co-express pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). These neurons are sensitive both to endocrine and to peripheral signals of energy balance. For instance, ghrelin, a gastric hormone, stimulates appetite and increases activity of NPY (Betley et al. 2015; Chen et al. 2015; Kamegai et al. 2000), while leptin, a adipose satiety hormone, stimulates POMC (Cowley et al. 2001; Elias et al. 1999) and inhibits NPY neurons (Morrison et al. 2005). Interestingly, these two populations project to PVN, the most important center of metabolic control. PVN results, thus, strongly implicated in the feeding circuit, since it integrates inputs from these two ARC cell populations. Moreover, PVN modulate feeding behavior also through the action of several pituitary hormones, including CRH and TRH, both indirectly via effects on energy expenditure, and directly through the HPA-axis (van Swieten et al. 2014).

Peptides released from NPY (AgRP) and POMC neurons (as α-melanocyte-stimulating hormone, α-MSH) in ARC may have opposed influence on parallel downstream targets, in particular on PVN (reviewed in (Webber, Bonci, and Krashes 2015)). A key element in the control of feeding behavior is melanocortin-4 receptors (MC4Rs), whose activation results in a reduction of fat mass via a suppression of food intake and elevation of energy expenditure (Benoit et al. 2000; Fan et al. 1997). Pharmacological studies identified the PVN as the putative site of action of MC4Rs. In fact AgRP released from NPY neurons antagonize (Hagan et al. 2000; Liu et al. 2012; Nijenhuis, Oosterom, and Adan 2001; Ollmann et al. 1997) and α-MSH released from POMC neurons agonize MC4Rs (Fan et al. 1997). MC4Rs are strongly expressed not only in the PVN, but also in other brain region, including the amygdaloid complex, the nucleus of the lateral olfactory tract, the dorsal medial hypothalamus (DM), and the lateral hypothalamus (LH)(Balthasar et al. 2005). A recent work showed that MC4R expressing neurons in PVN are the main second-order target for orexigenic circuit. In fact, both NPY and POMC projections from ARC play a significant role in activating satiety-promoting MC4R neurons within PVN (Garfield et al., 2015). Shan and colleagues (Shah et al. 2014), using a viral approach to determine the role of MC4Rs explicitly on PVN neurons, demonstrated that OXT, CRH and AVP neurons no express MCA4R, but certainly the PVN MC4R-expressing neurons that regulate feeding are $vGLUT^{+}$ and single-minded homolog 1 protein $(SIM1)^{+}$ (Shah et al. 2014). By the way, SIM1 is a transcription factor required for the proper development of the hypothalamus, and importantly, it is expressed in the large majority of neurons in the PVN. Very

interesting are the projections of these MC4R neurons. The third-order node through which the central melanocortin system regulates feeding behavior seems to be the lateral parabrachial nucleus (LPBN), an integration center for energetic balance visceral signals modulating food consumption within a number of contexts (Carter et al. 2013). Garfield and colleagues hypothesized that MC4R neurons of PVN promote satiety via their excitation of unidentified anorectic glutamatergic LPBN neurons (Garfield et al. 2015). Moreover, glutamatergic input of these MC4R neurons also reaches a small proportion of NTS. However, these projections are likely to contribute to other aspects of MC4R neurons of PVN regulated physiology since they have no effect on food consumption (Garfield et al. 2015).

NPY neurons from ARC project on OXT neurons in the PVN (Atasoy et al. 2012). Despite many evidences, the role of PVN OXT neurons in directly regulating the feeding behavior has recently been challenged (Garfield et al. 2015; Sutton et al. 2014; Wu, Clark, and Palmiter 2012). In fact, these neurons do not receive GABAergic input from NPY neurons (Garfield et al., 2015). Moreover, optogenetic stimulation of OXT neurons in PVN fails to occlude the feeding control from NPY neurons to PVN, suggesting that OXT neurons do not regulate feeding in general (Garfield et al. 2015). Probably the effect of OXT within food-intake control is mediated in part by projections to the NTS, where OXT release seems to lead to the consumption of smaller meals, enhancing the hindbrain response to gut-derived satiety signals such as cholecystokinin, CCK (Blevins and Ho 2013). Interestingly, mice lacking either OXT (Camerino 2009) or its receptor (Takayanagi et al. 2008) exhibit only a modest, late-onset obesity phenotype, and OXT neuron activation does not reduce food intake in fasted mice (Atasoy et al. 2012).

Other two neuronal populations of the PVN are involved in feeding control: CRH and TRH neurons. In rats, central injections of CRH inhibit food intake and reduce body weight (Vettor et al. 2002). In addition, leptin affects CRH expression. In fact leptin infusion increases CRH, but treatments with CRH antagonist attenuate the leptin-induced reduction of food intake and body weight (Masaki et al. 2003). Probably this inhibition of CRH in the PVN is due to direct or indirect ghrelin control (e.g. mediating by GABA) on NPY neurons. It is possible that ghrelin increases the release of NPY onto GABAergic nerve terminals adjacent to CRH neurons (Arvat et al. 2001).

Furthermore, injections of TRH reduce food intake (Masaki, 2003) and promote a negative energy balance (al-Arabi and Andrews 2003). Moreover, leptin enhances TRH secretion. In the

PVN, TRH neurons are activated by α-MSH and inhibited by NPY and AgRP. Interestingly, as TRH hyperactivity correlates with hypertension in rats, it has been suggested that the leptin-TRH pathway may be involved in the link between obesity and elevated blood pressure (Garcia et al. 2002).

The classical model is that PVN is implicated within food-intake circuit only as a satiety structure, however, molecular markers of TRH and pituitary adenylate cyclase activating polypeptide (PACAP) project to and synapse onto ARC with NPY neurons (Krashes et al. 2014). Moreover, chemogenetic activation of TRH or PACAP neurons in PVN produced significant food intake increases in sated animals, and viceversa simultaneous chemogenetic inhibition of downstream NPY neurons, demonstrating a hunger-promoting effect trough TRH-NPY neurons circuit. Finally, chemogenetic silencing of TRH neurons drastically reduced food intake during the dark cycle in calorically deficient mice (Krashes et al. 2014).

4. Interactions among gonadal steroids and PVN circuits

Reproduction is a physiological process vital for the survival of the species regulated by a neuroendocrine axis that involves the hypothalamus, the anterior pituitary gland and the gonads (HPG). This axis works concomitantly with the HPA axis in human and non-human primate. It is responsible for hormonal changes (*gonadarche*), which are the basis for the puberty onset and the reproductive ability acquirement. From perinatal period to childhood, HPG axis is under the control of both stimulatory and inhibitory mechanisms that respectively keep it active until neonatal phase (to permit the completely development of gonads), and inactive until juvenile period (Buck Louis et al. 2008). Human puberty onset, as well as in other mammals, is characterized by the re-activation of HPG axis induced by an increase of the GnRH pulsatility in the hypothalamus. GnRH secretion leads a cascade of events: primary it induces the secretion of gonadotropins FSH and LH from the anterior pituitary. Gonadotropins in turn stimulate gonads in both sexes: in females FSH induces follicle development, whereas LH stimulates maturation of the ovarian follicle and ovulation (in human these events finally trigger the start of menstrual cycle, menarche). In males FSH supports spermatogenesis and LH stimulates the synthesis and secretion of Androstenedione and Testosterone from testicular Leydig cells. As a result of their complete activation, gonads secrete gonadic hormones: estrogens, progestins and androgens. These gonadal steroids bind specific intracellular receptors that act as ligand-activated transcription factors (Evans 1988): the estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR). These receptors belong to the superfamily of steroid receptors that

includes also the glucocorticoid (GR), and the mineralocorticoid receptors (MR), Gonadal and adrenal steroid hormone receptors are well expressed within the neural circuitry of the PVN and this implies that the PVN is central to the HPG and HPA regulation, and that these two axis are related to each other. In fact, activation of the stress response and of PVN neurons is reported to be higher in females than males (Seale et al. 2004; Viau et al. 2005), and this could be due to variations in estrogen levels that occur across the estrous cycle of females (Iwasaki-Sekino et al. 2009; Viau and Meaney 1991).

The characterization of localization and phenotypes of neurons expressing the different forms of ER (ERß and ERα) in PVN is essential to understand how estradiol may modulate PVN circuits. In vivo and in vitro studies demonstrated that the action of estrogens is modulated by the ERß. This receptor is, in fact, involved in the up regulation of CRH mRNA transcripts in the PVN in gonadectomized male mice (Nomura et al. 2002), and in *in vitro* models, ERß bind to the CRH promoter following estradiol treatment (Chen et al. 2008) and to enhance CRH promoter activity (Miller et al. 2004). In the PVN ERß is expressed in some populations of CRH, AVP, and OXT neurons (Hrabovszky et al. 2004; Laflamme et al. 1998). CRH-ERß double labeled neurons are a smaller population in the medial parvocellular PVN (13% of CRH-ir neurons) and a larger one in the caudolateral PVN (60-90% of CRH-ir neurons (Laflamme et al. 1998)). In mice, the percentage of neurons that colocalize CRH-ERß is lower than in rats (Oyola et al. 2017).

ERß is also expressed in the maiority of AVP-ir neurons in the PVN. In fact, in female rat, a large number of AVP neurons express ERß, 66,14% (Suzuki and Handa 2004), and this percentage increase at 88-99% in ovarectomized rats (Hrabovszky et al. 2004). AVP-ERß co-expression is sexually dimorphic in the PVN of mice in which the number of colocalized neurons is higher in females than in males (Oyola et al. 2017). AVP and ERß co-expression is considered important for the control of water balance. In fact, in rats, ERß-ir levels in AVP neurons within PVN and SON are strongly decreased after cellular dehydration caused by salt loading (Somponpun and Sladek 2004).

ERß is also expressed in some OXT neurons (Hrabovszky et al. 2004). The percentage of OXT cells that are ERβ-positive in rat is currently debated. Previous studies indicated that a percentage of 40% of OXT cells in the ventromedial parvocellular PVN, of 15–20% in the caudal parvocellular PVN, while no cells were double labeled in the rostral PVN (Laflamme et al. 1998). However a recent study showed that the highest OXT/ERß colocalization is in the rostral PVN, a region composed of the medial and anterior parvicellular parts of the PVN (Oyola et al. 2017). In mice, 80% of OXT neurons in the PVN co-express ERß (Oyola et al. 2017).

Many studies explained this strong relationship between OXT and ERß. Studies of the 90's demonstrated that OXT promoter was under estrogen regulation (Burbach et al. 1994; Richard and Zingg 1990). Moreover, a more new study showed that the androgen metabolite and ERß selective ligand, 5α androstan-3ß, 17ß-diol (3ß-diol), increases OXT mRNA levels in the rat PVN and drives the OXT promoter *in vitro* (Hiroi et al. 2013). A possible effect of this relationship is to reduce anxiety-like behaviors and HPA axis responses to restraint stress in female rats (Kudwa, McGivern, and Handa 2014).

In addition, studies with antagonist of ERß (as tamoxifen) demonstrated that the activation of this receptor reduces the gain of the HPA axis response to an acute stressor (Oyola et al. 2012). Moreover, adrenal steroids can influence the expression of ERß. In fact, elevate adrenal steroid levels following a stressor may act to increase ERß expression within the PVN thereby dampening HPA axis reactivity to a subsequent stressor (Handa and Weiser 2014).

ERα mRNA is almost absent in the magnocellular nuclei (Laflamme et al. 1998) and also immunohistochemistry confirmed the presence of ERbeta and the absence of ERalfa (Merchenthaler et al. 2004). Nonetheless, ERα is present in brain regions projecting to the PVN: peri-PVN, BnST, medial preoptic area, lateral septum and hippocampus (Suzuki and Handa 2004). Notably, ERα acts on HPA axis activity. In fact, ERα agonist (as propylpyrazoletriol) treatment increases CORT and ACTH response to restraint stressors (Liu et al. 2012; Lund et al. 2005; Weiser and Handa 2009). However, Weiser and Handa (Liu et al. 2012; Lund et al. 2005; Weiser and Handa 2009) demonstrated that, in rat, ERα is localized in GABAergic peri-PVN neurons, where it is coexpressed with GAD67. These cells are likely to innervate the parvocellular cells of the PVN (Di et al. 2005). Recently, Oyola and colleagues (Oyola et al. 2017) demonstrated, in the PVN, also the presence of a small population of cells expressing ERα and that a low number of ERß cells co-express also ERα. The number of colocalized ERα/ERß cells is close to 30% in the cPVN, suggesting that these two receptors might be working at two different levels. The authors suggest that the ERα/ERß-EGFP cells detected at this level are non-neuroendocrine and hence presumably involved in modifying autonomic functions. A previous study showed that in rat approximately 50% of pre-autonomic neurons in PVN express ERß (Stern and Zhang 2003). These data on a different distribution of two subtype of ER and the low colocalization within PVN suggest that there might be circumstances where one receptor subtype is recruited more than another, perhaps due to differences in the type and availability of ligand, thereby shifting physiological responses in a context-dependent manner (Oyola et al. 2017).

Within the PVN, magnocellular and parvocellular neurons express, together with the classical ERs, also the more recently discovered G protein-coupled ER 1 (GPER 1), formerly referred to as G protein-coupled receptor 30 (GPR30) (Brailoiu et al. 2007; Carmeci et al. 1997; Filardo and Thomas 2012; Hazell et al. 2009; Srivastava and Evans 2013). This receptor is involved in physiological and pathological events regulated by estradiol in the central nervous, immune, reproductive and cardiovascular systems (Garcia et al. 2002). In fact, the GPCR activation in the PVN, but also in SON, has been demonstrated in a number of ways. These include increases in neuronal immediate early gene (e.g., c-fos) activation, changes in electrophysiological characteristics or in neuropeptide mRNA or protein levels (e.g., by ISHH, ICH or content of push–pull perfusates or microdialysates), and alterations in a number of physiological end-points such as plasma VP, OXT, CRH and ACTH release, water and energy homeostasis, cardiovascular parameters, nociception and behavior (for a review, see (Hazell et al. 2012)). Many studies suggest that the expression of GPCRs is largely related to the co-expression of different neuropeptides or enzymes within PVN, as with 5-HT_{1A}R, OXT, CRH, nNOS in the PVN (Grassi et al. 2017; Hazell et al. 2009; Xu et al. 2009). Effectively, experimental studies demonstrated that GPER1 plays a role in the estradiol-modulated activity and release of these hormones. For instance, the selective GPER1 agonist G1 is sufficient to produce the desensitization of 5-HT_{1A}R signaling and knocking down GPER1 prevents estradiol-induced desensitization (Xu et al. 2009) Furthermore, GPER involvement in the regulation of nNOS activity, together with the classical ERs, was observed by analyzing NADPH-diaphorase in the SON and PVN with use of a selective GPER agonist (G1) or in combination with G15, a selective GPER antagonist (Grassi et al. 2017).

References

al-Arabi, A., and J. F. Andrews. 2003. 'Thyrotropin releasing hormone (TRH) potentiates the metabolic effect of norepinephrine (NE) in warm-acclimated lean and obese rats', *Biomed Sci Instrum*, 39: 547-53.

Antoni, F. A., M. Palkovits, G. B. Makara, E. A. Linton, P. J. Lowry, and J. Z. Kiss. 1983. 'Immunoreactive corticotropin-releasing hormone in the hypothalamoinfundibular tract', *Neuroendocrinology*, 36: 415-23.

Armstrong, W. E., S. Warach, G. I. Hatton, and T. H. McNeill. 1980. 'Subnuclei in the rat hypothalamic paraventricular nucleus: a cytoarchitectural, horseradish peroxidase and immunocytochemical analysis', *Neuroscience*, 5: 1931-58.

Arvat, E., M. Maccario, L. Di Vito, F. Broglio, A. Benso, C. Gottero, M. Papotti, G. Muccioli, C. Dieguez, F. F. Casanueva, R. Deghenghi, F. Camanni, and E. Ghigo. 2001. 'Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone', *J Clin Endocrinol Metab*, 86: 1169-74.

Atasoy, D., J. N. Betley, H. H. Su, and S. M. Sternson. 2012. 'Deconstruction of a neural circuit for hunger', *Nature*, 488: 172-7.

Bale, T. L., and W. W. Vale. 2004. 'CRF and CRF receptors: role in stress responsivity and other behaviors', *Annu Rev Pharmacol Toxicol*, 44: 525-57.

Balthasar, N., L. T. Dalgaard, C. E. Lee, J. Yu, H. Funahashi, T. Williams, M. Ferreira, V. Tang, R. A. McGovern, C. D. Kenny, L. M. Christiansen, E. Edelstein, B. Choi, O. Boss, C. Aschkenasi, C. Y. Zhang, K. Mountjoy, T. Kishi, J. K. Elmquist, and B. B. Lowell. 2005. 'Divergence of melanocortin pathways in the control of food intake and energy expenditure', *Cell*, 123: 493-505.

Benarroch, E. E. 2005. 'Paraventricular nucleus, stress response, and cardiovascular disease', *Clin Auton Res*, 15: 254-63.

Benoit, S. C., M. W. Schwartz, J. L. Lachey, M. M. Hagan, P. A. Rushing, K. A. Blake, K. A. Yagaloff, G. Kurylko, L. Franco, W. Danhoo, and R. J. Seeley. 2000. 'A novel selective melanocortin-4 receptor agonist reduces food intake in rats and mice without producing aversive consequences', *J Neurosci*, 20: 3442-8.

Betley, J. N., S. Xu, Z. F. H. Cao, R. Gong, C. J. Magnus, Y. Yu, and S. M. Sternson. 2015. 'Neurons for hunger and thirst transmit a negative-valence teaching signal', *Nature*, 521: 180-85.

Biag, J., Y. Huang, L. Gou, H. Hintiryan, A. Askarinam, J. D. Hahn, A. W. Toga, and H. W. Dong. 2012. 'Cyto- and chemoarchitecture of the hypothalamic paraventricular nucleus in the C57BL/6J male mouse: a study of immunostaining and multiple fluorescent tract tracing', *J Comp Neurol*, 520: 6-33.

Bilezikjian, L. M., and W. W. Vale. 1987. 'Regulation of ACTH secretion from corticotrophs: the interaction of vasopressin and CRF', *Ann N Y Acad Sci*, 512: 85-96.

Blair, M. L., D. Piekut, A. Want, and J. A. Olschowka. 1996. 'Role of the hypothalamic paraventricular nucleus in cardiovascular regulation', *Clin Exp Pharmacol Physiol*, 23: 161-5.

Blevins, J. E., and J. M. Ho. 2013. 'Role of oxytocin signaling in the regulation of body weight', *Rev Endocr Metab Disord*, 14: 311-29.

Boudaba, C., K. Szabo, and J. G. Tasker. 1996. 'Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus', *J Neurosci*, 16: 7151-60.

Brailoiu, E., S. L. Dun, G. C. Brailoiu, K. Mizuo, L. A. Sklar, T. I. Oprea, E. R. Prossnitz, and N. J. Dun. 2007. 'Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system', *J Endocrinol*, 193: 311-21.

Buck Louis, G. M., L. E. Gray, Jr., M. Marcus, S. R. Ojeda, O. H. Pescovitz, S. F. Witchel, W. Sippell, D. H. Abbott, A. Soto, R. W. Tyl, J. P. Bourguignon, N. E. Skakkebaek, S. H. Swan, M. S. Golub, M. Wabitsch, J. Toppari, and S. Y. Euling. 2008. 'Environmental factors and puberty timing: expert panel research needs', *Pediatrics*, 121 Suppl 3: S192-207.

Burbach, J. P., S. Lopes da Silva, J. J. Cox, R. A. Adan, A. J. Cooney, M. J. Tsai, and S. Y. Tsai. 1994. 'Repression of estrogen-dependent stimulation of the oxytocin gene by chicken ovalbumin upstream promoter transcription factor I', *J Biol Chem*, 269: 15046-53.

Calas, A. 1985. 'Morphological correlates of chemically specified neuronal interactions in the hypothalamo-hypophyseal area', *Neurochem Int*, 7: 927-40.

Camerino, C. 2009. 'Low sympathetic tone and obese phenotype in oxytocin-deficient mice', *Obesity (Silver Spring)*, 17: 980-4.

Carmeci, C., D. A. Thompson, H. Z. Ring, U. Francke, and R. J. Weigel. 1997. 'Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer', *Genomics*, 45: 607-17.

Carter, M. E., M. E. Soden, L. S. Zweifel, and R. D. Palmiter. 2013. 'Genetic identification of a neural circuit that suppresses appetite', *Nature*, 503: 111-4.

Ceccatelli, S., A. Cintra, T. Hokfelt, K. Fuxe, A. C. Wikstrom, and J. A. Gustafsson. 1989. 'Coexistence of glucocorticoid receptor-like immunoreactivity with neuropeptides in the hypothalamic paraventricular nucleus', *Exp Brain Res*, 78: 33-42.

Chen, Q., and H. L. Pan. 2006. 'Regulation of synaptic input to hypothalamic presympathetic neurons by GABA(B) receptors', *Neuroscience*, 142: 595-606.

Chen, R., X. Wu, L. Jiang, and Y. Zhang. 2017. 'Single-Cell RNA-Seq Reveals Hypothalamic Cell Diversity', *Cell Rep*, 18: 3227-41.

Chen, X. N., H. Zhu, Q. Y. Meng, and J. N. Zhou. 2008. 'Estrogen receptor-alpha and -beta regulate the human corticotropin-releasing hormone gene through similar pathways', *Brain Res*, 1223: 1-10.

Chen, X. Q., J. Z. Du, and Y. S. Wang. 2004. 'Regulation of hypoxia-induced release of corticotropin-releasing factor in the rat hypothalamus by norepinephrine', *Regul Pept*, 119: 221-8.

Chen, Y., Y. C. Lin, T. W. Kuo, and Z. A. Knight. 2015. 'Sensory detection of food rapidly modulates arcuate feeding circuits', *Cell*, 160: 829-41.

Cowley, M. A., J. L. Smart, M. Rubinstein, M. G. Cerdan, S. Diano, T. L. Horvath, R. D. Cone, and M. J. Low. 2001. 'Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus', *Nature*, 411: 480-4.

Cullinan, W. E., J. P. Herman, and S. J. Watson. 1993. 'Ventral subicular interaction with the hypothalamic paraventricular nucleus: evidence for a relay in the bed nucleus of the stria terminalis', *J Comp Neurol*, 332: 1-20.

Cunningham, E. T., Jr., and P. E. Sawchenko. 1988. 'Anatomical specificity of noradrenergic inputs to the paraventricular and supraoptic nuclei of the rat hypothalamus', *J Comp Neurol*, 274: 60-76.

Cyr, N. E., A. M. Toorie, J. S. Steger, M. M. Sochat, S. Hyner, M. Perello, R. Stuart, and E. A. Nillni. 2013. 'Mechanisms by which the orexigen NPY regulates anorexigenic alpha-MSH and TRH', *Am J Physiol Endocrinol Metab*, 304: E640-50.

Dabrowska, J., R. Hazra, T. H. Ahern, J. D. Guo, A. J. McDonald, F. Mascagni, J. F. Muller, L. J. Young, and D. G. Rainnie. 2011. 'Neuroanatomical evidence for reciprocal regulation of the corticotrophin-releasing factor and oxytocin systems in the hypothalamus and the bed nucleus of the stria terminalis of the rat: Implications for balancing stress and affect', *Psychoneuroendocrinology*, 36: 1312-26.

Dabrowska, J., R. Hazra, J. D. Guo, S. Dewitt, and D. G. Rainnie. 2013. 'Central CRF neurons are not created equal: phenotypic differences in CRF-containing neurons of the rat paraventricular hypothalamus and the bed nucleus of the stria terminalis', *Front Neurosci*, 7: 156.

de Souza, L. M., and C. R. Franci. 2010. 'Differential immunoreactivity of glucocorticoid receptors and vasopressin in neurons of the anterior and medial parvocellular subdvisions of the hypothalamic paraventricular nucleus', *Brain Res Bull*, 82: 271-8.

Decavel, C., and A. N. Van den Pol. 1990. 'GABA: a dominant neurotransmitter in the hypothalamus', *J Comp Neurol*, 302: 1019-37.

Di, S., R. Malcher-Lopes, V. L. Marcheselli, N. G. Bazan, and J. G. Tasker. 2005. 'Rapid glucocorticoid-mediated endocannabinoid release and opposing regulation of glutamate and gamma-aminobutyric acid inputs to hypothalamic magnocellular neurons', *Endocrinology*, 146: 4292-301.

Dolen, G., A. Darvishzadeh, K. W. Huang, and R. C. Malenka. 2013. 'Social reward requires coordinated activity of nucleus accumbens oxytocin and serotonin', *Nature*, 501: 179-84.

Dong, H. W., G. D. Petrovich, and L. W. Swanson. 2001. 'Topography of projections from amygdala to bed nuclei of the stria terminalis', *Brain Res Brain Res Rev*, 38: 192-246.

Elias, C. F., C. Aschkenasi, C. Lee, J. Kelly, R. S. Ahima, C. Bjorbaek, J. S. Flier, C. B. Saper, and J. K. Elmquist. 1999. 'Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area', *Neuron*, 23: 775-86.

Eliava, M., M. Melchior, H. S. Knobloch-Bollmann, J. Wahis, M. da Silva Gouveia, Y. Tang, A. C. Ciobanu, R. Triana Del Rio, L. C. Roth, F. Althammer, V. Chavant, Y. Goumon, T. Gruber, N. Petit-Demouliere, M. Busnelli, B. Chini, L. L. Tan, M. Mitre, R. C. Froemke, M. V. Chao, G. Giese, R. Sprengel, R. Kuner, P. Poisbeau, P. H. Seeburg, R. Stoop, A. Charlet, and V. Grinevich. 2016. 'A New Population of Parvocellular Oxytocin Neurons Controlling Magnocellular Neuron Activity and Inflammatory Pain Processing', *Neuron*, 89: 1291-304.

Evans, R. M. 1988. 'The steroid and thyroid hormone receptor superfamily', *Science*, 240: 889-95.

Fan, W., B. A. Boston, R. A. Kesterson, V. J. Hruby, and R. D. Cone. 1997. 'Role of melanocortinergic neurons in feeding and the agouti obesity syndrome', *Nature*, 385: 165-8.

Fekete, C., and R. M. Lechan. 2007. 'Negative feedback regulation of hypophysiotropic thyrotropin-releasing hormone (TRH) synthesizing neurons: role of neuronal afferents and type 2 deiodinase', *Front Neuroendocrinol*, 28: 97-114.

Fekete, C., E. Mihaly, L. G. Luo, J. Kelly, J. T. Clausen, Q. Mao, W. M. Rand, L. G. Moss, M. Kuhar, C. H. Emerson, I. M. Jackson, and R. M. Lechan. 2000. 'Association of cocaine- and amphetamine-regulated transcript-immunoreactive elements with thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and its role in the regulation of the hypothalamic-pituitary-thyroid axis during fasting', *J Neurosci*, 20: 9224-34.

Fekete, C., S. Sarkar, W. M. Rand, J. W. Harney, C. H. Emerson, A. C. Bianco, A. Beck-Sickinger, and R. M. Lechan. 2002. 'Neuropeptide Y1 and Y5 receptors mediate the effects of neuropeptide Y on the hypothalamic-pituitary-thyroid axis', *Endocrinology*, 143: 4513-9.

Ferguson, A. V., K. J. Latchford, and W. K. Samson. 2008. 'The paraventricular nucleus of the hypothalamus - a potential target for integrative treatment of autonomic dysfunction', *Expert Opin Ther Targets*, 12: 717-27.

Filardo, E. J., and P. Thomas. 2012. 'Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology', *Endocrinology*, 153: 2953-62.

Garcia, S. I., M. S. Landa, P. I. Porto, A. L. Alvarez, M. Schuman, S. Finkielman, and C. J. Pirola. 2002. 'Thyrotropin-releasing hormone decreases leptin and mediates the leptin-induced pressor effect', *Hypertension*, 39: 491-5.

Garcia-Iglesias, B. B., M. E. Mendoza-Garrido, G. Gutierrez-Ospina, C. Rangel-Barajas, M. Noyola-Diaz, and J. A. Terron. 2013. 'Sensitization of restraint-induced corticosterone secretion after chronic restraint in rats: involvement of 5-HT(7) receptors', *Neuropharmacology*, 71: 216-27.

Garfield, A. S., C. Li, J. C. Madara, B. P. Shah, E. Webber, J. S. Steger, J. N. Campbell, O. Gavrilova, C. E. Lee, D. P. Olson, J. K. Elmquist, B. A. Tannous, M. J. Krashes, and B. B. Lowell. 2015. 'A neural basis for melanocortin-4 receptor-regulated appetite', *Nat Neurosci*, 18: 863-71.

Gillies, G. E., E. A. Linton, and P. J. Lowry. 1982. 'Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin', *Nature*, 299: 355-7.

Grassi, D., N. Lagunas, H. Pinos, G. Panzica, L. M. Garcia-Segura, and P. Collado. 2017. 'NADPH-Diaphorase Colocalizes with GPER and Is Modulated by the GPER Agonist G1 in the Supraoptic and Paraventricular Nuclei of Ovariectomized Female Rats', *Neuroendocrinology*, 104: 94-104.

Gray, T. S., M. E. Carney, and D. J. Magnuson. 1989. 'Direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus: possible role in stress-induced adrenocorticotropin release', *Neuroendocrinology*, 50: 433-46.

Gu, G., B. Roland, K. Tomaselli, C. S. Dolman, C. Lowe, and J. S. Heilig. 2013. 'Glucagon-like peptide-1 in the rat brain: distribution of expression and functional implication', *J Comp Neurol*, 521: 2235-61.

Guillemin, R. 2005. 'Hypothalamic hormones a.k.a. hypothalamic releasing factors', *J Endocrinol*, 184: 11-28.

Hagan, M. M., P. A. Rushing, L. M. Pritchard, M. W. Schwartz, A. M. Strack, L. H. Van Der Ploeg, S. C. Woods, and R. J. Seeley. 2000. 'Long-term orexigenic effects of AgRP-(83---132) involve mechanisms other than melanocortin receptor blockade', *Am J Physiol Regul Integr Comp Physiol*, 279: R47-52.

Hallbeck, M., and A. Blomqvist. 1999. 'Spinal cord-projecting vasopressinergic neurons in the rat paraventricular hypothalamus', *J Comp Neurol*, 411: 201-11.

Handa, R. J., and M. J. Weiser. 2014. 'Gonadal steroid hormones and the hypothalamo-pituitary-adrenal axis', *Front Neuroendocrinol*, 35: 197-220.

Hazell, G. G., C. C. Hindmarch, G. R. Pope, J. A. Roper, S. L. Lightman, D. Murphy, A. M. O'Carroll, and S. J. Lolait. 2012. 'G protein-coupled receptors in the hypothalamic paraventricular and supraoptic nuclei--serpentine gateways to neuroendocrine homeostasis', *Front Neuroendocrinol*, 33: 45-66.

Hazell, G. G., S. T. Yao, J. A. Roper, E. R. Prossnitz, A. M. O'Carroll, and S. J. Lolait. 2009. 'Localisation of GPR30, a novel G protein-coupled oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues', *J Endocrinol*, 202: 223-36.

Healy, D. P., and M. P. Printz. 1984. 'Autoradiographic localization of angiotensin II binding sites in the brain, pituitary, kidney and adrenal gland of the rat', *J Hypertens Suppl*, 2: S57-61.

Hermes, M. L., M. Kolaj, P. Doroshenko, E. Coderre, and L. P. Renaud. 2009. 'Effects of VPAC2 receptor activation on membrane excitability and GABAergic transmission in subparaventricular zone neurons targeted by suprachiasmatic nucleus', *J Neurophysiol*, 102: 1834-42.

Hiroi, R., A. F. Lacagnina, L. R. Hinds, D. G. Carbone, R. M. Uht, and R. J. Handa. 2013. 'The androgen metabolite, 5alpha-androstane-3beta,17beta-diol (3beta-diol), activates the oxytocin promoter through an estrogen receptor-beta pathway', *Endocrinology*, 154: 1802-12.

Hornby, P. J., and D. T. Piekut. 1989. 'Opiocortin and catecholamine input to CRF-immunoreactive neurons in rat forebrain', *Peptides*, 10: 1139-46.

Hosoya, Y., Y. Sugiura, N. Okado, A. D. Loewy, and K. Kohno. 1991. 'Descending input from the hypothalamic paraventricular nucleus to sympathetic preganglionic neurons in the rat', *Exp Brain Res*, 85: 10-20.

Hrabovszky, E., I. Kallo, A. Steinhauser, I. Merchenthaler, C. W. Coen, S. L. Petersen, and Z. Liposits. 2004. 'Estrogen receptor-beta in oxytocin and vasopressin neurons of the rat and human hypothalamus: Immunocytochemical and in situ hybridization studies', *J Comp Neurol*, 473: 315-33.

Ishikawa, K., Y. Taniguchi, K. Inoue, K. Kurosumi, and M. Suzuki. 1988. 'Immunocytochemical delineation of thyrotrophic area: origin of thyrotropin-releasing hormone in the median eminence', *Neuroendocrinology*, 47: 384-8.

Iwasaki-Sekino, A., A. Mano-Otagiri, H. Ohata, N. Yamauchi, and T. Shibasaki. 2009. 'Gender differences in corticotropin and corticosterone secretion and corticotropin-releasing factor mRNA expression in the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala in response to footshock stress or psychological stress in rats', *Psychoneuroendocrinology*, 34: 226-37.

Jamieson, B. B., B. B. Nair, and K. J. Iremonger. 2017. 'Regulation of Hypothalamic CRH Neuron Excitability by Oxytocin', *J Neuroendocrinol*.

Jansen, A. S., M. W. Wessendorf, and A. D. Loewy. 1995. 'Transneuronal labeling of CNS neuropeptide and monoamine neurons after pseudorabies virus injections into the stellate ganglion', *Brain Res*, 683: 1-24.

Kadar, A., E. Sanchez, G. Wittmann, P. S. Singru, T. Fuzesi, A. Marsili, P. R. Larsen, Z. Liposits, R. M. Lechan, and C. Fekete. 2010. 'Distribution of hypophysiotropic thyrotropin-releasing hormone (TRH)-synthesizing neurons in the hypothalamic paraventricular nucleus of the mouse', *J Comp Neurol*, 518: 3948-61.

Kadekaro, M. 2004. 'Nitric oxide modulation of the hypothalamo-neurohypophyseal system', *Braz J Med Biol Res*, 37: 441-50.

Kamegai, J., H. Tamura, T. Shimizu, S. Ishii, H. Sugihara, and I. Wakabayashi. 2000. 'Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression', *Endocrinology*, 141: 4797-800.

Kantzides, A., and E. Badoer. 2005. 'nNOS-containing neurons in the hypothalamus and medulla project to the RVLM', *Brain Res*, 1037: 25-34.

Kennedy, M. B. 2000. 'Signal-processing machines at the postsynaptic density', *Science*, 290: 750-4.

Kim, E. M., M. K. Grace, E. O'Hare, C. J. Billington, and A. S. Levine. 2002. 'Injection of alpha-MSH, but not beta-endorphin, into the PVN decreases POMC gene expression in the ARC', *Neuroreport*, 13: 497-500.

Kiss, J. Z. 1985. 'Anatomical studies of cholecystokinin in neurons and pathways involved in neuroendocrine regulation', *Ann N Y Acad Sci*, 448: 144-51.

Kiss, J. Z., J. Martos, and M. Palkovits. 1991. 'Hypothalamic paraventricular nucleus: a quantitative analysis of cytoarchitectonic subdivisions in the rat', *J Comp Neurol*, 313: 563-73.

Kitazawa, S., S. Shioda, and Y. Nakai. 1987. 'Catecholaminergic innervation of neurons containing corticotropin-releasing factor in the paraventricular nucleus of the rat hypothalamus', *Acta Anat (Basel)*, 129: 337-43.

Knobloch, H. S., A. Charlet, L. C. Hoffmann, M. Eliava, S. Khrulev, A. H. Cetin, P. Osten, M. K. Schwarz, P. H. Seeburg, R. Stoop, and V. Grinevich. 2012. 'Evoked axonal oxytocin release in the central amygdala attenuates fear response', *Neuron*, 73: 553-66.

Krashes, M. J., B. P. Shah, J. C. Madara, D. P. Olson, D. E. Strochlic, A. S. Garfield, L. Vong, H. Pei, M. Watabe-Uchida, N. Uchida, S. D. Liberles, and B. B. Lowell. 2014. 'An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger', *Nature*, 507: 238-42.

Kudwa, A. E., R. F. McGivern, and R. J. Handa. 2014. 'Estrogen receptor beta and oxytocin interact to modulate anxiety-like behavior and neuroendocrine stress reactivity in adult male and female rats', *Physiol Behav*, 129: 287-96.

Laflamme, N., R. E. Nappi, G. Drolet, C. Labrie, and S. Rivest. 1998. 'Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype', *J Neurobiol*, 36: 357-78.

Landgraf, R., and I. D. Neumann. 2004. 'Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication', *Front Neuroendocrinol*, 25: 150-76.

Lechan, R. M., and I. M. Jackson. 1982. 'Immunohistochemical localization of thyrotropin-releasing hormone in the rat hypothalamus and pituitary', *Endocrinology*, 111: 55-65.

Lee, K. S., T. H. Han, J. Y. Jo, G. Kang, S. Y. Lee, P. D. Ryu, J. H. Im, B. H. Jeon, and J. B. Park. 2008. 'Serotonin inhibits GABA synaptic transmission in presympathetic paraventricular nucleus neurons', *Neurosci Lett*, 439: 138-42.

Leng, G., C. H. Brown, and J. A. Russell. 1999. 'Physiological pathways regulating the activity of magnocellular neurosecretory cells', *Prog Neurobiol*, 57: 625-55.

Li, D. P., S. R. Chen, T. F. Finnegan, and H. L. Pan. 2004. 'Signalling pathway of nitric oxide in synaptic GABA release in the rat paraventricular nucleus', *J Physiol*, 554: 100-10.

Li, Y., W. Zhang, and J. E. Stern. 2003. 'Nitric oxide inhibits the firing activity of hypothalamic paraventricular neurons that innervate the medulla oblongata: role of GABA', *Neuroscience*, 118: 585-601.

Liu, T., D. Kong, B. P. Shah, C. Ye, S. Koda, A. Saunders, J. B. Ding, Z. Yang, B. L. Sabatini, and B. B. Lowell. 2012. 'Fasting activation of AgRP neurons requires NMDA receptors and involves spinogenesis and increased excitatory tone', *Neuron*, 73: 511-22.

Luiten, P. G., G. J. ter Horst, H. Karst, and A. B. Steffens. 1985. 'The course of paraventricular hypothalamic efferents to autonomic structures in medulla and spinal cord', *Brain Res*, 329: 374-8.

Lund, T. D., T. Rovis, W. C. Chung, and R. J. Handa. 2005. 'Novel actions of estrogen receptor-beta on anxiety-related behaviors', *Endocrinology*, 146: 797-807.

Mack, S. O., P. Kc, M. Wu, B. R. Coleman, F. P. Tolentino-Silva, and M. A. Haxhiu. 2002. 'Paraventricular oxytocin neurons are involved in neural modulation of breathing', *J Appl Physiol (1985)*, 92: 826-34.

Markakis, E. A. 2002. 'Development of the neuroendocrine hypothalamus', *Front Neuroendocrinol*, 23: 257-91.

Masaki, T., G. Yoshimichi, S. Chiba, T. Yasuda, H. Noguchi, T. Kakuma, T. Sakata, and H. Yoshimatsu. 2003. 'Corticotropin-releasing hormone-mediated pathway of leptin to regulate feeding, adiposity, and uncoupling protein expression in mice', *Endocrinology*, 144: 3547-54.

Merchenthaler, I., M. V. Lane, S. Numan, and T. L. Dellovade. 2004. 'Distribution of estrogen receptor alpha and beta in the mouse central nervous system: in vivo autoradiographic and immunocytochemical analyses', *J Comp Neurol*, 473: 270-91.

Merchenthaler, I., and Z. Liposits. 1994. 'Mapping of thyrotropin-releasing hormone (TRH) neuronal systems of rat forebrain projecting to the median eminence and the OVLT. Immunocytochemistry combined with retrograde labeling at the light and electron microscopic levels', *Acta Biol Hung*, 45: 361-74.

Miller, W. J., S. Suzuki, L. K. Miller, R. Handa, and R. M. Uht. 2004. 'Estrogen receptor (ER)beta isoforms rather than ERalpha regulate corticotropin-releasing hormone promoter activity through an alternate pathway', *J Neurosci*, 24: 10628-35.

Milner, T. A., D. J. Reis, V. M. Pickel, S. A. Aicher, and R. Giuliano. 1993. 'Ultrastructural localization and afferent sources of corticotropin-releasing factor in the rat rostral ventrolateral medulla: implications for central cardiovascular regulation', *J Comp Neurol*, 333: 151-67.

Morrison, C. D., G. J. Morton, K. D. Niswender, R. W. Gelling, and M. W. Schwartz. 2005. 'Leptin inhibits hypothalamic Npy and Agrp gene expression via a mechanism that requires phosphatidylinositol 3-OH-kinase signaling', *Am J Physiol Endocrinol Metab*, 289: E1051-7.

Nijenhuis, W. A., J. Oosterom, and R. A. Adan. 2001. 'AgRP(83-132) acts as an inverse agonist on the human-melanocortin-4 receptor', *Mol Endocrinol*, 15: 164-71.

Nomura, M., E. McKenna, K. S. Korach, D. W. Pfaff, and S. Ogawa. 2002. 'Estrogen receptor-beta regulates transcript levels for oxytocin and arginine vasopressin in the hypothalamic paraventricular nucleus of male mice', *Brain Res Mol Brain Res*, 109: 84-94.

Oldfield, E. H., G. P. Chrousos, H. M. Schulte, M. Schaaf, P. E. McKeever, A. G. Krudy, G. B. Cutler, Jr., D. L. Loriaux, and J. L. Doppman. 1985. 'Preoperative lateralization of ACTH-secreting pituitary microadenomas by bilateral and simultaneous inferior petrosal venous sinus sampling', *N Engl J Med*, 312: 100-3.

Ollmann, M. M., B. D. Wilson, Y. K. Yang, J. A. Kerns, Y. Chen, I. Gantz, and G. S. Barsh. 1997. 'Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein', *Science*, 278: 135-8.

Oyola, M. G., W. Portillo, A. Reyna, C. D. Foradori, A. Kudwa, L. Hinds, R. J. Handa, and S. K. Mani. 2012. 'Anxiolytic effects and neuroanatomical targets of estrogen receptor-beta (ERbeta) activation by a selective ERbeta agonist in female mice', *Endocrinology*, 153: 837-46.

Oyola, M. G., M. K. Thompson, A. Z. Handa, and R. J. Handa. 2017. 'Distribution and chemical composition of estrogen receptor beta neurons in the paraventricular nucleus of the female and male mouse hypothalamus', *J Comp Neurol*.

Pacak, K. 2000. 'Stressor-specific activation of the hypothalamic-pituitary-adrenocortical axis', *Physiol Res*, 49 Suppl 1: S11-7.

Perello, M., R. C. Stuart, and E. A. Nillni. 2006. 'The role of intracerebroventricular administration of leptin in the stimulation of prothyrotropin releasing hormone neurons in the hypothalamic paraventricular nucleus', *Endocrinology*, 147: 3296-306.

Petersson, M. 2002. 'Cardiovascular effects of oxytocin', *Prog Brain Res*, 139: 281-8.

Plotsky, P. M., and P. E. Sawchenko. 1987. 'Hypophysial-portal plasma levels, median eminence content, and immunohistochemical staining of corticotropin-releasing factor, arginine vasopressin, and oxytocin after pharmacological adrenalectomy', *Endocrinology*, 120: 1361-9.

Potts, P. D., J. Ludbrook, T. A. Gillman-Gaspari, J. Horiuchi, and R. A. Dampney. 2000. 'Activation of brain neurons following central hypervolaemia and hypovolaemia: contribution of baroreceptor and non-baroreceptor inputs', *Neuroscience*, 95: 499-511.

Pyner, S. 2009. 'Neurochemistry of the paraventricular nucleus of the hypothalamus: implications for cardiovascular regulation', *J Chem Neuroanat*, 38: 197-208.

Pyner, S., and J. H. Coote. 2000. 'Identification of branching paraventricular neurons of the hypothalamus that project to the rostroventrolateral medulla and spinal cord', *Neuroscience*, 100: 549-56.

Qadri, F., T. Arens, E. C. Schwarz, W. Hauser, A. Dendorfer, and P. Dominiak. 2003. 'Brain nitric oxide synthase activity in spontaneously hypertensive rats during the development of hypertension', *J Hypertens*, 21: 1687-94.

Reichlin, S. 1989. 'Neuroendocrinology of the pituitary gland', *Toxicol Pathol*, 17: 250-5.

Rho, J. H., and L. W. Swanson. 1989. 'A morphometric analysis of functionally defined subpopulations of neurons in the paraventricular nucleus of the rat with observations on the effects of colchicine', *J Neurosci*, 9: 1375-88.

Rhodes, C. H., J. I. Morrell, and D. W. Pfaff. 1981. 'Immunohistochemical analysis of magnocellular elements in rat hypothalamus: distribution and numbers of cells containing neurophysin, oxytocin, and vasopressin', *J Comp Neurol*, 198: 45-64.

Richard, S., and H. H. Zingg. 1990. 'The human oxytocin gene promoter is regulated by estrogens', *J Biol Chem*, 265: 6098-103.

Riphagen, C. L., and Q. J. Pittman. 1989. 'Mechanisms underlying the cardiovascular responses to intrathecal vasopressin administration in rats', *Can J Physiol Pharmacol*, 67: 269-75.

Rivier, C., and W. Vale. 1983. 'Interaction of corticotropin-releasing factor and arginine vasopressin on adrenocorticotropin secretion in vivo', *Endocrinology*, 113: 939-42.

Roland, B. L., and P. E. Sawchenko. 1993. 'Local origins of some GABAergic projections to the paraventricular and supraoptic nuclei of the hypothalamus in the rat', *J Comp Neurol*, 332: 123-43.

Ross, H. E., C. D. Cole, Y. Smith, I. D. Neumann, R. Landgraf, A. Z. Murphy, and L. J. Young. 2009. 'Characterization of the oxytocin system regulating affiliative behavior in female prairie voles', *Neuroscience*, 162: 892-903.

Rossi, D. V., Y. Dai, P. Thomas, G. A. Carrasco, L. L. DonCarlos, N. A. Muma, and Q. Li. 2010. 'Estradiol-induced desensitization of 5-HT1A receptor signaling in the paraventricular nucleus of the hypothalamus is independent of estrogen receptor-beta', *Psychoneuroendocrinology*, 35: 1023-33.

Saper, C. B., L. W. Swanson, and W. M. Cowan. 1976. 'The efferent connections of the ventromedial nucleus of the hypothalamus of the rat', *J Comp Neurol*, 169: 409-42.

Sarkar, S., C. Fekete, G. Legradi, and R. M. Lechan. 2003. 'Glucagon like peptide-1 (7-36) amide (GLP-1) nerve terminals densely innervate corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus', *Brain Res*, 985: 163-8.

Sawchenko, P. E., E. R. Brown, R. K. Chan, A. Ericsson, H. Y. Li, B. L. Roland, and K. J. Kovacs. 1996. 'The paraventricular nucleus of the hypothalamus and the functional neuroanatomy of visceromotor responses to stress', *Prog Brain Res*, 107: 201-22.

Sawchenko, P. E., and L. W. Swanson. 1981. 'Central noradrenergic pathways for the integration of hypothalamic neuroendocrine and autonomic responses', *Science*, 214: 685-7.

Sawchenko, P. E. 1983. 'The organization of forebrain afferents to the paraventricular and supraoptic nuclei of the rat', *J Comp Neurol*, 218: 121-44.

Sawchenko, P. E., L. W. Swanson, and W. W. Vale. 1984. 'Corticotropin-releasing factor: co-expression within distinct subsets of oxytocin-, vasopressin-, and neurotensin-immunoreactive neurons in the hypothalamus of the male rat', *J Neurosci*, 4: 1118-29.

Schlosser, S. F., O. F. Almeida, V. K. Patchev, A. Yassouridis, and J. Elands. 1994. 'Oxytocin-stimulated release of adrenocorticotropin from the rat pituitary is mediated by arginine vasopressin receptors of the V1b type', *Endocrinology*, 135: 2058-63.

Seale, J. V., S. A. Wood, H. C. Atkinson, M. S. Harbuz, and S. L. Lightman. 2004. 'Gonadal steroid replacement reverses gonadectomy-induced changes in the corticosterone pulse profile and stress-induced hypothalamic-pituitary-adrenal axis activity of male and female rats', *J Neuroendocrinol*, 16: 989-98.

Shafton, A. D., A. Ryan, and E. Badoer. 1998. 'Neurons in the hypothalamic paraventricular nucleus send collaterals to the spinal cord and to the rostral ventrolateral medulla in the rat', *Brain Res*, 801: 239-43.

Shah, B. P., L. Vong, D. P. Olson, S. Koda, M. J. Krashes, C. Ye, Z. Yang, P. M. Fuller, J. K. Elmquist, and B. B. Lowell. 2014. 'MC4R-expressing glutamatergic neurons in the paraventricular hypothalamus regulate feeding and are synaptically connected to the parabrachial nucleus', *Proc Natl Acad Sci U S A*, 111: 13193-8.

Shapiro, R. E., and R. R. Miselis. 1985. 'The central neural connections of the area postrema of the rat', *J Comp Neurol*, 234: 344-64.

Sofroniew, M. V., and U. Schrell. 1982. 'Long-term storage and regular repeated use of diluted antisera in glass staining jars for increased sensitivity, reproducibility, and convenience of single- and two-color light microscopic immunocytochemistry', *J Histochem Cytochem*, 30: 504-11.

Somponpun, S. J., and C. D. Sladek. 2004. 'Depletion of oestrogen receptor-beta expression in magnocellular arginine vasopressin neurones by hypovolaemia and dehydration', *J Neuroendocrinol*, 16: 544-9.

Srivastava, D. P., and P. D. Evans. 2013. 'G-protein oestrogen receptor 1: trials and tribulations of a membrane oestrogen receptor', *J Neuroendocrinol*, 25: 1219-30.

Stern, J. E. 2004. 'Nitric oxide and homeostatic control: an intercellular signalling molecule contributing to autonomic and neuroendocrine integration?', *Prog Biophys Mol Biol*, 84: 197-215.

Stern, J. E., and W. Zhang. 2003. 'Preautonomic neurons in the paraventricular nucleus of the hypothalamus contain estrogen receptor beta', *Brain Res*, 975: 99-109.

Sutton, A. K., H. Pei, K. H. Burnett, M. G. Myers, Jr., C. J. Rhodes, and D. P. Olson. 2014. 'Control of food intake and energy expenditure by Nos1 neurons of the paraventricular hypothalamus', *J Neurosci*, 34: 15306-18.

Suzuki, S., and R. J. Handa. 2004. 'Regulation of estrogen receptor-beta expression in the female rat hypothalamus: differential effects of dexamethasone and estradiol', *Endocrinology*, 145: 3658-70.

Suzuki, S., L. C. Solberg, E. E. Redei, and R. J. Handa. 2001. 'Prepro-thyrotropin releasing hormone 178-199 immunoreactivity is altered in the hypothalamus of the Wistar-Kyoto strain of rat', *Brain Res*, 913: 224-33.

Swanson, L. W. 1977. 'Immunohistochemical evidence for a neurophysin-containing autonomic pathway arising in the paraventricular nucleus of the hypothalamus', *Brain Res*, 128: 346-53.

Swanson, L. W. 1995. 'Mapping the human brain: past, present, and future', *Trends Neurosci*, 18: 471-4.

Swanson, L. W., and H. G. Kuypers. 1980. 'The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods', *J Comp Neurol*, 194: 555-70.

Swanson, L. W., P. E. Sawchenko, A. Berod, B. K. Hartman, K. B. Helle, and D. E. Vanorden. 1981. 'An immunohistochemical study of the organization of catecholaminergic cells and terminal fields in the paraventricular and supraoptic nuclei of the hypothalamus', *J Comp Neurol*, 196: 271-85.

Szarek, E., P. S. Cheah, J. Schwartz, and P. Thomas. 2010. 'Molecular genetics of the developing neuroendocrine hypothalamus', *Mol Cell Endocrinol*, 323: 115-23.

Takayanagi, Y., Y. Kasahara, T. Onaka, N. Takahashi, T. Kawada, and K. Nishimori. 2008. 'Oxytocin receptor-deficient mice developed late-onset obesity', *Neuroreport*, 19: 951-5.

Tauchi, M., R. Zhang, D. A. D'Alessio, J. E. Stern, and J. P. Herman. 2008. 'Distribution of glucagon-like peptide-1 immunoreactivity in the hypothalamic paraventricular and supraoptic nuclei', *J Chem Neuroanat*, 36: 144-9.

Van de Kar, L. D., and M. L. Blair. 1999. 'Forebrain pathways mediating stress-induced hormone secretion', *Front Neuroendocrinol*, 20: 1-48.

van Swieten, M. M., R. Pandit, R. A. Adan, and G. van der Plasse. 2014. 'The neuroanatomical function of leptin in the hypothalamus', *J Chem Neuroanat*, 61-62: 207-20.

Vandesande, F., and K. Dierickx. 1975. 'Identification of the vasopressin producing and of the oxytocin producing neurons in the hypothalamic magnocellular neurosecretroy system of the rat', *Cell Tissue Res*, 164: 153-62.

Vettor, R., R. Fabris, C. Pagano, and G. Federspil. 2002. 'Neuroendocrine regulation of eating behavior', *J Endocrinol Invest*, 25: 836-54.

Viau, V., B. Bingham, J. Davis, P. Lee, and M. Wong. 2005. 'Gender and puberty interact on the stress-induced activation of parvocellular neurosecretory neurons and corticotropin-releasing hormone messenger ribonucleic acid expression in the rat', *Endocrinology*, 146: 137-46.

Viau, V., and M. J. Meaney. 1991. 'Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat', *Endocrinology*, 129: 2503-11.

Vrang, N., P. J. Larsen, and J. D. Mikkelsen. 1995. 'Direct projection from the suprachiasmatic nucleus to hypophysiotrophic corticotropin-releasing factor immunoreactive cells in the paraventricular nucleus of the hypothalamus demonstrated by means of Phaseolus vulgaris-leucoagglutinin tract tracing', *Brain Res*, 684: 61-9.

Watkins, N. D., S. C. Cork, and S. Pyner. 2009. 'An immunohistochemical investigation of the relationship between neuronal nitric oxide synthase, GABA and presympathetic paraventricular neurons in the hypothalamus', *Neuroscience*, 159: 1079-88.

Webber, E. S., A. Bonci, and M. J. Krashes. 2015. 'The elegance of energy balance: Insight from circuit-level manipulations', *Synapse*, 69: 461-74.

Weiser, M. J., and R. J. Handa. 2009. 'Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic-pituitary-adrenal axis via estrogen receptor alpha within the hypothalamus', *Neuroscience*, 159: 883-95.

Weiss, M. L., S. I. Chowdhury, K. P. Patel, M. J. Kenney, and J. Huang. 2001. 'Neural circuitry of the kidney: NO-containing neurons', *Brain Res*, 919: 269-82.

Whitnall, M. H., and H. Gainer. 1988. 'Major pro-vasopressin-expressing and pro-vasopressin-deficient subpopulations of corticotropin-releasing hormone neurons in normal rats. Differential distributions within the paraventricular nucleus', *Neuroendocrinology*, 47: 176-80.

Windle, R. J., N. Shanks, S. L. Lightman, and C. D. Ingram. 1997. 'Central oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats', *Endocrinology*, 138: 2829-34.

Wu, Q., M. S. Clark, and R. D. Palmiter. 2012. 'Deciphering a neuronal circuit that mediates appetite', *Nature*, 483: 594-7.

Xu, H., S. Qin, G. A. Carrasco, Y. Dai, E. J. Filardo, E. R. Prossnitz, G. Battaglia, L. L. Doncarlos, and N. A. Muma. 2009. 'Extra-nuclear estrogen receptor GPR30 regulates serotonin function in rat hypothalamus', *Neuroscience*, 158: 1599-607.

Zhang, K., and K. P. Patel. 1998. 'Effect of nitric oxide within the paraventricular nucleus on renal sympathetic nerve discharge: role of GABA', *Am J Physiol*, 275: R728-34.

Zhang, X., O. Esenturk, and R. A. Walker. 2001. 'Reduced polarity in protic solvents near hydrophobic solid surfaces', *J Am Chem Soc*, 123: 10768-9.

Zhang, Y., K. J. Damjanoska, G. A. Carrasco, B. Dudas, D. N. D'Souza, J. Tetzlaff, F. Garcia, N. R. Hanley, K. Scripathirathan, B. R. Petersen, T. S. Gray, G. Battaglia, N. A. Muma, and L. D. Van de Kar. 2002. 'Evidence that 5-HT2A receptors in the hypothalamic paraventricular nucleus mediate neuroendocrine responses to (-)DOI', *J Neurosci*, 22: 9635-42.

Kisspeptin system

Kisspeptins are a family of structurally related peptides, encoded by the *KISS1/Kiss1* gene were sequentially identified between 1996 and 2001 (Oakley, Clifton, and Steiner 2009) (Roa et al. 2008) (Tena-Sempere 2006) and called in this way to ensure everyone knew where it was discovered – in Hershey, Pennsylvania, the home of the US famous Hershey Kisses Chocolate. Originally these peptides were catalogued as metastasis suppressors. In fact, by the use of subtractive hybridization in melanoma cell lines with different metastatic capacity, *KISS1* mRNA was identified in 1996 as a selectively overexpressed transcript in tumor cells with low invasiveness (Lee et al. 1996). This initial finding was followed by further characterization of the antimetastatic potential of the *KISS1* transcript (Lee and Welch 1997) (Lee and Welch 1997) and the cloning and chromosomal localization of human *KISS1* gene (West et al. 1998). The full characterization of the peptide products of the *KISS1* gene (localized on human and murine chromosome 1) was obtained only in the 2001(Kotani et al. 2001) (Muir et al. 2001) (Ohtaki et al. 2001). Based on structural similarities and its common origin as KISS1- derived peptides, the term *kisspeptins* was coined to globally define this family of peptides (Kotani et al. 2001), a name that became popular within the field and displaced the initial terminology of *metastin*. In

2009, Gottsch *et al.*, based on the Human Genome Organization Gene Nomenclature Committee (HGNC), proposed a specific nomenclature to use, in which the terms KiSS1 and Kiss1 are used to name the primate and non-primate genes, respectively, whereas their peptide products are termed kisspeptins (kp), with a numeric extension indicating the number of amino acidic residual (Gottsch, Clifton, and Steiner 2009).

Kisspeptins are derived from the differential proteolytic processing of the common precursor *PrePro-Kisspeptin (West et al. 1998)*. In human, the *KiSS1* gene encoded a 145 amino acid precursor protein, with a putative 19-aminoacid signal sequence, two potential dibasic cleavage sites (at amino acids 57 and 67) and one putative site for terminal cleavage and amidation (at amino acids 121-124), which generates the biologically active kisspeptins (Fig. 5; (Pinilla et al. 2012)).

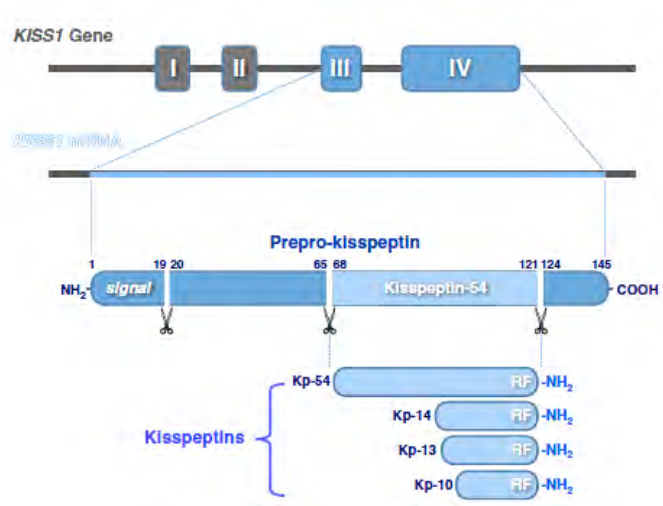

Figure 5: The products of the KiSS1 gene. Different kisspeptins are generated by proteolytic cleavage form a common precursor, prepro-kisspeptin, encoded by the KiSS1 gene (Pinillia et al., 2012).

The major peptide product of the KiSS1 gene appears to be a 54-amino-acid peptide, called kisspeptin-54 (kp-54), that derived from a post-translational process of the gene (Lee et al. 1996) (Kauffman et al. 2003). In rodent, Kiss1 gene, which is 46-52% homologous to the human *KiSS1* gene, encodes a 130-amino-acid precursor protein, which is processed to a 52- or 54-amino-acid mature kisspeptin peptide. In addition to kisspeptin-54 or -52, several other smaller peptide fragments have been identified (kp-14, -13, and -10); all kisspeptins share the C-terminal region of the metastin molecule, where they harbor an Arg-Phe-NH2 motif distinctive of the RF-amide peptide family, and each able to bind and to activate the GPR54 with similar efficacy (Kotani et al. 2001) (Muir et al. 2001) (Ohtaki et al. 2001).

In 2003, the interest on the physiological functions of KISS1-derived peptides increased extraordinarily after the observations that patients displaying inactivating mutations (*loss of function mutation*) of the GPR54 gene showed frequently hypogonadotropic hypogonadism (iHH) (de Roux et al. 2003) (Seminara et al. 2003). This sporadic disease is characterized by absence of GnRH-induced LH pulsation and low levels of gonadotropins and sex steroids, which bring out into typical symptoms such as failed puberty and sexual maturation, and abnormalities of gonads (Cerrato and Seminara 2007). Subsequently, it was demonstrated that activating mutation (*gain of function mutation*) of the same gene results in precocious puberty (Teles et al. 2008). These findings unveiled the previously unsuspected reproductive dimension of this signaling system, as a key player in the regulation of the gonadotropic axis. Finally, mice engineered to lack functional KISS1 or GPR54 genes show a phenotype totally comparable to that of affected humans (d'Anglemont de Tassigny et al. 2007) (Lapatto et al. 2007). This demonstrates the conserved roles of kisspeptins in the control of the HPG axis in mammals.

The characterization of the canonical receptor for kisspeptins preceded that of this family of peptides and in a totally unrelated context. Thus, Gpr54 was identified in the rat brain in 1999 as an orphan receptor with a significant sequence similarity (>40%) with the trans membrane regions of galanin receptors (Lee et al. 1999). Only in 2001 the GPR54, renamed kiss1r/KISS1R (Gottsch, Clifton, and Steiner 2009), was catalogued as putative receptor for KISS1-derived peptides (Muir et al. 2001) (Ohtaki et al. 2001); in fact, by use of heterologous cell systems (CHO K1 cells stably expressing this receptor) demonstrated that all kisspeptins efficiently activate GPR54, with the shorter 10-amino acid fragment (kisspeptin-10) retaining maximal activity in terms of receptor activation (Kotani et al. 2001). Conventional biochemical characterization demonstrated that, in human and in mouse, GPR54 is a seven transmembrane domain, Gq/11-coupled receptor, whose activation increases the intracellular Ca^{2+} levels ($[Ca^{2+}]i$) in a pertussis toxin-independent manner, without detectable changes in intracellular cAMP levels, therefore suggesting the lack of association with G_s and/or $G_{i/o}$ proteins (Kotani et al. 2001) (Muir et al. 2001).

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.

1. Distribution of Kisspeptin system

In rodents, ovine and primates (including humans) KiSS1 mRNA has been detected by either *in situ* hybridization or RT-PCR in hypothalamus; these analyses demonstrated (Bentsen et al. 2010; Clarkson et al. 2009; Estrada et al. 2006; Franceschini et al. 2006; Goodman et al. 2007; Irwig et al. 2004; Smith, Dungan, et al. 2005) the presence of a prominent population of Kiss1 neurons resides in the Arcuate Nucleus (ARC), or the equivalent infundibular region in primates. The phenotype of these ARC neurons has been later described by means of neuroanatomical studies in different species (sheep, mouse, monkey, and human) (Magee et al. 2009; Lehman, Coolen, and Goodman 2010). These studies demonstrated that in addition to kisspeptins, they express NKB and its putative receptor, NK3R, as well as dynorphyn (Dyn), therefore leading the proposal of the term *KNDy* to name this neuronal population (Lehman, Coolen, and Goodman 2010). In addition to this kisspeptin neurons population, studies in rodents have shown the presence of another population of Kiss1 neurons in the anteroventral periventricular nucleus (AVPV) or preoptic area in the sheep. Later studies, using mostly immunohistochemistry (IHC), documented that such *rostral* kiss system extends behind the AVPV, appearing as a *continuum* including adjacent areas, such as the preoptic periventricular nucleus (PeN). The region including AVPV and PeN has been later defined as rostral periventricular area of the third ventricle, RP3V (Clarkson and Herbison 2006) (Herbison 2008). A third population of Kiss1 neurons has been found in caudal portions of the posterodorsal area of the medial amygdala (MePD) (Pineda et al. 2017), the function of this kisspeptin neurons in the amygdala is unknown, but the amygdala has been implicated in numerous physiological and behavioral processes, including those related to reward, social behaviors and reproduction (Baxter and Murray 2002; Roozendaal, McEwen, and Chattarji 2009; Walf and Frye 2006). Moreover, the existence of scattered KiSS1 neurons has also been detected in the anterodorsal preoptic nucleus, the bed nucleus of the *stria terminalis*, the basal ganglia, the substantia nigra and hyppocampus (Adachi et al. 2007) (Clarkson et al. 2009) (Kauffman et al. 2007) (Smith et al. 2007). The physiological functions of KiSS1 population of these areas are uncertain (Gottsch, Clifton, and Steiner 2009), but where KiSS1 is expressed, at least in the amygdala, it seems to be under the control of sex steroids, which are the major regulators of KiSS1 mRNA levels at the ARC and RP3V (Kim et al. 2011).

In addition, during the last years a neuroanatomical mapping of Kiss fibers (and their relationship with GnRH neurons) has also been conducted in different mammalian species. Particularly, studies on rodents conducted by Clarkson and colleagues have identified an abundant presence of Kisspeptin fibers in various regions (including RP3V and ARC) and

synaptic contacts between KiSS1 cells originating from RP3V and GnRH neurons. Moreover, they created a detailed mapping of kisspeptin-IR fibers in the female mouse brain that documented the presence of abundant fibers within the hypothalamus in a diversity of nuclei, including the supraoptic (SON), paraventricular (PVN) and dorsomedial (DMN) nuclei, but were absent in the suprachiasmatic (SCN) and ventromedial (VMN) nuclei. In addition, a considerable number of fibers streams from the periventricular area into the lateral septum. Notably, in that study kisspeptin fibers were not detectable within the external zone of the mouse median eminence but were present in its internal zone and lateral margins (Clarkson et al. 2009)

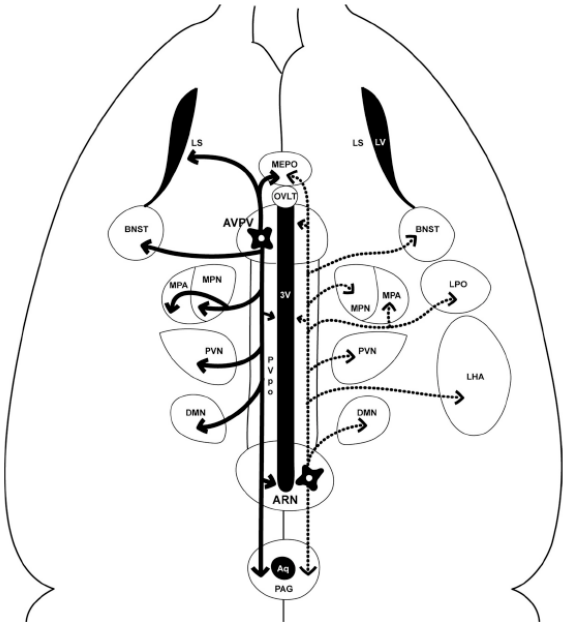

Figure 6: Schematic map of female mouse brain showing the organization of Kisspeptin neuronal projection by AVPV (left) and ARC (right) (Yeo and Herbison, 2011).

(Clarkson and Herbison 2006). A detailed neuroanatomical study in the mouse, using anterograde and retrograde tracing techniques, have revealed that the kisspeptin neurons of the ARC are found to project widely within both medial and lateral aspects of the hypothalamus and associated limbic structures, whereas RP3V kisspeptin neurons have a more medial projection pattern (Fig. 6). Also from the same work emerged that Kiss1 neurons in the rostral portion of the ARC, which may account for ~20% of the total ARC population, do project to the rostral POA, thus making highly plausible their direct interaction with GnRH neurons (Yeo and Herbison 2011). Moreover, it has been suggested that the ARC KiSS1 population may project to the ME where they establish axo axonic synaptic contacts with the GnRH nerve terminals (Smith et al. 2011).

Later, it was discovered that KiSS1 was abundantly expressed in several peripheral tissues: i.e.,

the placenta, ovary, testis, pancreas, small intestine, pancreas, and liver (Ohtaki et al. 2001). Since then, it has been shown that KiSS1 gene expression has potential role in other physiological functions, such as down-regulation of the activity of matrix metalloproteinase, which is though to be the mechanism by which KiSS1 suppresses cancer metastasis (Bilban et al. 2004); nociception and visceral regulation (Brailoiu et al. 2005); oxytocin release (Kotani et al. 2001); and perhaps the pathogenesis of the pre-eclampsia (Janneau et al. 2002).

More recently, essential aspects of kisspeptin distribution were studied using transgenic mice. These studies have refined our knowledge about the patterns of distribution of Kiss1 neurons initially defined by in situ hybridization and immunohistochemistry. Neuroanatomical studies in the model used by Cravo et al. (Cravo et al. 2011), a genetically engineered Kiss1-Cre mouse line to target green fluorescent protein (GFP) or β-galactosidase activity to Kiss1 neurons, and in Kiss1 -CreGFP knock-in mouse line reported by Gottsch et al. (Gottsch et al. 2011) have roughly replicated previous data of Kiss1 mRNA distribution in the mouse brain: prominent expression in the ARC and RPV3 (Smith, Dungan, et al. 2005; Smith, Cunningham, et al. 2005), as well as in different extrahypothalamic sites (Cota, Proulx, and Seeley 2007; Pinilla et al. 2012). The latter observation is partially in keeping with previous data showing the presence of additional groups of Kiss1 neurons in brain areas other than the hypothalamic ARC and RPV3, such the medial amygdala, the anterodorsal preoptic nucleus, and the bed nucleus of the stria terminalis, although the physiological roles of such Kiss1 neuronal populations remain largely unknown (Gottsch et al. 2004). A very recent study used a Kiss-CRE mouse line with CRE-activated tdTomato reporter mice to allow the fluorescence visualization of Kiss1 neurons in brain slices (Yeo et al. 2016). tdTomato positive neurons were colabelled with kisspeptin in the ARC and the AVPV and a small number of tdTomato-labelled neurons was also found in other locations, including the lateral septum, the anterodorsal preoptic nucleus, the amygdala, the dorsomedial and ventromedial hypothalamic nuclei, the periaquaductal grey, and the mammillary nucleus. The same work, using the CLARITY processing of whole brains, showed a higher numbers of Kiss1 neurons in the caudal region of the ARC compared to the rostral region and from ARC kisspeptin fiber projections arrive to several hypothalamic regions, including rostrally to the periventricular and preoptic areas and to the lateral hypothalamus (Fig. 7; (Yeo et al. 2016)).

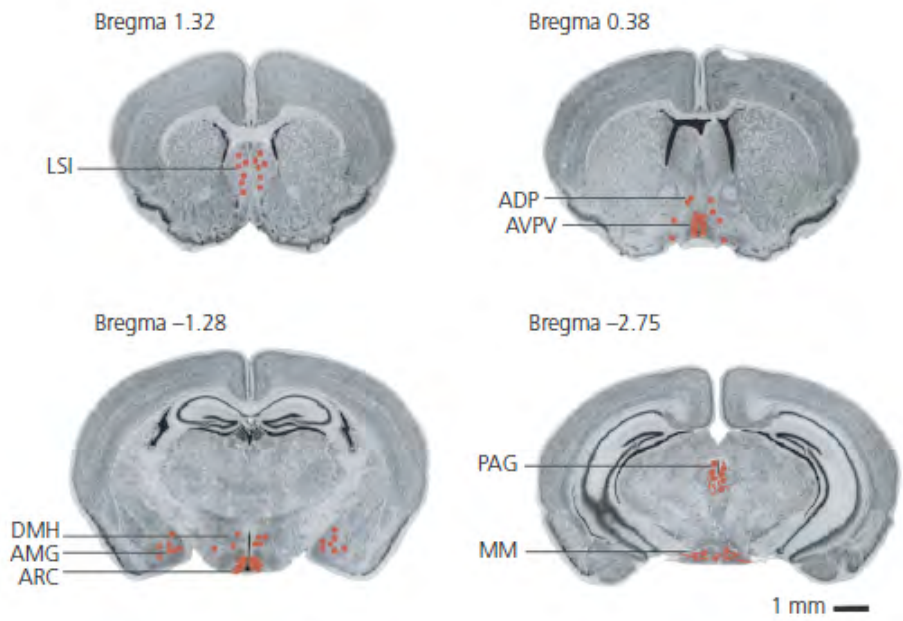

Figure 7: Distribution of Kiss-CRE/tdTomato labelled neurons in the female mouse brain. TdTomato positive cells bodies are represented by red circles and fibres represented by red lines. LSI, lateral septum; ADP, anterodorsal preoptic nucleus; AVPV, anteroventral periventricular nucleus; AMG, amygdala; DMH, dorsomedial hypothalamus; ARC, arcuete nucleus; PAG, periaqueductal grey; MM, medial mammillary nucleus (Yeo et al., 2016).

As for the distribution of GPR54 in different areas of the central nervous system, initial *in situ* hybridization analyses in the rat demonstrated expression of GPR54 mRNA in the hypothalamus, and specifically in GnRH neurons (Irwig et al. 2004). Studies by Herbison et al. (Herbison et al. 2010) using a transgenic GPR54 LacZ knock-in strategy have provided a reliable map of GPR54-positive neurons in different brain areas and confirmed the expression of this receptor in GnRH neurons at the rostral POA, but no GPR54 expression was apparently detected in the ARC or rostral periventricular. More recently, using in situ hybridisation combined with immunofluorescence, Higo and colleagues (Higo et al. 2016) have determined the whole brain map of Kiss1r mRNA. Neurons with strong Kiss1r signal were observed in the olfactory bulb, medial septum and diagonal band of Broca, around the OVLT, and throughout the preoptic area. Immunofluorescence staining revealed that most of the neuronal populations with strong Kiss1r signals in the rostral brain regions were also GnRH-immunoreactive. Moderate expression of Kiss1r was noted in the PVN, throughout the arcuate nucleus (ARC), in the caudal part of the dorsal raphe nucleus and in the inferior olive. In the PVN, Kiss1r-expressing neurons were sparsely distributed, mainly in the medial part of the nucleus. Neurons with relatively weak Kiss1r signals were observed in the supraoptic nucleus (SON), premammillary and supramammillary nuclei, periaqueductal grey and the inferior colliculus, in the hippocampus and dentate gyrus (Fig. 8; (Higo et al. 2016)).

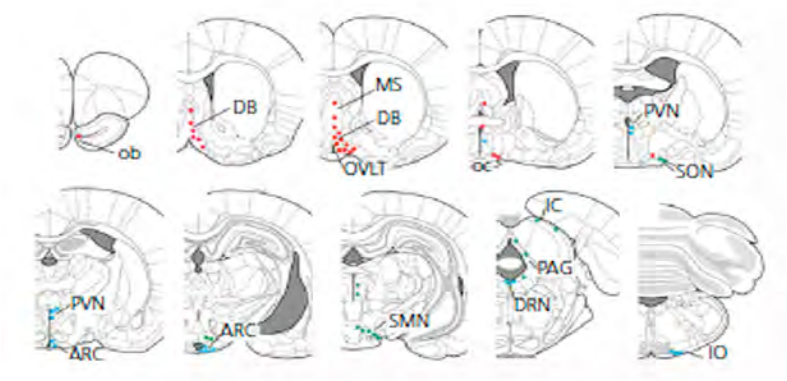

Figure 8: Distribution of Kiss1r-expressing neurons in female rat brain. ARC, arcuate nucleus; DB, diagonal band; DRN, dorsal raphe nucleus; IC, inferior colliculus; IO, inferior olive; MS, medial septum; Ob, olfactory bulb; OC, optic chiasm; OVLT, organum vasculosum of lamina terminalis; PAG, periaqueductal gray; PVN, Paraventricular nucleus; SMN, supramammillary nucleus; SON, supraoptic nucleus (modified from Higo et al., 2016).

2. Kisspeptin: physiological reproductive function, gonadal hormones and KiSS1 neurons

As described previously, Kisspeptin were originally characterized as potent anti-metastatic agents in breast cancer and malignant melanoma cells. The role of Kisspeptin and its receptor, GPR54, in the control of puberty and fertility remained unknown until late 2003, when de Roux and Seminara with their colleagues, independently, reported deletions and inactivating mutations of GPR54 in patients with idiopathic hypogonadotropic hypogonadism (IHH) (de Roux et al. 2003) (Seminara et al. 2003). The lack of overt defects in GnRH neuronal migration, GnRH synthesis or pituitary responsiveness to GnRH in individuals with impaired kisspeptin signaling led to the hypothesis that the Kiss1/GPR54 system should be an essential, excitatory upstream regulator of GnRH neurons so that absence of kisspeptin signaling would result in suppressed GnRH secretion (Pinilla et al. 2012). In fact, mutations in Kiss/GPR54 system that interfere with Kisspeptin signaling prevent normal pubertal development in humans and mice and the failure of the GPR54 and KiSS1 mutant mice to ovulate has led to the suggestion that Kisspeptin signaling may be required for the pre-ovulatory luteinizing hormone (LH) surge (Colledge and d'Anglemont de Tassigny 2010).

Since 2003, many papers, describing this neuroendocrine networks in different animal models such as: rodents, sheep, primates and pony mares, have demonstrated that (a) KiSS1 neurons and fibers are clearly present in RP3V and ARC nuclei of mice and rats' brain (Dungan, Clifton, and Steiner 2006) (Tena-Sempere 2006); (b) KiSS1 fibers belonging to these nuclei send direct projections to the GPR54-expressing GnRH neurons in the POA and that an increase in KiSS1 fibers immunoreactivity is evident a few days before puberty onset in mice (Clarkson et al. 2010) (Wintermantel et al. 2006); and (c) RP3V and ARC KiSS1 neurons express ERα, ERβ, and AR

and (d) that gonadal steroids differentially regulate kisspeptin expression in different hypothalamic neuronal populations. Altogether these findings have contributed to strengthen the role of kisspeptin in reproduction, which can be summarized as follows (Roa et al. 2008):

1. Kisspeptin is universally recognized as an essential activator of the gonadotropin axis: with key roles in puberty onset and control of gonadotropin secretion;
2. novel aspects of Kiss/GPR54 physiology have emerged, including their involvement in the neuroendocrine control of ovulation;
3. Kisspeptin plays an essential role in the metabolic regulation of fertility (as fundamental gatekeeper of reproduction).

Recent evidences from functional genomic studies support the view, anticipated on the basis of initial expression and electro-physiological studies, that kisspeptins ultimately modulate the output of GnRH neurons to directly conduct their potent stimulatory effects on the reproductive system, since selective elimination of GPR54 from GnRH cells recapitulates the hypogonadal phenotype of global GPR54 null animals (Kirilov et al. 2013) (Novaira et al. 2014), whereas rescue of GPR54 signaling in GnRH neurons appears to be sufficient to attain puberty and fertility (Kirilov et al. 2013). Yet, a recent study conducted by Leon and colleagues demonstrate that this direct action appear to be insufficient to completely preserve proper functionality of gonadotropic axis, underling the complex mode of action of kisspeptin in the control of the HPG axis and suggesting a role of kisspeptin signaling outside GnRH cells (Leon et al. 2016).

The patterns of pubertal activation of the HPG axis and its subsequent function in adulthood are sexually dimorphic and this is at the base of their diversity in differentiated brain circuitries and neural mechanisms. Indeed, studies in rodents showed that an important and distinctive characteristic of KiSS1 neuronal populations is their sexual dimorphism (particularly apparent in rodents AVPV area), with adults' females having more KiSS1 cells than males (Clarkson and Herbison 2006) (Kauffman et al. 2007). *In situ* hybridization studies on rats have shown that the number of KiSS1 mRNA-expressing neurons in the AVPV of adult females is as much as 25 times greater than in males (Clarkson et al. 2009), and similar sex differences in kisspeptin protein levels in the same area have also been reported by IHC studies in adult mice and rats (Adachi et al. 2007) (Clarkson and Herbison 2006). Although sex steroids in adulthood modulate KiSS1 gene expression in the AVPV, the sex difference in KiSS1 neurons is not attributable to sex difference in circulating levels of T or E in adulthood. Thus, exposure to different levels of sex steroids during perinatal life might induce a masculinization or feminization of KiSS1 neuronal populations. Gonadectomized male and female rats receiving equal sex steroids

treatments as adults still display strongly sexual dimorphism in KiSS1 expression in the AVPV (Kauffman et al. 2007); while withdrawal of T by neonatal gonadectomized male rats (not replaced by sex steroids injection) apparently feminizes the population of KiSS1 neurons at the AVPV and makes them able to respond to the positive feedback of estradiol (Homma et al. 2009). On the contrary, perinatal sex hormone's exposure dramatically affects the sex difference in KiSS1 neurons: female rats perinatally treated with a single injection of androgen (to mimic male normal development) display, when adult, a male-like AVPV Kiss1 system, suggesting that increasing levels of T in males are responsible for the virtual absence of KiSS1 neurons in this brain area (Kauffman et al. 2007). Additionally, studies on aromatase null and hypogonadal mice suggest that the sexual dimorphic differentiation of AVPV is not a simple procedure, in fact the complete functional feminization of these neurons is not only a defective process, but requires some degree of estrogenic input (Gill et al. 2010) (Navarro et al. 2009) (Gonzalez-Martinez et al. 2008).

The ARC, although not so well studied as the AVPV, also displays several sex differences in neuronal parameters, probably why the visualization of Kiss1 neuron cell bodies is difficult in the ARC region because of the high density of kisspeptin fibers in this region. Older studies described the lack of sex dimorphism for the Kisspeptin system of the ARC or organizational effects of early sex steroids exposure (Kauffman et al. 2007), however, more recent evidences suggest that the ARC population of KiSS1 neurons is larger in female rodents, in parallel with data from sheep and human (Iijima et al. 2011). In addition, neonatal administration of estrogens to male and female rats affects hypothalamic kisspeptin expression, confirming that ARC population is also likely sensitive to the organizational effects of sex steroids (Navarro et al. 2009).

Sex steroids provide feedback loops that allow the gonads to communicate with the hypothalamus to regulate GnRH release. However, sex steroids achieve this indirectly since GnRH neurons do not express androgen or estrogen (ERα) receptors (Herbison and Theodosis 1992) (Huang and Harlan 1993). It is now thought that kisspeptin neurons mediate the actions of sex steroids on GnRH neurons. The majority of kisspeptin neurons express estrogen receptor alpha (ERα of ~90%) (Franceschini et al. 2006; Smith, Dungan, et al. 2005), the androgen receptor (~65%) (Smith, Dungan, et al. 2005), and the progesterone receptor (~86%) (Smith et al. 2007), consistent with their role as mediators of sex steroid feedback on the reproductive axis. In rodents, sex steroids differentially regulate kisspeptin expression to decrease expression in the

AVPV region (Kauffman et al. 2007) (Smith, Dungan, et al. 2005) and increase expression in the ARC (Smith, Dungan, et al. 2005). These changes are reversed by either testosterone or estradiol replacement (Smith, Dungan, et al. 2005) (Fig. 9).

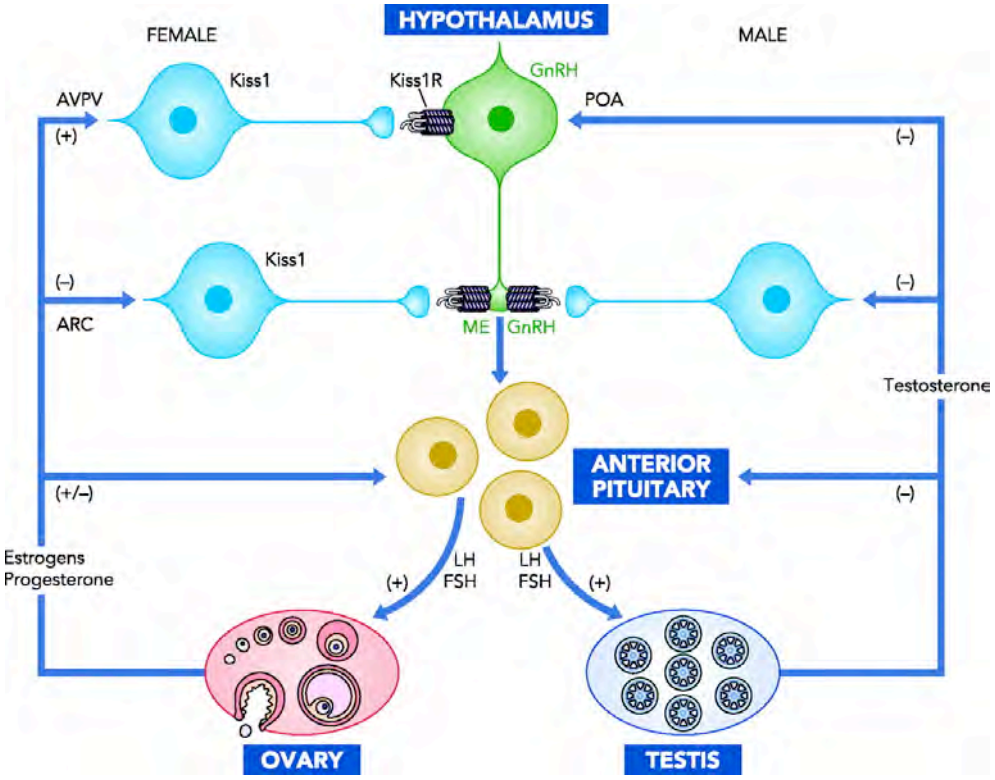

Figure 9: The mammalian hypothalamic pituitary gonadal axis. Pulsatile secretion of gonadotrophic releasing hormone (GnRH) stimulates the anterior pituitary to release the gonadotrophic hormones, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). These act on the gonads to promote gamete formation and the production of gonadal steroid hormones, which form feedback loops to regulate GnRH, LH, and FSH release. Kisspeptin (Kiss1) neurons act as a principal relay for steroid feedback on GnRH secretion. In females, high levels of estrogens and progesterone stimulate kisspeptin neurons of the AVPV to induce the preovulatory surge of GnRH/LH, whereas they inhibit KISS1 expression in the arcuate nucleus (ARC). In the male, GnRH and gonadotrophic hormone release are negatively regulated by circulating testosterone, partly through the activity of kisspeptin neurons of the ARC. POA, preoptic area; AVPV, anteroventral periventricular nucleus; ME, median eminence (d'Anglemont de Tassigny and Colledge, 2010).

- *Negative feedback: role of ARC KiSS1 neurons*

In rodents, ovine, and primates, the ARC (or the equivalent nucleus of the infundibular region) has been proposed to contain the KiSS1 neural substrate mediating the negative feedback action of sex steroids on GnRH/gonadotropin secretion (Roa et al. 2009). In agreement with this view, gonadectomized animals, in which sex steroids are low or absent, has been shown to elevate KiSS1 mRNA levels in ARC, conversely, sex steroids (estradiol or testosterone) replacement prevented such increases. Accordingly, there is low expression of KiSS1 mRNA in the ARC in the afternoon of proestrus phase (Kauffman 2009) (Smith, Dungan, et al. 2005) when estradiol reaches its peak. Furthermore, the ability of gonadectomy to remove the negative feedback

signaling to GnRH neurons, and in this manner stimulating the reproductive axis, is not detected in gonadectomized GPR54 KO mice. Thus, although KiSS1 gene expression in the ARC of this mice model increases after gonadectomy (in a similar manner to wild type mice), GPR54 KO mice do not display a post-ovariectomy rise in plasma LH, suggesting that GnRH neurons in these animals are not stimulated after gonadectomy (Funes et al. 2003).

The effects of sex steroids in mice ARC are mediated via ERα. Accordingly, KiSS1 neurons do express ERα in the ARC, and functional genomic studies performed using a mouse model lacking ERα in KiSS1 neurons evidenced the importance of ERα-mediated pathways in this hypothalamic nucleus for the inhibitory effect of estrogens on GnRH/gonadotropin release, even before puberty (Mayer et al. 2010). Other studies were performed using mice lacking ERE-mediated ERα signaling in DNA. These animals cannot activate the classical ERα genomic pathways, but they retain other (such as non-classical) aspects of ERα signaling, suggesting that the estrogen-negative feedback in ARC can take place in the congenital absence of classical ERα pathway (Gottsch, Clifton, and Steiner 2009).

Apparently this peculiarity contrasts with the mechanism of the estrogen regulation of KiSS1 expression at the RP3V (see section below). A further difference with that population is that ARC KiSS1 neurons co-express NKB and dynorphin in rodents, sheep, monkeys and humans (Goodman et al. 2007) (Hrabovszky et al. 2000) (Lehman, Coolen, and Goodman 2010) (Navarro et al. 2009). In conclusion, these findings suggest that sex steroids inhibit KiSS1 neurons in the ARC, thereby inhibiting ARC-derived kisspeptin stimulation of GnRH secretion.

- *Positive feedback: role of RP3V KiSS1 neurons*

Whereas negative feedback effects of sex steroids on reproductive axis occur in both sexes, a positive feedback action of estradiol should act in females at a specific stage of their estrus/menstrual cycle, inducing stimulation of GnRH/LH secretion, which triggers ovulation. Several studies demonstrated that, in rodents, estrogen-responsive KiSS1 neurons located in the RP3V are important mediators to induce the generation of the preovulatory LH surge. In brief, these studies have shown that:

(a) it exists a direct relationship between kisppeptin-GPR54-GnRH, and that kisspeptin is a potent secretagogue for GnRH via GPR54 activation (Irwig et al. 2004) (Parhar, Ogawa, and Sakuma 2004);

(b) KiSS1 neurons in the RP3V are connected to GnRH cells by projecting their axonal fibers directly on GnRH cell bodies and processes (d'Anglemont de Tassigny et al. 2008);

(c) KiSS1 expression in the RP3V increases at the preovulatory period and during sex steroid-

primed LH surge, as evidenced by c-Fos expression (Gottsch et al. 2004) (Irwig et al. 2004) (Navarro, Castellano, Fernandez-Fernandez, Tovar, Roa, Mayen, Nogueiras, et al. 2005) (Matsui et al. 2004);

(d) estrogen up-regulates the expression of KiSS1 selectively via ERα, and virtually all KiSS1 neurons in the RP3V express this receptor isoform (Smith, Dungan, et al. 2005);

(e) central infusions of kisspeptin peptide *in vivo* don't block the propagation of the signal abolishing the LH surge (Gottsch et al. 2004) (Matsui et al. 2004) (Navarro, Castellano, Fernandez-Fernandez, Tovar, Roa, Mayen, Barreiro, et al. 2005) (Navarro et al. 2009);

(f) RP3V kisspeptin neurons integrate a circadian vasopressin input (Piet et al. 2015) with the fluctuating levels of estradiol across the estrous cycle to become fully activated only on the afternoon of pro-estrus to stimulate GnRH neurons that, in turn, trigger the preovulatory GnRH surge (Fig. X5) (Smarr, Morris, and de la Iglesia 2012) (Herbison 2008) (de la Iglesia and Schwartz 2006).

In conclusion, these data, with the proven existence of projections of KiSS1 neurons from RP3V to GnRH neurons (d'Anglemont de Tassigny et al. 2008), strongly suggest a crucial role of this population for integrating and relaying estrogen signal on GnRH/gonadotropin secretion (Kauffman 2009). The molecular mechanism depends via classical ERα-ERE pathways at the RP3V, as demonstrated by the use of a genetically modified mouse model lacking ERE-mediated ERα signaling in DNA in KiSS1 neurons (Gottsch, Clifton, and Steiner 2009).

Although all spontaneously ovulating mammals generate an LH surge, the mechanisms underlying its generation seem to be species specific (Herbison 2016). For example, sheep have a relatively small population of KiSS1 neurons in the AVPV/preoptic area which is not strongly regulated by sex steroids (Smith et al. 2007), on the contrary the ovine mediobasal hypothalamus (corresponding to ventromedial and ARC nuclei) is believed to contain the neuronal substrate mediating the positive feedback effects on GnRH neurons (Caraty et al. 1998) (Clarke et al. 2001) (Goubillon et al. 1999). Studies on ovine showed that the expression of KiSS1 in the ARC is regulated by sex steroids and is strongly increased prior to and during the preovulatory LH surge, suggesting that in the ewe both positive and negative feedback effects of sex steroids may be mediated by the same group of KiSS1 neurons in the mediobasal hypothalamus (Kauffman 2009). Furthermore, in contrast to the situation in rodents, the GnRH neurons activated at the time of the surge in sheep are not localized to one region but instead are found throughout the GnRH neuron continuum (Moenter, Karsch, and Lehman 1993). Although kisspeptin neuron inputs to sheep GnRH neurons have a prominent function in surge generation, a three-phase

model of neuronal activation involving several different neuronal phenotypes has been proposed to underlie the ovine GnRH surge (Plant 2015). Till now, it is not clear if these positive and negative actions are mediated by the same ARC KiSS1 neurons or by separate sub-populations of KiSS1 cells within the nucleus (Estrada et al. 2006) (Smith et al. 2006). Primates, including human, have a distribution of KiSS1 neurons also concentrated in the mediobasal hypothalamus/infundibular nucleus, but the role of the system in generating the preovulatory LH surge in primates has not completely been demonstrated (Jayasena et al. 2011) (Lehman, Coolen, and Goodman 2010).

Upon disclosure of the potential roles of the Kiss1 system in the control of the HPG axis, one of the first goals of the subsequent physiological studies was to address whether kisspeptins are able to modulate adult gonadotropin secretion, as surrogate marker of GnRH neuronal activation (Roa et al. 2008) (Tena-Sempere 2006). The fact that mutations in *KISS1* and *GPR54* can disrupt pulsatile secretion of LH in humans (Tenenbaum-Rakover et al. 2007) (Seminara et al. 2003) and rodents (Steyn et al. 2013) has suggested a key role for kisspeptin in pulse generation. Navarro and colleagues (Navarro et al. 2009) proposed that kisspeptin neurons within the ARC use the co-expressed neuropeptides neurokinin B (NKB) and dynorphin (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. This model has been supported by different studies which have shown that arcuate kisspeptin neurons only project to the distal dendrites and terminals of GnRH neurons in and around the median eminence of mice (Yip et al. 2015) and that kisspeptin administration to GnRH neuron projections in this area results in the secretion of GnRH (Glanowska and Moenter 2015). Furthermore, using an optogenetic approach, the synchronous activation of arcuate kisspeptin neurons *in vivo* generated pulsatile LH secretion in the mouse (Han et al. 2015) (Fig. 10).
This model of pulse generation is compatible with data obtained from primates (Plant 2006), although the patterns of neuropeptide co-expression in arcuate kisspeptin neurons seem to differ between humans and other species (Skrapits et al. 2015). Many key features of this pulse-generator model remain to be established, such as how the arcuate kisspeptin neurons might be synchronized to generate an oscillatory output; however, it remains a useful hypothesis. Given the ubiquity of pulse generation among mammals, and the presence of arcuate kisspeptin neurons in all of these species (Oakley, Clifton, and Steiner 2009), it is possible that this pulse-generation unit is a highly conserved central core of the GnRH neuronal network.

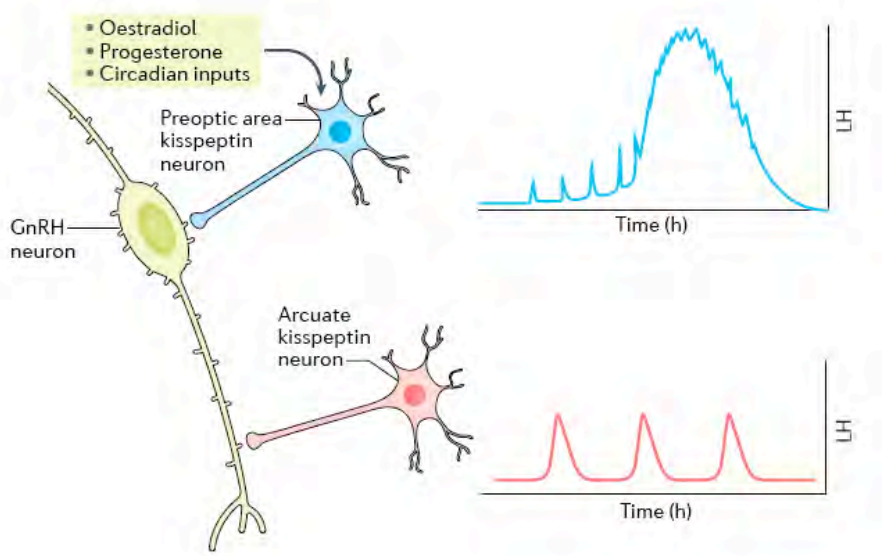

Figure 10: Regulation of GnRH neurons by kisspeptin neurons. The preoptic area kisspeptin neurons project to the cell body and proximal dendrites of gonadotropin-releasing hormone (GnRH) neurons. The arcuate kisspeptin neurons project to the distal dendron of the GnRH neuron and are involved in generating pulsatile secretion of luteinizing hormone (LH) (Herbison, 2016).

In addition to E_2 and T, progesterone (P) is a mediator of sex steroid feedback on the reproductive axis (Fig. X4). P is a major inhibitory brake in the luteal phase of the ovarian/menstrual cycle: it inhibits GnRH and LH secretion (McCartney et al. 2002). The administration of P before or concurrent with E_2 inhibits E_2 positive feedback and abolishes the preovulatory GnRH and gonadotrophin surge, and 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). Old studies have shown that an increase in circulatory level of P between estrous to metestrus induces the prolongation of estrous cycle length in rats (Everett 1948) (Nequin, Alvarez, and Schwartz 1979). These data were recently confirmed by a study demonstrating that exogenous administration of P during metestrus prolongs estrous cycle lengths, particularly the time spent in metestrus stage (He et al. 2017). Moreover, alterations in P inhibitory feedback have been implicated in infertility associated with enhanced GnRH/LH secretion (Molloy et al. 1984). Although P regulates GnRH secretion via its hypothalamic receptors, the lack of P receptors (PR) on GnRH neurons (Le et al. 1997) suggests that its action may involve interneurons expressing PR. The ability of E_2 to induce the LH surge is dependent on the presence and activation of PR (Chappell et al. 1999). In rodents, in AVPV while PR expression is under ERα regulation (Shughrue, Lane, and Merchenthaler 1997), progesterone down regulates ER (Simerly et al. 1996) and may counteract the induction of PR by E_2.

Therefore, P action to block the LH surge might be due to the presence of PR in the AVPV neurons. This could reduce the ability of E_2 to induce the LH surge. Mittelman-Smith et colleague hypothesized that AVPV/PeN Kiss neurons are a direct target for P and that integration of E2 and PR signaling in Kiss neurons is a critical component of the LH surge (Mittelman-Smith et al. 2017); in effect kisspeptin neurons are the most probable upstream afferents, expressing PR as well as ER (Fig. 11) (Clarkson et al. 2008).

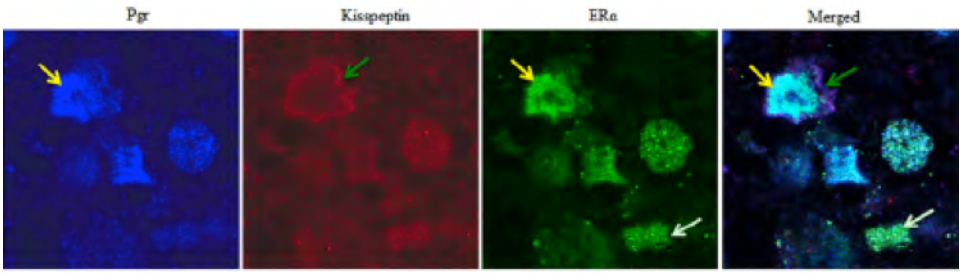

Figure 11: PR and ERα are co-localized AVPV kisspeptin neurons. Co-localization of PGR, ERα, and kisspeptin in kisspeptin neuron in the hypothalamic AVPV nucleus from a adult female C57BL/6 mouse in the estrus stage. PR (blue), kisspeptin (red), ERα (green) and merged image. Yellow arrows indicate nuclear PR, ERα, and merged PR/Erα; green cytoplasmic kisspeptin and white ERα positive nucleus (Gal et al., 2016).

Finally, PR knockout mice in kisspeptin neurons showed loss of LH surges, irregular estrous cycles, with persistent diestrus, and infertility (Gal et al. 2016) (Stephens et al. 2015). A recent *in vitro* study (Mittelman-Smith et al. 2017) showed that P facilitates the response of kiss neurons to E_2. Furthermore, co-culture and media sharing assays indicated that astrocyte-derived neuroP is sufficient to augment E_2 signaling in immortalized kiss neurons (Mittelman-Smith et al. 2017). In summary, their results demonstrate that E2 induction of PR at the membrane lead to kiss release. thus providing a mechanistic explanation of steroid signaling integration at the level of kiss cells.

PR in the ARC contributes to negative feedback of the axis. Microimplants of the P antagonist RU486 in the ARC, but not in the POA, blunt the negative feedback effects of P in ewes (Goodman et al. 2011), probably because KNDy neurons expressed PR (Goodman et al. 2004). These data are supported by study of He and colleagues (He et al. 2017), that demonstrated intra-ARC injection of RU486 significantly increased LH pulse frequency in P-treated rats. However, the role of hypothalamic PR in control of the LH surge is controversial. Previous studies have indicated that progesterone might act via its receptors in the mPOA to block the LH surge in rats (Banks and Freeman, 1980). In contrasts, 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). Therefore, the PR role in AVPV and ARC of progesterone negative feedback effect on pulsatile

LH secretion as well as the LH surge remains unclear.

3. Kisspeptin system: development and puberty

Until now, the mechanisms whereby sex steroids, or other modulators, affect the organization and development of KiSS1 system are not completely disclosed. In addition to the organizational phenomena that characterize kisspeptin cells development during the critical period, several data also indicate that the hypothalamic KiSS1 system undergoes a complex pattern of neuroanatomical maturation and functional activation during the course of puberty (Roa, Navarro, and Tena-Sempere 2011). Indeed, *in situ* hybridization and IHC studies have demonstrated a significant modification of kisspeptin during pubertal development. Early studies in mice showed that pre-prepubertal males have fewer Kiss1 mRNA expressing cells in the RP3V than adults (Han et al. 2005). Using IHC, Clarkson and Herbison, discovered that the number of kisspeptin neurons within the RP3V increases throughout postnatal development, reaching adult numbers at the time of puberty in both males and females (Clarkson and Herbison 2006). These investigations showed that AVPV/PeN *Kiss1* mRNA and kisspeptin protein are undetectable prior to PND 10 and 15, respectively (Clarkson et al. 2009), and then increase from PND 15 to adulthood, as assessed every 5 days of age (Clarkson et al. 2009). Similarly, an increase of mRNA and Kiss protein has been reported in the RP3V throughout pubertal development of rats and mice (Bentsen et al. 2010) (Mayer et al. 2010) (Takase et al. 2009) (Takumi, Iijima, and Ozawa 2011). A recent study has detailed day-by-day the *Kiss1* mRNA expression in female mice, demonstrating that *Kiss1* cell number, *Kiss1* mRNA per cell, and total *Kiss1* mRNA levels in the RP3V markedly, but consistently and gradually, increase from PND 15 through PND 30 (Fig. 12, A; (Semaan and Kauffman 2015)).

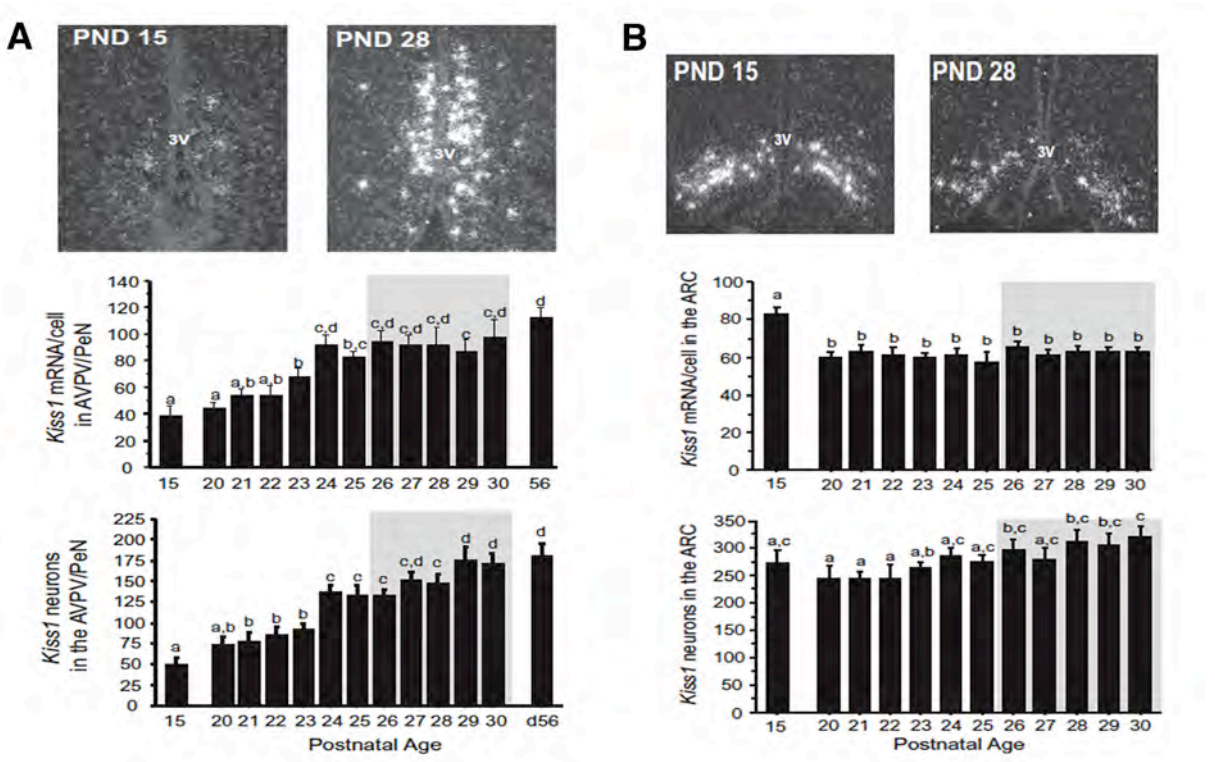

Figure 12: Kiss1 expression in the AVPV/PeN and ARC of female prepubertal and pubertal mice. Rapresentative images of kISS1 expression, determined by ISH, in the AVPV/PeN and ARC of female mice. Mean relative total Kiss1 mRNA and mean Kiss1 mRNA content per neuron in (A) AVPV/PeN and in (B) ARC of female mice between PND15 and PND30. 3V, third ventricle (modified from Semaan and Kauffman, 2015).

Contrary to the RP3V, ARC *Kiss1* expression is readily detectable prenatally and at birth in rodents, and continues to be expressed throughout postnatal development (Cao and Patisaul 2011) (Poling and Kauffman 2013). A study performed by Desroziers and colleagues demonstrated that in rat ARC kisspeptin neurons are born locally during an extended embryonic period and are able to synthesise kisspeptins rapidly after their birth. Moreover, a sex-independent decrease of kisspeptin-IR cell numbers was observed during the perinatal period, suggesting to authors important regulations of kisspeptin neurons around birth (Desroziers, Droguerre, et al. 2012). Data regarding peripubertal changes in ARC *Kiss1* gene expression are either lacking, incomplete, or conflicting. Probably because is difficult to distinguish, using IHC, the kisspeptin cell bodies amongst the dense plexus of kiss fibers labeling in the ARC. In the female rat, some studies demonstrated a small increase in ARC of kiss around early puberty, as *Kiss1* expression (Bentsen et al. 2010) (Takase et al. 2009) and as kisspeptin-IR fibre density (Desroziers, Mikkelsen, et al. 2012); a more dramatic pubertal increase has also recently been reported (Lomniczi et al. 2013). Similarly, Losa and colleagues (Losa et al. 2011) demonstrated that total kisspeptin immunoreactivity in the ARC increases throughout pubertal development in female but not male rats, however, in the same study, the number of kisspeptin immunoreactive cells and Kiss1 mRNA expressing cell bodies in the ARC did not change during pubertal

development and was not sexually dimorphic (Losa et al. 2011). A moderate increase of the *Kiss1* neurons' number and of the total levels of *Kiss1* mRNA in female mice ARC during the development has been reported by Semaan and Kauffman (Semaan and Kauffman 2015), the significant increases were first evident around PND 24–26. In the same study, the authors reported that the amount of *Kiss1* mRNA per cell in the ARC highest at PND 15; between PND 15 and PND 20, the level of *Kiss1* mRNA per cell dropped ~27% and, correspondingly, the total *Kiss1* mRNA in the ARC also decreased during this pre-pubertal stage (Fig. 12, B; (Semaan and Kauffman 2015)). The authors explain this decrease in *Kiss1* levels per cell to the high estradiol levels during puberty, since estradiol is known to repress *Kiss1* expression in the ARC (i.e., negative feedback; (Smith, Dungan, et al. 2005) {Semaan, 2015 #160). On the contrary, other studies have not reported differences in ARC *Kiss1* levels between juvenile and adult rodents (Gill et al. 2010). The results of these studies suggest that the increase in total immunoreactivity in the ARC during pubertal development is likely attributable to changes in kisspeptin fibers within the ARC rather than in the cell number. The origins of the kisspeptin immunoreactive fibers in the ARC remain unknown, however, recent tract-tracing studies conducted in adult mice demonstrate that at least some of the kisspeptin fibers within the ARC may originate from the RP3V kisspeptin neurons (Yeo and Herbison 2011). Kisspeptin neurons from within the ARC may also contribute to the ARC kisspeptin fiber network; however, this is difficult to determine using traditional tract-tracing techniques.

In parallel with developmental changes of kisspeptin neurons in RP3V and ARC, also the kisspeptin fibers within hypothalamus changed their density. Kisspeptin fiber appositions to GnRH neurons have been observed at the time of puberty in mice and ewes suggesting that kisspeptin neurons target the GnRH neurons to activate them (Clarkson and Herbison 2006) (Nestor et al. 2012), but the source of these fibers has not yet been definitively proven. In mice, the temporal correlation between the emergence of kisspeptin expression in the RP3V and the appearance of kisspeptin fibers in close proximity to GnRH neurons suggests that the RP3V may provide the inputs to GnRH neurons at puberty. However, it is also possible a contribute from the ARC, in fact, the coexpression of neurokinin B (NKB) and kisspeptin in fibers in close apposition with GnRH neurons demonstrate that the ARC kisspeptin neurons do project to the GnRH neurons in adult mice (Kallo et al. 2012). On the contrary, in gonadally intact ewes, the increase in the percentage of POA GnRH neurons exhibiting a close contact with kisspeptin fibers during pubertal development occurs in parallel with an increase in the number of kisspeptin- immunoreactive neurons in the ARC (Nestor et al. 2012). Even if it is not known

whether the increase in kisspeptin appositions with GnRH neurons at puberty results from an increase in synaptic contacts or from an increase in the content of kisspeptin peptide within the axons, the surge in kisspeptin terminal appositions with GnRH neurons suggests that kisspeptin is increasingly available to be released and to stimulate the GnRH neurons at the time of puberty (Clarkson and Herbison 2006) (Nestor et al. 2012). The involvement of kisspeptin is confirmed by the fact that puberty onset is delayed after inhibition of GPR54/Kiss1r signaling (Pineda et al. 2010).

Kisspeptin is also involved in pubertal maturation. In fact, Kisspeptin administration to prepubertal rodents and monkeys induces precocious puberty (Navarro et al. 2004) (Shahab et al. 2005), but low kisspeptin doses are less effective at stimulating gonadotropin secretion and GnRH neuronal firing activity in juvenile than adult rodents (Han et al. 2005), suggesting that kisspeptin has a reduced ability to activate the GnRH system before puberty. In rats of both sexes and in female monkeys, but not in male mice, hypothalamic Kiss1r expression is higher in adulthood than in juveniles (Han et al. 2005) (Navarro et al. 2004) (Shahab et al. 2005). Using transgenic mice, Herbison et al. (Herbison et al. 2010) reported that between PND 5 and PND 20 there was a significant increase in the number of GnRH cells expressing Kiss1r. On the contrary, Semaan and Kauffman (Semaan and Kauffman 2015) determined that the level of Kiss1r expression specifically in GnRH neurons of female mice is already at maximal adult levels by PND 15 and does not vary during any stage of the pubertal transition. These results suggest that in mice the developmental increase in Kiss1r-GnRH coexpression occurs before PND 15, because no changes were observed in mice after this age.

All these studies on the development of kisspeptin system, with an increase of expression mainly at the pubertal time, indicate that Kisspeptin signaling pathway is a requisite for the onset of puberty in mammals.

In rodents and humans, the *KiSS1-GPR54-GnRH system* is completely formed at birth, as well as the GnRH pulse generator is functioning and responsive to kisspeptin stimulation (Clarkson et al. 2010), however, the typical events of pubertal age and reproductive phase are not evident through all juvenile time. A two-step kisspeptin mechanism has been hypothesized to control the activation of the GnRH neuron at puberty: (a) the emergence of an estradiol-dependent kisspeptin input to GnRH neurons and (b) the maturation of the GnRH neuron electrical response to kisspeptin (Herbison et al. 2010).

The first step suggests that AVPV/PeN nuclei are not required for the initial activity of the HPG

axis, offering new cues to understand the mechanisms underlying the quiescent juvenile period. In fact, several studies demonstrated that γ-aminobutirric acid (GABA), glutamate and other neurotransmitters (Clarkson and Herbison 2006) (Terasawa and Fernandez 2001) (Vendel et al. 2006) play important roles in the regulation of GnRH neurons functions from birth to peripubertal phase. Neurotransmitters, converging with gonadal activation, may provide the initial activation of the GnRH neurons, which results in an increase in circulating E_2 levels. The rising levels of E_2 increase kisspeptin expression in the AVPV/PeN that then amplifies GnRH neuron activity in a positive feedback manner leading to the completion of puberty onset. After puberty onset, kisspeptin levels in the AVPV/PeN fluctuate with the cyclical levels of E_2 to drive the generation of the preovulatory GnRH/LH surge (Clarkson et al. 2010). According to this model, the AVPV/PeN kisspeptin neurons exist as part of an E_2-sensitive positive feedback pathway, activated at the beginning of puberty in response to the increasing E_2 levels, suggesting that these neurons are the most plausible candidates to explain the puberty onset (Fig. 13).

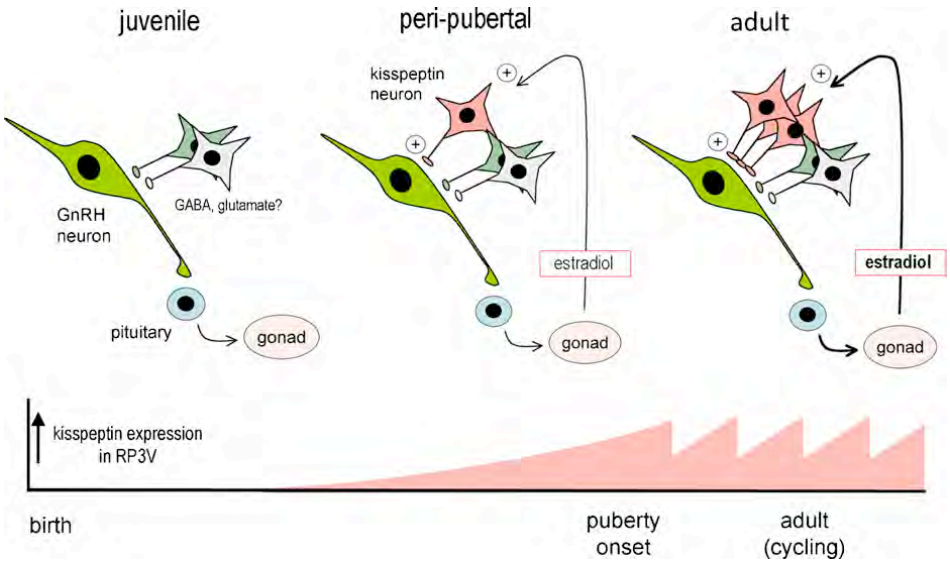

Figure 13: Schematic diagram demonstrating the proposed E2–kisspeptin–GnRH positive feedback circuit responsible for completing puberty onset in the female mouse. From birth to the early peripubertal period there is no kisspeptin expression in the RP3V (represented by the pink colour at the bottom of the figure). Neurotransmitters, such as GABA and glutamate, may provide the initial activation of the GnRH neurons, which results in an increase in circulating E_2 levels. The rising levels of E_2 act to increase kisspeptin expression the RP3V that then amplifies GnRH neuron activity in a positive feedback manner leading to the completion of puberty onset. After puberty onset, kisspeptin levels in the RP3V fluctuate with the cyclical levels of E_2 to drive the generation of the preovulatory GnRH/LH surge (Clarkson et al., 2010).

The second step is based on the maturation of the electrical properties of GnRH neuron in order to respond to the kisspeptin signals. In fact, electrophysiological investigations have detected an increase of electrical response of GnRH cells to kisspeptin during the pubertal and adult phase

compared to juvenile phase (Han et al. 2005). The stimulatory action results from the ability of GPR54 to influence multiple ion channels in the GnRH neuron including potassium and non-selective cation channels, while the prolonged nature of this response appears to be dependent upon the intrinsic properties of the GnRH neuron (Clarkson et al. 2010). In these studies (Han et al. 2005; Clarkson et al. 2010) demonstrated that kisspeptin exerted the same degree of membrane depolarization on different age, but the proportion of responding cells and the duration of their electrical response were smaller in prepubertal life than after puberty. Therefore, it is possible to hypothesize that the electrical response of GnRH neurons to GPR54 activation matures over the postnatal period, indicating that the GnRH neurons are ready to respond to stimuli sent by kisspeptin during embryonic and postnatal development, but that the GPR54 receptor expressed on these cells reach an adult-like state only around 2 weeks before the puberty onset in mice.

These observations demonstrate that kisspeptin neurons are a key excitatory input for the GnRH system. Furthermore, in the last decade many studies underlined that kisspeptin probably does not act alone, the existence of a direct projection of kisspeptin neurons to GnRH neurons is well documented, but functional data strongly suggest that kisspeptin may also partially operate indirectly on GnRH neurons to modulate gonadotropin secretion by glutamatergic, GABAergic or GnIH transmission. Moreover, it is possible that the kisspeptin is likely to be one of many signals that are important for the regulation of puberty and fertility, for example the individual's nutritional status and availability of energy stores can have profound impact in reproductive functions (Hill, Elmquist, and Elias 2008).

4. Kisspeptin: metabolic control of puberty and fertility

Many recent studies analyzed the role of metabolic conditions and the amount of energy reserves of the organism in the modulation of pubertal timing (reviewed in (Castellano and Tena-Sempere 2016; Manfredi-Lozano, Roa, and Tena-Sempere 2017)). In fact, especially in female, the tight connection between energy balance, puberty, and reproduction becomes evident both on conditions of energy insufficiency (e.g. anorexia) and on situations of energy excess (e.g. morbid obesity) (Castellano and Tena-Sempere 2016). Three basic elements for the metabolic control of puberty have been identified: the adipose hormone, *leptin;* the pancreatic hormone*, insulin* and the gastrointestinal hormone, *ghrelin*. Leptin is released within the circulatory system in proportion to the amount of fat tissue (Halaas et al. 1995) and acts as an anorexigenic and thermogenic factor within the hypothalamus to adjust energy requirements, fat reserves, and food

intake (Casanueva and Dieguez 1999). The indispensable role of leptin in the metabolic control of puberty and fertility (reviewed in (Castellano and Tena-Sempere 2016; Manfredi-Lozano, Roa, and Tena-Sempere 2017)) is clearly illustrated by the negative impact on reproductive maturation and function of leptin insufficiency (delay or absence of puberty and compromised fertility). Insulin conducts stimulatory/permissive actions on the HPG axis; conditions of low or null insulin levels, such as uncontrolled diabetes, are commonly associated with suppressed gonadotropin levels and reproductive defects (Pralong 2010). It is possible that part of these actions might be conducted via the capacity of insulin to stimulate leptin synthesis. While, ghrelin operates as an orexigenic factor in the central control of appetite and metabolism. Ghrelin is considered a relevant signal in the metabolic control of puberty and reproduction; its actions is (predominantly) inhibitory and opposite to that of leptin (which is permissive and stimulatory)(Tena-Sempere 2013). In fact, compelling evidence suggests the ability of leptin and ghrelin to regulate the function of the GnRH neurons (Sanchez-Garrido and Tena-Sempere 2013; Tena-Sempere 2013). However, there are many evidences that these neurons may not be targeted directly by these metabolic factors (Sanchez-Garrido and Tena-Sempere 2013) (Tena-Sempere 2013), because GnRH neurons seem to be devoid not only of the receptors for sex steroids (they only express estrogen receptor β) but also for metabolic signals. In fact, in rodents and primates the expression levels of leptin receptors (LepRs) in GnRH neurons in vivo are low or negligible (Hakansson et al. 1998; Quennell et al. 2009). Moreover, the selective ablation of LepRs (Guan et al. 1997) or of insulin receptor (Divall et al. 2010) from GnRH neurons did not have any impact on either reproductive maturation or reproductive functions in mice. About the ghrelin receptor, we know that it is present in several areas of the brain (Guan et al. 1997), but, so far, the potential expression in GnRH neurons has not been addressed. The lack of direct regulation of GnRH neurons from the peripheral metabolic hormones, leptin, insulin and ghrelin, has led to hypothesis, that the kisspeptin system may be the hypothalamic circuit that could transmit the metabolic information.

Two observations support the hypothesis of a role of kisspeptin in the metabolic control of puberty:

(a) kiss expression is altered in conditions of reproductive impairment linked to metabolic stress,

(b) normalization of the kiss contributes to improve the reproductive phenotype despite unfavorable metabolic conditions.

In fact, while extreme conditions of negative energy balance (disrupting reproductive maturation and function) induce a suppression of the hypothalamic Kiss1 system, postnatal overfeeding may

advance the age of VO and alters the kisspeptin system. In female rats subjected to chronic food restriction throughout puberty (20% for 7 days) the Kiss1 mRNA expression is suppressed in ARC (Roa et al. 2009). Always in female rats, another study showed that dams subjected to 50% food restriction during pregnancy delivered pups showing a delay of the age of VO that is associated with a significant decrease in the hypothalamic levels of Kiss1 mRNA at the juvenile age (Iwasa et al. 2010). However, this state of low gonadotropin levels and delayed puberty caused by chronic under nutrition in prepubertal female rats is reversed by the pharmacological 'replacement' (Castellano et al. 2005). In agreement, the timing of VO was also normalized in early-undernourished female rats by the chronic injection of kisspeptin during the prepubertal period (Castellano and Tena-Sempere 2016).

In conditions of early over nutrition female mice display a reduction in the number of kisspeptin projections from the ARC to the preoptic area (POA); no changes in the age of VO were detected in this study, but a perturbed estrous cyclicity and decreased fertility were observed at later ages (Caron et al. 2012). In contrast, a recent study in female rats subjected to a nutritional manipulation (maternal HFD) has shown a significant increase in the number of Kiss1-expressing neurons in the ARC, but not in the AVPV; these alterations were associated with a significant advance in the age of VO (Takumi et al. 2015). Moreover, conditions of long-term obesity linked to reproductive failure have also been associated to suppressed kisspeptin signaling in adulthood (Roa and Tena-Sempere 2014) (De Bond and Smith 2014).

In a very recent review, Castellano and Tena-Sempere (Castellano and Tena-Sempere 2016) developed a model that integrates the relevant neurohormonal and molecular mechanisms that influence the metabolic control of female puberty (Fig. 14).

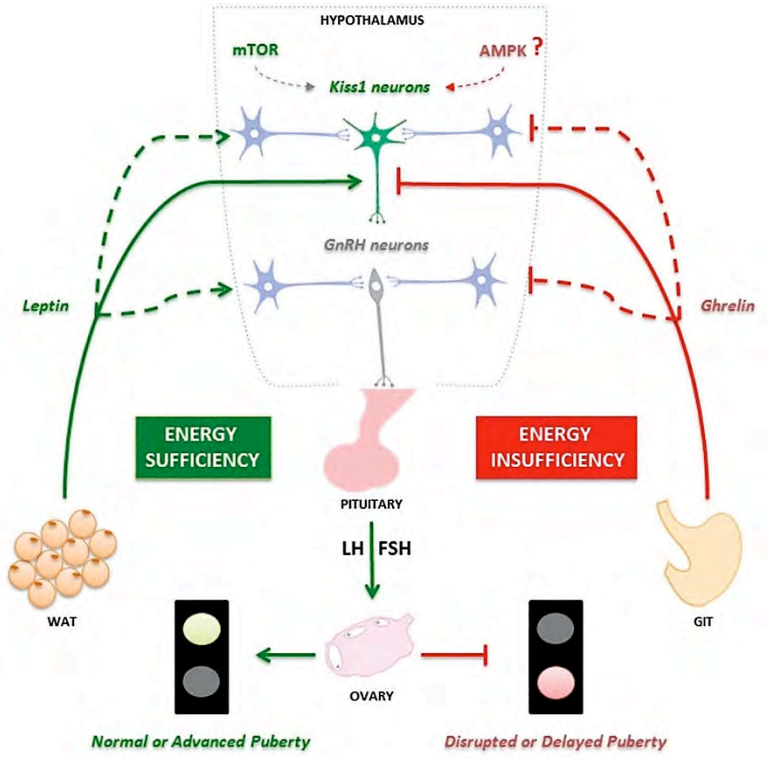

Figure 14: Model for the metabolic control of female puberty (Castellano and Tena-Sempere, 2016**).**

In this model leptin (as prominent signal for energy abundance; permissive/stimulatory factor) and ghrelin (as putative signal of energy insufficiency; inhibitory factor) have a strong role on indirect regulation of GnRH neurons; while, the major nodal role is represented by kisspeptin neurons, that integrate the different peripheral and central signals transmitting metabolic information onto reproductive centers. Many evidences suggest, that leptin can modulate directly the hypothalamic Kiss1 neurons at the time of puberty: (a) conditions of leptin deficiency reduced Kiss1 mRNA expression, especially in the ARC {Smith, 2006 #23} (Quennell et al. 2009), and the number of kisspeptin immunoreactive cells in the AVPV (Quennell et al. 2009); (b) leptin administration significantly increased the hypothalamic expression of Kiss1 mRNA in animal models of leptin deficiency and in neuronal cell lines (Sanchez-Garrido and Tena-Sempere 2013), (Roa and Tena-Sempere 2014), (De Bond and Smith 2014), (Smith et al. 2006) , (Backholer et al. 2010); (c) leptin stimulated ARC Kiss1 neurons through activation of transient receptor potential cation channels in guinea pigs (Qiu et al. 2011); (d) the mRNA encoding the functional LepR was detected in ARC Kiss1 neurons in mice and ewes (Smith et al. 2006) (Quennell et al. 2009); (e) hypothalamic colocalization of Kiss1 and LepR is very limited (Louis et al. 2011) and arises after completion of puberty (Cravo et al. 2013) (Qiu et al. 2015); and (f)

high levels of leptin have been associated with increased Kiss1/kisspeptin expression and early puberty onset (or vice versa) in female rats (Castellano et al. 2011). Furthermore, recent studies support also an indirect action of leptin on kisspeptin neurons suggesting the existence of a novel population of non-Kiss1 neurons, probably located in the hypothalamic premammillary nucleus (PMV), conveying the permissive effects of leptin on female puberty onset (Donato et al. 2009) (Donato et al. 2011). Only a fraction of PMV neurons expresses the leptin receptor and lesions studies seem to link the PMV at the kisspeptin signal (Donato et al. 2013).

Contrary to leptin, only a few studies have been conducted about the effect of ghrelin in the metabolic control of kiss1 neurons. The data suggest that ghrelin could act on hypothalamic Kiss1 neurons to modulate reproductive function. Some studies suggest that ghrelin may operate as putative regulator of Kiss1 expression in female rodents (Forbes et al. 2009) (Frazao et al. 2014). A very recent study conducted in OVX female mice demonstrated the expression of ghrelin receptor (GHSR) in a subset of kisspeptin neurons located at the ARC (Frazao et al. 2014). Recently, Smith and colleagues (Smith et al. 2013) suggested that the impact of ghrelin on Kiss1 expression at the POA might be indirect, since no expression of GHSR, the functional ghrelin receptor, was observed in the population of kisspeptin neurons located at the AVPV.

In the model proposed by Castellano and Tena-Sempere (Castellano and Tena-Sempere 2016) there are also reported two potential mediators of leptin actions on kiss1 neurons: the energy sensor, mTOR, and the adenosine monophosphateactivated protein kinase (AMPK) (Fig. 14). The mTOR signaling pathway has been proposed to be essential in keeping energy balance at the whole body level, among other mechanisms, by transmitting the anorectic effects of leptin (Cota et al. 2006) and seems to play a important role in the metabolic control of puberty (Roa et al. 2009). In fact, the blockade of central mTOR signaling, by means of repeated intracerebral administration of rapamycin, delayed the timing of puberty in female rats, as monitored by VO, reduced ovarian and uterus weights, perturbed ovarian follicular development, and suppressed ovulation (Roa et al. 2009). In the same study, the authors observed a decrease of the Kiss1 mRNA levels in the ARC and, to a lesser extent, in the AVPV of pubertal female rats, this was accompanied by a drastic reduction in circulating LH levels (Roa et al. 2009). Therefore, the hypotheses is that the function of mTOR as mediator of leptin effects might not take place directly within Kiss1 neurons, but rather occur in intermediate neuronal pathways with capacity to modulate Kiss1 cell function (Castellano and Tena-Sempere 2016). AMPK is a metabolite-sensing protein with the ability to detect changes in the AMP/ATP ratio and hence in the metabolic state of the cell (Canto and Auwerx 2009) (Naimi, Arous, and Van Obberghen 2010). AMPK signaling also plays a relevant role in the central control of energy balance and food

intake, as evidenced by its ability to stimulate appetite (Kahn et al. 2005) (Cota et al. 2006). In addition, AMPK is modulated by relevant metabolic hormones, such as leptin and ghrelin, which act as inhibitor and stimulator of AMPK activity, respectively (Andersson et al. 2004).

As previously reported, metabolic regulators, as leptin, insulin, and ghrelin, can induce an indirect regulation on GnRH neurons, acting on kisspeptin system. Furthermore, in the ARC there are two important sets of neuronal population with an anorexigenic action and orexigenic action that are essential first-order targets of these peripheral metabolic signals: proopiomelanocortin (POMC) and neuropeptide Y/agouti-related peptide (NPY/AgRP) neurons. In the last years, important studies showed that these two populations could interfere on reproductive functions through kisspeptin.

POMC neurons in the ARC act an anorexigenic action on food-intake control. These neurons co-express different neuropeptides, such as melanocortins, β -endorphin and cocaine and amphetamine-regulated transcript (CART), and neuro-transmitters, including γ -aminobutyric acid (GABA), glutamate (Meister et al. 2006) (Maolood and Meister 2008) (Hentges et al. 2009), and a wide variety of receptors, such as leptin receptors, insulin receptors, NPY Y1 receptors and also GPR54/kiss1r (Quennell et al. 2009) (Fuxe et al. 1997) (Konner et al. 2007) (Higo, Iijima, and Ozawa 2017). A very recent study in female rat, revealed that approximately 63±3% of Kiss1r-expressing neurons were also POMC-immunoreactive (Higo, Iijima, and Ozawa 2017). A pharmacological study by Fu et al. (Fu and van den Pol 2010) using wild-type mice suggested the possibility that kisspeptin has an excitatory effect on POMC neurons via the Kiss1r. Furthermore, in ARC mice the Kiss1r expression remains to be confirmed because of inconsistencies between reports using X-gal staining in heterozygous GPR54-IRES-LacZ transgenic mice (Liu and Herbison 2015), reverse transcriptase-PCR or electrophysiology (Fu and van den Pol 2010). However, it is noteworthy that, even if Kiss1r were not expressed in the mouse ARC, modulation of POMC neurons in this region by kisspeptin neurons is highly probable via a pathway independent of Kiss1r, such as one involving glutamatergic signal transduction (Nestor et al. 2016) or the neuropeptide FF receptors (Liu and Herbison 2015). Moreover, the neuroanatomical basis for this interrelation between POMC and kisspeptin is provided by the observation that POMC fibers are found in close apposition to ARC Kiss1 cell bodies in peripubertal rats (Manfredi-Lozano et al. 2016). POMC neurons in the ARC are known to co-express the neuropeptide, CART (Dhillo et al. 2002). Interestingly, True et al. (True et al. 2013) reported that CART might mediate part of leptin actions on Kiss1 neurons, as CART expression in POMC neurons is stimulated by leptin and CART has been shown to project

excitatory inputs to kisspeptin neurons. These results make kisspeptin system a potentially ideal integrator with POMC for the joint control of metabolism and reproductive function.

NPY act as orexigenic signals and have similar responses to fluctuations in energy availability, with increased expression in the ARC in response to fasting (Hahn et al. 1998). The ARC NPY population co-express the Agouti-related peptide, AgRP (Broberger et al. 1998), which operates as endogenous antagonist of melanocortin receptors 3 and 4. The strong interplay between POMC and NPY/AgRP is witnessed by an optogenetic study that showed as NPY/AgRP neuronal activation increases feeding independently of the suppression of melanocortin pathway (Aponte, Atasoy, and Sternson 2011), eventually by increasing the GABAergic transmission (Wu and Palmiter 2011). Leptin and insulin negatively regulate the NPY/AgRP neurons (Mayer and Belsham 2009) (Takahashi and Cone 2005). As POMC neurons, also this neuronal population might operate on reproductive control, but in a more direct way. In fact, ARC NPY/AgRP neurons project to GnRH perikarya and nerve terminals (Turi et al. 2003), and, indirectly, on the kisspeptin system. A very recent study demonstrated that the absence of AgRP neurons reduces GABAergic inputs to Kiss1 neurons. Additionally, in the same study, optogenetic and pharmacogenetic stimulation of AgRP neurons increased GABAergic transmission onto RP3V and ARC Kiss1 neurons *in vitro*, via $GABA_A$ receptors, and perturbed estrus cyclicity and conception rates *in vivo* (Padilla et al. 2017). Accordingly, it was proposed that increased AgRP transmission would be mechanistically relevant for the suppression of Kiss1, and hence, GnRH neurosecretion in conditions of negative energy balance (Padilla et al. 2017).

5. Kisspeptin and Genistein

The kisspeptin system is particularly vulnerable to Endocrine-disrupting chemicals (EDCs). In rodents, developmental exposure to estrogenic EDCs including PCBs, atrazine, BPA and GEN can perturb aspects of the sex-specific GnRH feedback system resulting in accelerated female puberty, irregular estrous cycles, subfertility, and premature anestrous (Patisaul and Belcher 2017). The fist studies on vulnerability of kisspeptin to EDCs were reported in 2009, using BPA and, immediately after, other works detected alterations of this system using PCBs. To date, very limited data has been generated regarding the possible impact of phytoestrogens on the kisspeptin networks. These studies have primarily used similar approaches to those for BPA and the PCBs, which focus on the impacts of acute exposure over discrete developmental periods (Patisaul and Belcher 2017). The developmental exposure to GEN has a masculinizing influence on the rat female kisspeptin system. Moreover, early life exposure to GEN advances female

puberty and produces estrous cycle irregularities (Bateman and Patisaul 2008), implying disrupted ontogeny of the HPG axis. In female rats, neonatal exposure to 10 mg/kg GEN, but not 1 mg/kg, resulted in a lower density of AVPV/PeN kisspeptin- ir fibers across the pubertal transition (Losa et al. 2011), an effect that persisted into adulthood and is indicative of masculinization. In the ARC, another region rich of kisspeptin, fiber density was unaltered by GEN but significantly lowered by developmental estrogen exposure (Bateman and Patisaul 2008) (Losa et al. 2011). In ovariectomized rats, early life GEN exposure resulted in impaired GnRH activation (Bateman and Patisaul 2008), an effect, which is consistent with masculinization of AVPV/PeN kisspeptin signaling pathways. Interestingly, equol, a phytoestrogen metabolite, did not confer a similar suite of effects on the female kisspeptin system when administered at the same dose (Bateman and Patisaul 2008), despite being considered a more potent estrogen agonist than GEN (Setchell and Clerici 2010). Moreover, in adult male no significant impacts were found on the kiss-ir system of postnatal GEN exposed rats (Patisaul et al. 2009). The collective effects of developmental GEN exposure are similar to those produced by PCBs and emphasize the sex-specific vulnerability of the kisspeptin system to estrogenic EDCs. Furthermore, while its role in GnRH regulation is well known, kisspeptin expression has been also found in numerous other peripheral tissues important for reproduction and energy balance including the gonads, adipose tissue, pancreas, kidney and pituitary. To date, however, virtually nothing is known about how EDCs might influence kisspeptin expression or activity in these tissues. It is hypothesized that EDC exposure could increase the risk of metabolic syndrome and other non-reproductive neuroendocrine diseases, it possible that endocrine disruption of the kisspeptin system may play an important, but as yet unappreciated, role in the etiology of these disorders.

References

Adachi, S., S. Yamada, Y. Takatsu, H. Matsui, M. Kinoshita, K. Takase, H. Sugiura, T. Ohtaki, H. Matsumoto, Y. Uenoyama, H. Tsukamura, K. Inoue, and K. Maeda. 2007. 'Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats', *J Reprod Dev*, 53: 367-78.

Andersson, U., K. Filipsson, C. R. Abbott, A. Woods, K. Smith, S. R. Bloom, D. Carling, and C. J. Small. 2004. 'AMP-activated protein kinase plays a role in the control of food intake', *J Biol Chem*, 279: 12005-8.

Aponte, Y., D. Atasoy, and S. M. Sternson. 2011. 'AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training', *Nat Neurosci*, 14: 351-5.

Backholer, K., J. T. Smith, A. Rao, A. Pereira, J. Iqbal, S. Ogawa, Q. Li, and I. J. Clarke. 2010. 'Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells', *Endocrinology*, 151: 2233-43.

Bateman, H. L., and H. B. Patisaul. 2008. 'Disrupted female reproductive physiology following neonatal exposure to phytoestrogens or estrogen specific ligands is associated with decreased GnRH activation and kisspeptin fiber density in the hypothalamus', *Neurotoxicology*, 29: 988-97.

Baxter, M. G., and E. A. Murray. 2002. 'The amygdala and reward', *Nat Rev Neurosci*, 3: 563-73.

Bentsen, A. H., L. Ansel, V. Simonneaux, M. Tena-Sempere, A. Juul, and J. D. Mikkelsen. 2010. 'Maturation of kisspeptinergic neurons coincides with puberty onset in male rats', *Peptides*, 31: 275-83.

Bilban, M., N. Ghaffari-Tabrizi, E. Hintermann, S. Bauer, S. Molzer, C. Zoratti, R. Malli, A. Sharabi, U. Hiden, W. Graier, M. Knofler, F. Andreae, O. Wagner, V. Quaranta, and G. Desoye. 2004. 'Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts', *J Cell Sci*, 117: 1319-28.

Brailoiu, G. C., S. L. Dun, M. Ohsawa, D. Yin, J. Yang, J. K. Chang, E. Brailoiu, and N. J. Dun. 2005. 'KiSS-1 expression and metastin-like immunoreactivity in the rat brain', *J Comp Neurol*, 481: 314-29.

Broberger, C., J. Johansen, C. Johansson, M. Schalling, and T. Hokfelt. 1998. 'The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice', *Proc Natl Acad Sci U S A*, 95: 15043-8.

Canto, C., and J. Auwerx. 2009. 'PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure', *Curr Opin Lipidol*, 20: 98-105.

Cao, J., and H. B. Patisaul. 2011. 'Sexually dimorphic expression of hypothalamic estrogen receptors alpha and beta and Kiss1 in neonatal male and female rats', *J Comp Neurol*, 519: 2954-77.

Caraty, A., C. Fabre-Nys, B. Delaleu, A. Locatelli, G. Bruneau, F. J. Karsch, and A. Herbison. 1998. 'Evidence that the mediobasal hypothalamus is the primary site of action of estradiol in inducing the preovulatory gonadotropin releasing hormone surge in the ewe', *Endocrinology*, 139: 1752-60.

Caron, E., P. Ciofi, V. Prevot, and S. G. Bouret. 2012. 'Alteration in neonatal nutrition causes perturbations in hypothalamic neural circuits controlling reproductive function', *J Neurosci*, 32: 11486-94.

Casanueva, F. F., and C. Dieguez. 1999. 'Neuroendocrine regulation and actions of leptin', *Front Neuroendocrinol*, 20: 317-63.

Castellano, J. M., A. H. Bentsen, M. A. Sanchez-Garrido, F. Ruiz-Pino, M. Romero, D. Garcia-Galiano, E. Aguilar, L. Pinilla, C. Dieguez, J. D. Mikkelsen, and M. Tena-Sempere. 2011. 'Early metabolic programming of puberty onset: impact of changes in postnatal feeding and rearing conditions on the timing of puberty and development of the hypothalamic kisspeptin system', *Endocrinology*, 152: 3396-408.

Castellano, J. M., V. M. Navarro, R. Fernandez-Fernandez, R. Nogueiras, S. Tovar, J. Roa, M. J. Vazquez, E. Vigo, F. F. Casanueva, E. Aguilar, L. Pinilla, C. Dieguez, and M. Tena-Sempere. 2005. 'Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition', *Endocrinology*, 146: 3917-25.

Castellano, J. M., and M. Tena-Sempere. 2016. 'Metabolic control of female puberty: potential therapeutic targets', *Expert Opin Ther Targets*, 20: 1181-93.

Cerrato, F., and S. B. Seminara. 2007. 'Human genetics of GPR54', *Rev Endocr Metab Disord*, 8: 47-55.

Chappell, P. E., J. S. Schneider, P. Kim, M. Xu, J. P. Lydon, B. W. O'Malley, and J. E. Levine. 1999. 'Absence of gonadotropin surges and gonadotropin-releasing hormone self-priming in ovariectomized (OVX), estrogen (E2)-treated, progesterone receptor knockout (PRKO) mice', *Endocrinology*, 140: 3653-8.

Clarke, I. J., S. Pompolo, C. J. Scott, J. A. Rawson, D. Caddy, A. E. Jakubowska, and A. M. Pereira. 2001. 'Cells of the arcuate nucleus and ventromedial nucleus of the ovariectomized ewe that respond to oestrogen: a study using Fos immunohistochemistry', *J Neuroendocrinol*, 13: 934-41.

Clarkson, J., W. C. Boon, E. R. Simpson, and A. E. Herbison. 2009. 'Postnatal development of an estradiol-kisspeptin positive feedback mechanism implicated in puberty onset', *Endocrinology*, 150: 3214-20.

Clarkson, J., X. d'Anglemont de Tassigny, A. S. Moreno, W. H. Colledge, and A. E. Herbison. 2008. 'Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge', *J Neurosci*, 28: 8691-7.

Clarkson, J., S. K. Han, X. Liu, K. Lee, and A. E. Herbison. 2010. 'Neurobiological mechanisms underlying kisspeptin activation of gonadotropin-releasing hormone (GnRH) neurons at puberty', *Mol Cell Endocrinol*, 324: 45-50.

Clarkson, J., and A. E. Herbison. 2006. 'Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons', *Endocrinology*, 147: 5817-25.

Colledge, W. H., and X. d'Anglemont de Tassigny. 2010. 'The role of kisspeptin signalling in the regulation of the GnRH-gonadotrophin ovarian axis in mice', *Ann Endocrinol (Paris)*, 71: 198-200.

Cota, D., K. Proulx, and R. J. Seeley. 2007. 'The role of CNS fuel sensing in energy and glucose regulation', *Gastroenterology*, 132: 2158-68.

Cota, D., K. Proulx, K. A. Smith, S. C. Kozma, G. Thomas, S. C. Woods, and R. J. Seeley. 2006. 'Hypothalamic mTOR signaling regulates food intake', *Science*, 312: 927-30.

Cravo, R. M., R. Frazao, M. Perello, S. Osborne-Lawrence, K. W. Williams, J. M. Zigman, C. Vianna, and C. F. Elias. 2013. 'Leptin signaling in Kiss1 neurons arises after pubertal development', *PLoS One*, 8: e58698.

Cravo, R. M., L. O. Margatho, S. Osborne-Lawrence, J. Donato, Jr., S. Atkin, A. L. Bookout, S. Rovinsky, R. Frazao, C. E. Lee, L. Gautron, J. M. Zigman, and C. F. Elias. 2011. 'Characterization of Kiss1 neurons using transgenic mouse models', *Neuroscience*, 173: 37-56.

d'Anglemont de Tassigny, X., L. A. Fagg, M. B. Carlton, and W. H. Colledge. 2008. 'Kisspeptin can stimulate gonadotropin-releasing hormone (GnRH) release by a direct action at GnRH nerve terminals', *Endocrinology*, 149: 3926-32.

d'Anglemont de Tassigny, X., L. A. Fagg, J. P. Dixon, K. Day, H. G. Leitch, A. G. Hendrick, D. Zahn, I. Franceschini, A. Caraty, M. B. Carlton, S. A. Aparicio, and W. H. Colledge. 2007. 'Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene', *Proc Natl Acad Sci U S A*, 104: 10714-9.

De Bond, J. A., and J. T. Smith. 2014. 'Kisspeptin and energy balance in reproduction', *Reproduction*, 147: R53-63.

de la Iglesia, H. O., and W. J. Schwartz. 2006. 'Minireview: timely ovulation: circadian regulation of the female hypothalamo-pituitary-gonadal axis', *Endocrinology*, 147: 1148-53.

de Roux, N., E. Genin, J. C. Carel, F. Matsuda, J. L. Chaussain, and E. Milgrom. 2003. 'Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54', *Proc Natl Acad Sci U S A*, 100: 10972-6.

Desroziers, E., M. Droguerre, A. H. Bentsen, V. Robert, J. D. Mikkelsen, A. Caraty, Y. Tillet, A. Duittoz, and I. Franceschini. 2012. 'Embryonic development of kisspeptin neurones in rat', *J Neuroendocrinol*, 24: 1284-95.

Desroziers, E., J. D. Mikkelsen, A. Duittoz, and I. Franceschini. 2012. 'Kisspeptin-immunoreactivity changes in a sex- and hypothalamic-region-specific manner across rat postnatal development', *J Neuroendocrinol*, 24: 1154-65.

Dhillo, W. S., C. J. Small, S. A. Stanley, P. H. Jethwa, L. J. Seal, K. G. Murphy, M. A. Ghatei, and S. R. Bloom. 2002. 'Hypothalamic interactions between neuropeptide Y, agouti-related protein, cocaine- and amphetamine-regulated transcript and alpha-melanocyte-stimulating hormone in vitro in male rats', *J Neuroendocrinol*, 14: 725-30.

Dierschke, D. J., T. Yamaji, F. J. Karsch, R. F. Weick, G. Weiss, and E. Knobil. 1973. 'Blockade by progesterone of estrogen-induced LH and FSH release in the rhesus monkey', *Endocrinology*, 92: 1496-501.

Divall, S. A., T. R. Williams, S. E. Carver, L. Koch, J. C. Bruning, C. R. Kahn, F. Wondisford, S. Radovick, and A. Wolfe. 2010. 'Divergent roles of growth factors in the GnRH regulation of puberty in mice', *J Clin Invest*, 120: 2900-9.

Donato, J., Jr., R. M. Cravo, R. Frazao, L. Gautron, M. M. Scott, J. Lachey, I. A. Castro, L. O. Margatho, S. Lee, C. Lee, J. A. Richardson, J. Friedman, S. Chua, Jr., R. Coppari, J. M. Zigman, J. K. Elmquist, and C. F. Elias. 2011. 'Leptin's effect on puberty in mice is relayed by the ventral premammillary nucleus and does not require signaling in Kiss1 neurons', *J Clin Invest*, 121: 355-68.

Donato, J., Jr., C. Lee, D. V. Ratra, C. R. Franci, N. S. Canteras, and C. F. Elias. 2013. 'Lesions of the ventral premammillary nucleus disrupt the dynamic changes in Kiss1 and GnRH expression characteristic of the proestrus-estrus transition', *Neuroscience*, 241: 67-79.

Donato, J., Jr., R. J. Silva, L. V. Sita, S. Lee, C. Lee, S. Lacchini, J. C. Bittencourt, C. R. Franci, N. S. Canteras, and C. F. Elias. 2009. 'The ventral premammillary nucleus links fasting-induced changes in leptin levels and coordinated luteinizing hormone secretion', *J Neurosci*, 29: 5240-50.

Dungan, H. M., D. K. Clifton, and R. A. Steiner. 2006. 'Minireview: kisspeptin neurons as central processors in the regulation of gonadotropin-releasing hormone secretion', *Endocrinology*, 147: 1154-8.

Estrada, K. M., C. M. Clay, S. Pompolo, J. T. Smith, and I. J. Clarke. 2006. 'Elevated KiSS-1 expression in the arcuate nucleus prior to the cyclic preovulatory gonadotrophin-releasing hormone/lutenising hormone surge in the ewe suggests a stimulatory role for kisspeptin in oestrogen-positive feedback', *J Neuroendocrinol*, 18: 806-9.

Everett, J. W. 1948. 'Progesterone and estrogen in the experimental control of ovulation time and other features of the estrous cycle in the rat', *Endocrinology*, 43: 389-405.

Forbes, S., X. F. Li, J. Kinsey-Jones, and K. O'Byrne. 2009. 'Effects of ghrelin on Kisspeptin mRNA expression in the hypothalamic medial preoptic area and pulsatile luteinising hormone secretion in the female rat', *Neurosci Lett*, 460: 143-7.

Franceschini, I., D. Lomet, M. Cateau, G. Delsol, Y. Tillet, and A. Caraty. 2006. 'Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha', *Neurosci Lett*, 401: 225-30.

Frazao, R., H. M. Dungan Lemko, R. P. da Silva, D. V. Ratra, C. E. Lee, K. W. Williams, J. M. Zigman, and C. F. Elias. 2014. 'Estradiol modulates Kiss1 neuronal response to ghrelin', *Am J Physiol Endocrinol Metab*, 306: E606-14.

Fu, L. Y., and A. N. van den Pol. 2010. 'Kisspeptin directly excites anorexigenic proopiomelanocortin neurons but inhibits orexigenic neuropeptide Y cells by an indirect synaptic mechanism', *J Neurosci*, 30: 10205-19.

Funes, S., J. A. Hedrick, G. Vassileva, L. Markowitz, S. Abbondanzo, A. Golovko, S. Yang, F. J. Monsma, and E. L. Gustafson. 2003. 'The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system', *Biochem Biophys Res Commun*, 312: 1357-63.

Fuxe, K., B. Tinner, L. Caberlotto, B. Bunnemann, and L. F. Agnati. 1997. 'NPY Y1 receptor like immunoreactivity exists in a subpopulation of beta-endorphin immunoreactive nerve cells in the arcuate nucleus: a double immunolabelling analysis in the rat', *Neurosci Lett*, 225: 49-52.

Gal, A., P. C. Lin, J. A. Cacioppo, P. R. Hannon, M. M. Mahoney, A. Wolfe, R. Fernandez-Valdivia, J. P. Lydon, C. F. Elias, and C. Ko. 2016. 'Loss of Fertility in the Absence of Progesterone Receptor Expression in Kisspeptin Neurons of Female Mice', *PLoS One*, 11: e0159534.

Gill, J. C., O. Wang, S. Kakar, E. Martinelli, R. S. Carroll, and U. B. Kaiser. 2010. 'Reproductive hormone-dependent and -independent contributions to developmental changes in kisspeptin in GnRH-deficient hypogonadal mice', *PLoS One*, 5: e11911.

Glanowska, K. M., and S. M. Moenter. 2015. 'Differential regulation of GnRH secretion in the preoptic area (POA) and the median eminence (ME) in male mice', *Endocrinology*, 156: 231-41.

Gonzalez-Martinez, D., C. De Mees, Q. Douhard, C. Szpirer, and J. Bakker. 2008. 'Absence of gonadotropin-releasing hormone 1 and Kiss1 activation in alpha-fetoprotein knockout mice: prenatal estrogens defeminize the potential to show preovulatory luteinizing hormone surges', *Endocrinology*, 149: 2333-40.

Goodman, R. L., L. M. Coolen, G. M. Anderson, S. L. Hardy, M. Valent, J. M. Connors, M. E. Fitzgerald, and M. N. Lehman. 2004. 'Evidence that dynorphin plays a major role in mediating progesterone negative feedback on gonadotropin-releasing hormone neurons in sheep', *Endocrinology*, 145: 2959-67.

Goodman, R. L., I. Holaskova, C. C. Nestor, J. M. Connors, H. J. Billings, M. Valent, M. N. Lehman, and S. M. Hileman. 2011. 'Evidence that the arcuate nucleus is an important site of progesterone negative feedback in the ewe', *Endocrinology*, 152: 3451-60.

Goodman, R. L., M. N. Lehman, J. T. Smith, L. M. Coolen, C. V. de Oliveira, M. R. Jafarzadehshirazi, A. Pereira, J. Iqbal, A. Caraty, P. Ciofi, and I. J. Clarke. 2007. 'Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B', *Endocrinology*, 148: 5752-60.

Gottsch, M. L., D. K. Clifton, and R. A. Steiner. 2009. 'From KISS1 to kisspeptins: An historical perspective and suggested nomenclature', *Peptides*, 30: 4-9.

Gottsch, M. L., M. J. Cunningham, J. T. Smith, S. M. Popa, B. V. Acohido, W. F. Crowley, S. Seminara, D. K. Clifton, and R. A. Steiner. 2004. 'A role for kisspeptins in the regulation of gonadotropin secretion in the mouse', *Endocrinology*, 145: 4073-7.

Gottsch, M. L., S. M. Popa, J. K. Lawhorn, J. Qiu, K. J. Tonsfeldt, M. A. Bosch, M. J. Kelly, O. K. Ronnekleiv, E. Sanz, G. S. McKnight, D. K. Clifton, R. D. Palmiter, and R. A. Steiner. 2011. 'Molecular properties of Kiss1 neurons in the arcuate nucleus of the mouse', *Endocrinology*, 152: 4298-309.

Goubillon, M., B. Delaleu, Y. Tillet, A. Caraty, and A. E. Herbison. 1999. 'Localization of estrogen-receptive neurons projecting to the GnRH neuron-containing rostral preoptic area of the ewe', *Neuroendocrinology*, 70: 228-36.

Guan, X. M., H. Yu, O. C. Palyha, K. K. McKee, S. D. Feighner, D. J. Sirinathsinghji, R. G. Smith, L. H. Van der Ploeg, and A. D. Howard. 1997. 'Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues', *Brain Res Mol Brain Res*, 48: 23-9.

Hahn, T. M., J. F. Breininger, D. G. Baskin, and M. W. Schwartz. 1998. 'Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons', *Nat Neurosci*, 1: 271-2.

Hakansson, M. L., H. Brown, N. Ghilardi, R. C. Skoda, and B. Meister. 1998. 'Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus', *J Neurosci*, 18: 559-72.

Halaas, J. L., K. S. Gajiwala, M. Maffei, S. L. Cohen, B. T. Chait, D. Rabinowitz, R. L. Lallone, S. K. Burley, and J. M. Friedman. 1995. 'Weight-reducing effects of the plasma protein encoded by the obese gene', *Science*, 269: 543-6.

Han, S. K., M. L. Gottsch, K. J. Lee, S. M. Popa, J. T. Smith, S. K. Jakawich, D. K. Clifton, R. A. Steiner, and A. E. Herbison. 2005. 'Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty', *J Neurosci*, 25: 11349-56.

Han, S. Y., T. McLennan, K. Czieselsky, and A. E. Herbison. 2015. 'Selective optogenetic activation of arcuate kisspeptin neurons generates pulsatile luteinizing hormone secretion', *Proc Natl Acad Sci U S A*, 112: 13109-14.

He, W., X. Li, D. Adekunbi, Y. Liu, H. Long, L. Wang, Q. Lyu, Y. Kuang, and K. T. O'Byrne. 2017. 'Hypothalamic effects of progesterone on regulation of the pulsatile and surge release of luteinising hormone in female rats', *Sci Rep*, 7: 8096.

Hentges, S. T., V. Otero-Corchon, R. L. Pennock, C. M. King, and M. J. Low. 2009. 'Proopiomelanocortin expression in both GABA and glutamate neurons', *J Neurosci*, 29: 13684-90.

Herbison, A. E. 2008. 'Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: the case for the rostral periventricular area of the third ventricle (RP3V)', *Brain Res Rev*, 57: 277-87.

Herbison, A. E. 2016. 'Control of puberty onset and fertility by gonadotropin-releasing hormone neurons', *Nat Rev Endocrinol*, 12: 452-66.

Herbison, A. E., Xd de Tassigny, J. Doran, and W. H. Colledge. 2010. 'Distribution and postnatal development of Gpr54 gene expression in mouse brain and gonadotropin-releasing hormone neurons', *Endocrinology*, 151: 312-21.

Herbison, A. E., and D. T. Theodosis. 1992. 'Immunocytochemical identification of oestrogen receptors in preoptic neurones containing calcitonin gene-related peptide in the male and female rat', *Neuroendocrinology*, 56: 761-4.

Higo, S., S. Honda, N. Iijima, and H. Ozawa. 2016. 'Mapping of Kisspeptin Receptor mRNA in the Whole Rat Brain and its Co-Localisation with Oxytocin in the Paraventricular Nucleus', *J Neuroendocrinol*, 28.

Higo, S., N. Iijima, and H. Ozawa. 2017. 'Characterisation of Kiss1r (Gpr54)-Expressing Neurones in the Arcuate Nucleus of the Female Rat Hypothalamus', *J Neuroendocrinol*, 29.

Hill, J. W., J. K. Elmquist, and C. F. Elias. 2008. 'Hypothalamic pathways linking energy balance and reproduction', *Am J Physiol Endocrinol Metab*, 294: E827-32.

Homma, T., M. Sakakibara, S. Yamada, M. Kinoshita, K. Iwata, J. Tomikawa, T. Kanazawa, H. Matsui, Y. Takatsu, T. Ohtaki, H. Matsumoto, Y. Uenoyama, K. Maeda, and H. Tsukamura. 2009. 'Significance of neonatal testicular sex steroids to defeminize anteroventral periventricular kisspeptin neurons and the GnRH/LH surge system in male rats', *Biol Reprod*, 81: 1216-25.

Hrabovszky, E., P. J. Shughrue, I. Merchenthaler, T. Hajszan, C. D. Carpenter, Z. Liposits, and S. L. Petersen. 2000. 'Detection of estrogen receptor-beta messenger ribonucleic acid and 125I-estrogen binding sites in luteinizing hormone-releasing hormone neurons of the rat brain', *Endocrinology*, 141: 3506-9.

Huang, X., and R. E. Harlan. 1993. 'Absence of androgen receptors in LHRH immunoreactive neurons', *Brain Res*, 624: 309-11.

Iijima, N., K. Takumi, N. Sawai, and H. Ozawa. 2011. 'An immunohistochemical study on the expressional dynamics of kisspeptin neurons relevant to GnRH neurons using a newly developed anti-kisspeptin antibody', *J Mol Neurosci*, 43: 146-54.

Irwig, M. S., G. S. Fraley, J. T. Smith, B. V. Acohido, S. M. Popa, M. J. Cunningham, M. L. Gottsch, D. K. Clifton, and R. A. Steiner. 2004. 'Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat', *Neuroendocrinology*, 80: 264-72.

Iwasa, T., T. Matsuzaki, M. Murakami, S. Fujisawa, R. Kinouchi, G. Gereltsetseg, A. Kuwahara, T. Yasui, and M. Irahara. 2010. 'Effects of intrauterine undernutrition on hypothalamic Kiss1 expression and the timing of puberty in female rats', *J Physiol*, 588: 821-9.

Janneau, J. L., J. Maldonado-Estrada, G. Tachdjian, I. Miran, N. Motte, P. Saulnier, J. C. Sabourin, J. F. Cote, B. Simon, R. Frydman, G. Chaouat, and D. Bellet. 2002. 'Transcriptional expression of genes involved in cell invasion and migration by normal and tumoral trophoblast cells', *J Clin Endocrinol Metab*, 87: 5336-9.

Jayasena, C. N., G. M. Nijher, A. N. Comninos, A. Abbara, A. Januszewki, M. L. Vaal, L. Sriskandarajah, K. G. Murphy, Z. Farzad, M. A. Ghatei, S. R. Bloom, and W. S. Dhillo. 2011. 'The effects of kisspeptin-10 on reproductive hormone release show sexual dimorphism in humans', *J Clin Endocrinol Metab*, 96: E1963-72.

Kahn, B. B., T. Alquier, D. Carling, and D. G. Hardie. 2005. 'AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism', *Cell Metab*, 1: 15-25.

Kallo, I., B. Vida, L. Deli, C. S. Molnar, E. Hrabovszky, A. Caraty, P. Ciofi, C. W. Coen, and Z. Liposits. 2012. 'Co-localisation of kisspeptin with galanin or neurokinin B in afferents to mouse GnRH neurones', *J Neuroendocrinol*, 24: 464-76.

Kasa-Vubu, J. Z., G. E. Dahl, N. P. Evans, L. A. Thrun, S. M. Moenter, V. Padmanabhan, and F. J. Karsch. 1992. 'Progesterone blocks the estradiol-induced gonadotropin discharge in the ewe by inhibiting the surge of gonadotropin-releasing hormone', *Endocrinology*, 131: 208-12.

Kauffman, A. S. 2009. 'Sexual differentiation and the Kiss1 system: hormonal and developmental considerations', *Peptides*, 30: 83-93.

Kauffman, A. S., M. L. Gottsch, J. Roa, A. C. Byquist, A. Crown, D. K. Clifton, G. E. Hoffman, R. A. Steiner, and M. Tena-Sempere. 2007. 'Sexual differentiation of Kiss1 gene expression in the brain of the rat', *Endocrinology*, 148: 1774-83.

Kauffman, E. C., V. L. Robinson, W. M. Stadler, M. H. Sokoloff, and C. W. Rinker-Schaeffer. 2003. 'Metastasis suppression: the evolving role of metastasis suppressor genes for regulating cancer cell growth at the secondary site', *J Urol*, 169: 1122-33.

Kim, J., S. J. Semaan, D. K. Clifton, R. A. Steiner, S. Dhamija, and A. S. Kauffman. 2011. 'Regulation of Kiss1 expression by sex steroids in the amygdala of the rat and mouse', *Endocrinology*, 152: 2020-30.

Kirilov, M., J. Clarkson, X. Liu, J. Roa, P. Campos, R. Porteous, G. Schutz, and A. E. Herbison. 2013. 'Dependence of fertility on kisspeptin-Gpr54 signaling at the GnRH neuron', *Nat Commun*, 4: 2492.

Konner, A. C., R. Janoschek, L. Plum, S. D. Jordan, E. Rother, X. Ma, C. Xu, P. Enriori, B. Hampel, G. S. Barsh, C. R. Kahn, M. A. Cowley, F. M. Ashcroft, and J. C. Bruning. 2007. 'Insulin action in AgRP-expressing neurons is required for suppression of hepatic glucose production', *Cell Metab*, 5: 438-49.

Kotani, M., M. Detheux, A. Vandenbogaerde, D. Communi, J. M. Vanderwinden, E. Le Poul, S. Brezillon, R. Tyldesley, N. Suarez-Huerta, F. Vandeput, C. Blanpain, S. N. Schiffmann, G. Vassart, and M. Parmentier. 2001. 'The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54', *J Biol Chem*, 276: 34631-6.

Kuang, Y., Q. Chen, Y. Fu, Y. Wang, Q. Hong, Q. Lyu, A. Ai, and Z. Shoham. 2015. 'Medroxyprogesterone acetate is an effective oral alternative for preventing premature luteinizing hormone surges in women undergoing controlled ovarian hyperstimulation for in vitro fertilization', *Fertil Steril*, 104: 62-70 e3.

Lapatto, R., J. C. Pallais, D. Zhang, Y. M. Chan, A. Mahan, F. Cerrato, W. W. Le, G. E. Hoffman, and S. B. Seminara. 2007. 'Kiss1-/- mice exhibit more variable hypogonadism than Gpr54-/- mice', *Endocrinology*, 148: 4927-36.

Le, W. W., B. Attardi, K. A. Berghorn, J. Blaustein, and G. E. Hoffman. 1997. 'Progesterone blockade of a luteinizing hormone surge blocks luteinizing hormone-releasing hormone Fos activation and activation of its preoptic area afferents', *Brain Res*, 778: 272-80.

Lee, D. K., T. Nguyen, G. P. O'Neill, R. Cheng, Y. Liu, A. D. Howard, N. Coulombe, C. P. Tan, A. T. Tang-Nguyen, S. R. George, and B. F. O'Dowd. 1999. 'Discovery of a receptor related to the galanin receptors', *FEBS Lett*, 446: 103-7.

Lee, J. H., M. E. Miele, D. J. Hicks, K. K. Phillips, J. M. Trent, B. E. Weissman, and D. R. Welch. 1996. 'KiSS-1, a novel human malignant melanoma metastasis-suppressor gene', *J Natl Cancer Inst*, 88: 1731-7.

Lee, J. H., and D. R. Welch. 1997. 'Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1', *Cancer Res*, 57: 2384-7.

Lehman, M. N., L. M. Coolen, and R. L. Goodman. 2010. 'Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion', *Endocrinology*, 151: 3479-89.

Leon, S., A. Barroso, M. J. Vazquez, D. Garcia-Galiano, M. Manfredi-Lozano, F. Ruiz-Pino, V. Heras, A. Romero-Ruiz, J. Roa, G. Schutz, M. Kirilov, F. Gaytan, L. Pinilla, and M. Tena-Sempere. 2016. 'Direct Actions of Kisspeptins on GnRH Neurons Permit Attainment of Fertility but are Insufficient to Fully Preserve Gonadotropic Axis Activity', *Sci Rep*, 6: 19206.

Liu, Q., X. M. Guan, W. J. Martin, T. P. McDonald, M. K. Clements, Q. Jiang, Z. Zeng, M. Jacobson, D. L. Williams, Jr., H. Yu, D. Bomford, D. Figueroa, J. Mallee, R. Wang, J. Evans, R. Gould, and C. P. Austin. 2001. 'Identification and characterization of novel mammalian neuropeptide FF-like peptides that attenuate morphine-induced antinociception', *J Biol Chem*, 276: 36961-9.

Liu, X., and A. Herbison. 2015. 'Kisspeptin regulation of arcuate neuron excitability in kisspeptin receptor knockout mice', *Endocrinology*, 156: 1815-27.

Lomniczi, A., A. Loche, J. M. Castellano, O. K. Ronnekleiv, M. Bosch, G. Kaidar, J. G. Knoll, H. Wright, G. P. Pfeifer, and S. R. Ojeda. 2013. 'Epigenetic control of female puberty', *Nat Neurosci*, 16: 281-9.

Losa, S. M., K. L. Todd, A. W. Sullivan, J. Cao, J. A. Mickens, and H. B. Patisaul. 2011. 'Neonatal exposure to genistein adversely impacts the ontogeny of hypothalamic kisspeptin signaling pathways and ovarian development in the peripubertal female rat', *Reprod Toxicol*, 31: 280-9.

Louis, G. W., M. Greenwald-Yarnell, R. Phillips, L. M. Coolen, M. N. Lehman, and M. G. Myers, Jr. 2011. 'Molecular mapping of the neural pathways linking leptin to the neuroendocrine reproductive axis', *Endocrinology*, 152: 2302-10.

Magee, C., C. D. Foradori, J. E. Bruemmer, J. A. Arreguin-Arevalo, P. M. McCue, R. J. Handa, E. L. Squires, and C. M. Clay. 2009. 'Biological and anatomical evidence for kisspeptin regulation of the hypothalamic-pituitary-gonadal axis of estrous horse mares', *Endocrinology*, 150: 2813-21.

Manfredi-Lozano, M., J. Roa, F. Ruiz-Pino, R. Piet, D. Garcia-Galiano, R. Pineda, A. Zamora, S. Leon, M. A. Sanchez-Garrido, A. Romero-Ruiz, C. Dieguez, M. J. Vazquez, A. E. Herbison, L. Pinilla, and M. Tena-Sempere. 2016. 'Defining a novel leptin-melanocortin-kisspeptin pathway involved in the metabolic control of puberty', *Mol Metab*, 5: 844-57.

Manfredi-Lozano, M., J. Roa, and M. Tena-Sempere. 2017. 'Connecting metabolism and gonadal function: Novel central neuropeptide pathways involved in the metabolic control of puberty and fertility', *Front Neuroendocrinol*.

Maolood, N., and B. Meister. 2008. 'Dynorphin in pro-opiomelanocortin neurons of the hypothalamic arcuate nucleus', *Neuroscience*, 154: 1121-31.

Matsui, H., Y. Takatsu, S. Kumano, H. Matsumoto, and T. Ohtaki. 2004. 'Peripheral administration of metastin induces marked gonadotropin release and ovulation in the rat', *Biochem Biophys Res Commun*, 320: 383-8.

Mayer, C., M. Acosta-Martinez, S. L. Dubois, A. Wolfe, S. Radovick, U. Boehm, and J. E. Levine. 2010. 'Timing and completion of puberty in female mice depend on estrogen receptor alpha-signaling in kisspeptin neurons', *Proc Natl Acad Sci U S A*, 107: 22693-8.

Mayer, C. M., and D. D. Belsham. 2009. 'Insulin directly regulates NPY and AgRP gene expression via the MAPK MEK/ERK signal transduction pathway in mHypoE-46 hypothalamic neurons', *Mol Cell Endocrinol*, 307: 99-108.

McCartney, C. R., M. B. Gingrich, Y. Hu, W. S. Evans, and J. C. Marshall. 2002. 'Hypothalamic regulation of cyclic ovulation: evidence that the increase in gonadotropin-releasing hormone pulse frequency during the follicular phase reflects the gradual loss of the restraining effects of progesterone', *J Clin Endocrinol Metab*, 87: 2194-200.

Meister, B., B. Gomuc, E. Suarez, Y. Ishii, K. Durr, and L. Gillberg. 2006. 'Hypothalamic proopiomelanocortin (POMC) neurons have a cholinergic phenotype', *Eur J Neurosci*, 24: 2731-40.

Mittelman-Smith, M. A., L. M. Rudolph, M. A. Mohr, and P. E. Micevych. 2017. 'Rodent Models of Non-classical Progesterone Action Regulating Ovulation', *Front Endocrinol (Lausanne)*, 8: 165.

Moenter, S. M., F. J. Karsch, and M. N. Lehman. 1993. 'Fos expression during the estradiol-induced gonadotropin-releasing hormone (GnRH) surge of the ewe: induction in GnRH and other neurons', *Endocrinology*, 133: 896-903.

Molloy, B. G., M. A. El Sheikh, C. Chapman, R. E. Oakey, K. W. Hancock, and M. R. Glass. 1984. 'Pathological mechanisms in polycystic ovary syndrome: modulation of LH pulsatility by progesterone', *Br J Obstet Gynaecol*, 91: 457-65.

Muir, A. I., L. Chamberlain, N. A. Elshourbagy, D. Michalovich, D. J. Moore, A. Calamari, P. G. Szekeres, H. M. Sarau, J. K. Chambers, P. Murdock, K. Steplewski, U. Shabon, J. E. Miller, S. E. Middleton, J. G. Darker, C. G. Larminie, S. Wilson, D. J. Bergsma, P. Emson, R. Faull, K. L. Philpott, and D. C. Harrison. 2001. 'AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1', *J Biol Chem*, 276: 28969-75.

Naimi, M., C. Arous, and E. Van Obberghen. 2010. 'Energetic cell sensors: a key to metabolic homeostasis', *Trends Endocrinol Metab*, 21: 75-82.

Navarro, V. M., J. M. Castellano, R. Fernandez-Fernandez, S. Tovar, J. Roa, A. Mayen, M. L. Barreiro, F. F. Casanueva, E. Aguilar, C. Dieguez, L. Pinilla, and M. Tena-Sempere. 2005. 'Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat', *Endocrinology*, 146: 1689-97.

Navarro, V. M., J. M. Castellano, R. Fernandez-Fernandez, S. Tovar, J. Roa, A. Mayen, R. Nogueiras, M. J. Vazquez, M. L. Barreiro, P. Magni, E. Aguilar, C. Dieguez, L. Pinilla, and M. Tena-Sempere. 2005. 'Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54', *Endocrinology*, 146: 156-63.

Navarro, V. M., R. Fernandez-Fernandez, J. M. Castellano, J. Roa, A. Mayen, M. L. Barreiro, F. Gaytan, E. Aguilar, L. Pinilla, C. Dieguez, and M. Tena-Sempere. 2004. 'Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54', *J Physiol*, 561: 379-86.

Navarro, V. M., M. A. Sanchez-Garrido, J. M. Castellano, J. Roa, D. Garcia-Galiano, R. Pineda, E. Aguilar, L. Pinilla, and M. Tena-Sempere. 2009. 'Persistent impairment of hypothalamic KiSS-1 system after exposures to estrogenic compounds at critical periods of brain sex differentiation', *Endocrinology*, 150: 2359-67.

Nequin, L. G., J. Alvarez, and N. B. Schwartz. 1979. 'Measurement of serum steroid and gonadotropin levels and uterine and ovarian variables throughout 4 day and 5 day estrous cycles in the rat', *Biol Reprod*, 20: 659-70.

Nestor, C. C., A. M. Briscoe, S. M. Davis, M. Valent, R. L. Goodman, and S. M. Hileman. 2012. 'Evidence of a role for kisspeptin and neurokinin B in puberty of female sheep', *Endocrinology*, 153: 2756-65.

Nestor, C. C., J. Qiu, S. L. Padilla, C. Zhang, M. A. Bosch, W. Fan, S. A. Aicher, R. D. Palmiter, O. K. Ronnekleiv, and M. J. Kelly. 2016. 'Optogenetic Stimulation of Arcuate Nucleus Kiss1 Neurons Reveals a Steroid-Dependent Glutamatergic Input to POMC and AgRP Neurons in Male Mice', *Mol Endocrinol*, 30: 630-44.

Novaira, H. J., M. L. Sonko, G. Hoffman, Y. Koo, C. Ko, A. Wolfe, and S. Radovick. 2014. 'Disrupted kisspeptin signaling in GnRH neurons leads to hypogonadotrophic hypogonadism', *Mol Endocrinol*, 28: 225-38.

Oakley, A. E., D. K. Clifton, and R. A. Steiner. 2009. 'Kisspeptin signaling in the brain', *Endocr Rev*, 30: 713-43.

Ohtaki, T., Y. Shintani, S. Honda, H. Matsumoto, A. Hori, K. Kanehashi, Y. Terao, S. Kumano, Y. Takatsu, Y. Masuda, Y. Ishibashi, T. Watanabe, M. Asada, T. Yamada, M. Suenaga, C. Kitada, S. Usuki, T. Kurokawa, H. Onda, O. Nishimura, and M. Fujino. 2001. 'Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor', *Nature*, 411: 613-7.

Oishi, S., R. Misu, K. Tomita, S. Setsuda, R. Masuda, H. Ohno, Y. Naniwa, N. Ieda, N. Inoue, S. Ohkura, Y. Uenoyama, H. Tsukamura, K. Maeda, A. Hirasawa, G. Tsujimoto, and N. Fujii. 2011. 'Activation of Neuropeptide FF Receptors by Kisspeptin Receptor Ligands', *ACS Med Chem Lett*, 2: 53-7.

Padilla, S. L., J. Qiu, C. C. Nestor, C. Zhang, A. W. Smith, B. B. Whiddon, O. K. Ronnekleiv, M. J. Kelly, and R. D. Palmiter. 2017. 'AgRP to Kiss1 neuron signaling links nutritional state and fertility', *Proc Natl Acad Sci U S A*, 114: 2413-18.

Parhar, I. S., S. Ogawa, and Y. Sakuma. 2004. 'Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish', *Endocrinology*, 145: 3613-8.

Patisaul, H. B., K. L. Todd, J. A. Mickens, and H. B. Adewale. 2009. 'Impact of neonatal exposure to the ERalpha agonist PPT, bisphenol-A or phytoestrogens on hypothalamic kisspeptin fiber density in male and female rats', *Neurotoxicology*, 30: 350-7.

Patisaul, Heather B., and Scott M. Belcher. 2017. *Endocrine disruptors, brain, and behaviors* (Oxford University Press: New York, NY).

Piet, R., A. Fraissenon, U. Boehm, and A. E. Herbison. 2015. 'Estrogen permits vasopressin signaling in preoptic kisspeptin neurons in the female mouse', *J Neurosci*, 35: 6881-92.

Pineda, R., D. Garcia-Galiano, A. Roseweir, M. Romero, M. A. Sanchez-Garrido, F. Ruiz-Pino, K. Morgan, L. Pinilla, R. P. Millar, and M. Tena-Sempere. 2010. 'Critical roles of kisspeptins in female puberty and preovulatory gonadotropin surges as revealed by a novel antagonist', *Endocrinology*, 151: 722-30.

Pineda, R., F. Plaisier, R. P. Millar, and M. Ludwig. 2017. 'Amygdala Kisspeptin Neurons: Putative Mediators of Olfactory Control of the Gonadotropic Axis', *Neuroendocrinology*, 104: 223-38.

Pinilla, L., E. Aguilar, C. Dieguez, R. P. Millar, and M. Tena-Sempere. 2012. 'Kisspeptins and reproduction: physiological roles and regulatory mechanisms', *Physiol Rev*, 92: 1235-316.

Plant, T. M. 2006. 'The role of KiSS-1 in the regulation of puberty in higher primates', *Eur J Endocrinol*, 155 Suppl 1: S11-6.

Plant, T. M. 2015. '60 YEARS OF NEUROENDOCRINOLOGY: The hypothalamo-pituitary-gonadal axis', *J Endocrinol*, 226: T41-54.

Poling, M. C., and A. S. Kauffman. 2013. 'Organizational and activational effects of sex steroids on kisspeptin neuron development', *Front Neuroendocrinol*, 34: 3-17.

Pralong, F. P. 2010. 'Insulin and NPY pathways and the control of GnRH function and puberty onset', *Mol Cell Endocrinol*, 324: 82-6.

Qiu, J., Y. Fang, M. A. Bosch, O. K. Ronnekleiv, and M. J. Kelly. 2011. 'Guinea pig kisspeptin neurons are depolarized by leptin via activation of TRPC channels', *Endocrinology*, 152: 1503-14.

Qiu, X., H. Dao, M. Wang, A. Heston, K. M. Garcia, A. Sangal, A. R. Dowling, L. D. Faulkner, S. C. Molitor, C. F. Elias, and J. W. Hill. 2015. 'Insulin and Leptin Signaling Interact in the Mouse Kiss1 Neuron during the Peripubertal Period', *PLoS One*, 10: e0121974.

Quennell, J. H., A. C. Mulligan, A. Tups, X. Liu, S. J. Phipps, C. J. Kemp, A. E. Herbison, D. R. Grattan, and G. M. Anderson. 2009. 'Leptin indirectly regulates gonadotropin-releasing hormone neuronal function', *Endocrinology*, 150: 2805-12.

Roa, J., E. Aguilar, C. Dieguez, L. Pinilla, and M. Tena-Sempere. 2008. 'New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function', *Front Neuroendocrinol*, 29: 48-69.

Roa, J., J. M. Castellano, V. M. Navarro, D. J. Handelsman, L. Pinilla, and M. Tena-Sempere. 2009. 'Kisspeptins and the control of gonadotropin secretion in male and female rodents', *Peptides*, 30: 57-66.

Roa, J., V. M. Navarro, and M. Tena-Sempere. 2011. 'Kisspeptins in reproductive biology: consensus knowledge and recent developments', *Biol Reprod*, 85: 650-60.

Roa, J., and M. Tena-Sempere. 2014. 'Connecting metabolism and reproduction: roles of central energy sensors and key molecular mediators', *Mol Cell Endocrinol*, 397: 4-14.

Roozendaal, B., B. S. McEwen, and S. Chattarji. 2009. 'Stress, memory and the amygdala', *Nat Rev Neurosci*, 10: 423-33.

Sanchez-Garrido, M. A., and M. Tena-Sempere. 2013. 'Metabolic control of puberty: roles of leptin and kisspeptins', *Horm Behav*, 64: 187-94.

Semaan, S. J., and A. S. Kauffman. 2015. 'Daily successive changes in reproductive gene expression and neuronal activation in the brains of pubertal female mice', *Mol Cell Endocrinol*, 401: 84-97.

Seminara, S. B., S. Messager, E. E. Chatzidaki, R. R. Thresher, J. S. Acierno, Jr., J. K. Shagoury, Y. Bo-Abbas, W. Kuohung, K. M. Schwinof, A. G. Hendrick, D. Zahn, J. Dixon, U. B. Kaiser, S. A. Slaugenhaupt, J. F. Gusella, S. O'Rahilly, M. B. Carlton, W. F. Crowley, Jr., S. A. Aparicio, and W. H. Colledge. 2003. 'The GPR54 gene as a regulator of puberty', *N Engl J Med*, 349: 1614-27.

Setchell, K. D., and C. Clerici. 2010. 'Equol: pharmacokinetics and biological actions', *J Nutr*, 140: 1363S-8S.

Shahab, M., C. Mastronardi, S. B. Seminara, W. F. Crowley, S. R. Ojeda, and T. M. Plant. 2005. 'Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates', *Proc Natl Acad Sci U S A*, 102: 2129-34.

Shughrue, P. J., M. V. Lane, and I. Merchenthaler. 1997. 'Regulation of progesterone receptor messenger ribonucleic acid in the rat medial preoptic nucleus by estrogenic and antiestrogenic compounds: an in situ hybridization study', *Endocrinology*, 138: 5476-84.

Simerly, R. B., A. M. Carr, M. C. Zee, and D. Lorang. 1996. 'Ovarian steroid regulation of estrogen and progesterone receptor messenger ribonucleic acid in the anteroventral periventricular nucleus of the rat', *J Neuroendocrinol*, 8: 45-56.

Skrapits, K., B. A. Borsay, L. Herczeg, P. Ciofi, Z. Liposits, and E. Hrabovszky. 2015. 'Neuropeptide co-expression in hypothalamic kisspeptin neurons of laboratory animals and the human', *Front Neurosci*, 9: 29.

Smarr, B. L., E. Morris, and H. O. de la Iglesia. 2012. 'The dorsomedial suprachiasmatic nucleus times circadian expression of Kiss1 and the luteinizing hormone surge', *Endocrinology*, 153: 2839-50.

Smith, J. T., B. V. Acohido, D. K. Clifton, and R. A. Steiner. 2006. 'KiSS-1 neurones are direct targets for leptin in the ob/ob mouse', *J Neuroendocrinol*, 18: 298-303.

Smith, J. T., C. M. Clay, A. Caraty, and I. J. Clarke. 2007. 'KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season', *Endocrinology*, 148: 1150-7.

Smith, J. T., M. J. Cunningham, E. F. Rissman, D. K. Clifton, and R. A. Steiner. 2005. 'Regulation of Kiss1 gene expression in the brain of the female mouse', *Endocrinology*, 146: 3686-92.

Smith, J. T., H. M. Dungan, E. A. Stoll, M. L. Gottsch, R. E. Braun, S. M. Eacker, D. K. Clifton, and R. A. Steiner. 2005. 'Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse', *Endocrinology*, 146: 2976-84.

Smith, J. T., Q. Li, K. S. Yap, M. Shahab, A. K. Roseweir, R. P. Millar, and I. J. Clarke. 2011. 'Kisspeptin is essential for the full preovulatory LH surge and stimulates GnRH release from the isolated ovine median eminence', *Endocrinology*, 152: 1001-12.

Smith, J. T., A. Reichenbach, M. Lemus, B. K. Mani, J. M. Zigman, and Z. B. Andrews. 2013. 'An eGFP-expressing subpopulation of growth hormone secretagogue receptor cells are distinct from kisspeptin, tyrosine hydroxylase, and RFamide-related peptide neurons in mice', *Peptides*, 47: 45-53.

Stephens, S. B., K. P. Tolson, M. L. Rouse, Jr., M. C. Poling, M. K. Hashimoto-Partyka, P. L. Mellon, and A. S. Kauffman. 2015. 'Absent Progesterone Signaling in Kisspeptin Neurons Disrupts the LH Surge and Impairs Fertility in Female Mice', *Endocrinology*, 156: 3091-7.

Steyn, F. J., Y. Wan, J. Clarkson, J. D. Veldhuis, A. E. Herbison, and C. Chen. 2013. 'Development of a methodology for and assessment of pulsatile luteinizing hormone secretion in juvenile and adult male mice', *Endocrinology*, 154: 4939-45.

Takahashi, K. A., and R. D. Cone. 2005. 'Fasting induces a large, leptin-dependent increase in the intrinsic action potential frequency of orexigenic arcuate nucleus neuropeptide Y/Agouti-related protein neurons', *Endocrinology*, 146: 1043-7.

Takase, K., Y. Uenoyama, N. Inoue, H. Matsui, S. Yamada, M. Shimizu, T. Homma, J. Tomikawa, S. Kanda, H. Matsumoto, Y. Oka, H. Tsukamura, and K. I. Maeda. 2009. 'Possible role of oestrogen in pubertal increase of Kiss1/kisspeptin expression in discrete hypothalamic areas of female rats', *J Neuroendocrinol*, 21: 527-37.

Takumi, K., N. Iijima, and H. Ozawa. 2011. 'Developmental changes in the expression of kisspeptin mRNA in rat hypothalamus', *J Mol Neurosci*, 43: 138-45.

Takumi, K., K. Shimada, N. Iijima, and H. Ozawa. 2015. 'Maternal high-fat diet during lactation increases Kiss1 mRNA expression in the arcuate nucleus at weaning and advances puberty onset in female rats', *Neurosci Res*, 100: 21-8.

Teles, M. G., S. D. Bianco, V. N. Brito, E. B. Trarbach, W. Kuohung, S. Xu, S. B. Seminara, B. B. Mendonca, U. B. Kaiser, and A. C. Latronico. 2008. 'A GPR54-activating mutation in a patient with central precocious puberty', *N Engl J Med*, 358: 709-15.

Tena-Sempere, M. 2006. 'GPR54 and kisspeptin in reproduction', *Hum Reprod Update*, 12: 631-9.

Tena-Sempere, M. 2013. 'Ghrelin, the gonadal axis and the onset of puberty', *Endocr Dev*, 25: 69-82.

Tenenbaum-Rakover, Y., M. Commenges-Ducos, A. Iovane, C. Aumas, O. Admoni, and N. de Roux. 2007. 'Neuroendocrine phenotype analysis in five patients with isolated hypogonadotropic hypogonadism due to a L102P inactivating mutation of GPR54', *J Clin Endocrinol Metab*, 92: 1137-44.

Terasawa, E., and D. L. Fernandez. 2001. 'Neurobiological mechanisms of the onset of puberty in primates', *Endocr Rev*, 22: 111-51.

True, C., S. Verma, K. L. Grove, and M. S. Smith. 2013. 'Cocaine- and amphetamine-regulated transcript is a potent stimulator of GnRH and kisspeptin cells and may contribute to negative energy balance-induced reproductive inhibition in females', *Endocrinology*, 154: 2821-32.

Turi, G. F., Z. Liposits, S. M. Moenter, C. Fekete, and E. Hrabovszky. 2003. 'Origin of neuropeptide Y-containing afferents to gonadotropin-releasing hormone neurons in male mice', *Endocrinology*, 144: 4967-74.

Vendel, A. C., M. D. Terry, A. R. Striegel, N. M. Iverson, V. Leuranguer, C. D. Rithner, B. A. Lyons, G. E. Pickard, S. A. Tobet, and W. A. Horne. 2006. 'Alternative splicing of the voltage-gated Ca2+ channel beta4 subunit creates a uniquely folded N-terminal protein binding domain with cell-specific expression in the cerebellar cortex', *J Neurosci*, 26: 2635-44.

Walf, A. A., and C. A. Frye. 2006. 'A review and update of mechanisms of estrogen in the hippocampus and amygdala for anxiety and depression behavior', *Neuropsychopharmacology*, 31: 1097-111.

West, A., P. J. Vojta, D. R. Welch, and B. E. Weissman. 1998. 'Chromosome localization and genomic structure of the KiSS-1 metastasis suppressor gene (KISS1)', *Genomics*, 54: 145-8.

Wintermantel, T. M., R. E. Campbell, R. Porteous, D. Bock, H. J. Grone, M. G. Todman, K. S. Korach, E. Greiner, C. A. Perez, G. Schutz, and A. E. Herbison. 2006. 'Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility', *Neuron*, 52: 271-80.

Wu, Q., and R. D. Palmiter. 2011. 'GABAergic signaling by AgRP neurons prevents anorexia via a melanocortin-independent mechanism', *Eur J Pharmacol*, 660: 21-7.

Yeo, S. H., and A. E. Herbison. 2011. 'Projections of arcuate nucleus and rostral periventricular kisspeptin neurons in the adult female mouse brain', *Endocrinology*, 152: 2387-99.

Yeo, S. H., V. Kyle, P. G. Morris, S. Jackman, L. C. Sinnett-Smith, M. Schacker, C. Chen, and W. H. Colledge. 2016. 'Visualisation of Kiss1 Neurone Distribution Using a Kiss1-CRE Transgenic Mouse', *J Neuroendocrinol*, 28.

Yip, S. H., U. Boehm, A. E. Herbison, and R. E. Campbell. 2015. 'Conditional Viral Tract Tracing Delineates the Projections of the Distinct Kisspeptin Neuron Populations to Gonadotropin-Releasing Hormone (GnRH) Neurons in the Mouse', *Endocrinology*, 156: 2582-94.

Endocrine Disruptors

Some natural or synthetic compounds may alter the hormonal and homeostatic systems of the body, they are known as Endocrine-disrupting chemicals (EDCs). The U.S. Environmental Protection Agency (EPA) defined an EDC as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" (Kavlock et al. 1996). This definition was updated in 2012 by the Endocrine Society to "an exogenous chemical, or mixture of chemicals, that can interfere with any aspect of hormone action" (Zoeller et al. 2012). At first, EDCs actions were mainly reported through the superfamily of nuclear hormone receptors (i.e. estrogen receptors (ERs), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs), and retinoid receptors, among others). Recently, many other molecular mechanisms have been identified for EDCs: nonnuclear steroid hormone receptors (*e.g.*, membrane ERs), nonsteroid receptors (*e.g.*, neurotransmitter receptors such as the serotonin receptor, dopamine receptor, norepinephrine receptor), orphan receptors (*e.g.*, aryl hydrocarbon receptor (AhR)-an orphan receptor), enzymatic pathways

involved in steroid biosynthesis and/or metabolism, all involved in the regulation of the endocrine and reproductive systems (Panzica et al. 2011) (Frye et al. 2012). Finally, exposure to EDCs during this period induces epigenetic marks that contribute to important morphological and functional changes that last an entire lifetime and might even generate transgenerational effects (Janesick, Shioda, and Blumberg 2014).

EDCs are a very heterogeneous group and includes synthetic chemicals used as industrial solvents/lubricants and their byproducts [polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins], plastics [bisphenol A (BPA)], plasticizers (phthalates), pesticides [methoxychlor, chlorpyrifos, dichlorodiphenyltrichloroethane (DDT)], fungicides (vinclozolin), and pharmaceutical agents [diethylstilbestrol (DES)]; but also natural compounds included in human and animal food (*e.g.*, phytoestrogens, including genistein and coumestrol).

The sources of exposure to EDCs are different and may widely vary around the world. The situation is constantly evolving because some EDCs were banned decades ago and others more recently, with significant differences between countries. In this respect, migrating people provide a model to study cessation and/or onset of exposure depending on contamination of the original and new milieus (Krysiak-Baltyn et al. 2010) (Parent et al. 2005). There are also several historical examples of toxic spills or pollution from PCBs and dioxins that show a direct causal relationship between a chemical and the manifestation of an endocrine or reproductive dysfunction in humans and wildlife. However, these cases of single exposure are not representative of more widespread persistent environmental exposure. In fact, environmental pollution is rarely due to a single compound, but rather a combination of them. Notably, the effects of different classes of EDCs may be additive or even synergistic (Crews 2003). Industrialized areas are typically characterized by contamination from a wide range of industrial chemicals that may leach into soil and groundwater. Exposure occurs through drinking contaminated water, breathing contaminated air, ingesting food, or contacting contaminated soil. These complex mixtures enter the food chain and accumulate in animals higher up the food chain such as humans. Additionally, people who work with pesticides, fungicides, and industrial chemicals are particularly high risk for exposure and thus for developing a reproductive or endocrine system (Diamanti-Kandarakis et al. 2009).

Some EDCs were designed to have long half-lives; this was beneficial for their industrial use, but it has turned out to be quite detrimental to wildlife and humans. Since these substances do

not decay easily, they may not be metabolized, or they may be metabolized or even broken down into compounds more toxic than the parent molecule. Consequently, high levels of substances that were banned decades ago are still present in the environment, and can be detected as part of the body burden of virtually every tested individual animal or human (Dickerson and Gore 2007). For this reason, some EDCs are detectable in so called "pristine" environments at remote distances from the site they were produced, used, or released due to water and air currents and via migratory animals that spend part of their life in a contaminated area, to be incorporated into the food chain in an otherwise uncontaminated region. Others, such as BPA, may not be as persistent (although recent evidences are suggesting longer half-life), anyhow, given their so widespread use, they continue to be a prevalent human source of exposure (Stahlhut et al. 2007).

The timing of exposure to EDCs is crucial to understand the consequences of exposure. In fact, there are critical developmental periods during which neuroendocrine systems are modulated by steroids and other hormones. Early life exposure to endogenous androgens or estrogens, particularly in fetal life and childhood, organizes the brain in a sexually dimorphic manner (e.g. resulting in morphological and functional differences between males and females) that became activated later in life (Gore 2008) (Schwarz and McCarthy 2008). Consequently, exposure to exogenous substances such as EDCs is likely to cause worse outcomes in developing organisms and/or in adults (Gore 2008). In other words, consequences of developmental exposure may not be immediately apparent early in life, but may arise in adulthood or during life, a concept now referred as the "fetal/developmental basis of adult disease", which includes the maternal environment (eutherian mammals), the egg (other vertebrates), and the external environment, interacting with the individual's genes to determine the propensity of that individual to develop a disease or dysfunctional later in life (Barker 2003). For example, in the case of HPG reproductive system, early life exposures to environmental EDCs can permanently alter sexual development, resulting in females that are masculinized or defeminized, and males that are feminized or demasculinized. All these effects of EDCs on brain sexual differentiation are manifested as changes in reproductive development or in the onset of puberty and may be detrimental to fertility and reproductive success.

1. Metabolism-disrupting chemicals (MDCs)

More and more epidemiological studies agree to establish a relationship between EDC exposures and the prevalence of obesity and type 2 diabetes mellitus (T2DM), and, in general, the Metabolic syndrome (MetS), a complex condition characterized by insulin resistance, abdominal

obesity, dyslipidemia, hypertension, and hyperglycemia. MetS is a risk factor for cardiovascular disease, T2D, stroke, chronic kidney disease and cancers (Grundy et al. 2004) (Alberti et al. 2009). Accordingly, in 2015 the Parma Consensus Statement proposed the term metabolism-disrupting chemicals (MDCs). MDC is any EDC that is able to promote metabolic changes that can result in obesity, T2D or fattyliver in animals including humans; these metabolic alterations may play an important role in the global epidemics of obesity, T2D and MetS, and includes the terms ‘obesogens’, ‘diabetogens’ and ‘diabesogens’ (Heindel et al. 2017).

The most common MDCs identified to date include diethylstilbestrol (DES), persistent organic pollutants (POPs), including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT) and its metabolites, perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and tetrabromobisphenol A (TBBPA), and the non-persistent MDCs, bisphenol A (BPA) and phthalates, mainly bis(2-ethylhexyl) phthalate (DEHP). Indeed, epidemiological studies indicate that we are exposed to a cocktail of MDCs (Gore et al. 2015). This cocktail can target several components of the energy balance equation simultaneously and, consequently, might lead to dysfunctional counter-regulatory mechanisms that could potentially alter energy homeostasis, thereby predisposing individuals to metabolic disorders (Nadal et al. 2017).

Evidences indicate that MDCs might regulate nutrient ingestion and metabolism by altering intestinal transport, secretion of gut peptides, composition of the gut microbiota and levels of hypothalamic neuropeptides that control food intake (Nadal et al. 2017). In fact, several studies have reported that MDCs can alter food intake, with different effects based on dose and duration of exposure (Angle et al. 2013) (Mackay et al. 2013) (Bo et al. 2016). In addition, recent evidence indicates that some common MDCs alter each of the components of total energy expenditure as is possible to see in Fig. 15.

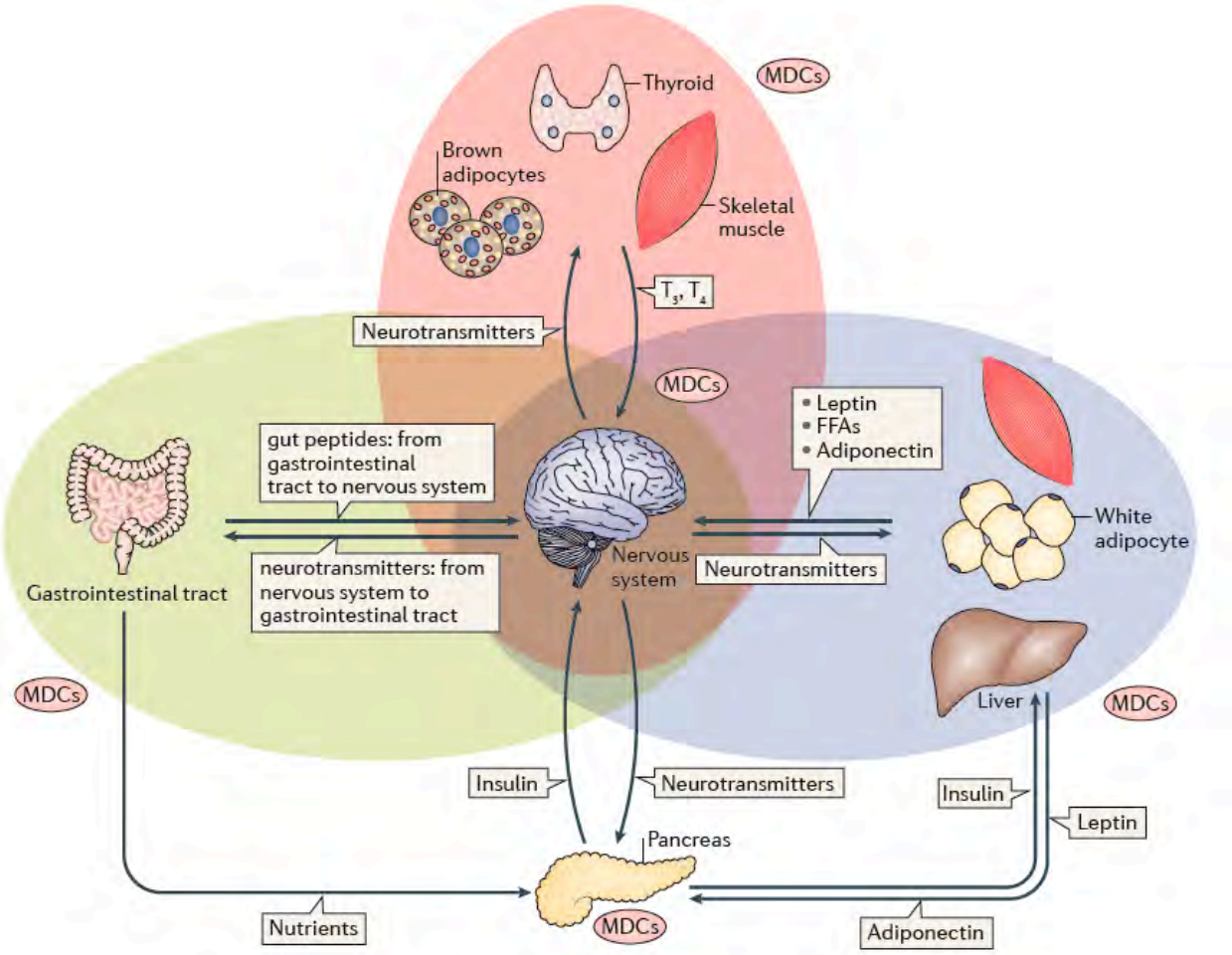

Figure 15: Action of metabolism-disrupting chemicals. Energy homeostasis is a balance between the control of energy intake and energy expenditure. MDCs can directly alter energy intake, modulating neuronal behaviour responsible for food intake, and indirectly, disrupting both epithelial transport in the intestine and the microbiota composition, which might alter serum levels of nutrients. Moreover, MDCs alter biosynthesis and probably also the release of gut peptides, which regulate food intake and energy balance. MDCs also affect insulin release from β cells, affecting both energy intake and energy storage. Energy storage might be disrupted as a consequence of the actions of MDCs on the brain, skeletal muscle, white adipose tissue and the liver. The disruption of insulin signalling in these organs or the direct action of MDCs on them disturbs the production and secretion of important signalling molecules, including free fatty acids (FFAs) and leptin, which have important roles in the endocrine pancreas and the brain. Energy output is disrupted by MDCs acting directly on the thyroid gland to modify serum levels of T3 and T4 and those that alter mitochondrial function in skeletal muscle and brown adipose tissue (Nadal et al., 2017).

As for EDCs, also for MDCs is the timing of the exposure during life is very important. Different studies analyzed MDCs effects on developmental stages. Perinatal exposure is without doubt extremely important, as organizational effects occur during development and induce permanent effects (Guillette et al. 1995). For example, exposure to BPA (10 µg/kg of body weight per day) during pregnancy increases the expression of many cell cycle-related genes, as well as pancreatic β-cell mass in young offspring (Garcia-Arevalo et al. 2016). These effects generate early hyperinsulinaemia and hyperleptinaemia that might be related to the increased susceptibility that these mice have to develop obesity and T2DM during adulthood (Garcia-Arevalo et al. 2016).

Notably, metabolic alterations of mothers' phenotype were demonstrated in pregnant mice treated with BPA (10 μg/kg of body weight per day) (Alonso-Magdalena et al. 2015). Between 6 and 7 months after delivery, BPA-treated dams were overweight, glucose intolerant, insulin resistant and exhibited notable disruption of β-cell mass and function compared to controls (Alonso-Magdalena et al. 2015). Moreover, adult exposure to MDCs should be considered with regard to the onset of metabolic disorders. Notably, exposure to MDCs during the perinatal period and adulthood modifies the cues that regulate energy homeostasis, such as serum levels of insulin, leptin and fatty acids, which are crucial mediators between the pancreas, WAT, liver, skeletal muscle, BAT and the brain (Stern, Rutkowski, and Scherer 2016).

The future works should increase the understanding of the mechanisms involved in the regulation of energy balance by MDCs and the identification of new MDCs.

2. Phytoestrogens

Phytoestrogens are plant-derived dietary compounds, diffused in a wide variety of foods, especially in soy. Since their chemical structure is very similarly to 17-β-oestradiol (E_2), the primary female sex hormone, they are able to act as natural endocrine disruptors. In plants, phytoestrogens perform a defensive role of through different means(Gang et al. 1999) including the recruitment of nitrogen fixing bacteria (Bladergroen and Spaink 1998), conferring resistance to fungi (Kessmann et al. 1990), and even controlling female fertility to prevent overpopulation and overgrazing by herbivore animals (Hughes 1988). The most diffused phytoestrogens in our diet are isoflavones (Table 1): they include genistein, daidzein, glycitein, formononetin and biochanin A, which are usually present in their conjugated forms like genistin, daidzin, puerarin, glycitin, ononin and sissotrin (Rietjens, Louisse, and Beekmann 2017). Isoflavones are biosynthesized in legumes, such as red clover, kidney beans, mung bean sprouts, navy beans, Japanese arrowroot (Kudzu), but are particularly abundant in soybeans. For instance, isoflavones in soybeans are mainly represented by the free and conjugate forms of genistein and daidzein, which make up, respectively, to 60% and 30% of the total isoflavones.

Table 1: Isoflavone contents of food (mg per 100g) by USDA (Zaheer , 2017).

Items	Total Isoflavones	Daidzein	Genestein
Soybeans	128.34	46.46	73.76
Ingredients:			
Okara	13.51	5.39	6.48
Soybean chips (paste)	54.16	26.71	27.45
Soy flour (full-fat)	171.89	96.83	71.19
Soy flour (textured)	148	59.62	78.62
Soy flour (defatted)	131.19	57.47	71.21
Soy protein (water)	102.07	43.04	59.59
Soy protein (alcohol ext)	12.47	6.63	5.33
Soy protein isolate	97.43	33.59	59.62
Food: non-fermented			
Soy milk	9.65	4.45	6.06
Soy drink	7.01	2.41	4.6
Tofu (firm)	22.70	8.00	12.75
Tofu (fried)	48.53	17.83	28.00
Tofu (soft)	29.24	8.59	20.65
Food: fermented			
Natto	58.93	21.85	29.04
Tempeh	43.52	17.58	24.85
Miso	42.55	16.13	24.56
Cheese (American)	17.95	5.75	8.75
Cheese, Monterey	18.70	7.80	8.80
Yogurt			
Food: 2nd generation products			
Vegetable burger	9.30	2.95	5.28
Soy hot dog	15.00	3.40	8.20
Bacon-meatless	12.10	2.8	6.9
Sausage, meatless	14.34	4.46	9.23

Phytoestrogens' structural similarity to E2 (Fig. 16) allows them to bind to the ER: estrogen receptor α (ERα) and estrogen receptor β (ERβ). These ER subtypes have different roles in gene regulation, cancer biology and therapy (Nilsson et al. 2001) (Williams et al. 2008) (Thomas and Gustafsson 2011). For instance, ERβ has been shown to counteract the ERα-mediated stimulation of cell proliferation (Bardin et al. 2004) (Stossi et al. 2004) (Sotoca et al. 2008) (Thomas and Gustafsson 2011). These different roles of ERα and ERβ in gene regulation, cell proliferation and related health effects, their changing ratio within tissues, are some of the factors that may influence the cellular response towards different phytoestrogens.

Figure 16: Chemical structures of E_2 and the isoflavones (modified from Rietjens et al, 2017)

The data in literature show for most phytoestrogens that have been investigated, that the IC_{50} (values from competitive binding assays)

values are higher for ERα than for ERβ, indicating a higher binding preference for the ERβ than for the ERα (Rietjens, Louisse, and Beekmann 2017). Remarkably, the EC_{50} (values from reporter gene assays) of genistein for ERβ activation is lower than that for ERα. Genistein concentration that activate ERα will, thus, at the same time activate ERβ counteracting at the same time the ERα-mediated effects on cell proliferation (Rietjens et al. 2013). Moreover, in addition to ERα and ERβ, many of the phytoestrogens including the soy isoflavone genistein are able to activate G protein coupled estrogen receptors (GPERs) as well (Prossnitz and Barton 2011), which may have a different role. In cell lines of thyroid, ovarian, endometrial and breast cancers, stimulation of GPERs with estrogens including genistein, activates a signaling pathway that promotes proliferation, although inhibition of proliferation has also been reported (Prossnitz and Barton 2011).

In addition to binding ERs, phytoestrogens have multiple modes of action including inhibition of tyrosine kinases at very high concentrations (Messina et al. 1994; Dixon and Ferreira 2002), and epigenetic activity including modulation of DNA methylation and chromatin configuration (Zhang and Chen 2011).

- *Phytoestrogens as EDCs*

The ability to bind the ER makes the phytoestrogens potent EDCs for numerous species, including rodents (Whitten and Naftolin 1992; Patisaul and Jefferson 2010), birds (Leopold et al. 1976; Viglietti-Panzica, Mura, and Panzica 2007), cheetahs (Setchell et al. 1987), various species of fish (Clotfelter and Rodriguez 2006; Sassi-Messai et al. 2009), and grazing mammals such as cattle, sheep and the southern white rhinoceros (Bennetts, Underwood, and Shier 1946; Adams 1995). In fact, adverse effects of phytoestrogens on fertility have been known for years, since the 1940s when sheep grazing on isoflavone-rich red clover fields in Western Australia showed fertility problems (Bennetts, Underwood, and Shier 1946) and this problem was related to the presence of soy isoflavone phytoestrogens in the standard animal diet (Setchell, Welsh, and Lim 1987). More recently, animal studies showed that soy diet accelerate female puberty (Patisaul and Jefferson 2010). Today, the endocrine disrupting properties of soy isoflavones and other phytoestrogens are well known also in humans. In particular, high amount of soy-rich diet causes menstrual cycle irregularities in young women (Chandrareddy et al. 2008) and lower serum testosterone levels in men (Gardner-Thorpe et al. 2003). Moreover, women raised with soy-based infant formula has been associated with a higher risk of menstrual cycle disorders and

uterine fibroids in pre-menopausal age (Strom et al. 2001) (D'Aloisio et al. 2010).

Briefly, phytoestrogen effect may change according to the administration paradigm (dose, administration route, treatment duration) and to the subject (species, sex, age) In summary, beneficial and detrimental effect of phytoestrogens have been reported in a number of studies, raising the interest for these compounds both as nutraceutics and as risk factors. Here is a summary of the main findings.

- *Phytoestrogens: effects*

Phytoestrogens are associated with an array of health benefits (Fig. 17). They are present in many dietary supplements and widely advertised as natural alternatives to estrogen replacement therapy for hot flashes and other menopausal symptoms. However, scientific studies have repeatedly and consistently failed to find reasonable evidence of phytoestrogens efficacy for that use (Setchell et al. 2001).

In addition, phytoestrogen exposure has been related to decreased risks of cardiovascular disease, obesity, metabolic syndrome and type2 diabetes, brain function disorders, a number of cancer (particularly breast, but also prostate, bowel and other form of cancer) (Hughes 1988) (Adlercreutz 2002) (Bhathena and Velasquez 2002) (Cederroth and Nef 2009) (Patisaul and Jefferson 2010) (Zhao and Mu 2011) (Jungbauer and Medjakovic 2014), although, a tumorigenic effect of some phytoestrogens have been proposed on other types of cancer. In particular, in human, a particular phenomenon called ‘Japanese Phenomenon’ has been observed the last decades. Many independent studies demonstrated that the incidence of specific chronic diseases in the Japanese population is lower than in Western Country, possibly due to a higher soy foods intake from early life onwards (Watanabe, Uesugi, and Kikuchi 2002; Korde, Calzone, and Zujewski 2004; Korde et al. 2009). Indeed, while in Asia, where fermented soy products are part of the traditional diet, isoflavone intake is about 15–50 mg isoflavones per day (Eisenbrand and Senate Commission on Food Safety of the German Research 2007), in Western industrial countries, isoflavone intake has been reported to be less than 2 mg isoflavones per day (Eisenbrand and Senate Commission on Food Safety of the German Research 2007).

However, the biological activity of phytoestrogens is involved in a plethora of negative effects.

Accordingly, the concern on the potential endocrine disrupting properties of isoflavonoids and other phytoestrogens is now growing, as soy consumption is rapidly increasing among all age

groups, especially infants and children (Strom et al. 2001; Setchell 2001; Cao et al. 2009). Most phytoestrogens readily cross the placenta, raising concerns about the potential outcomes of fetal exposure (Todaka et al. 2005). Total isoflavone content in soy infant formula varies, since the dosages recommended by the manufacturers may vary with the product, but is consistently higher in soy foods, averaging near 40μg total isoflavones per gram of formula (Setchell et al. 1987; Setchell et al. 1997; Franke, Custer, and Tanaka 1998; Johns, Dowlati, and Wargo 2003). Male offspring of pregnant women consuming a vegetarian diet during pregnancy have an increased incidence of congenital disorder of the urethra (North and Golding 2000). Furthermore, young adults who were fed with soy-based formulas as infants reported an increase in the use of medicines against allergy in both men and women, and longer menstrual bleeding and more discomfort during the menstrual cycle in women than their cow-based formula-fed counterparts (Strom et al. 2001).

Probably these conflictive results on developmental exposure to phytoestrogens suggesting some beneficial effects, but also adverse effects may depend on the timing of exposure, dose level, and examined end point (Jefferson, Padilla-Banks, and Newbold 2006). Certainly, a growing number of studies now warn that the health benefits commonly attributed to soy may not be entirely earned (Balk et al. 2005) (Sacks et al. 2006) (Jacobs et al. 2009), and that exposure may, in fact, pose a risk to some groups, particularly infants and the fetuses (Rozman et al. 2006).

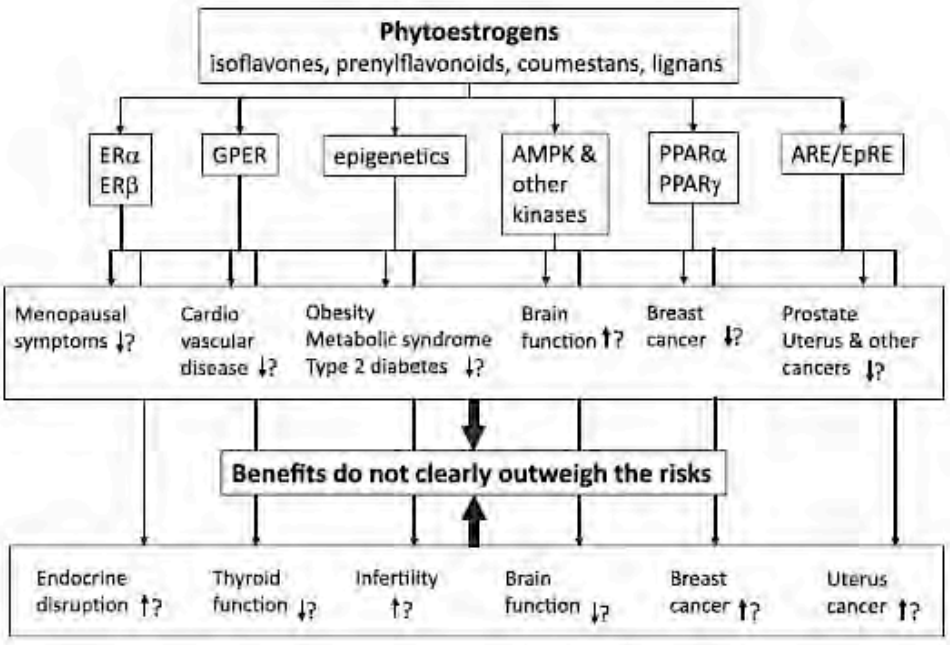

Figure 17: Schematic presentation summarizing the possible health effects of phytoestrogens and the potential underlying modes of action (Rietjens et al, 2017).

3. Genistein

Genistein (GEN) is an isoflavonoid compounds very abundant in the Leguminosae (Dixon and Ferreira 2002). It was first isolated in 1931 from an alcoholic extract of a soybean meal, when it was found that hydrolysis with hydrochloric acid produced 1 mole each of genistein and glucose. Its chemical structure (4', 5, 7-trihydroxyisoflavone) shares structural features with the strong estrogen estradiol-17β, particularly the phenolic ring and the distance between its 4'- and 7-hydroxyl groups (Dixon and Ferreira 2002) (Ganai and Farooqi 2015). These characteristics give GEN the ability to bind to sex hormone binding proteins and ERs (Polkowski and Mazurek 2000). Moreover, GEN shares structural similarities with tamoxifen, a compound used as chemo-preventive in women which is associated with increased risk of breast cancer, and with equol, a dietary isoflavonoid metabolite formed by gastrointestinal flora (Dixon and Ferreira 2002). Furthermore, GEN, can also have antiestrogenic activities by competitively binding to the same receptors as estradiol (Dixon and Ferreira 2002) (Sureda et al. 2017).

GEN, as other isoflavones, can promote symbiosis with rhizobacteria in order to defend the plant against pests and pathogens; it is a precursor of the antimicrobial compounds phytoalexins (synthesized following pathogen infection) and phytoanticipins (preformed compounds) (Dixon and Ferreira 2002). In our diet, isoflavonoids, as GEN, are almost limited to the Papilionoideae subfamily of the Leguminosae family. In particular, the major source of GEN is soybeans and soy-based foods. Though some foods contain only a small amount of isoflavones, when eaten regularly and from a wide range of sources, they can add reaching a cumulative dose that may cause long-term effects (Liggins, Grimwood, and Bingham 2000; Sureda et al. 2017).

As for other phytoestrogens, developmental exposure to GEN raises particular concern. During developmentb babies may be exposed to GEN in utero, as well as through lactation (Franke, Custer, and Tanaka 1998). High levels of phytoestrogens (6–9 mg/kg/day) are present in babies (Franke, Custer, and Tanaka 1998; Setchell et al. 1997) much higher than typical adult exposures (approximately 1 mg/kg/day) (Setchell et al. 1997). In fact, beside soy-based infant formulas, many soy-based foods are specifically labeled for children. Infants fed with soy-based formulas have high circulating levels of genistein (1–5 μM) indicating that this compound is readily absorbed (Setchell et al. 1997), and glucuronidated metabolites of GEN, as well as other GEN metabolites, in the urine (Hoey et al. 2004). Concern regarding the use of soy-based infant formula is emerging because epidemiology studies have associated its use with elevated risk of menstrual irregularities and uterine fibroids (D'Aloisio et al. 2010) (Strom et al. 2001).

4. Genistein, positive and negative effects: controversial issue

The central mechanism of GEN action is through ERs, but other modes of action have been described (Fig. 18). For instance, GEN is able to inhibit tyrosine kinases, enzymes involved in the control of many cellular processes (Akiyama et al. 1987). In addition, one of the key targets of GEN is an increase in nitric oxide production; however, this effect seems to be multi-factorial. It has been reported that different signaling pathways, such as ERK1/2, PI3 Kinase/Akt and AMP/protein kinase A, result in the phosphorylation of eNOS and consequently the activation of the enzyme (Martin et al. 2008). Moreover, GEN is able to suppress the expression of the p22phox NADPH oxidase subunit and angiotensin II type 1 (AT1) receptor through inhibition of the PPARγ signaling pathway resulting in decreased superoxide anion and peroxynitrite formation, leading to endothelial dysfunction (Xu et al. 2005). GEN is able also to induce epigenetic modification. It can inhibit the acetylation of histone and non-histone proteins and mediate chromatin remodeling through modulation of histone acetyl transferase (HATs) and histone deacetylase (HDACs) activity. These changes may be related to its health benefits (Vahid et al. 2015).

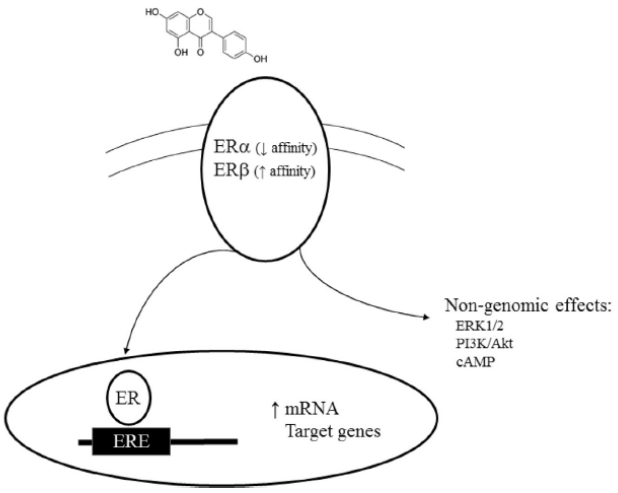

Figure 18: Estrogenic and endocrine-like properties/signaling of genistein. Genistein binds to estrogen receptors (ERs) and affect estrogen-regulated gene products containing estrogen response elements (ERE) and also mediates non-genomic effects via ERK1/2, PI3 Kinase/Akt and AMP/protein kinase A signaling pathways (Sureda 2017).

Public and scientific interest in GEN has increased because of its proposed beneficial effects. Several studies suggest that GEN possesses many physiological and pharmacological properties, ranging from antioxidant, antimutagenic and anticancer activities to cardioprotective and immunomodulatory capacities that make this compound a potential agent for the prevention and treatment of a number of chronic diseases (Sureda et al. 2017). In fact, GEN and daidzein intake

is associated with a decreased incidence of prostate cancer in humans through the reduction of androgen testosterone levels and inhibition of tumor development (Zhang and Chen 2011). Moreover, recent studies support the role of GEN in the suppression of breast cancer via the competition of phytoestrogen with natural estrogens, lowering their bioavailability and thereby inhibiting cancer cell growth (Rigalli et al. 2016).

In addition, the interest in GEN action has grown further because of its role as a factor, modulating processes involved in carbohydrate and lipid metabolism. These changes are often matched with metabolic changes and alterations in the hormonal status of the organism (as reviewed by (Szkudelska and Nogowski 2007). In fact, GEN affects insulin, thyroid hormones, adrenocortical hormones and leptin secretion. Briefly, GEN reduced blood insulin and perturbed the action of this hormone, by binding to its receptor. Moreover, GEN inhibits thyroid peroxidase (TPX) activity, binding to its active side. In the presence of iodide, the phytoestrogen is a substrate for TPX resulting in the formation of mono-, di- and triiodogenistein. The ability of GEN to affect thyroid function seems to be connected with high accumulation of the phytoestrogen in this gland. Furthermore, the adrenocortical function was also found to be affected by GEN. In vitro, cAMP-stimulated cortisol synthesis was depressed by GEN in adult adrenocortical cell line, H295, and GEN was shown to be a competitive inhibitor of adrenocortical 3ß-hydroxysteroid dehydrogenase and cytochrome P450 21-hydroxylase. As far as concerned the GEN action on leptin, it was demonstrated that it decreased the release of leptin from isolated adipocytes compared to control cells. It seems that the phytoestrogen cut leptin secretion mainly via the inhibition of glucose metabolism downstream the formation of pyruvate. Since adipocytes are the main source of circulating leptin, the direct limitation of its secretion from fat cells evoked by GEN may be the reason of reduced leptinemia in rats receiving soy or pure isoflavone (Szkudelska and Nogowski 2007).

Moreover, metabolic changes evoked by GEN involve blood, liver and muscle triglycerides, blood cholesterol, blood glucose and liver and muscle glycogen. GEN was also found to restrict lipogenesis and enhance lipolysis in adipocytes. Besides hormonal changes, isoflavones were found to induce numerous metabolic effects, which may contribute to decrease in body weight. Kim and co-workers (Kim et al. 2006) found that in ovariectomized female mice, orally administered GEN (1500 mg/kg, a very hight dose) reduced food intake and body weight. Notably, GEN did not alter serum glucose, insulin and leptin concentrations in these animals. The authors suggest that GEN is able to evoke apoptosis in adipocytes. Moreover, subcutaneous injections of GEN for 21–28 days or feeding a diet with this phytoestrogen for 12 days

substantially decreased adipose tissue weight in ovariectomized mice and reduced lipoprotein lipase gene expression in adipose tissue, contributing to reduce lipid uptake by fat cells (Naaz et al. 2003). In contrast with these data, in recent study of Cao and colleagues (Cao et al. 2015), rats under soy-diet gained less weight during pregnancy and, although they consumed more than dams on a soy-free diet during lactation, did not become heavier. Their offspring (both sexes), however, became significantly heavier (more pronounced in males) already before weaning. Soy also enhanced food intake and accelerated female pubertal onset in the offspring (Cao et al. 2015). Furthermore, postnatal oral administration (PND1 to PND22) of 50mg/kg GEN (dose that mimics blood GEN levels in human infants fed soy formula) in rat pups induced in female but not male increases of fat/lean mass ratio, fat mass, adipocyte size and number, and decreased muscle fiber perimeter (Strakovsky et al. 2014). Therefore, these contradictory data underlined the importance of considering the timing of exposure, the dose/concentration and the sex when establishing safety recommendations for dietary GEN intake, especially if in early-life.

GEN can potentially affect androgens and estrogens clearance rates and therefore their availability (Dixon and Ferreira 2002). The results of the administration of phytoestrogens to animals and humans can vary considerably, as GEN binds differently to the different ERs that are differentially expressed in different species. GEN is able to bind preferentially to ERß and activate ERdependent gene transcription (Zhao, Mao, and Brinton 2009), but GEN affinity is much lower in comparison to estradiol (Kuiper et al. 1998). Kuiper and colleagues measured the transcriptional activity of GEN (Kuiper et al. 1998). This activity is also lower for isoflavones than for estradiol although, at high concentrations, may be similar or even larger (Kuiper et al. 1998), in particular, GEN has the highest estrogen receptor binding affinity (RBA) (Kuiper et al. 1998). Probably this is due to exact position and number of the hydroxyl substituent; GEN, containing three hydroxyl groups, has a higher RBA to ERß than daidzein and biochanin A, which contain two hydroxyl groups (Kuiper et al. 1998).

Many studies, in fact, showed the properties of GEN as an EDC; indeed the soy is well recognized as a hormonally active food (Patisaul and Belcher 2017). As other EDCs the majority of GEN studies have focused on reproductive endpoints, because EDC-mediated alterations of HPG organization. In fact, studies in mice has revealed that GEN can interfere with ovarian differentiation resulting in ovarian malformations indicative of impaired fecundity such as multi-

oocyte follicles, and attenuated oocyte cell death (Jefferson, Padilla-Banks, and Newbold 2006) (Jefferson, Patisaul, and Williams 2012). Ovarian defects, including the absence of corpora lutea, the presence of large antral-like follicles with degenerating or no oocytes and numerous ovarian cysts have also been observed following neonatal GEN exposure in rats (Kouki et al. 2003). Moreover, the literature reports GEN effects on estrous cyclicity, fecundity, ovulation and female reproductive behavior (Jefferson, Padilla-Banks, and Newbold 2006) (Crain and Shen 2008).

Many effects of GEN are sex specific, probably for its ability to bind the ERs. Studies reported these sexual alterations particularly in hypothalamus, a region hormone sensitive. Neonatal GEN demasculinized (reduced) tyrosine hydroxylase immunoreactivity in the male AVPV (Patisaul, Fortino, and Polston 2006, 2007) and defeminized (reduced) kisspeptin fiber density in the female AVPV and ARC (Losa et al. 2011). A sexually dimorphic effect on vasopressin-ir (AVP) was observed in our recent study (Ponti et al. 2017). The early-life exposure to GEN, with a dose that mimic the soy infant formula, induced in mice an increase of signal in the female BnST but did not impact the sexually dimorphic AVP innervation of the LS (Ponti et al. 2017). In the same experiment, the sexually dimorphic density of AVP-ir neurons in the medial parvicellular part of the PVN (PaMP) was eliminated by postnatal GEN, with higher numbers in females and lower numbers in males (Ponti et al. 2017). Furthermore, a very recent review of Patisaul showed, analyzing different works, as GEN can alter pathways and behaviors coordinated by two important hypothalamic population, AVP and oxytocin (OT) (Patisaul and Belcher 2017). In this review is well reported as long-term consumption of soy-rich diets enhance aggression in male cynomolgus monkeys (Simon et al. 2004) and Syrian hamsters (Moore, Karom, and O'Farrell 2004), correlated to lower AVP expression in the lateral septum but higher in lateral hypothalamus. In addition, two dietary isoflavone supplements produced anxiolytic elevated plus maze behavior in proestrus female rats, but anxiogenic responses in gonadally intact males (Patisaul et al. 2005). Similarly, male rats maintained on a diet containing 150 μg/g GEN and daidzein displayed increased anxiety and elevated stress- induced plasma AVP and corticosterone levels (Hartley et al. 2003).

References

Adams, N. R. 1995. 'Organizational and activational effects of phytoestrogens on the reproductive tract of the ewe', *Proc Soc Exp Biol Med*, 208: 87-91.

Adlercreutz, H. 2002. 'Phytoestrogens and breast cancer', *J Steroid Biochem Mol Biol*, 83: 113-8.

Akiyama, T., J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya, and Y. Fukami. 1987. 'Genistein, a specific inhibitor of tyrosine-specific protein kinases', *J Biol Chem*, 262: 5592-5.

Alberti, K. G., R. H. Eckel, S. M. Grundy, P. Z. Zimmet, J. I. Cleeman, K. A. Donato, J. C. Fruchart, W. P. James, C. M. Loria, S. C. Smith, Jr., Epidemiology International Diabetes Federation Task Force on, Prevention, Lung Hational Heart, Institute Blood, Association American Heart, Federation World Heart, Society International Atherosclerosis, and Obesity International Association for the Study of. 2009. 'Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity', *Circulation*, 120: 1640-5.

Alonso-Magdalena, P., M. Garcia-Arevalo, I. Quesada, and A. Nadal. 2015. 'Bisphenol-A treatment during pregnancy in mice: a new window of susceptibility for the development of diabetes in mothers later in life', *Endocrinology*, 156: 1659-70.

Angle, B. M., R. P. Do, D. Ponzi, R. W. Stahlhut, B. E. Drury, S. C. Nagel, W. V. Welshons, C. L. Besch-Williford, P. Palanza, S. Parmigiani, F. S. vom Saal, and J. A. Taylor. 2013. 'Metabolic disruption in male mice due to fetal exposure to low but not high doses of bisphenol A (BPA): evidence for effects on body weight, food intake, adipocytes, leptin, adiponectin, insulin and glucose regulation', *Reprod Toxicol*, 42: 256-68.

Balk, E., M. Chung, P. Chew, S. Ip, G. Raman, B. Kupelnick, A. Tatsioni, Y. Sun, B. Wolk, D. DeVine, and J. Lau. 2005. 'Effects of soy on health outcomes', *Evid Rep Technol Assess (Summ)*: 1-8.

Bardin, A., N. Boulle, G. Lazennec, F. Vignon, and P. Pujol. 2004. 'Loss of ERbeta expression as a common step in estrogen-dependent tumor progression', *Endocr Relat Cancer*, 11: 537-51.

Barker, D. J. 2003. 'The developmental origins of adult disease', *Eur J Epidemiol*, 18: 733-6.

Bennetts, H. W., E. J. Underwood, and F. L. Shier. 1946. 'A specific breeding problem of sheep on subterranean clover pastures in Western Australia', *Br Vet J*, 102: 348-52.

Bhathena, S. J., and M. T. Velasquez. 2002. 'Beneficial role of dietary phytoestrogens in obesity and diabetes', *Am J Clin Nutr*, 76: 1191-201.

Bladergroen, M. R., and H. P. Spaink. 1998. 'Genes and signal molecules involved in the rhizobia-leguminoseae symbiosis', *Curr Opin Plant Biol*, 1: 353-9.

Bo, E., A. Farinetti, M. Marraudino, D. Sterchele, C. Eva, S. Gotti, and G. Panzica. 2016. 'Adult exposure to tributyltin affects hypothalamic neuropeptide Y, Y1 receptor distribution, and circulating leptin in mice', *Andrology*, 4: 723-34.

Cao, J., R. Echelberger, M. Liu, E. Sluzas, K. McCaffrey, B. Buckley, and H. B. Patisaul. 2015. 'Soy but not bisphenol A (BPA) or the phytoestrogen genistin alters developmental weight gain and food intake in pregnant rats and their offspring', *Reprod Toxicol*, 58: 282-94.

Cao, Y., A. M. Calafat, D. R. Doerge, D. M. Umbach, J. C. Bernbaum, N. C. Twaddle, X. Ye, and W. J. Rogan. 2009. 'Isoflavones in urine, saliva, and blood of infants: data from a pilot study on the estrogenic activity of soy formula', *J Expo Sci Environ Epidemiol*, 19: 223-34.

Cederroth, C. R., and S. Nef. 2009. 'Soy, phytoestrogens and metabolism: A review', *Mol Cell Endocrinol*, 304: 30-42.

Chandrareddy, A., O. Muneyyirci-Delale, S. I. McFarlane, and O. M. Murad. 2008. 'Adverse effects of phytoestrogens on reproductive health: a report of three cases', *Complement Ther Clin Pract*, 14: 132-5.

Clotfelter, E. D., and A. C. Rodriguez. 2006. 'Behavioral changes in fish exposed to phytoestrogens', *Environ Pollut*, 144: 833-9.

Crain, S. M., and K. F. Shen. 2008. 'Low doses of cyclic AMP-phosphodiesterase inhibitors rapidly evoke opioid receptor-mediated thermal hyperalgesia in naive mice which is converted to prominent analgesia by cotreatment with ultra-low-dose naltrexone', *Brain Res*, 1231: 16-24.

Crews, D. 2003. 'Sex determination: where environment and genetics meet', *Evol Dev*, 5: 50-5.

D'Aloisio, A. A., D. D. Baird, L. A. DeRoo, and D. P. Sandler. 2010. 'Association of intrauterine and early-life exposures with diagnosis of uterine leiomyomata by 35 years of age in the Sister Study', *Environ Health Perspect*, 118: 375-81.

Diamanti-Kandarakis, E., J. P. Bourguignon, L. C. Giudice, R. Hauser, G. S. Prins, A. M. Soto, R. T. Zoeller, and A. C. Gore. 2009. 'Endocrine-disrupting chemicals: an Endocrine Society scientific statement', *Endocr Rev*, 30: 293-342.

Dickerson, S. M., and A. C. Gore. 2007. 'Estrogenic environmental endocrine-disrupting chemical effects on reproductive neuroendocrine function and dysfunction across the life cycle', *Rev Endocr Metab Disord*, 8: 143-59.

Dixon, R. A., and D. Ferreira. 2002. 'Genistein', *Phytochemistry*, 60: 205-11.

Eisenbrand, G., and Foundation Senate Commission on Food Safety of the German Research. 2007. 'Isoflavones as phytoestrogens in food supplements and dietary foods for special medical purposes. Opinion of the Senate Commission on Food Safety (SKLM) of the German Research Foundation (DFG)-(shortened version)', *Mol Nutr Food Res*, 51: 1305-12.

Franke, A. A., L. J. Custer, and Y. Tanaka. 1998. 'Isoflavones in human breast milk and other biological fluids', *Am J Clin Nutr*, 68: 1466S-73S.

Frye, C. A., E. Bo, G. Calamandrei, L. Calza, F. Dessi-Fulgheri, M. Fernandez, L. Fusani, O. Kah, M. Kajta, Y. Le Page, H. B. Patisaul, A. Venerosi, A. K. Wojtowicz, and G. C. Panzica. 2012. 'Endocrine disrupters: a review of some sources, effects, and mechanisms of actions on behaviour and neuroendocrine systems', *J Neuroendocrinol*, 24: 144-59.

Ganai, A. A., and H. Farooqi. 2015. 'Bioactivity of genistein: A review of in vitro and in vivo studies', *Biomed Pharmacother*, 76: 30-8.

Gang, D. R., H. Kasahara, Z. Q. Xia, K. Vander Mijnsbrugge, G. Bauw, W. Boerjan, M. Van Montagu, L. B. Davin, and N. G. Lewis. 1999. 'Evolution of plant defense mechanisms. Relationships of phenylcoumaran benzylic ether reductases to pinoresinol-lariciresinol and isoflavone reductases', *J Biol Chem*, 274: 7516-27.

Garcia-Arevalo, M., P. Alonso-Magdalena, J. M. Servitja, T. Boronat-Belda, B. Merino, S. Villar-Pazos, G. Medina-Gomez, A. Novials, I. Quesada, and A. Nadal. 2016. 'Maternal Exposure to Bisphenol-A During Pregnancy Increases Pancreatic beta-Cell Growth During Early Life in Male Mice Offspring', *Endocrinology*, 157: 4158-71.

Gardner-Thorpe, D., C. O'Hagen, I. Young, and S. J. Lewis. 2003. 'Dietary supplements of soya flour lower serum testosterone concentrations and improve markers of oxidative stress in men', *Eur J Clin Nutr*, 57: 100-6.

Gore, A. C. 2008. 'Developmental programming and endocrine disruptor effects on reproductive neuroendocrine systems', *Front Neuroendocrinol*, 29: 358-74.

Gore, A. C., V. A. Chappell, S. E. Fenton, J. A. Flaws, A. Nadal, G. S. Prins, J. Toppari, and R. T. Zoeller. 2015. 'EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals', *Endocr Rev*, 36: E1-E150.

Grundy, S. M., H. B. Brewer, Jr., J. I. Cleeman, S. C. Smith, Jr., C. Lenfant, Lung National Heart, Institute Blood, and Association American Heart. 2004. 'Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition', *Arterioscler Thromb Vasc Biol*, 24: e13-8.

Guillette, L. J., Jr., D. A. Crain, A. A. Rooney, and D. B. Pickford. 1995. 'Organization versus activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife', *Environ Health Perspect*, 103 Suppl 7: 157-64.

Hartley, D. E., J. E. Edwards, C. E. Spiller, N. Alom, S. Tucci, P. Seth, M. L. Forsling, and S. E. File. 2003. 'The soya isoflavone content of rat diet can increase anxiety and stress hormone release in the male rat', *Psychopharmacology (Berl)*, 167: 46-53.

Heindel, J. J., B. Blumberg, M. Cave, R. Machtinger, A. Mantovani, M. A. Mendez, A. Nadal, P. Palanza, G. Panzica, R. Sargis, L. N. Vandenberg, and F. Vom Saal. 2017. 'Metabolism disrupting chemicals and metabolic disorders', *Reprod Toxicol*, 68: 3-33.

Hoey, L., I. R. Rowland, A. S. Lloyd, D. B. Clarke, and H. Wiseman. 2004. 'Influence of soya-based infant formula consumption on isoflavone and gut microflora metabolite concentrations in urine and on faecal microflora composition and metabolic activity in infants and children', *Br J Nutr*, 91: 607-16.

Hughes, C. L., Jr. 1988. 'Phytochemical mimicry of reproductive hormones and modulation of herbivore fertility by phytoestrogens', *Environ Health Perspect*, 78: 171-4.

Jacobs, A., U. Wegewitz, C. Sommerfeld, R. Grossklaus, and A. Lampen. 2009. 'Efficacy of isoflavones in relieving vasomotor menopausal symptoms - A systematic review', *Mol Nutr Food Res*, 53: 1084-97.

Janesick, A. S., T. Shioda, and B. Blumberg. 2014. 'Transgenerational inheritance of prenatal obesogen exposure', *Mol Cell Endocrinol*, 398: 31-5.

Jefferson, W. N., E. Padilla-Banks, and R. R. Newbold. 2006. 'Studies of the effects of neonatal exposure to genistein on the developing female reproductive system', *J AOAC Int*, 89: 1189-96.

Jefferson, W. N., H. B. Patisaul, and C. J. Williams. 2012. 'Reproductive consequences of developmental phytoestrogen exposure', *Reproduction*, 143: 247-60.

Johns, P., L. Dowlati, and W. Wargo. 2003. 'Determination of isoflavones in ready-to-feed soy-based infant formula', *J AOAC Int*, 86: 72-8.

Jungbauer, A., and S. Medjakovic. 2014. 'Phytoestrogens and the metabolic syndrome', *J Steroid Biochem Mol Biol*, 139: 277-89.

Kavlock, R. J., G. P. Daston, C. DeRosa, P. Fenner-Crisp, L. E. Gray, S. Kaattari, G. Lucier, M. Luster, M. J. Mac, C. Maczka, R. Miller, J. Moore, R. Rolland, G. Scott, D. M. Sheehan, T. Sinks, and H. A. Tilson. 1996. 'Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop', *Environ Health Perspect*, 104 Suppl 4: 715-40.

Kessmann, H., R. Edwards, P. W. Geno, and R. A. Dixon. 1990. 'Stress Responses in Alfalfa (Medicago sativa L.) : V. Constitutive and Elicitor-Induced Accumulation of Isoflavonoid Conjugates in Cell Suspension Cultures', *Plant Physiol*, 94: 227-32.

Kim, H. K., C. Nelson-Dooley, M. A. Della-Fera, J. Y. Yang, W. Zhang, J. Duan, D. L. Hartzell, M. W. Hamrick, and C. A. Baile. 2006. 'Genistein decreases food intake, body weight, and fat pad weight and causes adipose tissue apoptosis in ovariectomized female mice', *J Nutr*, 136: 409-14.

Korde, L. A., K. A. Calzone, and J. Zujewski. 2004. 'Assessing breast cancer risk: genetic factors are not the whole story', *Postgrad Med*, 116: 6-8, 11-4, 19-20.

Korde, L. A., A. H. Wu, T. Fears, A. M. Nomura, D. W. West, L. N. Kolonel, M. C. Pike, R. N. Hoover, and R. G. Ziegler. 2009. 'Childhood soy intake and breast cancer risk in Asian American women', *Cancer Epidemiol Biomarkers Prev*, 18: 1050-9.

Kouki, T., M. Kishitake, M. Okamoto, I. Oosuka, M. Takebe, and K. Yamanouchi. 2003. 'Effects of neonatal treatment with phytoestrogens, genistein and daidzein, on sex difference in female rat brain function: estrous cycle and lordosis', *Horm Behav*, 44: 140-5.

Krysiak-Baltyn, K., J. Toppari, N. E. Skakkebaek, T. S. Jensen, H. E. Virtanen, K. W. Schramm, H. Shen, T. Vartiainen, H. Kiviranta, O. Taboureau, S. Brunak, and K. M. Main. 2010. 'Country-specific chemical signatures of persistent environmental compounds in breast milk', *Int J Androl*, 33: 270-8.

Kuiper, G. G., J. G. Lemmen, B. Carlsson, J. C. Corton, S. H. Safe, P. T. van der Saag, B. van der Burg, and J. A. Gustafsson. 1998. 'Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta', *Endocrinology*, 139: 4252-63.

Leopold, A. S., M. Erwin, J. Oh, and B. Browning. 1976. 'Phytoestrogens: adverse effects on reproduction in California quail', *Science*, 191: 98-100.

Liggins, J., R. Grimwood, and S. A. Bingham. 2000. 'Extraction and quantification of lignan phytoestrogens in food and human samples', *Anal Biochem*, 287: 102-9.

Losa, S. M., K. L. Todd, A. W. Sullivan, J. Cao, J. A. Mickens, and H. B. Patisaul. 2011. 'Neonatal exposure to genistein adversely impacts the ontogeny of hypothalamic kisspeptin signaling pathways and ovarian development in the peripubertal female rat', *Reprod Toxicol*, 31: 280-9.

Mackay, H., Z. R. Patterson, R. Khazall, S. Patel, D. Tsirlin, and A. Abizaid. 2013. 'Organizational effects of perinatal exposure to bisphenol-A and diethylstilbestrol on arcuate nucleus circuitry controlling food intake and energy expenditure in male and female CD-1 mice', *Endocrinology*, 154: 1465-75.

Martin, N. I., W. T. Beeson, J. J. Woodward, and M. A. Marletta. 2008. 'N(G)-aminoguanidines from primary amines and the preparation of nitric oxide synthase inhibitors', *J Med Chem*, 51: 924-31.

Messina, M. J., V. Persky, K. D. Setchell, and S. Barnes. 1994. 'Soy intake and cancer risk: a review of the in vitro and in vivo data', *Nutr Cancer*, 21: 113-31.

Moore, T. O., M. Karom, and L. O'Farrell. 2004. 'The neurobehavioral effects of phytoestrogens in male Syrian hamsters', *Brain Res*, 1016: 102-10.

Naaz, A., S. Yellayi, M. A. Zakroczymski, D. Bunick, D. R. Doerge, D. B. Lubahn, W. G. Helferich, and P. S. Cooke. 2003. 'The soy isoflavone genistein decreases adipose deposition in mice', *Endocrinology*, 144: 3315-20.

Nadal, A., I. Quesada, E. Tuduri, R. Nogueiras, and P. Alonso-Magdalena. 2017. 'Endocrine-disrupting chemicals and the regulation of energy balance', *Nat Rev Endocrinol*, 13: 536-46.

Nilsson, S., S. Makela, E. Treuter, M. Tujague, J. Thomsen, G. Andersson, E. Enmark, K. Pettersson, M. Warner, and J. A. Gustafsson. 2001. 'Mechanisms of estrogen action', *Physiol Rev*, 81: 1535-65.

North, K., and J. Golding. 2000. 'A maternal vegetarian diet in pregnancy is associated with hypospadias. The ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood', *BJU Int*, 85: 107-13.

Panzica, G. C., E. Bo, M. A. Martini, D. Miceli, E. Mura, C. Viglietti-Panzica, and S. Gotti. 2011. 'Neuropeptides and enzymes are targets for the action of endocrine disrupting chemicals in the vertebrate brain', *J Toxicol Environ Health B Crit Rev*, 14: 449-72.

Parent, A. S., G. Rasier, A. Gerard, S. Heger, C. Roth, C. Mastronardi, H. Jung, S. R. Ojeda, and J. P. Bourguignon. 2005. 'Early onset of puberty: tracking genetic and environmental factors', *Horm Res*, 64 Suppl 2: 41-7.

Patisaul, H. B., A. Blum, J. R. Luskin, and M. E. Wilson. 2005. 'Dietary soy supplements produce opposite effects on anxiety in intact male and female rats in the elevated plus-maze', *Behav Neurosci*, 119: 587-94.

Patisaul, H. B., A. E. Fortino, and E. K. Polston. 2006. 'Neonatal genistein or bisphenol-A exposure alters sexual differentiation of the AVPV', *Neurotoxicol Teratol*, 28: 111-8.

Patisaul, H. B. 2007. 'Differential disruption of nuclear volume and neuronal phenotype in the preoptic area by neonatal exposure to genistein and bisphenol-A', *Neurotoxicology*, 28: 1-12.

Patisaul, H. B., and W. Jefferson. 2010. 'The pros and cons of phytoestrogens', *Front Neuroendocrinol*, 31: 400-19.

Patisaul, Heather B., and Scott M. Belcher. 2017. *Endocrine disruptors, brain, and behaviors* (Oxford University Press: New York, NY).

Polkowski, K., and A. P. Mazurek. 2000. 'Biological properties of genistein. A review of in vitro and in vivo data', *Acta Pol Pharm*, 57: 135-55.

Ponti, G., A. Rodriguez-Gomez, A. Farinetti, M. Marraudino, F. Filice, B. Foglio, G. Sciacca, G. C. Panzica, and S. Gotti. 2017. 'Early postnatal genistein administration permanently affects nitrergic and vasopressinergic systems in a sex-specific way', *Neuroscience*, 346: 203-15.

Prossnitz, E. R., and M. Barton. 2011. 'The G-protein-coupled estrogen receptor GPER in health and disease', *Nat Rev Endocrinol*, 7: 715-26.

Rietjens, I. M., A. M. Sotoca, J. Vervoort, and J. Louisse. 2013. 'Mechanisms underlying the dualistic mode of action of major soy isoflavones in relation to cell proliferation and cancer risks', *Mol Nutr Food Res*, 57: 100-13.

Rietjens, Imcm, J. Louisse, and K. Beekmann. 2017. 'The potential health effects of dietary phytoestrogens', *Br J Pharmacol*, 174: 1263-80.

Rigalli, J. P., G. N. Tocchetti, M. R. Arana, S. S. Villanueva, V. A. Catania, D. Theile, M. L. Ruiz, and J. Weiss. 2016. 'The phytoestrogen genistein enhances multidrug resistance in breast cancer cell lines by translational regulation of ABC transporters', *Cancer Lett*, 376: 165-72.

Rozman, K. K., J. Bhatia, A. M. Calafat, C. Chambers, M. Culty, R. A. Etzel, J. A. Flaws, D. K. Hansen, P. B. Hoyer, E. H. Jeffery, J. S. Kesner, S. Marty, J. A. Thomas, and D. Umbach. 2006. 'NTP-CERHR expert panel report on the reproductive and developmental toxicity of genistein', *Birth Defects Res B Dev Reprod Toxicol*, 77: 485-638.

Sacks, F. M., A. Lichtenstein, L. Van Horn, W. Harris, P. Kris-Etherton, M. Winston, and Committee American Heart Association Nutrition. 2006. 'Soy protein, isoflavones, and cardiovascular health: an American Heart Association Science Advisory for professionals from the Nutrition Committee', *Circulation*, 113: 1034-44.

Sassi-Messai, S., Y. Gibert, L. Bernard, S. Nishio, K. F. Ferri Lagneau, J. Molina, M. Andersson-Lendahl, G. Benoit, P. Balaguer, and V. Laudet. 2009. 'The phytoestrogen genistein affects zebrafish development through two different pathways', *PLoS One*, 4: e4935.

Schwarz, J. M., and M. M. McCarthy. 2008. 'Steroid-induced sexual differentiation of the developing brain: multiple pathways, one goal', *J Neurochem*, 105: 1561-72.

Setchell, K. D. 2001. 'Soy isoflavones--benefits and risks from nature's selective estrogen receptor modulators (SERMs)', *J Am Coll Nutr*, 20: 354S-62S; discussion 81S-83S.

Setchell, K. D., N. M. Brown, P. Desai, L. Zimmer-Nechemias, B. E. Wolfe, W. T. Brashear, A. S. Kirschner, A. Cassidy, and J. E. Heubi. 2001. 'Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements', *J Nutr*, 131: 1362S-75S.

Setchell, K. D., S. J. Gosselin, M. B. Welsh, J. O. Johnston, W. F. Balistreri, L. W. Kramer, B. L. Dresser, and M. J. Tarr. 1987. 'Dietary estrogens--a probable cause of infertility and liver disease in captive cheetahs', *Gastroenterology*, 93: 225-33.

Setchell, K. D., M. B. Welsh, and C. K. Lim. 1987. 'High-performance liquid chromatographic analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical and thermospray mass spectrometric detection', *J Chromatogr*, 386: 315-23.

Setchell, K. D., L. Zimmer-Nechemias, J. Cai, and J. E. Heubi. 1997. 'Exposure of infants to phyto-oestrogens from soy-based infant formula', *Lancet*, 350: 23-7.

Simon, N. G., J. R. Kaplan, S. Hu, T. C. Register, and M. R. Adams. 2004. 'Increased aggressive behavior and decreased affiliative behavior in adult male monkeys after long-term consumption of diets rich in soy protein and isoflavones', *Horm Behav*, 45: 278-84.

Sotoca, A. M., H. van den Berg, J. Vervoort, P. van der Saag, A. Strom, J. A. Gustafsson, I. Rietjens, and A. J. Murk. 2008. 'Influence of cellular ERalpha/ERbeta ratio on the ERalpha-agonist induced proliferation of human T47D breast cancer cells', *Toxicol Sci*, 105: 303-11.

Stahlhut, R. W., E. van Wijngaarden, T. D. Dye, S. Cook, and S. H. Swan. 2007. 'Concentrations of urinary phthalate metabolites are associated with increased waist circumference and insulin resistance in adult U.S. males', *Environ Health Perspect*, 115: 876-82.

Stern, J. H., J. M. Rutkowski, and P. E. Scherer. 2016. 'Adiponectin, Leptin, and Fatty Acids in the Maintenance of Metabolic Homeostasis through Adipose Tissue Crosstalk', *Cell Metab*, 23: 770-84.

Stossi, F., D. H. Barnett, J. Frasor, B. Komm, C. R. Lyttle, and B. S. Katzenellenbogen. 2004. 'Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) alpha or ERbeta in human osteosarcoma cells: distinct and common target genes for these receptors', *Endocrinology*, 145: 3473-86.

Strakovsky, R. S., S. Lezmi, J. A. Flaws, S. L. Schantz, Y. X. Pan, and W. G. Helferich. 2014. 'Genistein exposure during the early postnatal period favors the development of obesity in female, but not male rats', *Toxicol Sci*, 138: 161-74.

Strom, B. L., R. Schinnar, E. E. Ziegler, K. T. Barnhart, M. D. Sammel, G. A. Macones, V. A. Stallings, J. M. Drulis, S. E. Nelson, and S. A. Hanson. 2001. 'Exposure to soy-based formula in infancy and endocrinological and reproductive outcomes in young adulthood', *JAMA*, 286: 807-14.

Sureda, A., A. Sanches Silva, D. I. Sanchez-Machado, J. Lopez-Cervantes, M. Daglia, S. F. Nabavi, and S. M. Nabavi. 2017. 'Hypotensive effects of genistein: From chemistry to medicine', *Chem Biol Interact*, 268: 37-46.

Szkudelska, K., and L. Nogowski. 2007. 'Genistein--a dietary compound inducing hormonal and metabolic changes', *J Steroid Biochem Mol Biol*, 105: 37-45.

Thomas, C., and J. A. Gustafsson. 2011. 'The different roles of ER subtypes in cancer biology and therapy', *Nat Rev Cancer*, 11: 597-608.

Todaka, E., K. Sakurai, H. Fukata, H. Miyagawa, M. Uzuki, M. Omori, H. Osada, Y. Ikezuki, O. Tsutsumi, T. Iguchi, and C. Mori. 2005. 'Fetal exposure to phytoestrogens--the difference in phytoestrogen status between mother and fetus', *Environ Res*, 99: 195-203.

Vahid, F., H. Zand, E. Nosrat-Mirshekarlou, R. Najafi, and A. Hekmatdoost. 2015. 'The role dietary of bioactive compounds on the regulation of histone acetylases and deacetylases: a review', *Gene*, 562: 8-15.

Viglietti-Panzica, C., E. Mura, and G. Panzica. 2007. 'Effects of early embryonic exposure to genistein on male copulatory behavior and vasotocin system of Japanese quail', *Horm Behav*, 51: 355-63.

Watanabe, S., S. Uesugi, and Y. Kikuchi. 2002. 'Isoflavones for prevention of cancer, cardiovascular diseases, gynecological problems and possible immune potentiation', *Biomed Pharmacother*, 56: 302-12.

Whitten, P. L., and F. Naftolin. 1992. 'Effects of a phytoestrogen diet on estrogen-dependent reproductive processes in immature female rats', *Steroids*, 57: 56-61.

Williams, C., K. Edvardsson, S. A. Lewandowski, A. Strom, and J. A. Gustafsson. 2008. 'A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells', *Oncogene*, 27: 1019-32.

Xu, Y., M. Gen, L. Lu, J. Fox, S. O. Weiss, R. D. Brown, D. Perlov, H. Ahmad, P. Zhu, C. Greyson, C. S. Long, and G. G. Schwartz. 2005. 'PPAR-gamma activation fails to provide myocardial protection in ischemia and reperfusion in pigs', *Am J Physiol Heart Circ Physiol*, 288: H1314-23.

Zhang, Y., and H. Chen. 2011. 'Genistein, an epigenome modifier during cancer prevention', *Epigenetics*, 6: 888-91.

Zhao, E., and Q. Mu. 2011. 'Phytoestrogen biological actions on Mammalian reproductive system and cancer growth', *Sci Pharm*, 79: 1-20.

Zhao, L., Z. Mao, and R. D. Brinton. 2009. 'A select combination of clinically relevant phytoestrogens enhances estrogen receptor beta-binding selectivity and neuroprotective activities in vitro and in vivo', *Endocrinology*, 150: 770-83.

Zoeller, R. T., T. R. Brown, L. L. Doan, A. C. Gore, N. E. Skakkebaek, A. M. Soto, T. J. Woodruff, and F. S. Vom Saal. 2012. 'Endocrine-disrupting chemicals and public health protection: a statement of principles from The Endocrine Society', *Endocrinology*, 153: 4097-110.

Microscopy technique: a new method of seeing the PVN

In all my experiments present in this thesis, I used a particular method to better visualize the kisspeptin fibers within the PVN. In fact, as possible to see in Fig. 19 at low magnification (20 x objective) the immunoreactivity for kisspeptin in a PVN of female mice is present but the image is not clear and it is impossible to discriminate the fibers to perform quantitative analysis.

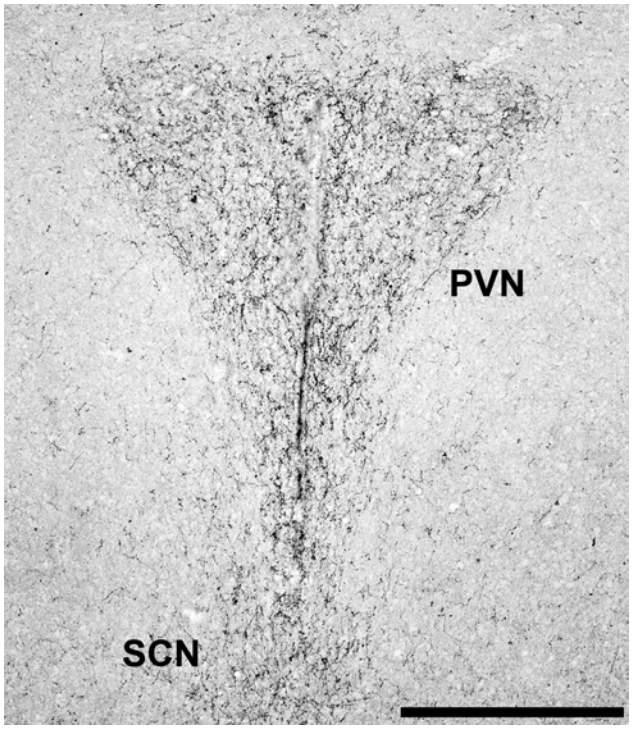

Figure 19: Kisspeptin innervation of PVN in female mice acquired with a NIKON Digital Sight DS-Fi1 video camera connected to a NIKON Eclipse 80i microscope (Nikon Italia S.p.S., Firenze, Italy) at 20x objective.

Scale bar =100µm

For this reason I choose a different approach. Images of the PVN were recorded at higher magnification (40 x objective) subdividing the nucleus into two regions, dorsal and ventral. Each single field was acquired with four different planes of focus (see Fig. 20). The images must be of the size. In this way it is possible to reconstruct a single image from the 8 original images.

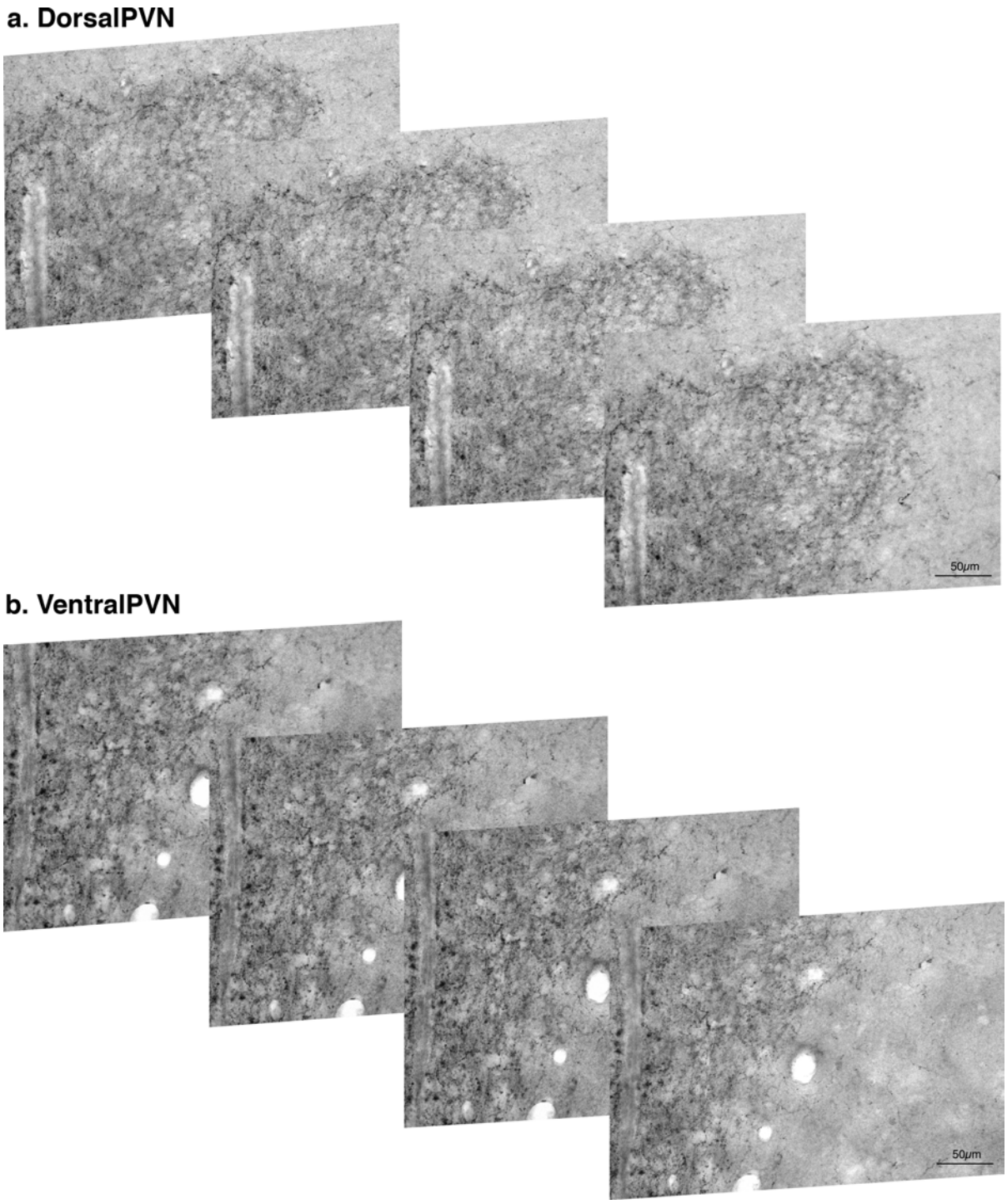

Figure 20: Kisspeptin innervation of PVN in female mice acquired with a NIKON Digital Sight DS-Fi1 video camera connected to a NIKON Eclipse 80i microscope (Nikon Italia S.p.S., Firenze, Italy) at 40x objective. In (a) the acquisitions of dorsal parte and in 4(b) the ventral part of PVN at four different focus.

To do this, I used the Z Project command of the software *Image J*. The Z Project is a method of analyzing a stack by applying different projection methods to the pixels within the stack. This process may be used to highlight specific data from the stack, as the kisspeptin fibers in this case. In fact, using 'Z project' it is possible to create a new stack consisting of the images currently displayed in separate windows. The command projects an image stack along the perpendicular axis to an image plane (the so-called "z" axis). With ImageJ is possible to use different ways of projection, in particular, I used the 'minimum intensity' that creates an output image each of whose pixels contains the minimum value over all images in the stack at the particular pixel location.
At this point I reconstituted an image of the whole nucleus by Photomerge of the software *Photoshop* that in automatic manner attempts produce the best photomerge. The resulting image of monolateral PVN was then analyzed to detect the fractional area (FA) covered by the kisspeptin fibers.

Kisspeptin immureactivity was measured by calculating in binary transformations (threshold function of the *Image J* software) the fractional area (percentage of pixels) covered by immunoreactive elements of the images. 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.
As possible to see in Fig. 21, using a multifocal image the resolution is better in terms of sharpness of the image, that turns into a cleaner signal to measure. In fact choosing a fixed value of threshold, I measured the FA in each images, and albeit with small variations, the final measurement result higher, because the fibers are more defined.

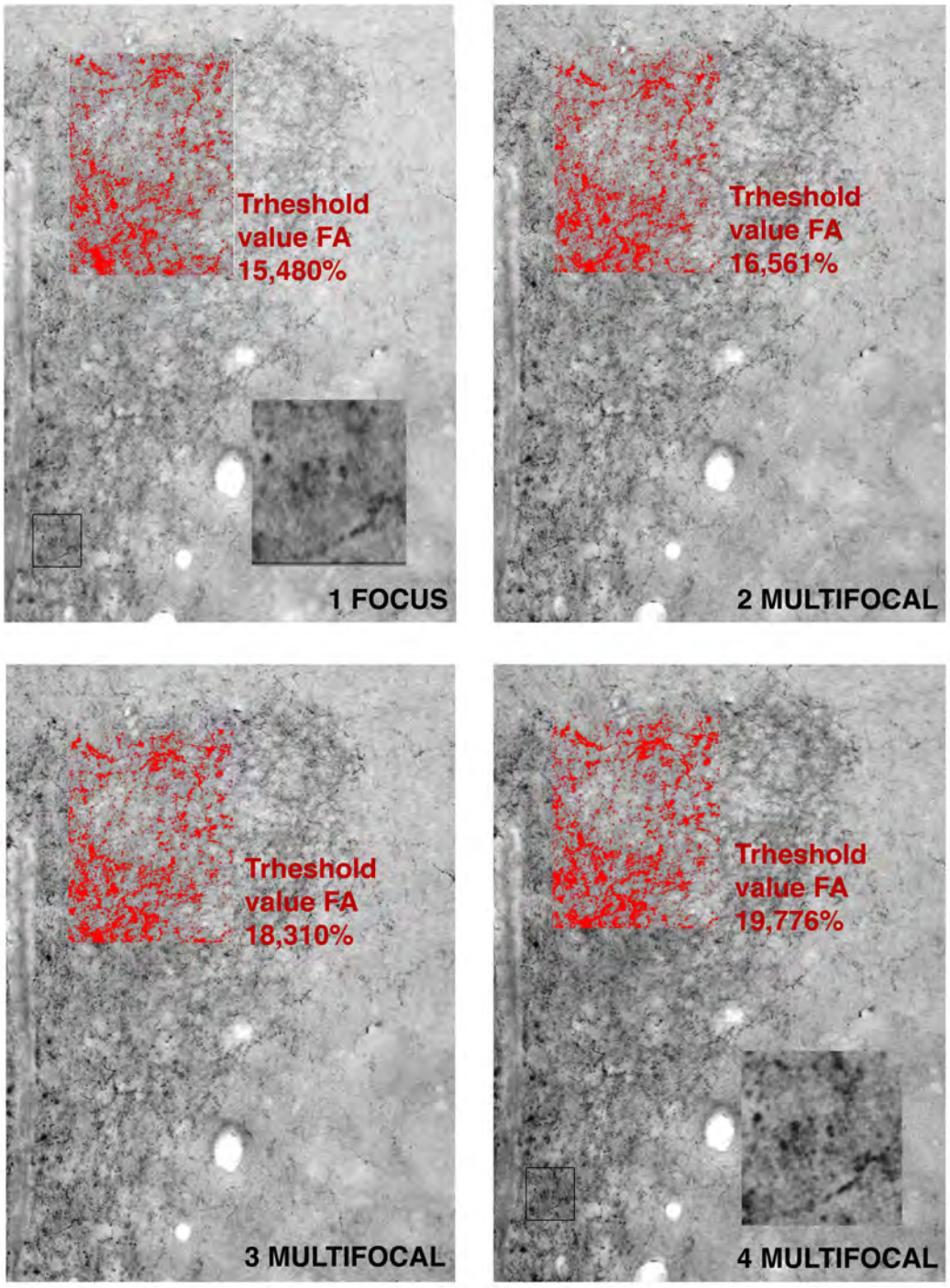

Figure 21: Kisspeptin innervation of monolateral PVN reconstituted by before Image with Z project, using 1, 2, 3 and 4 images at 4 different focus to create a single image of dorsal and ventral PVN and after with Photoshop to create a single image of whole PVN. Reported the different value of FA measured by ImageJ and a higher magnification of a small part of the nucleus.

CHAPTER 2

Aim of Thesis

As discussed in the introduction, the control of energy homeostasis in an organism is essential for the regulation of reproduction. Several investigations showed that the metabolic conditions and the amount of energy reserves of the organism are indispensable for the modulation of pubertal timing. In the last two decades, several studies underlined the role of the Kisspeptin system as an important regulator of the GnRH neurons for both puberty and fertility. Within the hypothalamus, the kisspeptin neurons are located, in a sexually dimorphic manner, in two different regions: the anterior periventricular region (RP3V) [that include the anterior ventral periventricular region (AVPV) and the periventricular nucleus (PeN)], and the arcuate nucleus (ARC) projecting mainly to gonadotropin-releasing hormone (GnRH) neurons and to different hypothalamic areas including the hypothalamic paraventricular nucleus (PVN) that seems to be one of the major targets of the system, as mentioned in several studies.

The PVN is one of the most important autonomic control centers in the brain. It is a very complex nucleus, organized into subdivisions associated with specific functions. The neurons located within the PVN play essential roles in controlling stress, growth, reproduction, immune and other more traditional autonomic functions (gastrointestinal, renal and cardiovascular), but also metabolism. Moreover, two different neuronal populations localized in the ARC (sensitive both to endocrine and to peripheral signals of energy balance, which regulate the central control of food-intake) project directly to the PVN. The orexigenic neurons are characterized by the co-expression of agouti-related peptide (AgRP) and neuropeptide Y (NPY) while the anorexigenic neurons co-express pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). In the PVN, fibers arising from these two neuronal populations modulate feeding behavior also through the action of several pituitary hormones, including corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH), both indirectly via effects on energy expenditure, and directly through the HPA-axis.

The PVN results, thus, strongly implicated in the feeding circuits, since it integrates inputs from these two ARC cell populations, but also in the reproductive circuit, because kisspeptin system, the most important regulator of GnRH, projects to this hypothalamic nucleus. However, unlike the ARC, in literature there aren't evidences that demonstrated a correlation between metabolic and reproductive regulation within PVN. For this reason I focused my attention to investigate the nature of this kisspeptin innervation in PVN and to understand if there was a strong neuroanatomical correlation between the reproductive and metabolic control through kisspeptin projections within PVN. For this reason in the first part of my thesis I wanted:

(a) to analyze the distribution of kisspeptin fibers within the PVN in adult male and female mice, investigating if also in this nucleus the innervation is dimorphic, and if there were variations of kisspeptin distribution during the estrous cycle.
(b) to study the development of the kisspeptin system of female mice, in AVPV, ARC, and in the PVN, concentrating the analysis in the peripubertal period, a temporal window interesting for pubertal organization.
(c) To investigate the role played of E_2 and P (alone or together) on the kisspeptin system of gonadectomized female mice.
(d) To analyze the possible correlation between kiss fiber and some of the cellular systems localized within the PVN, as Oxytocin neurons, catecholaminergic neurons and thyreotroping releasing-hormonne (TRH) producing neurons.

Kisspeptin system, being regulated by gonadal steroids, is a preferential target for Endocrine disrupting chemicals (EDCs), including Genistein (GEN). GEN is a phytoestrogen, and it is present in our diet: soybeans and soy-based foods are the major source of GEN. This phytoestrogen may interfere with the synthesis, metabolism and action of endogenous hormones; moreover it could have an obesogenic effect or may contribute to the development of metabolic syndrome. To evaluate if the relation between metabolism and reproduction could be altered by this phytoestrogen present in the food in the second part of this thesis I wanted:
(a) To study the effect of early postnatal administration of GEN, in an oral dose comparable to the exposure of babies fed with soy-based formulas on the development of kisspeptin system and also of two important hypothalamic systems (POMC and Orexin) implicated in the control of food intake and metabolic regulation.
(b) To measure different peripheral metabolic and reproductive parameters to explore the possible alterations caused by GEN treatment. Using this experimental design, I compared mice of both sexes in order to understand possible sexually differentiated *organizational effects* of GEN.
(c) To explore which type of estrogen receptor (ERα, ERβ or GPR30) mediates the GEN effects. For this, I evaluated if GEN, like estradiol, could affect processes' outgrowth (neuritogenesis) in hypothalamic neurons in culture, if this action could be sexually differentiated and what estrogen receptor (ER) is likely to be implicated.

CHAPTER 3

Experiment 1

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

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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 gonadotropin-releasing hormone (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: anterior ventral periventricular region; arcuate nucleus; diestrus; estrus; Kiss1; PVN.

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 gonadotropin-releasing hormone (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 (Gottsch et al. 2004; Smith et al. 2005a; Clarkson & Herbison, 2006), 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 (Lehman et al. 2010; Grachev et al. 2014). A second population of kisspeptin-positive cells is located in the rostral periventricular area of the third ventricle [RP3V, which includes the anterior ventral periventricular region (AVPV) and the periventricular nucleus (PeN)] of mice (Gottsch

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et al. 2004; Smith et al. 2005b; Clarkson & Herbison, 2006), 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,b). Several studies performed in RP3V of adult rodents showed a peculiar dimorphism, with females displaying the highest kisspeptin expression (Clarkson & Herbison, 2006; Kauffman, 2009). Recently, a few studies showed that rodent's kisspeptin system is also dimorphic within the 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 & 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 & 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 the PVN contains magnocellular neurons chiefly projecting to the posterior pituitary [where they release oxytocin (OT) and arginine vasopressin (AVP) into the blood], the medial part of the 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), AVP (Caldwell et al. 2008) and somatostatin (Tan et al. 2013)].

In the present study, we describe for the first time the sexual dimorphism of kisspeptin-immunoreactive (kiss-ir) 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 three males or three females in 45 × 25 × 15 cm polypropylene mouse cages at 22 ± 2 °C, under a 12 : 12 h light : dark cycle (light on at 08:00 h). 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): six female in diestrus and six male mice at postnatal day 60 (PND60);
experiment 2 (estrous cycle observation): 10 adult female mice (PND90), four mice in estrus and six mice in diestrus phase.
experiment 3 (interaction between kisspeptin and different neuronal populations of PVN): four adult female mice (PND90) in estrus phase.

Animal care and handling were according to the European Union Council Directive of 22 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-day estrous cycles, female mice were killed in diestrus for experiment 1; instead, for experiments 2 and 3, at PND90, a group of female mice ($n = 4$ for experiment 2; and $n = 4$ for experiment 3) was killed in estrus, and the others ($n = 6$ for experiment 2) in diestrus.

Male and female mice were deeply anesthetized with a mixture of ketamine–xylazine (respectively, 100 mg mL^{-1} and 20 mg mL^{-1}) 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 & 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 experiments 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 biotinylated goat anti-rabbit IgG (Vector Laboratories) 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). The peroxidase activity was visualized with a solution containing 0.400 mg mL^{-1} of 3,3′-diamino-benzidine (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 (Franceschini et al. 2006; Clarkson et al. 2009).

The AC#566 antibody was raised against the 10 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 bovine serum albumin (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 (Franceschini et al. 2006; Clarkson et al. 2009).

We performed the following additional 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.

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 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) and 1% BSA (Sigma–Aldrich). 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).

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.

Quantitative analysis (experiments 1 and 2)

Selected standardized sections of comparable levels covering the ARC (bregma −1.46 to 1.70 mm), the AVPV (bregma 0.50–0.02 mm), the PeN (bregma 0.14–0.22 mm) and the PVN (bregma −0.58 to 0.94 mm) were chosen (Paxinos & 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 10 × (AVPV and PeN) or a 20 × (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 40 × (PVN and ARC) or a 20 × (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 the 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

Table 1 Primary and secondary antibodies used in the double-label immunofluorescence assays.

Primary Abs					
Kisspeptin	AC053	*A. Caraty*	*Sheep, pc*	1 : 2000	Poling et al. (2013)
NkB	T4450	*Peninsula*	*Rabbit, pc*	1 : 2000	Taziaux et al. (2012)
AVP	64717	*ICN*	*Rabbit, pc*	1 : 8000	Ferris et al. (1997)
nNOS	24287	*DiaSorin*	*Rabbit, pc*	1 : 3000	Gillespie et al. (2005)
OT	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	

AVP, arginine vasopressin; mc, monoclonal antibody; NkB, neurokinin B; nNOS, neural nitric oxide synthase; OT, oxytocin; pc, polyclonal antibody; TH, tyrosine hydroxylase.

by immunoreactive elements of the images (as previously performed in our laboratory; Viglietti-Panzica et al. 1994; Plumari et al. 2002; Pierman et al. 2008). 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 (± SEM) values. The statistical analysis was performed, using the SPSS 22.0 statistic software (SPSS, Chicago, IL, USA), and was undertaken using ANOVA with Student's *t*-test to analyze 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 kiss-ir 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 RP3V; Herbison, 2008), and ARC nuclei show the larger clusters of kisspeptin-expressing cell bodies in females compared with 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 it difficult to precisely discern the limits of AVPV and PeN nuclei. 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 bed nucleus of the stria terminalis; on the contrary, only few kisspeptin fibers were present within the medial septum.

A second large population of kisspeptin-positive cell bodies was observed caudally, within the ARC. The kiss-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 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 ($P < 0.001$; Figs 2A,B and 3), but also for the ARC ($P < 0.05$; Figs 2E,F and 3), and for the amount of immunoreactive fibers in the PVN ($P < 0.001$; Figs 2C,D and 3).

Experiment 2: estrous cycle observation

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

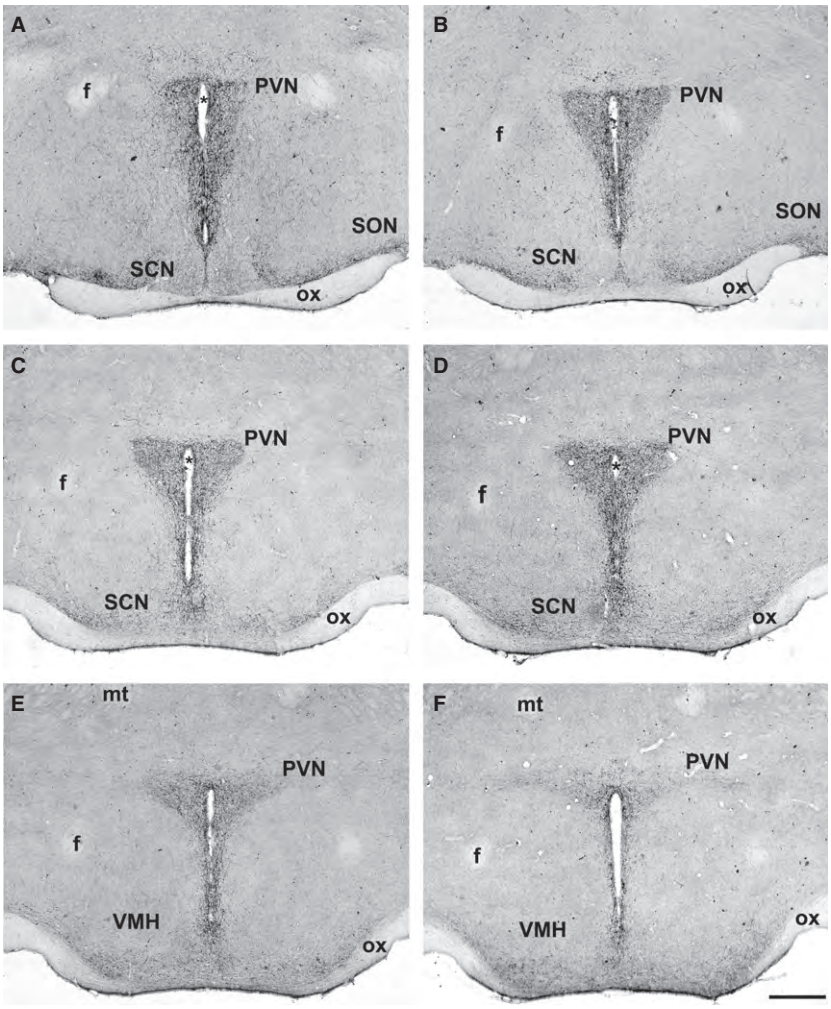

Fig. 1 Distribution of kisspeptin-immunoreactive (kiss-ir) fibers within the paraventricular nucleus (PVN) of CD1 (diestrus) female mice, from rostral to caudal sections. Kisspeptin fibers outline the boundaries of the PVN and run along the wall of the third ventricle towards the optic chiasm (ox). A moderate innervation is present also within the supraoptic nucleus (SON), while the suprachiasmatic nucleus (SCN) is almost totally empty of immunoreactivity, as well as the ventromedial hypothalamic nucleus (VMH). f, fornix; *, third ventricle; mt, mammillothalamic tract. Scale bar: 100 μm.

was similar to that described in experiments 1 and 2. In fact, within the PVN the immunofluorescence appeared not homogeneous with a higher concentration of kiss-ir fibers in the medial compared with the lateral PVN (Figs 5A and 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-ir within the 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 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 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 OT cells were observed both in the lateral and medial PVN, including the ventro-medial part (Fig. 6C). Interactions with kisspeptin fibers were possible both in the medial and lateral part. The TH-ir cell bodies were also scattered within the PVN; however, they were particularly clustered in the 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 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

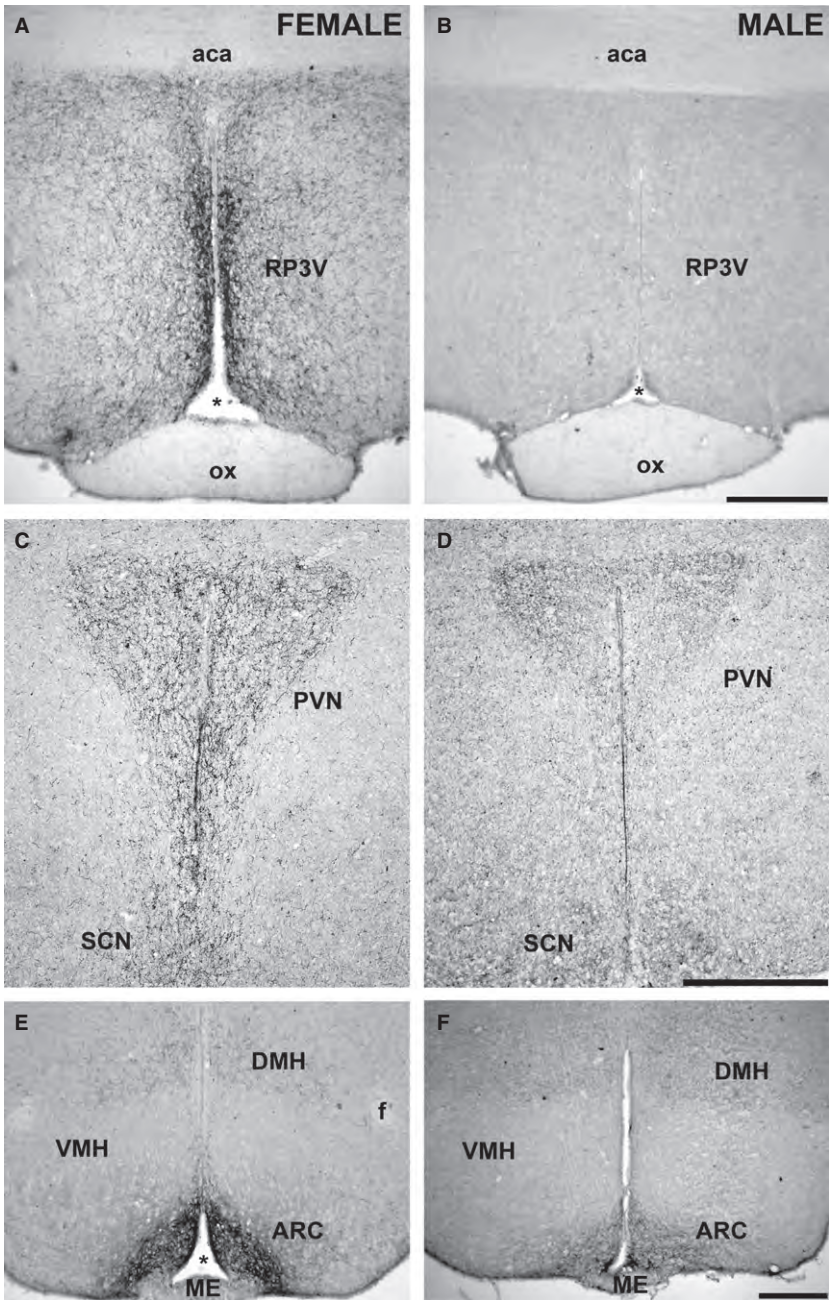

Fig. 2 Dimorphic kisspeptin system in the hypothalamic nuclei. Comparison of the distribution of kisspeptin fibers in diestrus female (left panels) and male (right panels) CD1 mice. The expression of kisspeptin is higher in females than in males. (A, B) Rostral periventricular area of the third ventricle (RP3V). (C, D) Paraventricular nucleus (PVN). (E, F) Arcuate hypothalamic nucleus (ARC). ox, optic chiasm; aca, anterior commissure; f, fornix; *, third ventricle; SCN, suprachiasmatic nucleus; VMH, ventromedial hypothalamic nucleus; DMH, dorsomedial hypothalamic nucleus; ME, median eminence. Scale bar: 100 μm.

as well as in the ARC. Previous studies (Clarkson & 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 & 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), kiss-ir of female CD1 mice changes during the estrous cycle in a different way in the RP3V and the ARC, showing the 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 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 (Roseweir & Millar, 2009; d'Anglemont de

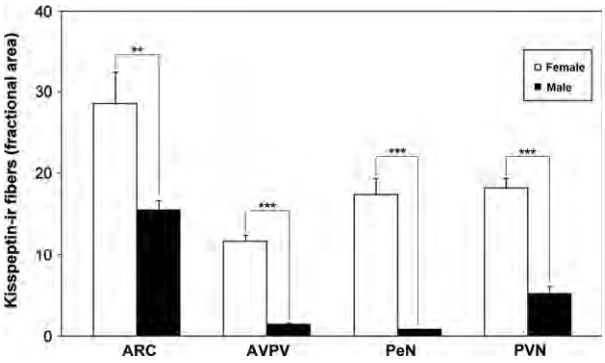

Fig. 3 Quantitative study of kisspeptin system in the hypothalamic nuclei. Histograms representing the fractional area covered by kisspeptin-immunoreactive (kiss-ir) structures (mean ± SEM) in the arcuate hypothalamic nucleus (ARC), anterior ventral periventricular nucleus (AVPV), periventricular nucleus (PeN) and paraventricular nucleus (PVN) of male (black bars) and female (white bars) CD1 mice. Males showed a lower immunoreactivity in comparison with the female group. $^{**}P < 0.01$, $^{***}P < 0.001$ different from males ($P < 0.05$; Student's *t*-test).

Tassigny & Colledge, 2010; Tsutsui et al. 2010; Navarro & Tena-Sempere, 2011). 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 (Lehman et al. 2010; Grachev et al. 2014); moreover, a higher co-expression of kisspeptin and NkB within the 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 kiss-ir in the PVN. This confirms the hypothesis that the kisspeptin fibers observed in the 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

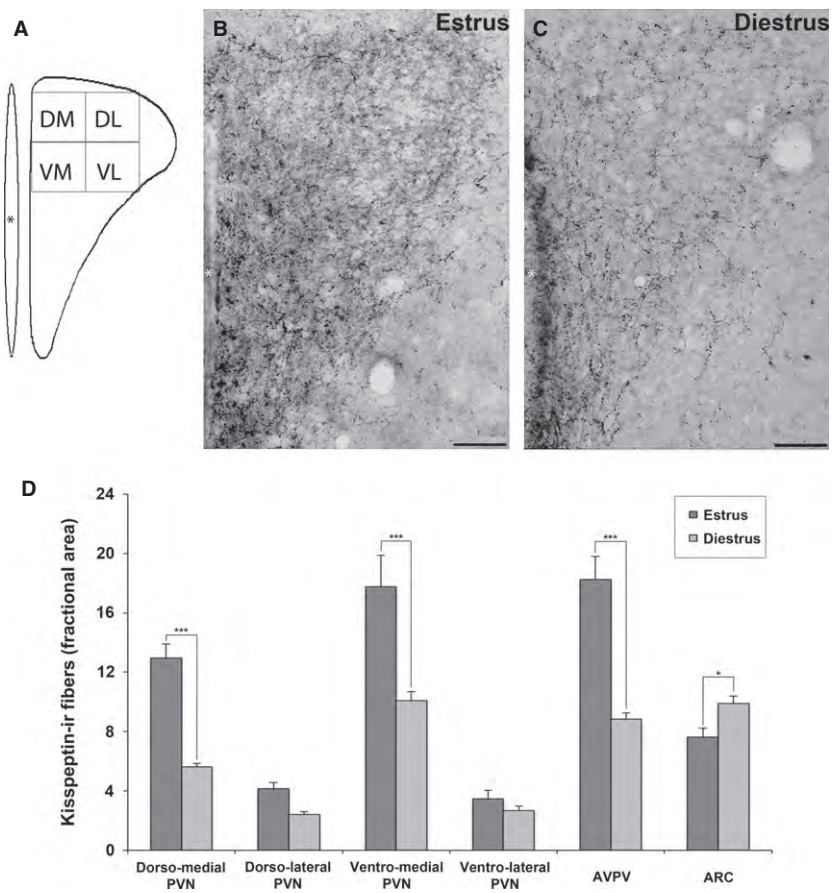

Fig. 4 Effect of estrous cycle on the kisspeptin immunoreactivity (kiss-ir) in adult mice. (A) The representative subdivision of the paraventricular nucleus (PVN) in four quadrants (DM, dorso-medial; DL, dorso-lateral; VM, ventro-medial; VL, ventro-lateral). (B, C) The comparison of kiss-ir fibers within the PVN of female CD1 mice in estrus (B) and diestrus (C) phases, respectively. (D) Histograms representing the fractional area covered by kiss-ir structures (mean ± SEM) in the PVN (DM, DL, VM, VL), anterior ventral periventricular nucleus (AVPV) and arcuate hypothalamic nucleus (ARC) of female adult mice in estrus (dark gray) and diestrus (light gray) phases. $^{*}P < 0.05$, $^{***}P < 0.001$ different from diestrus ($P < 0.05$, Bonferroni test). Scale bar: 50 μm.

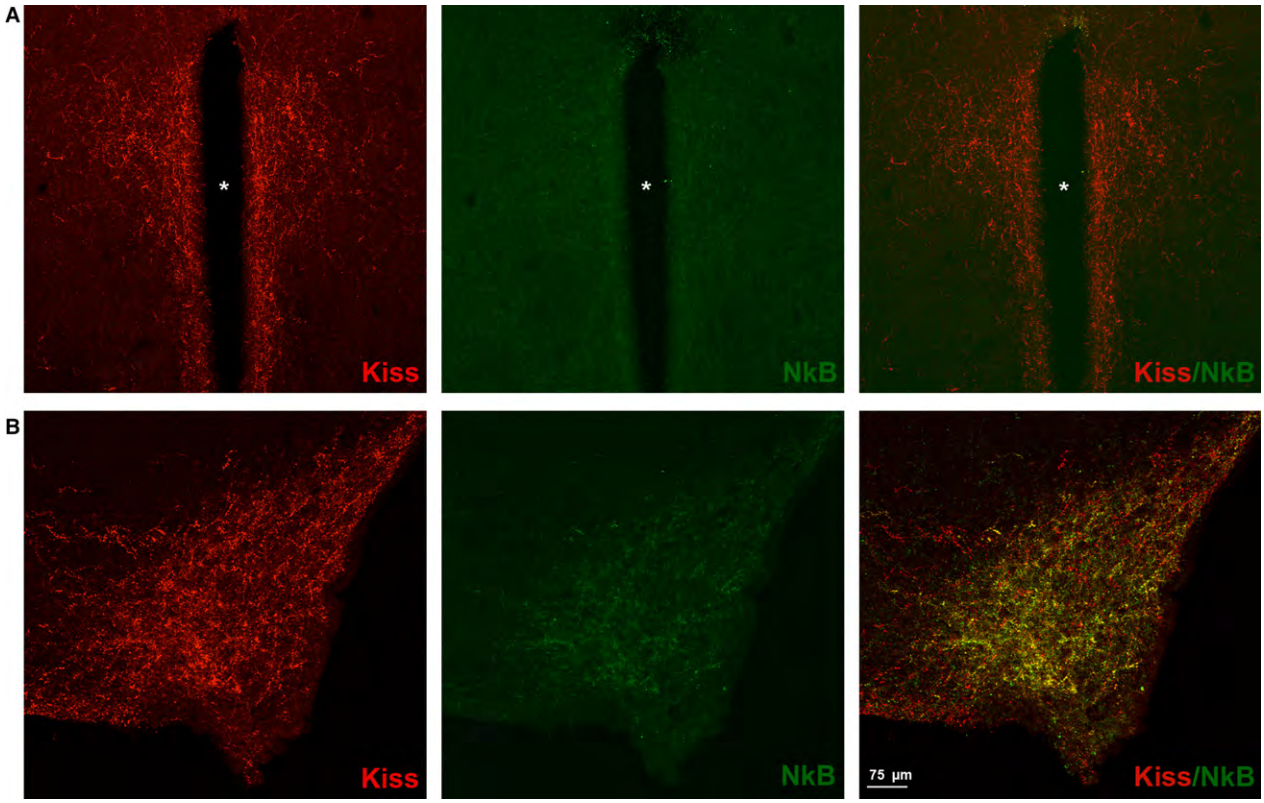

Fig. 5 Kisspeptin (Kiss) and neurokinin B (NkB) expression within the paraventricular nucleus (PVN) and arcuate hypothalamic nucleus (ARC) in adult CD1 female mice. Kiss (red) and NkB (green) immunoreactivity in a coronal section of the PVN (A) and ARC (B) of the adult CD1 female mice in estrus phase. A dense Kiss expression is evident in both nuclei, with respect to the NkB signal: is clearly present only in ARC; in PVN the NkB signal is very low. The merge indicates that the Kiss and Nkb immunoreactivity co-localize with the ARC nucleus.

(Swanson & Sawchenko, 1980; Maniam & Morris, 2012; Bosch, 2013; Kovács, 2013; Handa & 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 lateral PVN. While the lateral part of the PVN contains magnocellular neurons chiefly projecting to the posterior pituitary (where they release OT and AVP into the blood), the medial part of the 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. CRH; Wang et al. 2011; TRH; Kadar et al. 2010; TH; Ruggiero et al. 1984; nNOS; Gotti et al. 2004, 2005; AVP; 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 OT neurons of the medial part of the PVN (Higo et al. 2016). Moreover, in AVPV more than half of kiss-ir neurons express also TH-ir (Clarkson & 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. To 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

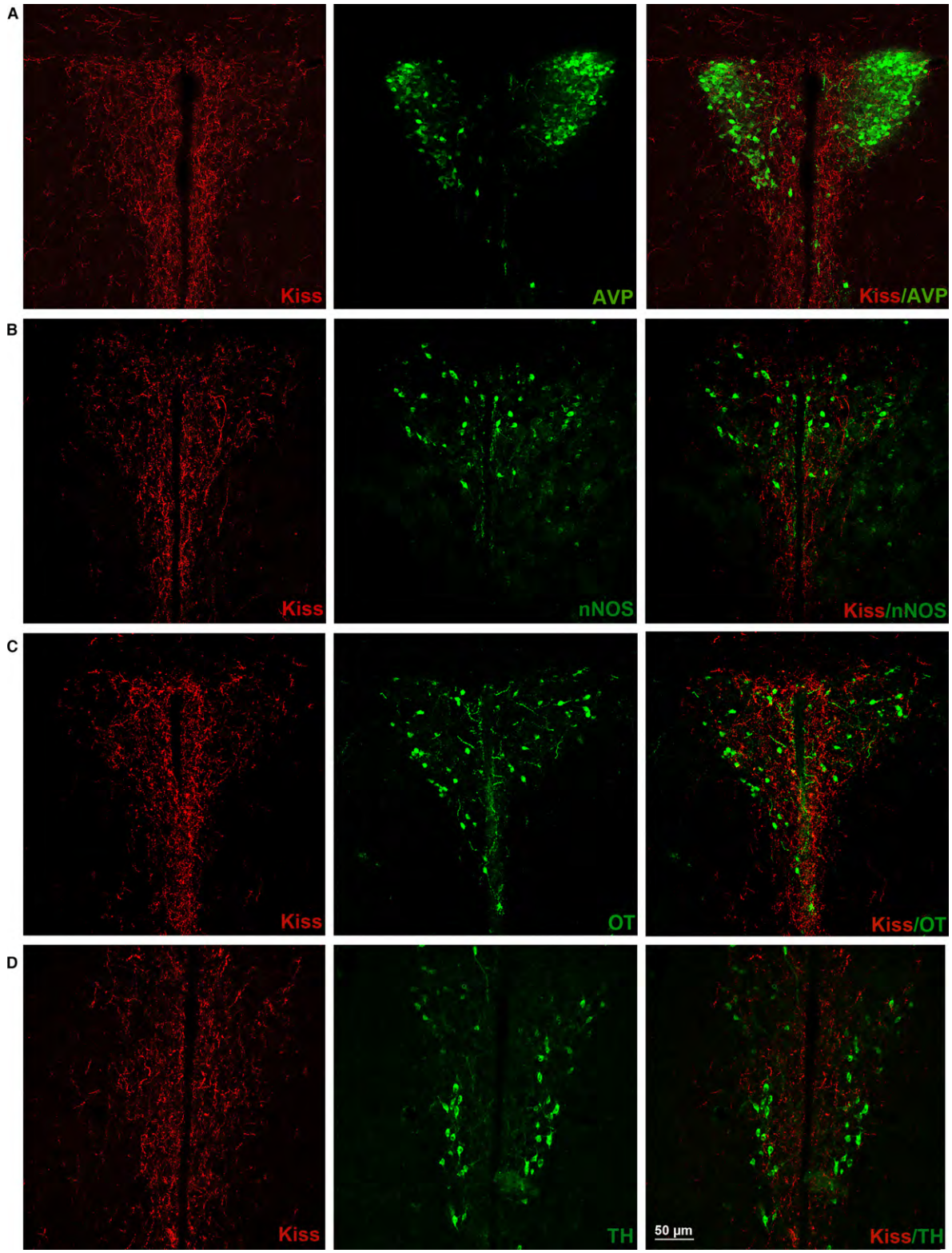

Fig. 6 Kisspeptin (Kiss) and paraventricular nucleus (PVN) cellular populations [arginine vasopressin (AVP), neural nitric oxide synthase (nNOS), oxytocin (OT), tyrosine hydroxylase (TH)]. Coronal section of the adult CD1 female mice PVN in estrus phase. It is possible to observe the relations with Kiss (red) and different PVN neuronal populations: (A) AVP (green); (B) nNOS (green); (C) OT (green); (D) TH (green). Note that the total AVP and the majority of nNOS neuronal cell bodies were distributed in lateral PVN, where the concentration of Kiss fibers was lower; in the medial PVN instead, a conspicuous number of OT and TH cell bodies were present.

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 GnRH neurons are located, 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.

Acknowledgements

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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.

References

Adachi S, Yamada S, Takatsu Y, et al. (2007) Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. *J Reprod Dev* **53**, 367–378.

d'Anglemont de Tassigny X, Colledge WH (2010) The role of kisspeptin signaling in reproduction. *Physiology (Bethesda)* **25**, 207–217.

Bean JC, Lin TW, Sathyamurthy A, et al. (2014) Genetic labeling reveals novel cellular targets of schizophrenia susceptibility gene: distribution of GABA and non-GABA ErbB4-positive cells in adult mouse brain. *J Neurosci* **34**, 13549–13566.

Becker JB, Arnold AP, Berkley KJ, et al. (2005) Strategies and methods for research on sex differences in brain and behavior. *Endocrinology* **146**, 1650–1673.

Bosch OJ (2013) Maternal aggression in rodents: brain oxytocin and vasopressin mediate pup defence. *Philos Trans R Soc Lond B Biol Sci* **368**, 20130085.

Brailoiu GC, Dun SL, Ohsawa M, et al. (2005) KiSS-1 expression and metastin-like immunoreactivity in the rat brain. *J Comp Neurol* **481**, 314–329.

Caldwell HK, Lee HJ, Macbeth AH, et al. (2008) Vasopressin: behavioral roles of an "original" neuropeptide. *Prog Neurobiol* **84**, 1–24.

Castellano J, Bentsen A, Mikkelsen J, et al. (2010) Kisspeptins: bridging energy homeostasis and reproduction. *Brain Res* **1364**, 129–138.

Clarkson J, Herbison AE (2006) Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology* **147**, 5817–5825.

Clarkson J, Herbison AE (2011) Dual phenotype kisspeptin-dopamine neurones of the rostral periventricular area of the third ventricle project to gonadotrophin-releasing hormone neurones. *J Neuroendocrinol* **23**, 293–301.

Clarkson J, d'Anglemont de Tassigny X, Colledge WH, et al. (2009) Distribution of kisspeptin neurones in the adult female mouse brain. *J Neuroendocrinol* **21**, 673–682.

Daadi MM, Weiss S (1999) Generation of tyrosine hydroxylase-producing neurons from precursors of the embryonic and adult forebrain. *J Neurosci* **19**, 4484–4497.

Decourt C, Tillet Y, Caraty A, et al. (2008) Kisspeptin immunoreactive neurons in the equine hypothalamus: interactions with GnRH neuronal system. *J Chem Neuroanat* **36**, 131–137.

Ferguson AV, Latchford KJ, Samson WK (2008) The paraventricular nucleus of the hypothalamus – a potential target for integrative treatment of autonomic dysfunction. *Expert Opin Ther Targets* **12**, 717–727.

Ferris CF, Melloni RH Jr, Koppel G, et al. (1997) Vasopressin/serotonin interactions in the anterior hypothalamus control aggressive behavior in golden hamsters. *J Neurosci* **17**, 4331–4340.

Franceschini I, Lomet D, Cateau M, et al. (2006) Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha. *Neurosci Lett* **401**, 225–230.

Franceschini I, Yeo SH, Beltramo M, et al. (2013) Immunohistochemical evidence for the presence of various kisspeptin isoforms in the mammalian brain. *J Neuroendocrinol* **25**, 839–851.

Gillespie JI, Markerink-van Ittersum M, de Vente J (2005) Expression of neuronal nitric oxide synthase (nNOS) and nitric-oxide-induced changes in cGMP in the urothelial layer of the guinea pig bladder. *Cell Tissue Res* **321**, 341–351.

Goldman JM, Murr AS, Cooper RL (2007) The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Res B Dev Reprod Toxicol* **80**, 84–97.

Gotti S, Chiavegatto S, Sica M, et al. (2004) Alteration of NO-producing system in the basal forebrain and hypothalamus of Ts65Dn mice: an immunohistochemical and histochemical study of a murine model for Down syndrome. *Neurobiol Dis* **16**, 563–571.

Gotti S, Sica M, Viglietti Panzica C, et al. (2005) Distribution of nitric oxide sythase immunoreactivity in the mouse brain. *Microsc Res Tech* **68**, 13–35.

Gottsch ML, Cunningham MJ, Smith JT, et al. (2004) A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology* **145**, 4073–4077.

Grachev P, Li XF, Hu MH, et al. (2014) Neurokinin B signaling in the female rat: a novel link between stress and reproduction. *Endocrinology* **155**, 2589–2601.

Greives TJ, Mason AO, Scotti MA, et al. (2007) Environmental control of kisspeptin: implications for seasonal reproduction. *Endocrinology* **148**, 1158–1166.

Han SK, Gottsch ML, Lee KJ, et al. (2005) Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. *J Neurosci* **25**, 11 349–11 356.

Handa R, Weiser M (2014) Gonadal steroid hormones and the hypothalamo-pituitary-adrenal axis. *Front Neuroendocrinol* **35**, 197–220.

Herbison AE (2008) Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: the case for the rostral periventricular area of the third ventricle (RP3V). *Brain Res Rev* **57**, 277–287.

Herbison AE, de Tassigny X, Doran J, et al. (2010) Distribution and postnatal development of Gpr54 gene expression in mouse brain and gonadotropin-releasing hormone neurons. *Endocrinology* **151**, 312–321.

Higo S, Honda S, Iijima N, et al. (2016) Mapping of kisspeptin receptor mRNA in the whole rat brain and its co-localization with oxytocin in the paraventricular nucleus. *J Neuroendocrinol* **28**, 10.1111/jne.12356.

Irwig MS, Fraley GS, Smith JT, et al. (2004) Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. *Neuroendocrinology* **80**, 264–272.

Kadar A, Sanchez E, Wittmann G, et al. (2010) Distribution of hypophysiotropic thyrotropin-releasing hormone (TRH)-synthesizing neurons in the hypothalamic paraventricular nucleus of the mouse. *J Comp Neurol* **518**, 3948–3961.

Kauffman AS (2009) Sexual differentiation and the Kiss1 system: hormonal and developmental considerations. *Peptides* **30**, 83–93.

Kauffman AS, Gottsch ML, Roa J, et al. (2007) Sexual differentiation of Kiss1 gene expression in the brain of the rat. *Endocrinology* **148**, 1774–1783.

Knoll JG, Clay CM, Bouma GJ, et al. (2013) Developmental profile and sexually dimorphic expression of kiss1 and kiss1r in the fetal mouse brain. *Front Endocrinol (Lausanne)* **4**, 140.

Kotani M, Detheux M, Vandenbogaerde A, et al. (2001) The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem* **276**, 34 631–34 636.

Kovács K (2013) CRH: the link between hormonal-, metabolic- and behavioral responses to stress. *J Chem Neuroanat* **54**, 25–33.

Kumar D, Candlish M, Periasamy V, et al. (2015) Specialized subpopulations of kisspeptin neurons communicate with GnRH neurons in female mice. *Endocrinology* **156**, 32–38.

Lee DK, Nguyen T, O'Neill GP, et al. (1999) Discovery of a receptor related to the galanin receptors. *FEBS Lett* **446**, 103–107.

Lehman MN, Coolen LM, Goodman RL (2010) Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology* **151**, 3479–3489.

Lehman MN, Hileman SM, Goodman RL (2013) Neuroanatomy of the kisspeptin signaling system in mammals: comparative and developmental aspects. *Adv Exp Med Biol* **784**, 27–62.

Liu Q, Guan XM, Martin WJ, et al. (2001) Identification and characterization of novel mammalian neuropeptide FF-like peptides that attenuate morphine-induced antinociception. *J Biol Chem* **276**, 36 961–36 969.

Maniam J, Morris MJ (2012) The link between stress and feeding behaviour. *Neuropharmacology* **63**, 97–110.

McLean AC, Valenzuela N, Fai S, et al. (2012) Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *J Vis Exp* **4389**.

Navarro VM, Tena-Sempere M (2011) Kisspeptins and the neuroendocrine control of reproduction. *Front Biosci (Schol Ed)* **3**, 267–275.

Navarro VM, Gottsch ML, Chavkin C, et al. (2009) Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J Neurosci* **29**, 11 859–11 866.

Oishi S, Misu R, Tomita K, et al. (2011) Activation of neuropeptide FF receptors by kisspeptin receptor ligands. *ACS Med Chem Lett* **2**, 53–57.

Overgaard A, Tena-Sempere M, Franceschini I, et al. (2013) Comparative analysis of kisspeptin-immunoreactivity reveals genuine differences in the hypothalamic Kiss1 systems between rats and mice. *Peptides* **45**, 85–90.

Paxinos G, Franklin KBJ (2001) *The Mouse Brain in Stereotaxic Coordinates*. San Diego: Academic Press.

Pierman S, Sica M, Allieri F, et al. (2008) Activational effects of estradiol and dihydrotestosterone on social recognition and the arginine-vasopressin immunoreactive system in male mice lacking a functional aromatase gene. *Horm Behav* **54**, 98–106.

Pineda R, Sabatier N, Ludwig M, et al. (2016) A direct neurokinin B projection from the arcuate nucleus regulates magnocellular vasopressin cells of the supraoptic nucleus. *J Neuroendocrinol* **28**, 10.1111/jne.12342.

Pinilla L, Aguilar E, Dieguez C, et al. (2012) Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiol Rev* **92**, 1235–1316.

Plumari L, Viglietti Panzica C, Allieri F, et al. (2002) Changes in the arginine-vasopressin immunoreactive systems in male mice lacking a functional aromatase gene. *J Neuroendocrinol* **14**, 971–978.

Poling MC, Quennell JH, Anderson GM, et al. (2013) Kisspeptin neurones do not directly signal to RFRP-3 neurones but RFRP-3 may directly modulate a subset of hypothalamic kisspeptin cells in mice. *J Neuroendocrinol* **25**, 876–886.

Pyner S (2014) The paraventricular nucleus and heart failure. *Exp Physiol* **99**, 332–339.

Rao YS, Mott NN, Pak TR (2011) Effects of kisspeptin on parameters of the HPA axis. *Endocrine* **39**, 220–228.

Rometo AM, Krajewski SJ, Voytko ML, et al. (2007) Hypertrophy and increased kisspeptin gene expression in the hypothalamic infundibular nucleus of postmenopausal women and ovariectomized monkeys. *J Clin Endocrinol Metab* **92**, 2744–2750.

Roseweir AK, Millar RP (2009) The role of kisspeptin in the control of gonadotrophin secretion. *Hum Reprod Update* **15**, 203–212.

de Roux N, Genin E, Carel JC, et al. (2003) Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci USA* **100**, 10 972–10 976.

Ruggiero DA, Baker H, Joh TH, et al. (1984) Distribution of catecholamine neurons in the hypothalamus and preoptic region of mouse. *J Comp Neurol* **223**, 556–582.

Seminara SB, Messager S, Chatzidaki EE, et al. (2003) The GPR54 gene as a regulator of puberty. *N Engl J Med* **349**, 1614–1627.

Shahab M, Mastronardi C, Seminara SB, et al. (2005) Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. *Proc Natl Acad Sci USA* **102**, 2129–2134.

Sladek CD, Michelini LC, Stachenfeld NS, et al. (2015) Endocrine-autonomic linkages. *Comp Physiol* **5**, 1281–1323.

Smith JT, Cunningham MJ, Rissman EF, et al. (2005a) Regulation of KiSS-1 gene expression in the brain of the female mouse. *Endocrinology* **146**, 3686–3692.

Smith JT, Dungan HM, Stoll EA, et al. (2005b) Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology* **146**, 2976–2984.

Smith JT, Popa SM, Clifton DK, et al. (2006) Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. *J Neurosci* **26**, 6687–6694.

Smith JT, Clay CM, Caraty A, et al. (2007) KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season. *Endocrinology* **148**, 1150–1157.

Smith JT, Shahab M, Pereira A, et al. (2010) Hypothalamic expression of KISS1 and gonadotropin inhibitory hormone genes during the menstrual cycle of a non-human primate. *Biol Reprod* **83**, 568–577.

Stengel A, Wang L, Goebel-Stengel M, et al. (2011) Centrally injected kisspeptin reduces food intake by increasing meal intervals in mice. *NeuroReport* **22**, 253–257.

Swanson LW, Sawchenko PE (1980) Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology* **31**, 410–417.

van Swieten MM, Pandit R, Adan RA, et al. (2014) The neuroanatomical function of leptin in the hypothalamus. *J Chem Neuroanat* **61–62**, 207–220.

Tan HY, Huang L, Simmons D, et al. (2013) Hypothalamic distribution of somatostatin mRNA expressing neurones relative to pubertal and adult changes in pulsatile growth hormone secretion in mice. *J Neuroendocrinol* **25**, 910–919.

Taziaux M, Swaab DF, Bakker J (2012) Sex differences in the neurokinin B system in the human infundibular nucleus. *J Clin Endocrinol Metab* **97**, E2210–E2220.

Tena-Sempere M (2006) KiSS-1 and reproduction: focus on its role in the metabolic regulation of fertility. *Neuroendocrinology* **83**, 275–281.

Tsutsui K, Bentley GE, Kriegsfeld LJ, et al. (2010) Discovery and evolutionary history of gonadotrophin-inhibitory hormone and kisspeptin: new key neuropeptides controlling reproduction. *J Neuroendocrinol* **22**, 716–727.

Viglietti-Panzica C, Aste N, Balthazart J, et al. (1994) Vasotocinergic innervation of sexually dimorphic medial preoptic nucleus of the male Japanese quail: influence of testosterone. *Brain Res* **657**, 171–184.

Wang L, Goebel-Stengel M, Stengel A, et al. (2011) Comparison of CRF-immunoreactive neurons distribution in mouse and rat brains and selective induction of Fos in rat hypothalamic CRF neurons by abdominal surgery. *Brain Res* **1415**, 34–46.

Watson RE, Wiegand SJ, Clough RW, et al. (1986) Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. *Peptides* **7**, 155–159.

Yeo SH, Herbison AE (2011) Projections of arcuate nucleus and rostral periventricular kisspeptin neurons in the adult female mouse brain. *Endocrinology* **152**, 2387–2399.

Yip SH, Boehm U, Herbison AE, et al. (2015) Conditional viral tract tracing delineates the projections of the distinct kisspeptin neuron populations to gonadotropin-releasing hormone (GnRH) neurons in the mouse. *Endocrinology* **156**, 2582–2594.

CHAPTER 4

Experiment 2

'Distribution of kisspeptin immunoreactivity in the hypothalamic paraventricular nucleus in female mice: post-natal developmental study'

Distribution of kisspeptin immunoreactivity in the hypothalamic paraventricular nucleus in female mice: post-natal developmental study

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Abstract

Introduction: Kisspeptin is a hypothalamic peptide regulating the gonadotrophin releasing hormone system and its signaling pathway is a requisite for the onset of mammalian puberty. Immunohistochemical and *in situ* hybridization studies demonstrated kisspeptin positive neurons in two main groups located in the anteroventral periventricular region (AVPV) and in the arcuate nucleus (ARC). Both cell groups show a strong sex dimorphism, as as the innervation of the paraventricular nucleus (PVN), one of the major targets of the system. While the kisspeptin system development is well known for the AVPV, scarce information are for the development of the ARC and nothing is known for the PVN.

Materials and Methods: In the present study we investigated by immunohistochemistry the distribution of the kisspeptin innervation within the PVN and the variations within the AVPV and the ARC during the postnatal development (from PND12 to PND30) of CD1 female mice.

Results and Conclusion: In mice, the time window of puberty occurs at PND26-30, but it may occur also before. In our study, all the analyzed nuclei (AVPV, ARC and PVN) show an increase of the kisspeptin immunoreactivity before puberty age, at PND18. In AVPV and PVN we observed a small increase up to PND30, while in ARC, after the peak at PND18, the kisspeptin immunoreactivity decreased significantly. This longitudinal study demonstrates that the development of the kisspeptin system within the hypothalamus show a similar increasing trend in both AVPV and ARC, as well in the PVN, until the time of puberty. Differences among AVPV (and PVN) and ARC were observed in the late postnatal development.

Introduction

From a physiological point of view, the kisspeptin (protein encoded by *Kiss1* gene) has been identified as the most potent known regulator of GnRH (Irwig et al. 2004); moreover, it is implicated in the timing of puberty onset (Han et al. 2005). The neuroanatomical locations of kisspeptin-synthesizing cell populations is similar in mammalian species. In mice a large population of kisspeptin neurons has been identified in the Arcuate hypotalamic nucleus (ARC) and a second population of kisspeptin-positive cells has been detected in the anteroventral periventricular region, AVPV (Smith et al. 2005). Kisspeptin fibers from these two groups of cell bodies reach different hypothalamic areas, including the paraventricular nucleus (PVN), the major autonomic output area of the hypothalamus and critical for energy homeostasis (Sutton et al. 2014).

The hypothalamic kisspeptin system undergoes a complex neuroanatomical maturation and functional activation during the course of puberty (Roa, Navarro, and Tena-Sempere 2011). *In situ* hybridization and immunohistochemistry (IHC) studies have demonstrated a significant modification of kisspeptin during pubertal development, showing that in mice AVPV *Kiss1* mRNA and kisspeptin protein are undetectable prior to post natal day (PND) 10 and 15, respectively, and then they increase from PND 15 to adulthood (Clarkson et al. 2009; Clarkson and Herbison 2006). Unlike the AVPV, ARC *Kiss1* expression is readily detectable prenatally and at birth in rodents, and continues to be express throughout postnatal development (Cao and Patisaul 2011) (Poling and Kauffman 2013). However, previous data regarding peripubertal changes in ARC *Kiss1* gene expression are either lacking, incomplete, or conflicting. While nothing we know about the development of kiss innervation within the PVN.

In the present study, we analyzed the development of kisspeptin distribution in CD1 female mice in three different hypothalamic nuclei (AVPV, ARC and PVN), with a particular focus on the PVN, during postnatal development from PND12 to PND30.

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 Department of Neuroscience, 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. Food

and water were provided *ad libitum* (standard mouse chow 4RF21, Mucedola srl, Settimo Milanese, Italy).

For this experiment we used 20 female CD1 mice killed at different age (PND12, PND15, PND18, PND21, PND30). Animal care and handling were according to the European Union Council Directive of 24th November 1986 (86/609/EEC); the Italian Ministry of Health and Ethical Committee of the University of Torino approved all the procedures reported in the present study.

Fixation and tissue sampling

Before the sacrifice, female mice belonging to PND30 group were inspected by daily vaginal smears in order to minimize the potential variations of kisspeptin expression due to the estrus cycle's phase (Adachi et al. 2007) and sacrificed in diestrus.

Animals were deeply anesthetized with ketamine–xylazine (respectively, 100 mg mL^{-1} and 20 mg mL^{-1}) and perfused, through the heart, with saline solution (0.9%) until vessels were completely blood-free, followed by the fixative solution (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. 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) at -20°C. Every fourth section one was processed for kisspeptin immunohistochemistry using the free-floating technique.

Kisspeptin immunohistochemistry

Briefly, after overnight washing in PBS, sections were exposed to Triton X-100 (0.2% in PBS) for 30 min and then treated for blocking endogenous peroxidase activity (PBS solution containing methanol/hydrogen peroxide, 20 min, at room temperature). Sections were then incubated with normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30 minutes and incubated overnight at 4 °C with a polyclonal rabbit kisspeptin antibody (AC#566; diluted 1:10.000 in PBS-Triton X-100 0.2%). The next 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 collected on slides pre-treated with chrome alum, air-dried, washed with xylene and cover slipped with Entellan mounting medium (Merck, Milano, Italy).

The kisspeptin antibody is highly specific to 10 amino acid peptide corresponding to 43–52 residues of mouse kisspeptin (kp-10); the specificity (production and characterization) was reported in previous studies (Clarkson et al. 2009; Franceschini et al. 2006). We have performed the following 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.

Quantitative analysis

For quantitative analysis, selected standardized sections of comparable levels (according the mouse brain atlas of Paxinos and Franklin 2001) covering the arcuate hypothalamic nucleus (ARC, Bregma -1.46 -1.70 mm), the anteroventral periventricular nucleus (AVPV, Bregma 0.50 - 0.02 mm) and the paraventricular hypothalamic nucleus (PVN, bregma -0.58 - 0.94 mm) were chosen. For each animal, three 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), by using a 40x (PVN) or a 20x (ARC and AVPV) objective. 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). For ARC and AVP the ROI was a rectangular box of fixed size and shape covering a large part of each considered nucleus standardized for each age. As in our previous study (Marraudino et al. 2017), the PVN was subdivided in four ROI that covered the full side of the nucleus (see fig. 3). The boxes divided the nucleus in medial and in lateral portion and then each portion is further subdivided in dorsal and in ventral part. To follow the changes in size and extension of the nucleus during the animal's development, the four boxes were standardized for each age. The measure of total PVN was a mean of signal measured in each four boxes.

Kp-ir fibers (in PVN) and structures (cell bodies and processes, in ARC and AVPV) were measured by calculating in binary transformations of the images (threshold function of the software) the fractional area, FA (percentage of pixels) covered by immunoreactive elements

(Pierman et al. 2008; Viglietti-Panzica et al. 1994). Due to differences in the immunostaining, the range of the threshold was individually adjusted for each section. The results obtained from each nucleus were grouped to provide mean (± S.E.M.) values.

Statistical analysis

Quantitative data were examined with SPSS statistic software (SPSS inc., Chicago, USA). The FA covered by kiss-ir fibers for lateral and medial PVN was analyzed by two-way analysis of variance (ANOVA) for repeated measures with the position (lateral vs. medial) and the age as the two independent variables and the antero-posterior neuroanatomical levels as repeated factor. While we used one-way ANOVA to analyze the other nuclei (AVPV and ARC) and for each single part of PVN (medial, lateral, DM, DL, VM, VL), followed, when appropriate, by a post-hoc Bonferroni test. Differences were considered statistically significant for values of $p<0.05$.

Results

Based on previous developmental studies (Clarkson and Herbison 2006), we started our study of the postnatal development of the kisspeptin system in mice from the postnatal day 12 (PND12). We examined the animals at different ages from juvenile to adult: PND12, PND15, PND18, PND21, PND30. The Kisspeptin signal was quantified in three hypothalamic nuclei, PVN, ARC and AVPV, for a complete view of system development.

The one-way ANOVA for AVPV, ARC and PVN (being the postnatal day the independent variable) reported a significant effect of the age on the kisspeptin immunoreactivity (see table 1 and 2).

In the AVPV, the Fig.1a shows an increase of the kisspeptin immunoreactivity from earlier stages to PND30. The comparisons by Bonferroni's test showed that no significant difference was present between PND12 and PND15, but after this age the kiss innervation increase at PND18 (vs. PND12 and PND15, $p<0,001$), that then stabilizes in a "plateau" between PND21 and PND30 (PND18 vs. PND30, $p=0,018$) (Fig. 1a).

In the ARC, the Fig. 1b shows at first an increase of the immunoreactivity (as in the AVPV), however, after the plateau between PND18 and PND21 we observed a decrease to the PND30. The Bonferroni's test confirms that these differences are statistically significant: significant increase from PND15 to PND18 ($p<0,001$) and significant decrease from PND 21 to PND 30 PND21 ($p=0,001$)

	PND12 MEAN±SEM	PND15 MEAN±SEM	PND18 MEAN±SEM	PND21 MEAN±SEM	PND30 MEAN±SEM	ANOVA 1 WAY F	p
AVPV	1,58±0,06	2,25±0,17	6,42±0,40	7,44±0,26	8,72±0,77	53,115	<0,001
ARC	6,05±0,37	5,40±0,42	17,58±0,20	18,33±0,22	16,00±0,30	425,000	<0,001

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

In the PVN the Fig. 2b shows a constant increase from PND12 to PND18, than a plateau between day PND18 and 21, followed by a moderate increase up to day PND30. The Bonferroni's test confirms, showing a significant increase from PND12 to PND18 ($p<0,001$) and no significant differences from PND18 to PND30.

In addition, we separately analyzed the changes in the immunoreactivity in the four different part of the PVN (Fig. 3). In this case we can observe a strong difference in the postnatal changes of kissppeptin immunoreactivity in the lateral and in the medial part of the PVN. The two-way ANOVA with the position (lateral vs. medial) and the age as the two independent variables demonstrated a general significant effect ($F= 17,447$; $p<0,001$). Moreover, the ANOVA revealed a strong significant effect of the position (lateral vs. medial) ($F=266,473$; $p<0,001$). The analysis with Bonferroni's test showed that the signal was higher in medial portion of PVN with a significant increase up to PND18 (vs. PND12, $p<0,001$; vs. PND15, $p=0,015$). While in lateral portion of PVN the fractional area covered by kisspeptin-ir fibers was lower and fairly constant with only a significant peak, compared to all other age respect to PND12, at PND21 (vs. PND12, $p=0,021$) (see Table 2). A more detailed analysis of the four part of the nucleus revealed that the most innervated part of PVN in the whole development is the VM (Fig. 3c). The values of one way ANOVA are reported in Table 1 for each part of the nucleus analyzed. In DM, the post hoc Bonferroni's test, showed that from PND18 to adult there was a significant increase of kiss innervation respect to PND12 (PND12 vs. PND18, $p=0,015$; vs. PND21, $p=0,005$; vs. PND30, $p<0,000$) (Fig. 3a). The situation is totally different in DL, where only at PND21 a significant peak was present (vs. PND12, $p=0,016$) (Fig. 3b). While, in VM the higher FA value was at PND18 (vs. PND12, $p=0,009$) and in adult (vs. PND12, $p=0,002$) (Fig. 3c). No significant differences were observed in the VL part of the PVN (Fig. 3d).

% Kisspeptin-ir fibers within PVN							
	PND12	PND15	PND18	PND21	PND30	ANOVA 1 WAY	
	MEAN±SEM	MEAN±SEM	MEAN±SEM	MEAN±SEM	MEAN±SEM	F	p
PVN	2,95±0,31	4,86±0.75	7,06±0,28	6,75±0,36	7,42±0,65	13,661	<0,001
Medial PVN	3,73±0,34	6,47±0,94	10,70±0,45	9,33±0,55	11,71±1,21	17,920	<0,001
Lateral PVN	2,17±0,39	3,26±0,65	3,41±0,22	4,16±0,26	3,13±0,30	3,224	0,043
DM	3,20±0,40	4,02±0,82	8,10±0,64	8,80±0,88	9,64±0,74	12,045	<0,001
DL	2,69±0,46	3,19±1,00	4,41±0,28	5,95±0,55	4,22±0,46	4,400	0,015
VM	4,25±0,36	8,91±1,22	13,30±0,70	9,87±0,30	13,78±1,70	7,449	0,002
VL	1,65±0,37	3,33±0,47	2,41±0,33	2,36±0,08	2,05±0,19	3,735	0,027

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

Discussion

Many neurotransmitters and neuropeptides have been implicated in the activation of Gonadotropin-releasing hormone (GnRH) neurons at puberty, including kisspeptin (Roa, Navarro, and Tena-Sempere 2011). In fact, mutation or deletion of kisspeptin receptor, GPR54 (also known as Kiss1r) resulted in failed puberty and infertility (de Roux et al. 2003) (Seminara et al. 2003). Moreover, *Kiss1* deletion or loss of function mutations in both mice and humans exhibit similar pubertal perturbations and infertility as the GPR54 knockout mice (d'Anglemont de Tassigny et al. 2007; Lapatto et al. 2007). In rodents and humans, the *KiSS1-GPR54-GnRH system* is completely formed at birth, as well as the GnRH pulse generator is functioning and responsive to kisspeptin stimulation (Clarkson et al. 2010), but investigations showed that AVPV *Kiss1* mRNA and kisspeptin protein are undetectable prior to PND10 and 15, respectively (Clarkson et al. 2009), and then they increase from PND 15 to adulthood. In fact, examining in both male and female mice the kisspeptin neurons during the development, it seems that the number of kisspeptin neurons within the rostral periventricular area of the third ventricule (RP3V, which includes the AVPV and the periventricular nucleus, PeN) increases throughout postnatal development, reaching adult numbers at the time of puberty in males and females (Clarkson and Herbison 2006). A recent study detailed day-by-day until puberty the *Kiss1* mRNA expression in female mice, demonstrating that *Kiss1* cell number, *Kiss1* mRNA per cell,

and total *Kiss1* mRNA levels in the RP3V consistently and gradually increase from PND 15 through PND 30 (Semaan and Kauffman 2015). Our data are perfectly in line with this, we also found a moderate increase at PND15, which become stronger at PND18 and after continued with a gradual increase up to PND30.

In contrast, ARC *Kiss1* expression is detectable prenatally and at birth in rodents, and continues to be express throughout postnatal development (Cao and Patisaul 2011; Poling and Kauffman 2013). However, previous data regarding peripubertal changes in ARC *Kiss1* gene expression are either lacking, incomplete, or conflicting. In the female rat, some studies demonstrated a small increases in ARC *Kiss1* expression around early puberty (Bentsen et al. 2010; Takase et al. 2009; Semaan and Kauffman 2015), while a more dramatic pubertal increase (Lomniczi et al. 2013). Similarly, Losa and colleagues (2011) demonstrated that the total kisspeptin immunoreactivity in the ARC increases throughout pubertal development in female but not male rats (Losa et al. 2011), but the number of kisspeptin immunoreactive cells and Kiss1 mRNA expression in the ARC does not change during pubertal development (Losa et al. 2011). The results of these studies suggest that the increases in total immunoreactivity in the ARC during pubertal development is likely attributable to changes in kisspeptin fibers within the ARC. In our study we analyzed the FA, which includes immunoreactive cell bodies and processes. We showed that an interesting and strong peak of kiss-ir was present at PND18, in comparison with the first two ages analyzed, PND12 and PND 15; after this peak, the signal was slightly decreased until PND30. Therefore, our data suggest the presence of a strong increase of kisspeptin before the puberty in ARC.

As expected, developmental changes are not restricted to the two locations where the majority of kisspeptin cell bodies are located (e.g. AVPV and ARC), but involves also their projection sites. In particular we analyzed the development of kisspeptin innervation of the PVN. As demonstrated our previous study the PVN present a large kisspeptin innervation, and represents, probably, one of the major targets of the kisspeptin system (Marraudino et al. 2017).

In general the changes of kisspeptin fibers density in the PVN are very similar to that observed in the AVPV and ARC, with a moderate increase from PND15, a further stronger increase at PND18 and a gradual, no significant increase, up to PND30. This temporal correlation between the development of kisspeptin fibers in PVN and changes in the immunoreactivity in the two nuclei, confirm the hypothesis that both nuclei contribute to the PVN innervation. Moreover, we confirm here our previous observation of a significant difference in the innervation of the lateral versus the medial part of the PVN (Marraudino et al. 2017). The separate analysis of the different portions of the PVN shows that the two regions of the nucleus, medial and lateral,

follow two different trends for the development of the kisspeptin innervation.

During the postnatal development, the medial part of the PVN showed a higher density of kisspeptin fibers in comparison to the lateral part. Changes in the medial part are similar to those observed in the AVPV and ARC, as well as to that observed when examining the PVN as an unique entity. On the contrary, the innervation of the lateral part is always lower and we have not observed any strongly significant changes in this part of the PVN, with the exception of the dorsolateral part, where we observed a significant peak at PND21.

These two patters of development probably are based on the possible interrelations between kisspeptin and neuronal subpopulations allocated within the medial part of the PVN. In fact, while the lateral part of the PVN contains magnocellular neurons (i.e. AVT and OXY producing neurons), the medial part of the PVN is characterized by the presence of different types of parvocellular neurons (CRH, TRH, but also AVP and OXT). In our previous study we showed that in the PVN there is a possible close relationship between kisspeptin fibers and OXT (Marraudino et al. 2017). OXT magno- and parvo-cellular neurons are mainly located in the DM and DL part of the PVN. In these two regions, the highest level of kisspeptin fibers was reached at PND21. Two different works of same authors (Miller et al. 1989; Chibbar et al. 1990)demonstrated that in female rats neuronal oxytocin mRNA levels increased during pubertal development and this increase was detected in OXT magnocellular and parvocellular neurons (Miller et al. 1989). Moreover, the pubertal increase of OXT mRNA is largely dependent upon circulating gonadal steroids, but it seems that is not correlated with neither testosterone nor estradiol, suggesting that oxytocinergic neurons do not yet express the appropriate receptors and/or that neural maturation is a necessary requisite for steroid sensitivity (Chibbar et al. 1990). It is possible to speculate that, before the puberty, kisspeptin, sensitive to estrogen, could be responsible of the increase of the OXT expression.

In conclusion, development of kisspeptin system within hypothalamus, with an increase of expression especially at puberal time, shows that kiss signaling pathway is a requisite for the onset of mammalian puberty. In mice, the time window of puberty occurs at PND26-30, but it extends also before. In our study the all nuclei analyzed (AVPV, ARC and PVN) increased the kiss-ir just before puberty age, at PND18. In AVPV and PVN this stabilized in small and constant increase up to PND30, while in ARC, after the peak at PND18, the kisspeptin decreased significantly.

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References

Adachi, S., S. Yamada, Y. Takatsu, H. Matsui, M. Kinoshita, K. Takase, H. Sugiura, T. Ohtaki, H. Matsumoto, Y. Uenoyama, H. Tsukamura, K. Inoue, and K. Maeda. 2007. 'Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats', *J Reprod Dev*, 53: 367-78.

Bentsen, A. H., L. Ansel, V. Simonneaux, M. Tena-Sempere, A. Juul, and J. D. Mikkelsen. 2010. 'Maturation of kisspeptinergic neurons coincides with puberty onset in male rats', *Peptides*, 31: 275-83.

Cao, J., and H. B. Patisaul. 2011. 'Sexually dimorphic expression of hypothalamic estrogen receptors alpha and beta and Kiss1 in neonatal male and female rats', *J Comp Neurol*, 519: 2954-77.

Chibbar, R., J. G. Toma, B. F. Mitchell, and F. D. Miller. 1990. 'Regulation of neural oxytocin gene expression by gonadal steroids in pubertal rats', *Mol Endocrinol*, 4: 2030-8.

Clarkson, J., W. C. Boon, E. R. Simpson, and A. E. Herbison. 2009. 'Postnatal development of an estradiol-kisspeptin positive feedback mechanism implicated in puberty onset', *Endocrinology*, 150: 3214-20.

Clarkson, J., S. K. Han, X. Liu, K. Lee, and A. E. Herbison. 2010. 'Neurobiological mechanisms underlying kisspeptin activation of gonadotropin-releasing hormone (GnRH) neurons at puberty', *Mol Cell Endocrinol*, 324: 45-50.

Clarkson, J., and A. E. Herbison. 2006. 'Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons', *Endocrinology*, 147: 5817-25.

d'Anglemont de Tassigny, X., L. A. Fagg, J. P. Dixon, K. Day, H. G. Leitch, A. G. Hendrick, D. Zahn, I. Franceschini, A. Caraty, M. B. Carlton, S. A. Aparicio, and W. H. Colledge. 2007. 'Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene', *Proc Natl Acad Sci U S A*, 104: 10714-9.

de Roux, N., E. Genin, J. C. Carel, F. Matsuda, J. L. Chaussain, and E. Milgrom. 2003. 'Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54', *Proc Natl Acad Sci U S A*, 100: 10972-6.

Franceschini, I., D. Lomet, M. Cateau, G. Delsol, Y. Tillet, and A. Caraty. 2006. 'Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha', *Neurosci Lett*, 401: 225-30.

Han, S. K., M. L. Gottsch, K. J. Lee, S. M. Popa, J. T. Smith, S. K. Jakawich, D. K. Clifton, R. A. Steiner, and A. E. Herbison. 2005. 'Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty', *J Neurosci*, 25: 11349-56.

Irwig, M. S., G. S. Fraley, J. T. Smith, B. V. Acohido, S. M. Popa, M. J. Cunningham, M. L. Gottsch, D. K. Clifton, and R. A. Steiner. 2004. 'Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat', *Neuroendocrinology*, 80: 264-72.

Lapatto, R., J. C. Pallais, D. Zhang, Y. M. Chan, A. Mahan, F. Cerrato, W. W. Le, G. E. Hoffman, and S. B. Seminara. 2007. 'Kiss1-/- mice exhibit more variable hypogonadism than Gpr54-/- mice', *Endocrinology*, 148: 4927-36.

Lomniczi, A., A. Loche, J. M. Castellano, O. K. Ronnekleiv, M. Bosch, G. Kaidar, J. G. Knoll, H. Wright, G. P. Pfeifer, and S. R. Ojeda. 2013. 'Epigenetic control of female puberty', *Nat Neurosci*, 16: 281-9.

Losa, S. M., K. L. Todd, A. W. Sullivan, J. Cao, J. A. Mickens, and H. B. Patisaul. 2011. 'Neonatal exposure to genistein adversely impacts the ontogeny of hypothalamic kisspeptin signaling pathways and ovarian development in the peripubertal female rat', *Reprod Toxicol*, 31: 280-9.

Marraudino, M., D. Miceli, A. Farinetti, G. Ponti, G. Panzica, and S. Gotti. 2017. 'Kisspeptin innervation of the hypothalamic paraventricular nucleus: sexual dimorphism and effect of estrous cycle in female mice', *J Anat*, 230: 775-86.

Miller, F. D., G. Ozimek, R. J. Milner, and F. E. Bloom. 1989. 'Regulation of neuronal oxytocin mRNA by ovarian steroids in the mature and developing hypothalamus', *Proc Natl Acad Sci U S A*, 86: 2468-72.

Paxinos G., Franklin K.B.J. 2001. 'The Mouse Brain in Stereotaxic Coordinates', San Diego: Academic Press.

Pierman, S., M. Sica, F. Allieri, C. Viglietti-Panzica, G. C. Panzica, and J. Bakker. 2008. 'Activational effects of estradiol and dihydrotestosterone on social recognition and the arginine-vasopressin immunoreactive system in male mice lacking a functional aromatase gene', *Horm Behav*, 54: 98-106.

Poling, M. C., and A. S. Kauffman. 2013. 'Organizational and activational effects of sex steroids on kisspeptin neuron development', *Front Neuroendocrinol*, 34: 3-17.

Roa, J., V. M. Navarro, and M. Tena-Sempere. 2011. 'Kisspeptins in reproductive biology: consensus knowledge and recent developments', *Biol Reprod*, 85: 650-60.

Semaan, S. J., and A. S. Kauffman. 2015. 'Daily successive changes in reproductive gene expression and neuronal activation in the brains of pubertal female mice', *Mol Cell Endocrinol*, 401: 84-97.

Seminara, S. B., S. Messager, E. E. Chatzidaki, R. R. Thresher, J. S. Acierno, Jr., J. K. Shagoury, Y. Bo-Abbas, W. Kuohung, K. M. Schwinof, A. G. Hendrick, D. Zahn, J. Dixon, U. B. Kaiser, S. A. Slaugenhaupt, J. F. Gusella, S. O'Rahilly, M. B. Carlton, W. F. Crowley, Jr., S. A. Aparicio, and W. H. Colledge. 2003. 'The GPR54 gene as a regulator of puberty', *N Engl J Med*, 349: 1614-27.

Smith, J. T., H. M. Dungan, E. A. Stoll, M. L. Gottsch, R. E. Braun, S. M. Eacker, D. K. Clifton, and R. A. Steiner. 2005. 'Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse', *Endocrinology*, 146: 2976-84.

Sutton, A. K., H. Pei, K. H. Burnett, M. G. Myers, Jr., C. J. Rhodes, and D. P. Olson. 2014. 'Control of food intake and energy expenditure by Nos1 neurons of the paraventricular hypothalamus', *J Neurosci*, 34: 15306-18.

Takase, K., Y. Uenoyama, N. Inoue, H. Matsui, S. Yamada, M. Shimizu, T. Homma, J. Tomikawa, S. Kanda, H. Matsumoto, Y. Oka, H. Tsukamura, and K. I. Maeda. 2009. 'Possible role of oestrogen in pubertal increase of Kiss1/kisspeptin expression in discrete hypothalamic areas of female rats', *J Neuroendocrinol*, 21: 527-37.

Viglietti-Panzica, C., N. Aste, J. Balthazart, and G. C. Panzica. 1994. 'Vasotocinergic innervation of sexually dimorphic medial preoptic nucleus of the male Japanese quail: influence of testosterone', *Brain Res*, 657: 171-84.

Watson R.E., Wiegand S.J., Clough R.W., and Hoffman G.E. 1986. ‘Use of cryoprotectant to maintain long-term peptide immunoreactivity and tissue morphology’, *Peptides,* 7: 155–159

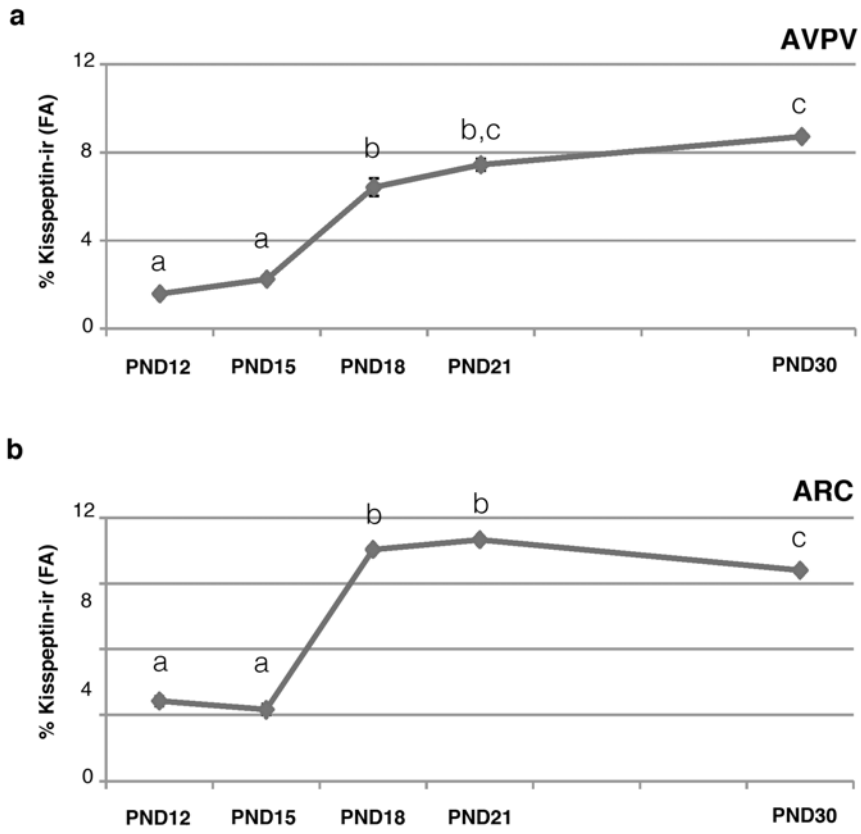


Figure 1. Kisspeptin in development of female mice in AVPV and ARC.

Graph Lines represent the fractional area covered by kisspeptin immunoreactive structures (**a**) in anteroventral periventricular nucleus (AVPV) and (**b**) in arcuate nucleus (Arc) in famale CD1 mice at different ages sacrificed (expressed as mean±SEM).

Different letters indicate significant difference from one another ($p \leq 0.05$); PND = post natal day.

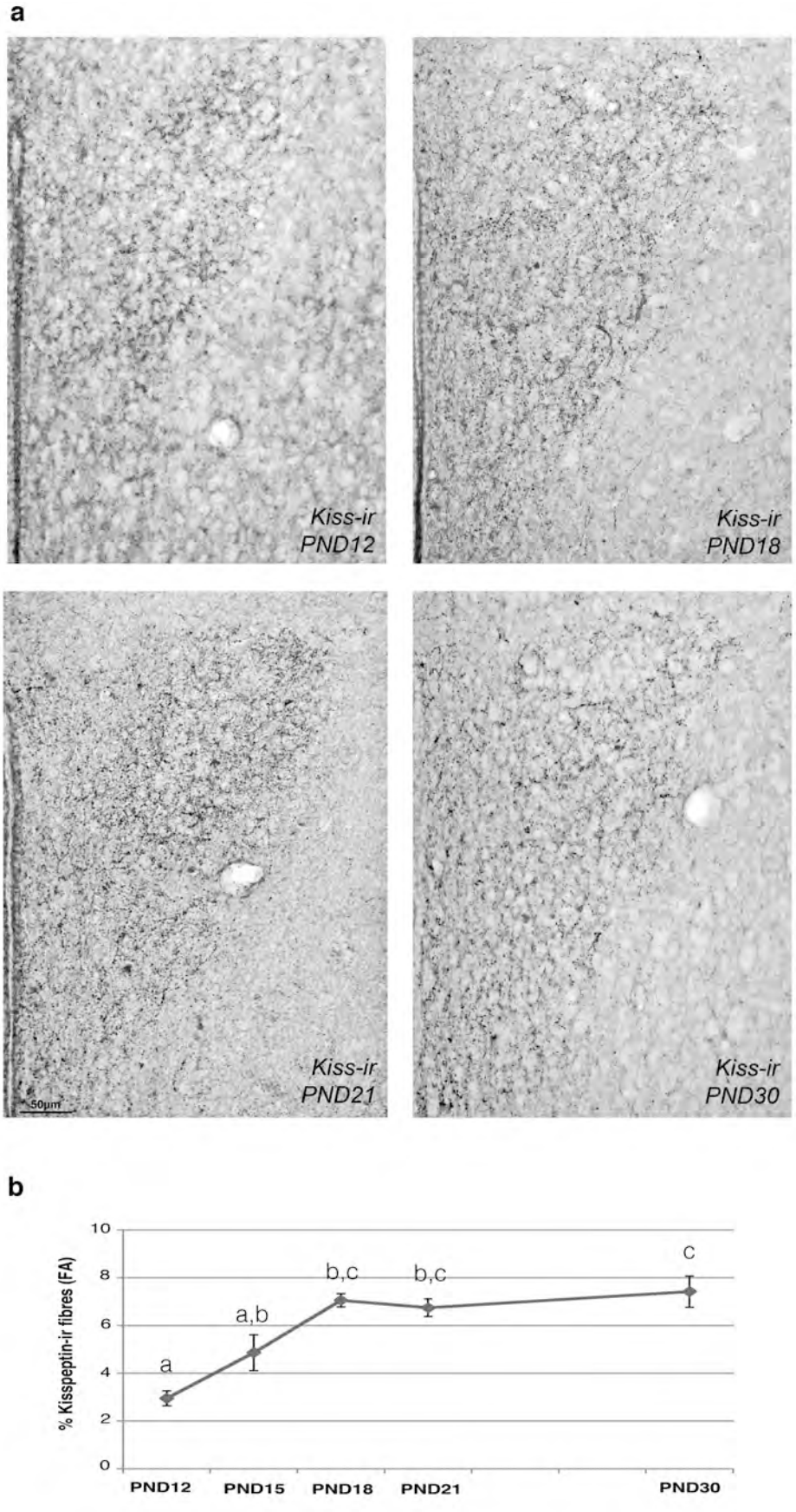
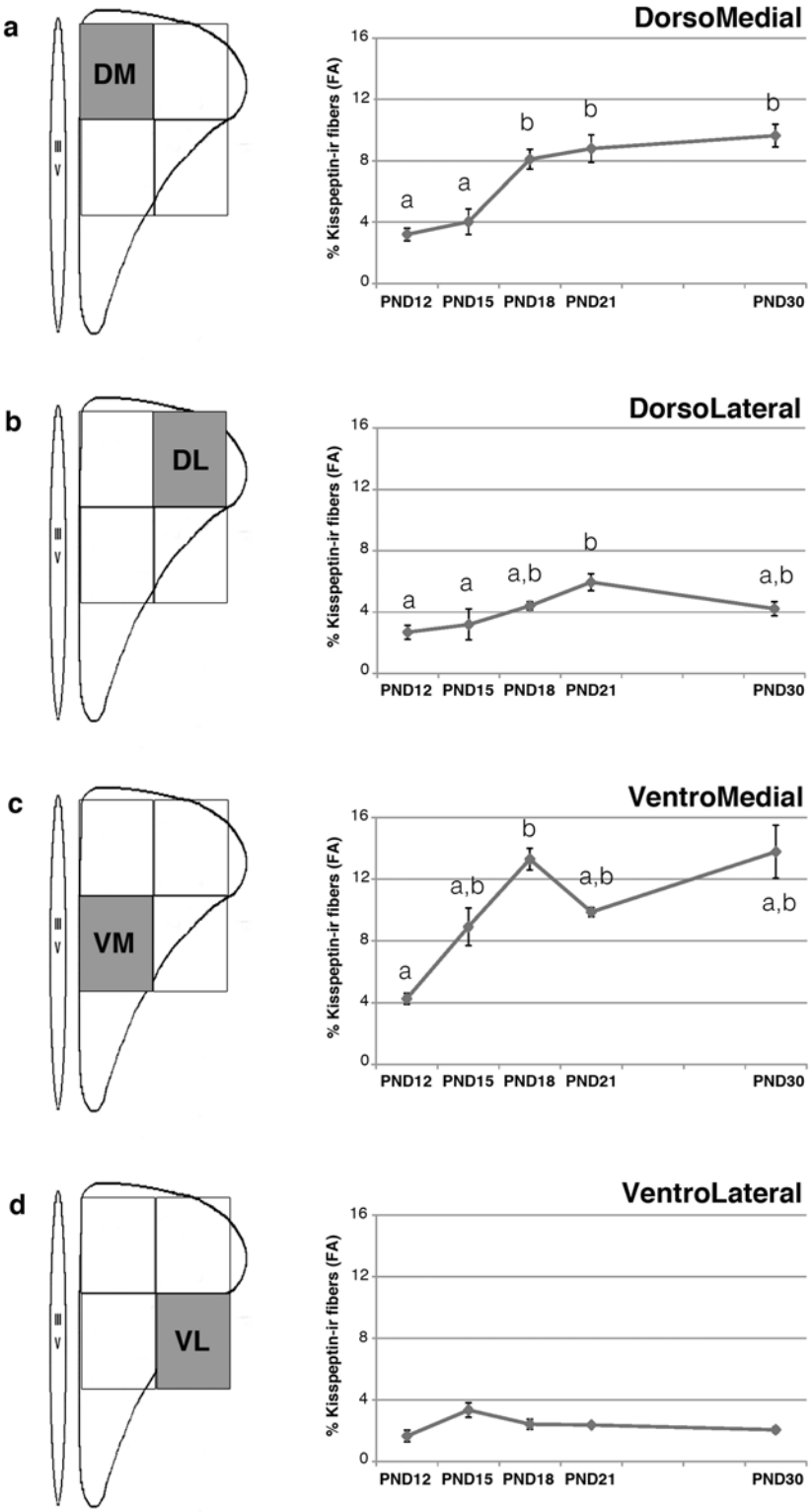

a
Kiss-ir
PND12
Kiss-ir
PND18
Kiss-ir
PND21
50µm
Kiss-ir
PND30
b
% Kisspeptin-ir fibres (FA)
10
8
6
4
2
0
a
a,b
b,c
b,c
c
PND12
PND15
PND18
PND21
PND30

Figure 2. Kisspeptin fibers within PVN in development of female mice.

(a) Photomicrographs representing the kisspeptin immunoreactivite fibers in Paraventricular nucleus (PVN) at postnatal day (PND) 12, PND18, PND21 and PND30. (**b**) Graph line represents the percentage of area covered by kisspeptin immunopositive fibers in PVN of female CD1 mice at different ages sacrificed (expressed as mean±SEM).

Different letters indicate significant difference from one another ($p \leq 0.05$); PND = post natal day. Scale bar = 50µm.


a
DM
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PND15
PND18
PND21
PND30
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DL
DorsoLateral
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a
a,b
b
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VM
VentroMedial
a
a,b
b
a,b
a,b
d
VL
VentroLateral

Figure 3. Kisspeptin development of female mice in different parts of PVN.

On left the scheme represent the four quadrants that divide the paraventricular nucleus (dorsomedial, DM; dorsolateral, DL; ventromedial, VM; ventrolateral, VL); on right the graph line representing the fractional area **(**expressed as mean±SEM**)** covered by kisspeptin immunoreactive structures in (**a**) DM, (**b**) DL, (**c**) VM and (**d**) VL of PVN of female CD1 mice at different ages sacrificed.

Different letters indicate significant difference from one another ($p \leq 0.05$); PND = post natal day.

CHAPTER 5

Experiment 3

'Effects of estradiol and progesterone on regulation of the Kisspeptin system in ovariectomized CD1 mice'

Effects of estradiol and progesterone on regulation of the Kisspeptin system in ovariectomized CD1 mice

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Abstract

Introduction: 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 at 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.

Materials and Methods: 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.

Results and Conclusion: 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 was not affecting 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 the source of these fibers is probably depending on both cell groups.

Introduction

In the cycling rodents, the gonadal hormone levels changed 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, and 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).

The Kisspeptin system (kiss) is an essential excitatory regulator of GnRH neurons; the absence or mutations of kisspeptin signaling would result in suppressed GnRH secretion, and in alterations of normal pubertal development (Pinilla et al. 2012). Consistent with the role of 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 nuclei: the rostral periventricular region (RP3V) and the arcuate nucleus (ARC). Previous studies showed that in female rodents kisspeptin immunoreactivity (kiss-ir) changes during the estrous cycle in different way 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 target of the kisspeptin system is the paraventricular nucleus (PVN, (Marraudino et al. 2017). 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).

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). Furthermore, a recent study hypothesized that kiss-ir neurons in RP3V 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). To distinguish between the role played by E_2 and P on kisspeptin system, not only in RP3V, but also in ARC and PVN, we analyzed here, in gonadectomized female mice, the effects of E_2 and P (alone or together) administration, following a timing that emulates the different phases of the estrous cycle (Martini et al, 2011).

Materials and Methods

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 1 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 responsive by the second week of priming and testing, two 4-day cycles of hormonal administration have been performed.

	Experimental design			
	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 (Edwards 1970). On two consecutive cycles of 4 days each, animals of the group E_2 received four injections of 10 µg of EB

(Sigma, St. Louis) and those of the group P received one injection of 500 μg of P (Sigma, St. Louis). 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 in the number of two 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

The 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 (Franklin and Paxinos 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 and, after, were 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) (for details of production and

characterization of the antibody see (Clarkson et al. 2009; Franceschini et al. 2006). 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 3,30- 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 (Franceschini et al. 2006; Clarkson et al. 2009).
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 μm^2 for ARC; 104.048 μm^2 for RP3V). While the PVN, in each selected section, was divided into fourteen squares (40,150 μm^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 statistic software (SPSS inc., Chicago, USA) by one-way analysis of variance (ANOVA), followed, when appropriate, by a post-hoc Bonferroni test. Differences were considered statistically significant for values of $p<0.05$.

Results

In order to stimulate female sexual receptivity, we have performed two cycles of hormonal administration of 4 days each one. In the original experiment females were tested for lordosis behavior, and they were receptive only after the second cycle of treatment (see Table 2 in (Martini, Pradotto, and Panzica 2011). We mimicked the hormonal situation typical of the late proestrus in the Group E_2+P, with high levels of E_2 and P, the early proestrus in the Group E_2 and the diestrus in Group P. In these different hormonal situations we analyzed the Kiss expression in three hypothalamic nuclei: ARC, RP3V and PVN. The pattern of distribution of kiss-ir structures observed within the hypothalamic region of control animals was according to previous observations in rodents (Clarkson and Herbison 2006) and was comparable to our previous study performed with a different (paraformaldehyde) fixative (Marraudino et al. 2017).

Kiss-ir of female CD1 mice changes during the estrous cycle in different way in the RP3V and the ARC (Smith et al. 2006), in fact the effect of E_2, P or E_2+P 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 FA within RP3V (see Table 2). The subsequent analysis by Bonferroni's test reported no differences among OIL and P groups (p=1.00), and among E_2+P and E_2 groups (p=1.00), 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 Bonferroni's 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=0.107, and OIL vs. P, p<0.001 (Fig. 1c).

For the ARC, the highest value for the FA covered by kiss-ir was instead reported in gonadectomized female treated with only Progesterone (Fig. 2a). The analysis by one-way ANOVA resulted statistically significant (Table 2) and the two-by two comparison (Bonferroni's test) demonstrated that the FA in P group was significantly higher than all other groups (p<0.001) in ARC. No difference was observed in comparison between the control group vs. E_2+P (p=1) 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 2: 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 3) and the Bonferroni's 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 revealed always a significant effect (table 3),

however the post-hoc analysis revealed strong differences among the different parts. In the medial subdivisions (DM and VM) the Bonferroni's test demonstrated that all the treated groups increased significantly the kiss-ir (OIL vs. E2+P, $p<0.001$; OIL vs. E2, $p<0.001$ and OIL vs. P, $p<0.001$). On the contrary, in DL and VL part only OIL vs. E_2 was always significant ($p=0.02$ 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 3: 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.

Discussion

This study demonstrates that E_2 and P play a different role on Kisspeptin system in the three hypothalamic nuclei analyzed. In fact, mimicking the hormonal situation typical of the late proestrus, the early proestrus and the diestrus, we show that P plays an important role on ARC kiss neurons, whereas E_2 (alone or together at P) actives RP3V kiss neurons. While, the highest PVN kisspeptin innervation observed in medial part of nucleus seems to be an effect of E_2, alone or together with P.

Sex steroids provide feedback loops that allow the gonads to communicate with the hypothalamus to regulate GnRH release. This regulation results indirect, since GnRH neurons do not express androgen (AR) or estrogen (ER) receptors (Herbison and Theodosis 1992; Huang and Harlan 1993). The mediator of the actions of sex steroids on GnRH neurons is Kisspeptin, whose neurons express estrogen receptor alpha, ERα (~90%) (Franceschini et al. 2006; Smith et al. 2005), the AR (~65%) (Smith et al. 2005), and also 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).

In rodents, while PR expression is likely under ERα regulation in AVPV (Shughrue, Lane, and Merchenthaler 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 seem 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 a highest density of kisspeptin in 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 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. So, E_2 could increase the release of Dyn and NKB in ARC, which inhibits the kiss expression during the estrus phase; but in diestrus, when increase the P concentration, the inhibitory effect of Dyn is annulated and consequentially there is an increase of kiss-ir in 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 the estrus the kiss-ir within PVN strongly increases in the medial part, suggesting that kiss innervation arrive mostly from RP3V (Marraudino et al. 2017). However, observations of the changes in the kisspeptin innervation of the PVN during the development clarified that also the ARC cells may have a role in the innervation of the PVN (see chapter IV). Present data confirm this hypothesis, 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), or only with P (mimicking the diestrus). In proestrus, the more rostral kisspeptin neurons (RP3V) are strongly activated by E_2, while more caudal neurons (ARC) are inactive. In diestrus, 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 innervate 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 [as TRH, (Kadar et al. 2010)] may have some close relation with kisspeptin and, as a result are indirectly regulated by E_2.

In conclusion, we demonstrated that in gonadectomized CD1 female mice E_2 and P played a different role on 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 ARC. All the treatments significantly affected the kisspeptin innervation of the PVN, with regional differences, suggesting that the source of these fibers is probably depending on both cell groups.

References

Clarkson, J., W. C. Boon, E. R. Simpson, and A. E. Herbison. 2009. 'Postnatal development of an estradiol-kisspeptin positive feedback mechanism implicated in puberty onset', *Endocrinology*, 150: 3214-20.

Clarkson, J., and A. E. Herbison. 2006. 'Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons', *Endocrinology*, 147: 5817-25.

Dierschke, D. J., T. Yamaji, F. J. Karsch, R. F. Weick, G. Weiss, and E. Knobil. 1973. 'Blockade by progesterone of estrogen-induced LH and FSH release in the rhesus monkey', *Endocrinology*, 92: 1496-501.

Downer, J. B., L. A. Jones, J. A. Katzenellenbogen, and M. J. Welch. 2001. 'Effect of administration route on FES uptake into MCF-7 tumors', *Nucl Med Biol*, 28: 397-9.

Edwards, D.A. 1970. 'Induction of estrus in female mice: estrogen–progesterone interactions', *Horm. Behav,* 1: 299-304.

Franceschini, I., D. Lomet, M. Cateau, G. Delsol, Y. Tillet, and A. Caraty. 2006. 'Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha', *Neurosci Lett*, 401: 225-30.

Goodman, R. L., I. Holaskova, C. C. Nestor, J. M. Connors, H. J. Billings, M. Valent, M. N. Lehman, and S. M. Hileman. 2011. 'Evidence that the arcuate nucleus is an important site of progesterone negative feedback in the ewe', *Endocrinology*, 152: 3451-60.

Goodman, R. L., M. N. Lehman, J. T. Smith, L. M. Coolen, C. V. de Oliveira, M. R. Jafarzadehshirazi, A. Pereira, J. Iqbal, A. Caraty, P. Ciofi, and I. J. Clarke. 2007. 'Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B', *Endocrinology*, 148: 5752-60.

Herbison, A. E., and D. T. Theodosis. 1992. 'Immunocytochemical identification of oestrogen receptors in preoptic neurones containing calcitonin gene-related peptide in the male and female rat', *Neuroendocrinology*, 56: 761-4.

Higo, S., S. Honda, N. Iijima, and H. Ozawa. 2016. 'Mapping of Kisspeptin Receptor mRNA in the Whole Rat Brain and its Co-Localisation with Oxytocin in the Paraventricular Nucleus', *J Neuroendocrinol*, 28.

Huang, X., and R. E. Harlan. 1993. 'Absence of androgen receptors in LHRH immunoreactive neurons', *Brain Res*, 624: 309-11.

Kadar, A., E. Sanchez, G. Wittmann, P. S. Singru, T. Fuzesi, A. Marsili, P. R. Larsen, Z. Liposits, R. M. Lechan, and C. Fekete. 2010. 'Distribution of hypophysiotropic thyrotropin-releasing hormone (TRH)-synthesizing neurons in the hypothalamic paraventricular nucleus of the mouse', *J Comp Neurol*, 518: 3948-61.

Kasa-Vubu, J. Z., G. E. Dahl, N. P. Evans, L. A. Thrun, S. M. Moenter, V. Padmanabhan, and F. J. Karsch. 1992. 'Progesterone blocks the estradiol-induced gonadotropin discharge in the ewe by inhibiting the surge of gonadotropin-releasing hormone', *Endocrinology*, 131: 208-12.

Kauffman, A. S., M. L. Gottsch, J. Roa, A. C. Byquist, A. Crown, D. K. Clifton, G. E. Hoffman, R. A. Steiner, and M. Tena-Sempere. 2007. 'Sexual differentiation of Kiss1 gene expression in the brain of the rat', *Endocrinology*, 148: 1774-83.

Kuang, Y., Q. Chen, Y. Fu, Y. Wang, Q. Hong, Q. Lyu, A. Ai, and Z. Shoham. 2015. 'Medroxyprogesterone acetate is an effective oral alternative for preventing premature luteinizing hormone surges in women undergoing controlled ovarian hyperstimulation for in vitro fertilization', *Fertil Steril*, 104: 62-70 e3.

Le, W. W., B. Attardi, K. A. Berghorn, J. Blaustein, and G. E. Hoffman. 1997. 'Progesterone blockade of a luteinizing hormone surge blocks luteinizing hormone-releasing hormone Fos activation and activation of its preoptic area afferents', *Brain Res*, 778: 272-80.

Marraudino, M., D. Miceli, A. Farinetti, G. Ponti, G. Panzica, and S. Gotti. 2017. 'Kisspeptin innervation of the hypothalamic paraventricular nucleus: sexual dimorphism and effect of estrous cycle in female mice', *J Anat*, 230: 775-86.

Martini, M., M. Pradotto, and G. Panzica. 2011. 'Synergic effects of estradiol and progesterone on regulation of the hypothalamic neuronal nitric oxide synthase expression in ovariectomized mice', *Brain Res*, 1404: 1-9.

McCartney, C. R., M. B. Gingrich, Y. Hu, W. S. Evans, and J. C. Marshall. 2002. 'Hypothalamic regulation of cyclic ovulation: evidence that the increase in gonadotropin-releasing hormone pulse frequency during the follicular phase reflects the gradual loss of the restraining effects of progesterone', *J Clin Endocrinol Metab*, 87: 2194-200.

Mittelman-Smith, M. A., L. M. Rudolph, M. A. Mohr, and P. E. Micevych. 2017. 'Rodent Models of Non-classical Progesterone Action Regulating Ovulation', *Front Endocrinol (Lausanne)*, 8: 165.

Molloy, B. G., M. A. El Sheikh, C. Chapman, R. E. Oakey, K. W. Hancock, and M. R. Glass. 1984. 'Pathological mechanisms in polycystic ovary syndrome: modulation of LH pulsatility by progesterone', *Br J Obstet Gynaecol*, 91: 457-65.

Paxinos G., Franklin K.B.J. 2001. 'The Mouse Brain in Stereotaxic Coordinates', San Diego: Academic Press.

Pinilla, L., E. Aguilar, C. Dieguez, R. P. Millar, and M. Tena-Sempere. 2012. 'Kisspeptins and reproduction: physiological roles and regulatory mechanisms', *Physiol Rev*, 92: 1235-316.

Shughrue, P. J., M. V. Lane, and I. Merchenthaler. 1997. 'Regulation of progesterone receptor messenger ribonucleic acid in the rat medial preoptic nucleus by estrogenic and antiestrogenic compounds: an in situ hybridization study', *Endocrinology*, 138: 5476-84.

Simerly, R. B., A. M. Carr, M. C. Zee, and D. Lorang. 1996. 'Ovarian steroid regulation of estrogen and progesterone receptor messenger ribonucleic acid in the anteroventral periventricular nucleus of the rat', *J Neuroendocrinol*, 8: 45-56.

Smith, J. T., B. V. Acohido, D. K. Clifton, and R. A. Steiner. 2006. 'KiSS-1 neurones are direct targets for leptin in the ob/ob mouse', *J Neuroendocrinol*, 18: 298-303.

Smith, J. T., C. M. Clay, A. Caraty, and I. J. Clarke. 2007. 'KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season', *Endocrinology*, 148: 1150-7.

Smith, J. T., H. M. Dungan, E. A. Stoll, M. L. Gottsch, R. E. Braun, S. M. Eacker, D. K. Clifton, and R. A. Steiner. 2005. 'Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse', *Endocrinology*, 146: 2976-84.

Watson, R. E., S. J. Wiegand, R. W. Clough, and G. E. Hoffman. 1986. 'Use of Cryoprotectant to Maintain Long-Term Peptide Immunoreactivity and Tissue Morphology', *Peptides*, 7: 155-59.

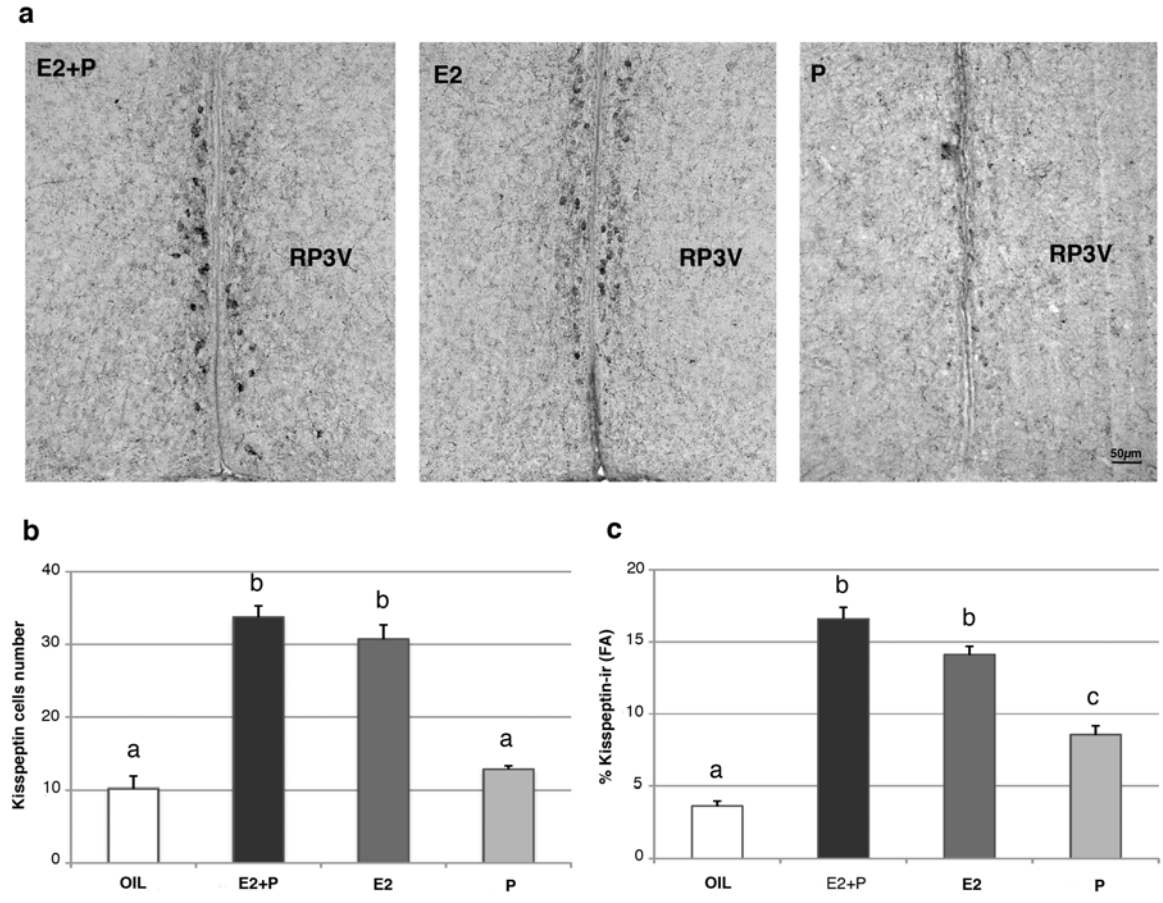

Figure 1. (a) Effects of estradiol+progesterone (E_2+P), estradiol (E_2) and progesterone (P) administration on Kisspeptin system in rostral periventricular region of the third ventricle (RP3V). **(c and b)** Histograms representing the number of cells and the fractional area (FA) **(**expressed as mean±SEM) 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 Bonferroni test; $p \leq 0.05$) are denoted by a, b or c. Scale bar = 50µm.

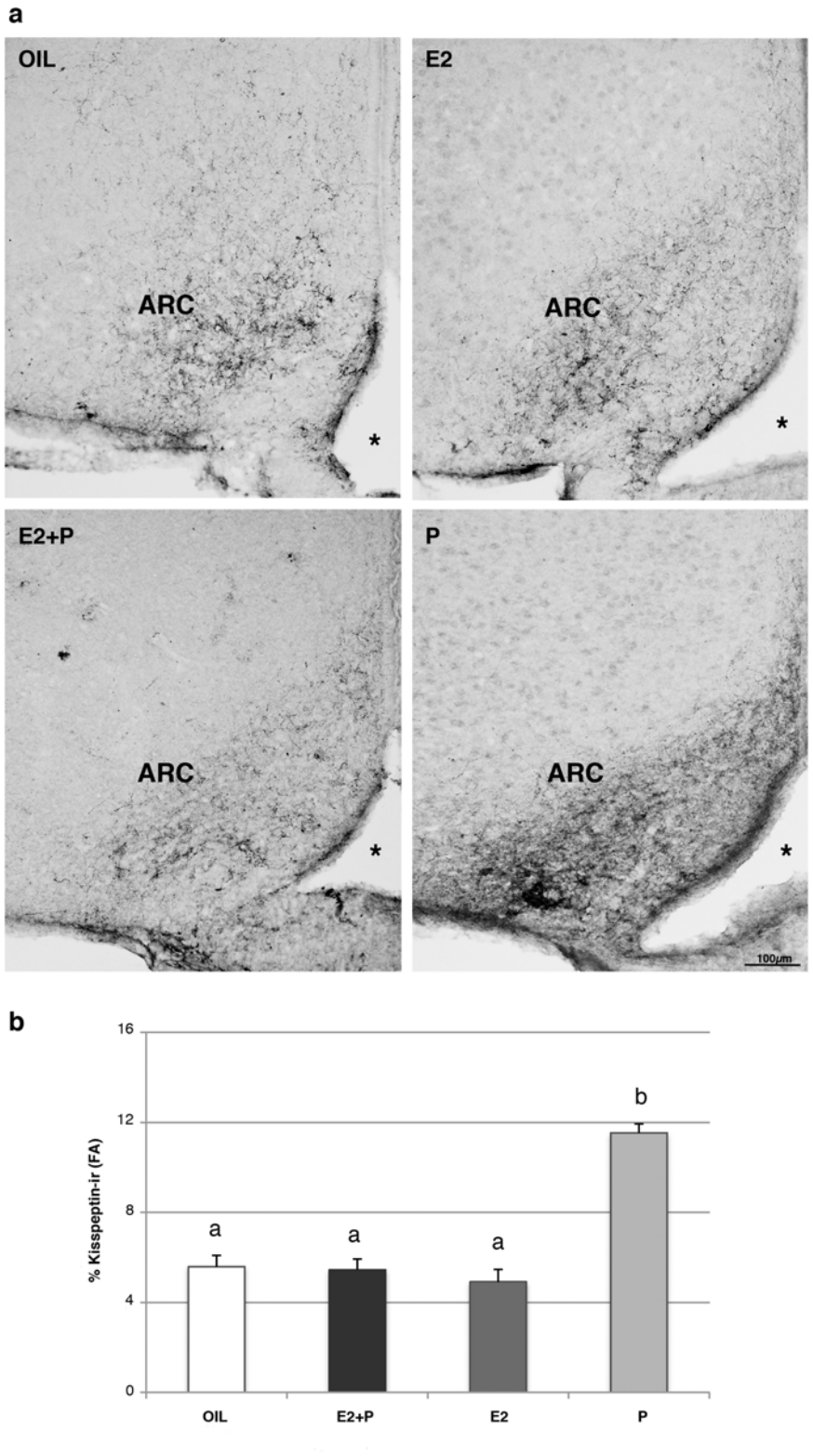

Figure 2. (a) Photomicrographs representing the effects of estradiol+progesterone (E_2+P), estradiol (E_2) and progesterone (P) administration on Kisspeptin system in arcuate nucleus (ARC) in comparison with control group (OIL). **(b)** Histogram representing the fractional area (FA) **(**expressed as 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 Bonferroni test; $p \leq 0.05$) are denoted by a or b. * = third ventricle. Scale bar = 100µm.

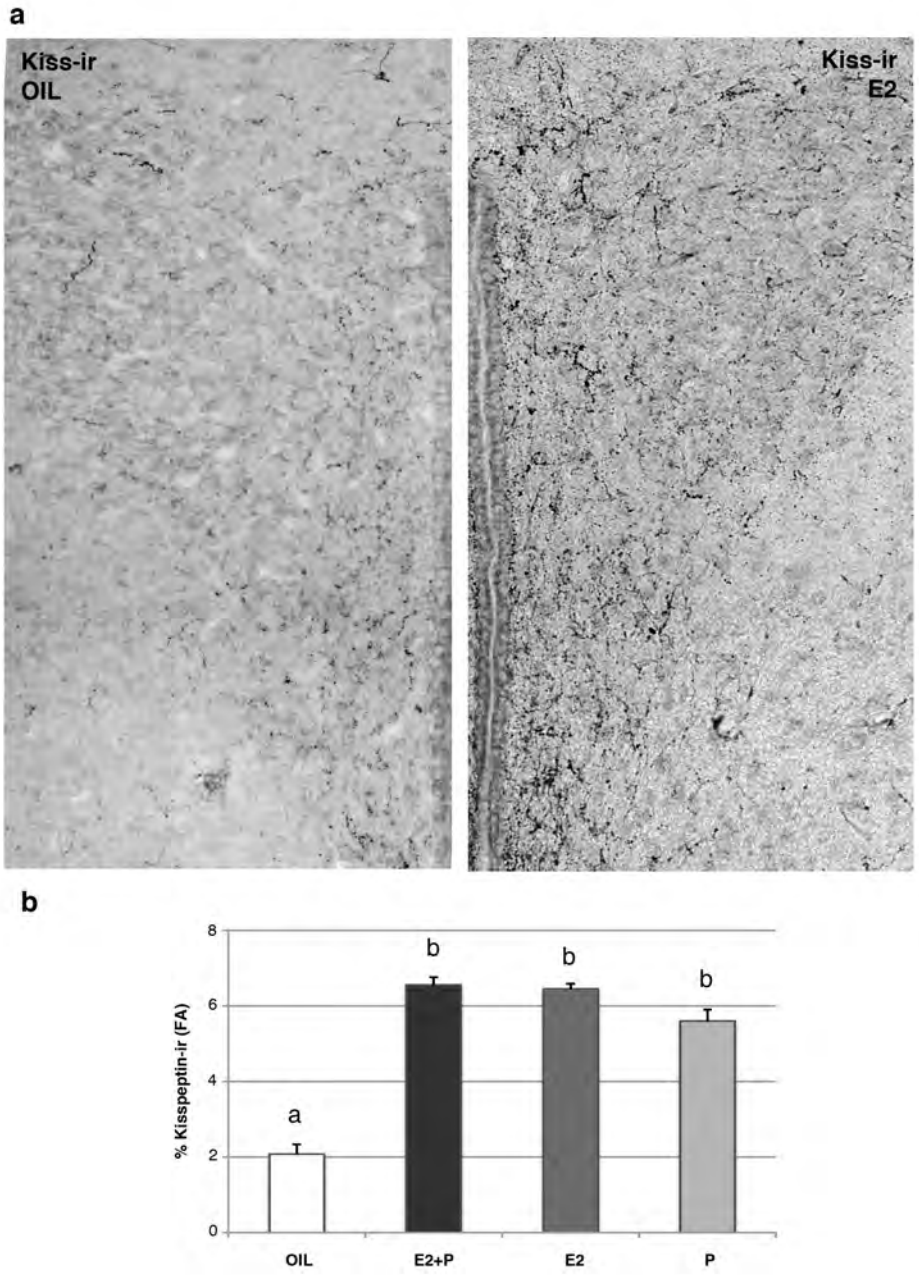

Figure 3. (a) Photomicrographs representing the comparison between control group (OIL) and the effect of the estradiol (E_2) administration on Kisspeptin system in Paraventricular Nucleus (PVN) of female mice. **(b)** Histogram representing the fractional area (FA) (mean±SEM) covered by kisspeptin-immunoreactive 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 Bonferroni test; $p \leq 0.05$) are denoted by a or b. Scale bar = 50µm.

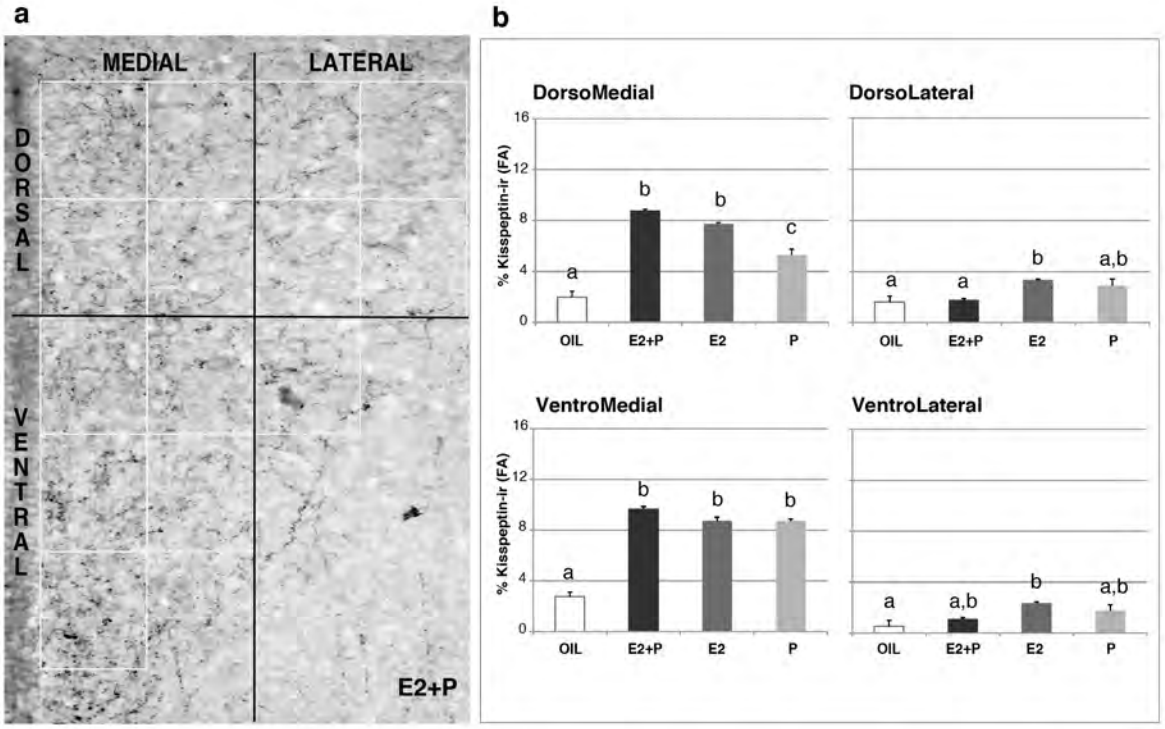

Figure 4. (a) The representative subdivision in the Paraventricular Nucleus (PVN) of female mice treated with estradiol+progesterone (E_2+P) in fourteen quadrants to identify the four parts of nucleus (DM, dorso-medial; DL, dorsolateral; VM, ventro-medial; VL, ventro-lateral). **(b)** Histograms representing the fractional area (FA) covered by kiss-ir fibers (mean±SEM) in the PVN (DM, DL, VM, VL) in ovariectomized mice treated with estradiol (E_2), progesterone (P) and estradiol+progesterone (E_2+P). Significant differences (ANOVA followed by the Bonferroni test; $p \leq 0.05$) are denoted by a, b or c.

CHAPTER 6

Experiment 4

'Thyreotropin-releasing hormone and kisspeptin in the hypothalamic paraventricular nucleus'

Thyreotropin-releasing hormone and kisspeptin in the hypothalamic paraventricular nucleus

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Abstract

Introduction: The kisspeptin system controls the reproduction via the control of GnRH, from the rostral periventricular area of the third ventricle (RP3V) and the arcuate nucleus (ARC), two regions where are allocated the main clusters of kiss neurons. Outside the GnRH system, the hypothalamic paraventricular nucleus (PVN) represents one of the major targets of the system. This kiss innervation within PVN is dimorphic, higher in female than in male mice, and heterogeneous. The medial part of PVN contains the highest concentration of kiss fibers, in the same region there are the thyrotropin-releasing hormone (TRH) producing neurons, best known as the central regulator of the hypothalamic-pituitary-thyroid (HPT) axis, that regulates many physiological functions linked to the metabolism.

Material and Methods: In the present study we investigated by double-labeling immunofluorescence the relationship between kisspeptin fibers and TRH neurons within PVN in male and female adult CD1 mice.

Results and Conclusion: In mice, the cell bodies of TRH neurons are located in the mid-level of the PVN, we demonstrated that in males and females there is a close relationship between kisspeptin fibers and TRH neurons within PVN. This suggests that kisspeptin could regulate the TRH system in PVN, creating a strong correlation between reproductive and metabolic control.

Introduction

Kisspeptin neurons in rostral periventricular area of the third ventricle (RP3V, which includes the AVPV and the periventricular nucleus, PeN) and arcuate nucleus (ARC) have been directly related to the control of reproduction via the control of GnRH system (Navarro et al. 2005; Roseweir et al. 2009; Tsutsui et al. 2010); however, the large, sexually dimorphic innervation of the paraventricular nucleus (PVN) can not be directly related to reproduction, sexual behavior or puberty control. The PVN is, in fact, involved in the control of several vegetative functions (food intake, cardiovascular control, metabolism control, osmoregulation, and many others). The distribution of positive fibers along the entire extension of the PVN, including both magnocellular and parvocellular regions, indicates that kisspeptin could be implicated in the regulation of the multiple physiological activities of the PVN (Marraudino et al. 2017).

Thyrotropin-releasing hormone (TRH), regulator of many physiological functions, is best known as the central regulator of the hypothalamic-pituitary-thyroid (HPT) axis (Reichlin 1989). In mice, the cell bodies of TRH neurons are located in the compact part and in the neighboring region in the mid-level of the PVN (Kadar et al. 2010), while their axons reach the median eminence (Fekete et al. 2000; Ishikawa et al. 1988; Merchenthaler and Liposits 1994). There, they release TRH into the portal capillary system, which is conveyed to the anterior pituitary gland (Fekete and Lechan 2007), where TRH regulates the TSH release.

In this study we would like to show if there are possible close relationships between kisspeptin fibers and TRH neurons within PVN in adult male and female mice.

Materials and Methods

Animals

The experiment was carried out on adult (90 day old), male and female, CD1 mice. Animals were maintained in an automatically controlled room, with a temperature of 22°C ± 2°C under a 12 h light/12 h dark cycle (lights on at 08:00) with food and water *ad libitum*. For this study, animal care and handling practices were approved by the Local Ethics Committees of Department of Psychobiology, Universidad Nacional de Educación a Distancia, Madrid, Spain and were in accordance with the European Union Directive, (2010/63/UE) and Spanish Government Directive (R.D. 53/2013).

Tissue preparation

At postnatal day 90, 4 male and 4 female CD1 mice were anesthetized (ketamine: 50 lg/g; xylazine: 10 lg/g body weight, i.p.) and intracerebro-ventricularly treated with colchicine (1 lg/g BW) by injection through a 26-g needle placed into the lateral ventricle under stereotaxic control (coordinates from Bregma: anteroposterior, -0.2 mm; lateral, -1 mm; dorsoventral, -2.5 mm) (Paxinos 2001). Female mice were inspected by vaginal smears and sacrificed in estrus in order to minimize the potential variations of kisspeptin expression due to the estrus cycle's phase (Adachi et al. 2007).

Twenty hours after the colchicine treatment, the animals were anesthetized with ketamine–xylazine (respectively, 100 mg mL^{-1} and 20 mg mL^{-1}) and perfused, through the heart, with saline solution (0.9%), followed by 30 ml 1% acrolein/ 3% paraformaldehyde (PFA) in PBS (pH 7.3), and finally 20 ml 3% PFA in PBS. The brains were removed and were cryoprotected in 30% sucrose solution in PBS at 4°C overnight, 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 m 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 2001). Sections were collected in a cryoprotectant solution (Watson 1986) at -20°C. Every fourth section one was processed for double-labeling immunofluorescence using the free-floating technique.

Double-labeling immunofluorescence

Floating sections were removed from storing solution, washed in PBS and incubated, at room temperature, in the dark, for 30 minutes with 1% sodiumborohydride diluted in deionized H_2O. The sections are then washed with PBS, until all the sodium borohydride solution is removed, followed by a solution of 0.5% Triton-X-100 diluted in PBS. Sections were incubated for 15 minutes with 10% of normal horse serum (NHS) diluted in PBS at room temperature, followed by a solution of primary sheep anti-TRH antibody (1:500; a generous gift of Dr. Csaba Fekete, Budapest, Hungary, (Wittmann et al. 2009)) and rabbit anti-Kiss antibody (1:4000; AC#566, a generous gift of Dr M. Keller, Tours, France, (Franceschini et al. 2006)) in 2% NHS and PBS, for 36 hours at 4°C. The third day, sections were washed and incubated with solutions of secondary biotinilated goat anti-sheep IgG (1:250; Vector Laboratories, Burlingame, CA, USA), 2% NHS and PBS for two hours, and then with secondary donkey anti-rabbit IgG (1:500; Anti-rabbit Alexa Fluor 555), Texas Red Avidin (1:500; Vector), and DAPI. Sections were then cover-slipped with antifade mounting medium Mowiol (Sigma–Aldrich).

Qualitative analysis

Selected standardized sections of comparable levels covering the PVN (bregma -0.58 to 0.94 mm, Paxinos 2001) were chosen. For each animal, four 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.

Results

In adult CD1 mice the distribution of kisspeptin fluorescence immunoreactivity within PVN was similar to that described in our previous experiment (Marraudino et al. 2017). In fact, the concentration of kiss fibers was strongly higher in females than males. Moreover, in male and in female mice the distribution of kiss-ir innervation was heterogeneous, denser in the medial than the lateral PVN (Fig. 1a). While, the distribution of TRH neurons was according the description provided by Kadar et al. (Kadar et al. 2010). In all parts of the PVN TRH immunoreactive neurons were present, but the density of positive cells bodies was highest at the anterior and mid-levels of the nucleus, particularly in the compact part of the PVN (Fig. 1b).

Close apposition of kisspeptin fibers to TRH neurons were observed in both the medial and the lateral part, but they were particularly clustered in the medial part, with no differences among male and female CD1 adult mice (Fig. 2).

TRH negative cells stained with DAPI and surrounded by kisspeptin fibers and terminals were also observed.

Discussion

The presence of positive fibers along the entire extension of the PVN suggested in our previous study (Marraudino et al. 2017) that kisspeptin could be implicated in the regulation of many of the physiological activities controlled by the PVN. This innervation of PVN by kisspeptin fibers is sexually dimorphic and heterogeneous. In fact, the density of kisspeptin fibers is higher in the medial than in lateral PVN (Marraudino et al. 2017). In the lateral part of PVN are located magnocellular neurons, vasopressin (AVP) and oxytocin (OXT), that project to the posterior pituitary; while, in the medial part of PVN are present different types of parvocellular neurons:

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; Gotti et al. 2005), vasopressin (Caldwell et al. 2008), somatostatin (Tan et al. 2013). Previously, we demonstrated that AVP- and nNOS-containing neurons were not strongly related to kisspeptin system, while a close relationships were observed with OXT- and TH-positive neurons in the medial PVN (Marraudino et al. 2017). In this work we analyzed the relationships of Kisspeptin and the hypothalamic neuronal population of TRH neurons.

TRH has an important role in the regulation of energy homeostasis not only for the control of thyroid function, but also through effects on feeding behavior, and on energy consuming activities as thermogenesis or activation of locomotion (for a review see (Lechan and Fekete 2006). The TRH neurons receive direct inputs from Melanocyte-stimulating hormone (MSH) and Neuropeptide Y (NPY) containing neurons of the hypothalamic arcuate nucleus (Fekete et al. 2000; Broberger et al. 1999). MSH promotes weight loss and increases energy expenditure, whereas NPY promotes weight gain and reduce energy expenditure (van Swieten et al. 2014; Myers and Olson 2012). TRH neurons are located within the medial subdivision of PVN, an area chiefly related to the control of energy metabolism and food intake, where we observed the highest density of kisspeptin fibers and close relationships among these two systems.

Many data suggest that the onset of puberty is strictly related to the regulation of body metabolism, in fact, the kiss expression is altered in conditions of reproductive impairment linked to metabolic stress and the normalization of the kiss contributes to improving the reproductive phenotype despite unfavorable metabolic conditions (Castellano et al. 2011; Castellano and Tena-Sempere 2016; Roa et al. 2009). Therefore a direct, sexually dimorphic, link among kisspeptin system and the PVN circuits, could represent the anatomical base of this functional relationship. In addition, kisspeptin neurons of the ARC nucleus express the receptor for leptin (Donato et al. 2011), and studies on transgenic mice demonstrated that the leptin receptor is present in about 10% of kisspeptin neurons in the ARC nucleus, but in only a few scattered elements of the RP3V population (Cravo et al. 2011). TRH secretion is continuously regulated by leptin. During fasting, thyroid activity decreases to reduce energy expenditure in humans and mice, and this is associated to low leptin levels with a reduction of TRH gene expression and TRH formation (Perello, Stuart, and Nillni 2006). We know that TRH neurons are activated by MSH and inhibited by NPY, we could speculate that also kisspeptin neurons may modulate the TRH.

In addition, the distribution of different forms of estrogen receptors (ERß and ER) in PVN is

essential to understand how estradiol may modulate PVN circuits. *In vivo* and *in vitro* studies demonstrated that the action of estrogens in PVN is modulated essentially by the ERß, immunohistochemistry showed the presence of ERß and the absence of ER (Merchenthaler et al. 2004). Many paraventricular neuronal population express ERß (e.g. CRH, AVP, and OXT neurons (Hrabovszky et al. 2004; Laflamme et al. 1998; Suzuki and Handa 2004), but no TRH. Contrarily, it is well demonstrated that the gonadal steroids differentially regulate kisspeptin expression in ARC and RP3V, in fact kiss neurons express ER, ER, and also androgen receptor (AR). Therefore, it is possible that the kisspeptin could have a role as mediator of the gonadal steroids on TRH neurons.

In conclusion, we show here that in adult male and female CD1 mice there is a close relationship between kisspeptin fibers and TRH neurons within the PVN, this could represent the neuroanatomical link to connect the gonadal hormones' signals and the metabolic control generated by TRH neurons.

Acknowledgements. We want to acknowledge Dr. Csaba Fekete (Department of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary) that kindly supplied the sheep anti-TRH antibody, Dr. Matthieu Keller (INRA, Tours, France) that kindly supplied the rabbit anti-kiss antibody, and the Dr. Ivan Enrico Repetto for his help in performing the experiments.

References

Adachi, S., S. Yamada, Y. Takatsu, H. Matsui, M. Kinoshita, K. Takase, H. Sugiura, T. Ohtaki, H. Matsumoto, Y. Uenoyama, H. Tsukamura, K. Inoue, and K. Maeda. 2007. 'Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats', *J Reprod Dev*, 53: 367-78.

Broberger, C., T. J. Visser, M. J. Kuhar, and T. Hokfelt. 1999. 'Neuropeptide Y innervation and neuropeptide-Y-Y1-receptor-expressing neurons in the paraventricular hypothalamic nucleus of the mouse', *Neuroendocrinology*, 70: 295-305.

Caldwell, H. K., H. J. Lee, A. H. Macbeth, and W. S. Young, 3rd. 2008. 'Vasopressin: behavioral roles of an "original" neuropeptide', *Prog Neurobiol*, 84: 1-24.

Castellano, J. M., A. H. Bentsen, M. A. Sanchez-Garrido, F. Ruiz-Pino, M. Romero, D. Garcia-Galiano, E. Aguilar, L. Pinilla, C. Dieguez, J. D. Mikkelsen, and M. Tena-Sempere. 2011. 'Early metabolic programming of puberty onset: impact of changes in postnatal feeding and rearing conditions on the timing of puberty and development of the hypothalamic kisspeptin system', *Endocrinology*, 152: 3396-408.

Castellano, J. M., and M. Tena-Sempere. 2016. 'Metabolic control of female puberty: potential therapeutic targets', *Expert Opin Ther Targets*, 20: 1181-93.

Cravo, R. M., L. O. Margatho, S. Osborne-Lawrence, J. Donato, Jr., S. Atkin, A. L. Bookout, S. Rovinsky, R. Frazao, C. E. Lee, L. Gautron, J. M. Zigman, and C. F. Elias. 2011. 'Characterization of Kiss1 neurons using transgenic mouse models', *Neuroscience*, 173: 37-56.

Donato, J., Jr., R. M. Cravo, R. Frazao, L. Gautron, M. M. Scott, J. Lachey, I. A. Castro, L. O. Margatho, S. Lee, C. Lee, J. A. Richardson, J. Friedman, S. Chua, Jr., R. Coppari, J. M. Zigman, J. K. Elmquist, and C. F. Elias. 2011. 'Leptin's effect on puberty in mice is relayed by the ventral premammillary nucleus and does not require signaling in Kiss1 neurons', *J Clin Invest*, 121: 355-68.

Fekete, C., and R. M. Lechan. 2007. 'Negative feedback regulation of hypophysiotropic thyrotropin-releasing hormone (TRH) synthesizing neurons: role of neuronal afferents and type 2 deiodinase', *Front Neuroendocrinol*, 28: 97-114.

Fekete, C., E. Mihaly, L. G. Luo, J. Kelly, J. T. Clausen, Q. Mao, W. M. Rand, L. G. Moss, M. Kuhar, C. H. Emerson, I. M. Jackson, and R. M. Lechan. 2000. 'Association of cocaine- and amphetamine-regulated transcript-immunoreactive elements with thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus and its role in the regulation of the hypothalamic-pituitary-thyroid axis during fasting', *J Neurosci*, 20: 9224-34.

Franceschini, I., D. Lomet, M. Cateau, G. Delsol, Y. Tillet, and A. Caraty. 2006. 'Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha', *Neurosci Lett*, 401: 225-30.

Gotti, S., S. Chiavegatto, M. Sica, C. Viglietti-Panzica, R. J. Nelson, and G. Panzica. 2004. 'Alteration of NO-producing system in the basal forebrain and hypothalamus of Ts65Dn mice: an immunohistochemical and histochemical study of a murine model for Down syndrome', *Neurobiol Dis*, 16: 563-71.

Gotti, S., M. Sica, C. Viglietti-Panzica, and G. Panzica. 2005. 'Distribution of nitric oxide synthase immunoreactivity in the mouse brain', *Microsc Res Tech*, 68: 13-35.

Hrabovszky, E., I. Kallo, A. Steinhauser, I. Merchenthaler, C. W. Coen, S. L. Petersen, and Z. Liposits. 2004. 'Estrogen receptor-beta in oxytocin and vasopressin neurons of the rat and human hypothalamus: Immunocytochemical and in situ hybridization studies', *J Comp Neurol*, 473: 315-33.

Ishikawa, K., Y. Taniguchi, K. Inoue, K. Kurosumi, and M. Suzuki. 1988. 'Immunocytochemical delineation of thyrotrophic area: origin of thyrotropin-releasing hormone in the median eminence', *Neuroendocrinology*, 47: 384-8.

Kadar, A., E. Sanchez, G. Wittmann, P. S. Singru, T. Fuzesi, A. Marsili, P. R. Larsen, Z. Liposits, R. M. Lechan, and C. Fekete. 2010. 'Distribution of hypophysiotropic thyrotropin-releasing hormone (TRH)-synthesizing neurons in the hypothalamic paraventricular nucleus of the mouse', *J Comp Neurol*, 518: 3948-61.

Laflamme, N., R. E. Nappi, G. Drolet, C. Labrie, and S. Rivest. 1998. 'Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype', *J Neurobiol*, 36: 357-78.

Lechan, R. M., and C. Fekete. 2006. 'The TRH neuron: a hypothalamic integrator of energy metabolism', *Prog Brain Res*, 153: 209-35.

Marraudino, M., D. Miceli, A. Farinetti, G. Ponti, G. Panzica, and S. Gotti. 2017. 'Kisspeptin innervation of the hypothalamic paraventricular nucleus: sexual dimorphism and effect of estrous cycle in female mice', *J Anat*, 230: 775-86.

Merchenthaler, I., M. V. Lane, S. Numan, and T. L. Dellovade. 2004. 'Distribution of estrogen receptor alpha and beta in the mouse central nervous system: in vivo autoradiographic and immunocytochemical analyses', *J Comp Neurol*, 473: 270-91.

Merchenthaler, I., and Z. Liposits. 1994. 'Mapping of thyrotropin-releasing hormone (TRH) neuronal systems of rat forebrain projecting to the median eminence and the OVLT. Immunocytochemistry combined with retrograde labeling at the light and electron microscopic levels', *Acta Biol Hung*, 45: 361-74.

Myers, M. G., Jr., and D. P. Olson. 2012. 'Central nervous system control of metabolism', *Nature*, 491: 357-63.

Navarro, V. M., J. M. Castellano, R. Fernandez-Fernandez, S. Tovar, J. Roa, A. Mayen, M. L. Barreiro, F. F. Casanueva, E. Aguilar, C. Dieguez, L. Pinilla, and M. Tena-Sempere. 2005. 'Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone

secretion in the rat', *Endocrinology*, 146: 1689-97.

Perello, M., R. C. Stuart, and E. A. Nillni. 2006. 'The role of intracerebroventricular administration of leptin in the stimulation of prothyrotropin releasing hormone neurons in the hypothalamic paraventricular nucleus', *Endocrinology*, 147: 3296-306.

Reichlin, S. 1989. 'Neuroendocrinology of the pituitary gland', *Toxicol Pathol*, 17: 250-5.

Roa, J., D. Garcia-Galiano, L. Varela, M. A. Sanchez-Garrido, R. Pineda, J. M. Castellano, F. Ruiz-Pino, M. Romero, E. Aguilar, M. Lopez, F. Gaytan, C. Dieguez, L. Pinilla, and M. Tena-Sempere. 2009. 'The mammalian target of rapamycin as novel central regulator of puberty onset via modulation of hypothalamic Kiss1 system', *Endocrinology*, 150: 5016-26.

Roseweir, A. K., A. S. Kauffman, J. T. Smith, K. A. Guerriero, K. Morgan, J. Pielecka-Fortuna, R. Pineda, M. L. Gottsch, M. Tena-Sempere, S. M. Moenter, E. Terasawa, I. J. Clarke, R. A. Steiner, and R. P. Millar. 2009. 'Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation', *J Neurosci*, 29: 3920-9.

Ruggiero, D. A., H. Baker, T. H. Joh, and D. J. Reis. 1984. 'Distribution of catecholamine neurons in the hypothalamus and preoptic region of mouse', *J Comp Neurol*, 223: 556-82.

Suzuki, S., and R. J. Handa. 2004. 'Regulation of estrogen receptor-beta expression in the female rat hypothalamus: differential effects of dexamethasone and estradiol', *Endocrinology*, 145: 3658-70.

Tan, H. Y., L. Huang, D. Simmons, J. D. Veldhuis, F. J. Steyn, and C. Chen. 2013. 'Hypothalamic distribution of somatostatin mRNA expressing neurones relative to pubertal and adult changes in pulsatile growth hormone secretion in mice', *J Neuroendocrinol*, 25: 910-9.

Tsutsui, K., G. E. Bentley, L. J. Kriegsfeld, T. Osugi, J. Y. Seong, and H. Vaudry. 2010. 'Discovery and evolutionary history of gonadotrophin-inhibitory hormone and kisspeptin: new key neuropeptides controlling reproduction', *J Neuroendocrinol*, 22: 716-27.

van Swieten, M. M., R. Pandit, R. A. Adan, and G. van der Plasse. 2014. 'The neuroanatomical function of leptin in the hypothalamus', *J Chem Neuroanat*, 61-62: 207-20.

Wang, L., M. Goebel-Stengel, A. Stengel, S. V. Wu, G. Ohning, and Y. Tache. 2011. 'Comparison of CRF-immunoreactive neurons distribution in mouse and rat brains and selective induction of Fos in rat hypothalamic CRF neurons by abdominal surgery', *Brain Res*, 1415: 34-46.

Wittmann, G., T. Fuzesi, P. S. Singru, Z. Liposits, R. M. Lechan, and C. Fekete. 2009. 'Efferent projections of thyrotropin-releasing hormone-synthesizing neurons residing in the anterior parvocellular subdivision of the hypothalamic paraventricular nucleus', *J Comp Neurol*, 515: 313-30.

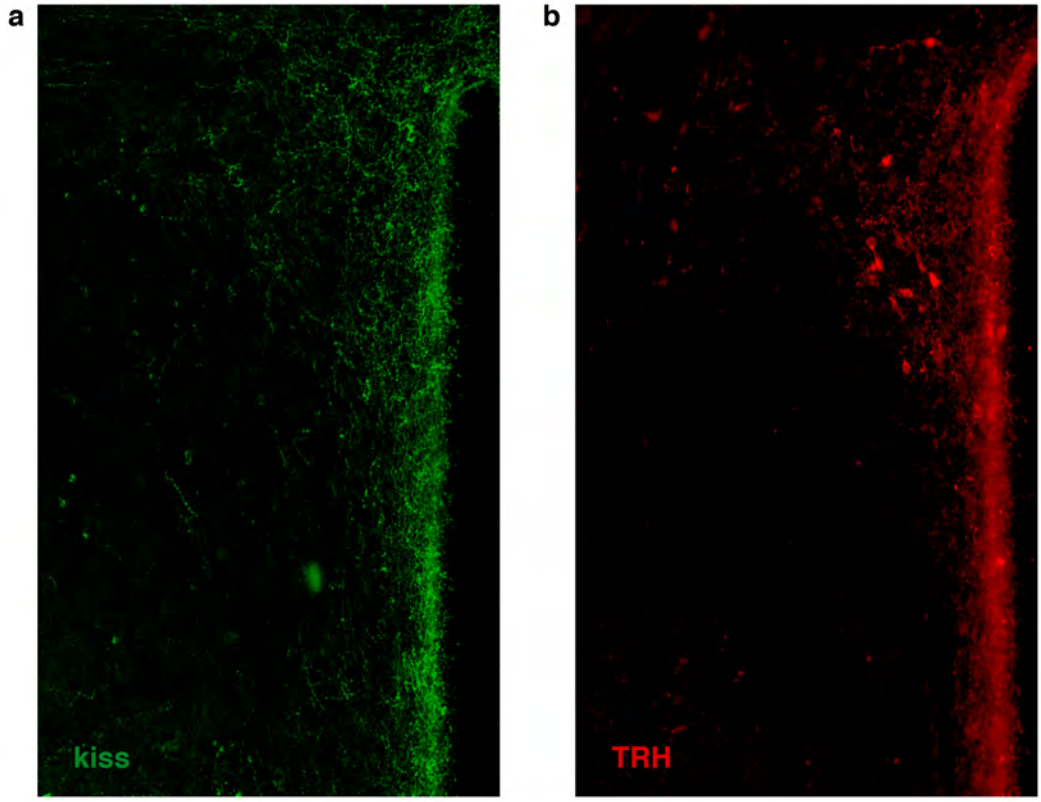

Figure 1. Distribution of **(a)** kisspeptin fibers (kiss, green) and **(b)** TRH structures (TRH, red) within PVN in adult female CD1 mice.

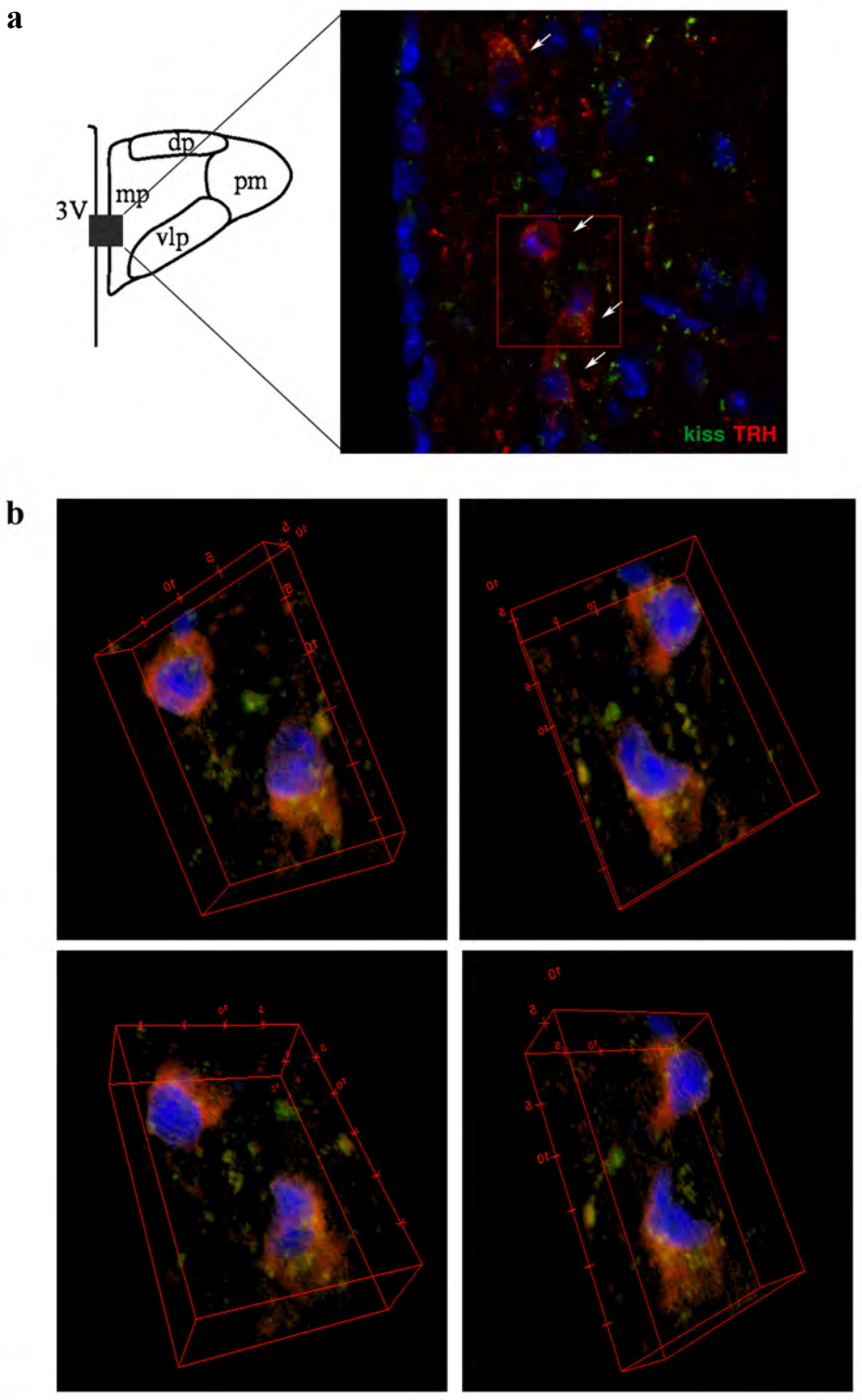

Figure 2. (**a**) On left, a representative scheme of PVN devise in dorsoparvocellular (dp), medialparvocellular (mp), posteriormagnocellular (pm) and ventrolateralparvocellular (vlp). On right, the co-localization of kisspeptin fibers (kiss, green) and TRH neurons (TRH, red) in medial part of PVN (indicated with gray quadrant in mp portion of PVN scheme) in adult female CD1 mice. (**b**) Reconstructed volume 3D view of homolog present in red quadrant in panel **a** (right) generated by rotating it clockwise about the y-axis.

CHAPTER 7

Experiment 5

'Sexual dimorphic organizational effect of early postnatal genistein administration on mice hypothalamic neuroendocrine circuits'

Sexual dimorphic organizational effect of early postnatal genistein administration on mice hypothalamic neuroendocrine circuits

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Abstract

Introduction: Genistein (GEN), a phytoestrogen contained in soy and other legumes, may interfere with the endocrine system in multiple ways, including permanent morphological alterations of estrogen sensitive circuits in adult brain. Several estrogen-sensitive systems are influencing food intake and energy expenditure (NPY, POMC, Orexin). Among them, the Kisspeptin system, originally identified as regulator of puberty and fertility, is an important target for neuroendocrine disruption.

Material and methods: We analyzed the effects during development of an early postnatal treatment (from PND1 to PND8) with GEN (50 mg/kg body weight, a dose comparable to the exposure level in babies fed with soy-based formulas) on adult CD1 mice of both sexes. We have immunohistochemically evidenced the expression of the POMC, Orexin and Kisspeptin within different hypothalamic nuclei. In addition, we tested different physiological parameters related to metabolism and reproductive system (steroid hormones concentration, mammary gland, gonads, uterus, vaginal opening).

Results: Postnatal exposure to GEN induced sexually dimorphic effects, which were more severe in females. Body weight increase and plasmatic leptin and T3 concentration decrease was limited to females while POMC, Orexin and Kisspeptin immunoreactivity were significantly altered in animal

treated with GEN, during development, but especially in adult and in female. Moreover, GEN treated males showed only a minor decrease of testicles' weight and a significant decrease of fecal testosterone concentration. In females, GEN treatment induced an advanced pubertal onset (premature vaginal opening) and an altered estrous cycle: in fact, the concentration of progesterone increased in the plasma and the mammary gland present more tertiary branches.

Conclusion: Early postnatal exposure of CD1 mice to GEN determines long-term sex specific organizational effects. It impairs reproductive system and have an obesogenic effect, which is due, at least partly, to alteration of metabolic regulation, thus GEN may be classified as a Metabolism disrupting chemical.

Introduction

Genistein (GEN; 4', 5, 7-trihydroxyisoflavone) is an isoflavonoid compound of the Leguminosae (Dixon and Ferreira 2002). Its chemical structure shares structural features with the potent estrogen estradiol-17β, including the ability to bind estrogen receptors. In fact, GEN has a strong estrogenic activity, and is, thus, considered as a phytoestrogen (Polkowski et al. 2000). The main sources of GEN, in our diet, are soybeans and soy-based foods. Although most foods only contain a small amount of isoflavones, when eaten regularly and from a range of sources, they can reach a cumulative dose that may contribute to long-term effects (Liggins et al. 2000; Sureda et al. 2017). Infants may be exposed to higher levels of phytoestrogens (6–9 mg/kg/day) than typical adult exposures (approximately 1 mg/kg/day) (Setchell et al. 1997). In fact, in addition to soy-based infant formulas also many soy-based foods that are specifically aimed at children (Franke, Custer, and Tanaka 1998) (Setchell et al. 1997). Indeed soy is well recognized as a hormonally active food (Patisaul and Belcher 2017) which high use is increasingly raising concern. Studies have associated its use with elevated risk of menstrual irregularities and uterine fibroids (D'Aloisio et al. 2010) (Strom et al. 2001). Moreover, many studies demonstrated a high endocrine disrupting activity of GEN. Sex specific alterations were reported particularly in hypothalamus, a region hormone sensitive (Patisaul 2007; Patisaul 2006; Losa et al. 2011; Ponti et al. 2017), as orexigenic and anorexigenic systems that influence food intake and energy expenditure.

Two different neuronal populations localized in the arcuate nucleus (ARC) regulate the central control of food-intake. The anabolic, orexigenic, neurons are characterized by the co-expression of agouti-related peptide (AgRP) and neuropeptide Y (NPY), while the catabolic, anorexigenic, neurons co-express pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). These neurons are sensitive both to endocrine and to peripheral signals of energy balance. For instance, leptin, an adipose satiety hormone, stimulates POMC (Cowley et al. 2001; Elias et al. 1999) and inhibits NPY neurons (Morrison et al. 2005). High concentrations of leptin tend to hyperpolarize orexin-A/hypocretin-1 (OX) neurons clustered in lateral hypothalamus (LH) and implicated in feeding behavior and energy homeostasis. OX modulate energy balance according to food intake in response to physiological variation in glucose levels between meals (Messina et al. 2014). Moreover, OX signaling promotes obesity resistance by enhancing spontaneous physical activity and energy expenditure (Chieffi et al. 2017). Interestingly, all those system converge to the paraventricular nucleus (PVN), the most important centre of metabolic control. Peptides released

from NPY (AgRP) and POMC neurons (as α-melanocyte-stimulating hormone, α-MSH) are directly released in PVN. Beside them, leptin acts indirectly through kisspeptin (kiss) neurons. Kiss neurons are located in the ARC and in rostral periventricular area of the third ventricle (RP3V) and project to PVN. Kiss neurons seem to have a major nodal role, integrating the different peripheral and central signals transmitting metabolic information onto reproductive centres (Castellano and Tena-Sempere 2016).

Many evidences suggest that GEN or other soy phytoestrogens could have an obesogenic effect (Newbold 2010) or might counteract aspects of metabolic syndrome (Jungbauer and Medjakovic 2014). Studies in rats reveal that treatment with GEN change body weight, but the effect depend to sex, age, and hormonal status (Cao et al. 2015; Ruhlen et al. 2008; Slikker et al. 2001).

In the present study we analyzed the effect of postnatal administration of GEN, in a dose comparable to exposure of babies fed with soy-based formulas (Cimafranca et al. 2010) on the metabolism and reproductive system. We examined both peripheral parameters and the three hypothalamic systems (POMC, OX and kiss) implicated on control of food intake and metabolic regulation.

Materials and methods

Animals

In this experiment we used 20 female and 10 male adult virgin CD-1 mice purchased from Janvier Breeding Centre (Le Genest Saint-Isle, France) and maintained as an outbreed colony at INRA, Tours, France. All animal studies were performed in accordance with the French and European legal requirements (Decree 2010/63/UE).

The animals housed in monosexual groups of 3 mice in conventional polycarbonate cages (45x25x15 cm) with water and food (Standard diet 150 low phytoestrogen certificate, SAFE, France) ad libitum. After 2 weeks of adaptation period on animal house, females were housed with males in groups of 2 females and 1 male for one night, beginning at 18:00 h (at the end of the light phase of the 12-h light/dark cycle); after the mating, verified by the presence of a vaginal plug (generally 3-5 days), the females were placed in single cages.

Genistein treatment

The day after parturition, litters were reduced at 8 pups, 4 males and 4 females; pups were allocated randomly to three groups and subjected to oral administration of vehicle (sesame oil) or genistein (GEN 50 mg/Kg body weight; cat. Number G6649, Sigma-Aldrich, Milan, Italy) diluted in sesame oil. This protocol mimics the exposure of babies fed with soy-based formulas (Cimafranca et al. 2010). Starting from post-natal day one (PDN1) to PDN8, mice were trained daily to spontaneously drink through a micropipette directly into the mouth (Ponti et al. 2017). Three weeks after birth (PND21), pups were weaned and housed in treatment-differentiated monosexual groups of 3-5 animals in polypropylene mouse cages.

We used in total 192 animals, we perfused 6 males and 6 females controls and treated at PND12, PND22, PND30 and PND60 (N=96) to analyze the effect of GEN on the brain circuits and we killed by decapitation the same number of animal (N=6 per group) to study the other parameters.

Peripheral parameters

Body weight, food consumption and Feed Efficiency (FE)

Body weight was recorded daily during the treatment and every two days from PND8 to sacrifice with an electronic precision balance. To eliminate differences in body weights due to the variability between animals randomly assigned to the groups, we transformed the absolute body weight into a percentage of the body weight of first day before the treatment (PND1) conventionally considered equal to 100%.

Animals were fed with a standard diet 150 low phytoestrogen certificate (SAFE, France) containing 3,264 Kcal/g of metabolizable energy with 21% as protein, 12,6% as lipid and 66.4% as carbohydrate. Mean food consumption (mean grams per mouse per day) was determined (Zammaretti, Panzica, and Eva 2007) every two days at 10.00 A.M. All animals from each group of mice were housed in standard cages (each containing 3 animals). In order to determine the amount of food consumption, we subtracted the weight of the residual food found in each cage from the total amount supplied to the animals. The obtained value was the total food consumption per cage per each period. To estimate the daily food consumption per animal, the total food consumption was divided for the number of mice housed in the cage and the number of days after the last measurement. After the measurement the mice were given fresh quantities of their food.

Daily energy intake was obtained by multiplying daily food intake by the caloric value of the chow (3,264 Kcal/g), and daily feed efficiency was expressed as body weight (g)/Kcal eaten (Heine et al. 2000).

Hormonal levels

Plasmatic progesterone, leptin and total triiodothyronine (T3) hormonal levels were measured in 4 groups of animals (N=6): control males (CON M), control females (CON F), treated males (GEN M) and treated females (GEN F). Samples for assay of plasmatic leptin were collected at PND12, PND22, PND31, PND60, while for progesterone and total triiodothyronine (T3) at PND30 and PND60. All animals were killed by decapitation and in particularly females were in diestrous phase (determined by examination of vaginal smears taken immediately before the sacrifice).

Samples were collected in the morning and centrifuged at 3000 g for 180'' /3' and freezed at -20°C until assayed. Samples were processed according to the manufacturer instructions with the following kits: progesterone EIA-1561, leptin ELI-4564, total triiodothyronine (T3) EIA-4569 (DRG Instruments GmbH, D).

We measured testosterone levels in feces at PND30 and PND60. Animals were isolated in a clean cage in the late morning. After 2 hours 1.7+/-0.3 ml of fecal pellet were collected and animals returned to their home cage. Pellets were stored at -80°C.

Extraction and determination of fecal testosterone were carried out as previously reported (Macchi et al. 2010). Briefly, samples were kiln-dried at 55°C for 24 h in an oven, thoroughly crushed and five parts (0.25 g each) of pulverized feces were put in extraction tubes with a Teflon-sealed cap. 3 mL of diethyl ether (Carlo Erba, Milan, Italy) were added to each tube and the mixture was vortexed for 10 min. The ether layer was recovered in another tube by decantation after freezing, and evaporated for 24 h. Extracted samples were brought to 2 mL liquid phase with 97–98% ethanol and PBS (pH 7.4 + 1% BSA) at a 1:1 ratio. Fecal testosterone level was determined using a multi-species testosterone enzyme immunoassay kit (K032; Arbor Assays, Ann Arbor, MI) validated for dried fecal extracts.

Vaginal opening and Estrous Cycle

Upon weaning, females (N =24 per group of treatment) were checked daily for day of vaginal opening (VO) from PND19 to PND29 in order to compare puberty onset in GEN treated and control mice (Fig. 1). In rodents, VO is a hallmark of puberty and was therefore used as a morphophysiological indicator of puberty and its modifications (anticipations or delays) are strong signals indicating an alteration of puberty onset.

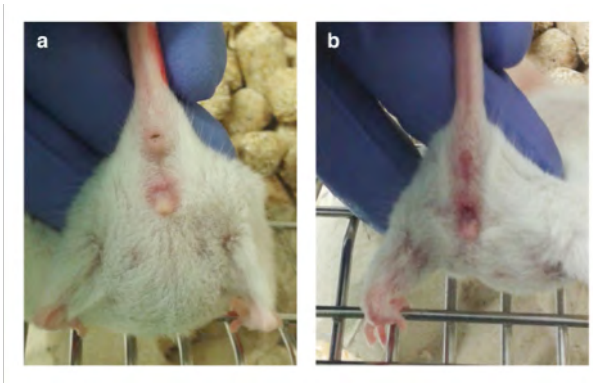

Fig. 1: The ano-genital region of female CD1 mice. Animal has still to reach the puberty onset (a); animal has reached puberty onset (b).

The estrous cycle was examined daily and identified under a microscope (×100) using a vaginal smear flushed with physiological saline for two weeks from PND40. Vaginal epithelial cell stained using toluidine blue. We measured as percentage the time spent in each phase of estrous cycle during these two weeks in CON F (N=6) and GEN F (N=6).

Uterus and testicles weight

The weight of reproductive organs, uterus and testicles, obtained from females (N=6) and males (N=6) mice were manually dissected and measured in each group at PND22, PN30 and PND60 after decapitation. Testicles weight was calculated summing the two testicles weights in each animal.

Gonads histology

Gonads (testicles, uterus and ovaries) were collected at PND22, PND30, PND60 from control and treated males (N=6 per group) and females (N=6 per group) in the diestrous phase. The gonads were removed after perfusion; they were fixed in PBS-paraformaldehyde (4%), pH 7.3, then processed and paraffin embedded according to histological routine procedures. Representative sections of each sample were stained with Hematoxylin and eosin (H&E) and evaluated at a light microscope.

Several classification systems are described in literature (Holstein, Schulze, and Davidoff 2003; Johnsen 1970; Nistal and Paniagua 1999; Silber et al. 1997) to analyze the scoring of spermatogenesis in testicles, all based on five main histological patterns of spermatogenesis: (i) absence of seminiferous tubules (tubular sclerosis); (ii) no germ cells within the seminiferous tubules (Sertoli cell-only syndrome); (iii) incomplete spermatogenesis, not beyond the spermatocyte stage (spermatogenic arrest); (iv) all germ cell stages present including spermatozoa, but there is a distinct decline in the number of germ cells (hypospermatogenesis); and (v) normal spermatogenesis.

Mammary gland analysis

Mammary glands were collected at PND22, PND30, PND60 from females in the diestrous phase of the cycle (as determined by vaginal smear).

Briefly, the fourth mammary gland (inguinal) was dissected from the skin, stretched on a glass slide and fixed in Carnoy's fixative at 4°C for 4 h. Whole mounts were gradually re-hydrated, stained with Carmine Alum (Stem Cell Technologies) overnight, distained for 2 hours in 70% EtOH with 2% HCl, progressively dehydrated, clarified in methylsalicilate overnight and photographed.

Whole mounts were photographed at 1x and 4x on a Leica S8AP0 stereomicroscope equipped with a Leica EC3 digital camera. Mammary gland length was measured as the distance between the start of the duct that arises from the nipple and the farther away ducts of the glands. Total number of branches at PND30 and PND60 were counted as the number of branches at 3 given distances from the upper tip of the lymphnode (-3,5 mm, 0 mm and 3,5 mm). The number of tertiary branches was arbitrarily estimated in a scale ranging from no branches (0) to high density (5).

Hypothalamic analysis

Fixation and sampling

We randomly divided the animals in 4 groups (N=6): control males (CON M), control females (CON F), treated males (GEN M) and treated females (GEN F). Mice were perfused at different ages: PND12, PND22, PND30 and PND60.

Animals were deeply anesthetized with intraperitoneal injection of tribromoethanol (250 mg/kg), monitored until the pedal reflex was abolished and killed by intracardiac perfusion with saline solution (NaCl 0,9%) followed by fixative (4% paraformaldehyde, PAF, in 0.1 M phosphate buffer,

pH 7.3). Females at PND30 and PND60 were in the same hormonal condition, they were killed in diestrus (tested by vaginal smear). Once, we removed and stored the brains in a freshly prepared PAF solution for 2 h at 4 °C, followed by washings in a 30% sucrose solution at 4°C overnight. Finally, brains were frozen in liquid isopentane pre-cooled in dry ice at -35 °C, and stored in a deep freezer at -80 °C until sectioning.

Brains were cut in coronal plane with a cryostat (Leica CM 1900) at 40 μm of thickness obtaining three series of adjacent serial sections, collected for free-floating procedure in multiwell plates, filled with a cryoprotectant solution (Watson et al. 1986) and kept at -20°C. We stained these three series respectively for kiss, POMC and OX immunohistochemistry. Brains were processed in groups containing both female and male sections of each treatment, so that between-assays variance could not cause systematic group differences. Sections were washed overnight in PBS at pH 7.3 before immunohistochemical processing. The following day, sections were permeabilized in PBS containing 0.2%Triton X-100 for 30 min then endogenous peroxidase activity was blocked with methanol/hydrogen peroxide solution (1:1) in PBS for 20 min at room temperature.

Kisspeptin immunohistochemistry

Sections were pre 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 biotinylated goat anti-rabbit IgG (Vector Laboratories) 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). The peroxidase activity was visualized with a solution containing 0.400 mg/mL of 3,30- 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).

Specificity of kiss antiserum for immunohistochemistry was reported in previous studies (Franceschini et al. 2006; Clarkson et al. 2009).

POMC immunohistochemistry

Sections were incubated with normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30 min and incubated overnight at 4 °C with the rabbit polyclonal antibody against POMC (POMC precursor 27-52, H029-30, Phoenix Pharmaceuticals, Inc.) diluted 1:5000 in PBS-Triton X-100 0,2%, pH 7.3–7.4. The following day, sections were incubated for 60 min in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) 1:250. The antigen-antibody reaction was revealed with the same protocol described for kiss immunohistochemistry.

Orexin immunohistochemistry

Sections were incubated in normal goat serum (Vector, California, USA, diluted 1:5 in PBS) for 30 min at room temperature. After we incubated the sections overnight in rabbit anti-orexin A primary antibody (Calbiochem) diluted 1:2000 in PBS at 4 °C. The second day, the sections were incubated before for 90 min with biotinylated anti-rabbit IgG serum (Pierce, Vector, California, USA, 1:200) and then with avidin-peroxidase complex (Immunopure ABC, Pierce, Vector, California, USA) for 60 min at room temperature. Finally, the presence of peroxidase activity was visualized with a solution containing 0.02g/ml diaminobenzidine (DAB, Aldrich, Madrid, Spain) and 0.025% hydrogen peroxidase in Tris-HCl, pH 7.6. The sections were mounted on gelatin-coated slides, ethanol dehydrated, washed in xylene and coverslipped with DPX (Surgipath Europe Ltd., Peterborough, UK).

Quantitative analysis

Three standardized serial sections of comparable level were selected for kiss system in three hypothalamic nuclei: arcuate nucleus (ARC; Bregma -1,58 mm; -1,70 mm; -1,82 mm), 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) according to the mouse brain atlas (Paxinos & Franklin, 2001). 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), by using a 40x (PVN) or a 20x (ARC and RP3V) objective. Digital images were processed and analyzed with IMAGEJ (version 1.47v; Wayne Rasband, NIH, Bethesda, MD, USA). Measurements were performed within predetermined fields (region of interest, ROI). The PVN, in each selected section, was divided into fourteen squares (each of 31,080 μm^2 at PND12; 37,057 μm^2 at PND22; 40,150 μm^2 at PND30 and PND60) 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 regions. The ROI for ARC changed during development (at PND12 551,574 μm^2; at PND22 602,454 μm^2 and in adult 889,728 μm^2). These were placed within the boundaries of the nuclei to cover the immunopositive region; we used as reference the third ventricle to position the ROI always in the same orientation. Positive kiss 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), by calculating, in binary transformation of the images (threshold function of Image J), the percentage of pixels covered by the positive structures in the predetermined ROIs (as previously performed in our laboratory; (Viglietti-Panzica et al. 1994)). Due to differences in the immunostaining, the range of the threshold was individually adjusted for each section. The results obtained were grouped to provide mean (± S.E.M.) values.

POMC system was analyzed in three selected standardized sections of comparable levels ARC adjacent to the ones stained for kiss (Paxinos and Franklin 2001). We measured the number of POMC positive cells and the FA. The methodology of acquisition, processing and quantification was identical to the one used for kiss system.

We measured the number of OX cells within four coronal sections through the region of lateral hypothalamic area (LH). The levels analyzed corresponded to Plates 40, 42, 44, and 48 from Paxinos's atlas of the mouse brain (Paxinos & Franklin 2001), which is where most OX-ir cells were found in the caudal hypothalamus. The sections were acquired by a digital camera (Olympus DP25) connected to Nikon eclipse E600 microscope using a 10x digitized images and digital images were processed and analyzed by Analyze Particles of IMAGEJ (version 1.47v; Wayne Rasband, NIH, Bethesda, MD, USA). All cells OX-ir were counted within a standardized area for all subjects that covered the entire hypothalamic region. The results obtained were grouped to provide mean (± S.E.M.) values.

Statistical analysis

Quantitative data were examined with SPSS statistic software (SPSS inc, Chicago, USA) by analysis of variance two-way ANOVA, where sex and treatment were considered independent variables,

or/and one-way ANOVA. When appropriated, we performed a multivariate test to compare groups. Differences between groups were considered significant for values of $p \leq 0.05$.

Results

Peripheral parameters

Body weight, food consumption and Feed Efficiency (FE)

We did not observe any acute effect of GEN administration during the treatment on the metabolic parameters considered. However, the body weight measured in grams of the control animals presented strong differences starting from PND30 (Table 1), the analysis by Tukey test for CON M vs. CON F reported a significant increase of weight in male (PND30, PND40, PND50 and PND60, p<0,001) (Fig.2).

Body weight (gr)

	CON M	GEN M	CON F	GEN F	ANOVA 1 WAY	
	MEAN±SEM	MEAN±SEM	MEAN±SEM	MEAN±SEM	F	p
PND1	1,83 ± 0,08	1,81 ± 0,05	1,71 ± 0,06	1,67 ± 0,07	0,695	0,566
PND8	4,88 3 ± 0,26	5,41 ± 0,16	4,28 ± 0,16	5,11 ± 0,16	3,084	0,051
PND12	6,63 ± 0,40	6,83 ± 0,30	6,13 ± 0,21	7,08 ± 0,17	0,974	0,424
PND18	9,10 ± 0,59	9,62 ± 0,50	8,27 ± 0,26	9,60 ± 0,33	1,015	0,407
PND22	13,18 ± 0,62	13,55 ± 0,80	12,11 ± 0,36	13,25 ± 0,43	0,579	0,635
PND30	26,08 ± 0,70	26,20 ± 0,69	20,32 ± 0,38	21,91 ± 0,10	15,614	0,001
PND40	32,05 ± 0,60	32,15 ± 0,55	23,67 ± 0,61	26,47 ± 0,24	33,589	0,001
PND50	34,32 ± 0,41	35,80 ± 0,71	25,13 ± 0,55	28,27 ± 0,35	45,681	0,001
PND60	36,30 ± 0,65	37,50 ± 0,58	25,7 ± 0,77	29,93 ± 0,65	34,64	0,001

Table 1: Body weight gain (expressed as grams) during the development for different groups of CD1 mice are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

Differences were observed also in the percentage of body weight gained since PND 0 (Table 2 and Fig.3a-b). Two way ANOVA for sex and treatment confirmed this result (respectively F=15,39; p= 0,001 and F=5,901; p=0,025). No differences were present between controls and treated males (Fig.3a), while a very interesting increase from PND40 was calculated in GEN F respect at the

control females. The GEN treatment caused a significant increase of percentage of body weight limited adult females (Tukey test, CON F vs. GEN F at PND40 p=0,007; at PND50 p=0,001; at PND60 p=0,005) (Fig.3b).

Body weight gain (%)

	CON M	GEN M	CON F	GEN F	ANOVA 1 WAY	
	MEAN±SEM	MEAN±SEM	MEAN±SEM	MEAN±SEM	F	p
PND1	100	100	100	100		
PND8	266,36 ± 14,37	298,17 ± 8,96	249,51 ± 9,42	307,00 ± 9,94	1,99	0,148
PND12	361,82 ± 22,04	376,15 ± 16,90	357,28 ± 12,43	425,00 ± 10,42	1,703	0,199
PND18	496,36 ± 32,22	529,36 ± 28,02	418,55 ± 15,21	576,00 ± 20,23	1,352	0,286
PND22	719,09 ± 33,68	745,87 ± 44,55	705,83 ± 20,88	759,00 ± 26,06	0,976	0,424
PND30	1422,73 ±33,22	1442,20 ± 38,44	1183,50 ± 22,19	1315,00 ± 6,33	7,501	0,001
PND40	1748,18 ± 33,22	1769,72 ± 30,81	1361,17 ± 35,82	1588,00 ± 14,35	18,14	0,001
PND50	1871,82 ± 22,59	1970,64 ± 39,12	1464,08 ± 32,37	1696,00 ± 20,948	29,788	0,001
PND60	1980,00 ± 35,54	2064,22 ± 31,75	1497,09 ± 44,95	1796,00 ± 38,96	17,134	0,001

Table 2: Body weight gain (expressed as percentage) during the development for different groups of CD1 mice are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

Daily food consumption per each animal during the weeks after weaning was calculated as explained above (see methods). Generally, we observed a reduction in females' food consumption respect to control males, but no differences were found between control and treated animals (Table 3 and Fig.3c). The two-way ANOVA for repented measures, being sex and treatment the independent variables and weekly food consumption the repeated measure, showed in fact an effect of sex (F=21,015 and p=0,001) but not of treatment (F=0,165 and p=0,689) on food consumption and of interaction between sex and treatment (F=0,056 and p=0,815).

Daily food eaten (gr)

	CON M	GEN M	CON F	GEN F	ANOVA 1 WAY	
	(mean±SEM)	(mean±SEM)	(mean±SEM)	(mean±SEM)	F	*p*
week1	3,13±0,24	3,25±0,34	2,99±0,23	3,11±0,34	0,227	0,877

week2	5,03±0,26	4,97±0,09	4,16±0,16	4,06±0,30	5,523	0,006
week3	6,26±0,19	6,26±0,46	5,63±0,32	5,49±0,38	1,354	0,285
week4	5,80±0,20	5,50±0,05	4,75±0,23	4,72±0,08	11,832	0,001
week5	5,50±0,32	5,25±0,24	4,55±0,32	4,56±0,21	3,095	0,050

Table 3: Daily food eaten (expressed as grams) during five weeks after weaning for different groups of CD1 mice are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

Feed efficiency (body weight gain/Kcal) was statistically analyzed by two-way ANOVA for repeated measures (with sex and treatment as independent variables and feed efficiency as repeated measure) demonstrating a global effect of sex (F=6,722 and p=0,0017) but no of treatment (F=0,704 and p=0,411). The multiple comparison between groups displayed that effectively there was significant increase CON M vs. CON F at last week (Tukey test, p=0,038), while no differences were observed in feed efficiency between control and treated animals, males and females (Table 4 and Fig.3d).

Daily feed efficiency (Body weight/Kcal)

	CON M	**GEN M**	**CON F**	**GEN F**	**ANOVA 1 WAY**	
	(mean±SEM)	(mean±SEM)	(mean±SEM)	(mean±SEM)	F	*p*
week1	1,23 ± 0,05	1,17 ± 0,06	1,19 ± 0,03	1,14 ± 0,04	0,245	0,864
week2	1,41 ± 0,05	1,44 ± 0,05	1,38 ± 0,02	1,39 ± 0,06	0,116	0,950
week3	1,40 ± 0,02	1,43 ± 0,03	1,24 ± 0,02	1,28 ± 0,04	4,064	0,021
week4	1,69 ± 0,03	1,78 ±0,03	1,50 ± 0,04	1,59 ± 0,03	5,959	0,004
week5	1,91 ± 0,02	2,08 ± 0,04	1,70 ± 0,03	1,78 ± 0,04	10,467	0,001

Table 4: Daily feed efficiency (expressed as body weight gain/Kcal) during five weeks after weaning for different groups of CD1 mice are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

Hormonal levels

Leptin blood levels were low in control animals at PND12 (females vs. males; p=0,875) and PND22 (p=1). While in control females, leptin levels stayed low at PND30 (PND30 vs. PND22; p=1) and significantly increased at PND60 (PND60 vs. PND30; p<0,001). In control males, leptin levels increased at PND30 (PND30 vs. PND22; p=0,001) then decreased at PND60 (PND60 vs. PND30;

p<0,001). Interestingly GEN treatment caused an increase in leptin level at PND12 (GEN M vs. CON M; p<0,001) and a decrease at both PND30 (GEN M vs. CON M; p=0,032) and P60, although to a lesser extent (GEN M vs. CON M; p=0,456) (Fig. 4a). On the other hand, at PND12 and PND22 there was no difference between GEN vs CON F (p=1,00) in plasmatic leptin concentration, but very interesting was an anticipated peak at PND30 in GEN females (GEN F vs. CON F; p<0,001) and present at PND60 in Control females (CON F vs. GEN F; p<0,001) (Fig. 4b).

Plasmatic Leptin (pg/ml)

	CON M (MEAN±SEM)	GEN M (MEAN±SEM)	CON F (MEAN±SEM)	GEN F (MEAN±SEM)
PND12	5,4±0,12	58,5±1,03	15,6±4,82	15,0±5,24
PND22	6,8±1,09	6,4±0,70	5,7±0,59	5,0±0,68
PND30	61,3±6,95	42,2±6,95	9,7±0,45	40,7±3,11
PND60	22,15±0,82	9,275±2,42	43,6±1,53	14,7±2,96

Table 5: Concentration of plasmatic leptin (expressed as pg/ml) at PND12, PND22, PND30 and PND60 for different groups of CD1 mice are reported in the other columns (Mean±SEM).

T3 blood levels in control young animals did not show a sexual dimorphism (PND30 CON F vs. PND30 CON M; p=1,00). T3 levels increased in adult females (PND60 CON F vs. PND30; p=0,001), while did not change significantly in males (PND60 CON M vs. PND30; p=0,491). Interestingly, GEN treatment did not affect T3 levels in males nor in young (PND30 GEN vs. PND30 CON; p=0,999) nor in adults (PND60 GEN vs. PND60 CON; p=1,00). However, GEN treatment had a pivotal role on T3 levels in females: it tended to increase it in young animals (PND30 GEN vs. PND30 CON; p=0,001), while it decreased significantly in adults (PND60 GEN vs. PND60 CON; p=0,055; Fig 5).

Plasmatic T3 (ng/ml)

	CON M (MEAN±SEM)	GEN M (MEAN±SEM)	CON F (MEAN±SEM)	GEN F (MEAN±SEM)
PND30	5,3±0,32	5,8±0,28	5,6±0,19	6,2±0,34
PND60	6,41±0,06	5,7±0,11	7,5±0,19	6,3±0,09

Table 6: Concentration of plasmatic T3 (expressed as ng/ml) at PND30 and PND60 for different groups of CD1 mice are reported in the other columns (Mean±SEM).

Progesterone levels, in control females did not change significantly from puberty to adulthood (PND30 CON 6.84±1.19; PND60 CON 6.73±0.90; p=0,565). GEN treatment did not alter progesterone levels at PND30 (4.41±0.46; p=0.41) while it strongly increases it in adults (PND60 GEN 14.12±1.77; p=0,007) (Fig. 6d).

Fecal testosterone levels did not display significant differences in females nor at PND30 (CON vs. GEN; p=1,00) nor at PND60 (CON vs. GEN; p=1,00), although adult females had slightly more testosterone than young ones (p=1,00). On the other hand, control males had higher levels of fecal testosterone, already at PND30 (CON M vs. CON F; p=0,024). At this age in male GEN treatment did not result in a significant difference of testosterone levels compared to controls (GEN vs. CON; p=1,00). Control males had higher testosterone levels at PND60 (CON vs. P30 CON; p<0,001) while GEN treated mice did not show this increase (PND60 GEN vs. PND30 GEN; p=0,992) resulting in significantly lower levels than in control adults (p=0,003) (Fig.6f).

Fecal Testosterone (ng/gr)

	CON M (MEAN±SEM)	**GEN M** (MEAN±SEM)	**CON F** (MEAN±SEM)	**GEN F** (MEAN±SEM)
PND30	0,62±0,07	0,59±0,10	0,1±0,03	0,04±0,02
PND60	1,12±0,09	0,5±0,16	0,17±0,04	0,14±0,05

Table 7: Concentration of fecal testosterone (expressed as ng/gr) at PND30 and PND60 for different groups of CD1 mice are reported in the other columns (Mean±SEM).

Vaginal Opening and Estrous Cycle

Vaginal opening (VO) is one of the parameters employed to evaluate the potential effects of endocrine chemical disruptors on the physiological puberty onset in rodents. Compared by Student's t-test with the control animals, the GEN administered altered the timing of VO (CON F= 27,8 ± 0,29; GEN F= 26,5 ± 0,30), in fact VO was significantly advanced (p=0,005) (Fig. 6a).

The GEN treatment caused an alteration on estrous cycle of females; GEN F, in fact spent a lot of time in Estrus and Diestrus phases compared to CON F (Estrus=30% and Diestrus=43% vs. 35% and 51% respectively in GEN F) with a co respective significant reduction on Proestrus phase (t-Test, p=0,03; CON F= 16,67 vs. GEN F= 6,67) (Fig.6c).

Uterus and testicles weight

At PND22 the uterus weight of GEN F was significantly higher than CON F compared by Student's t-test (p=0,035), but these difference was not present during adult ages (PND30, p=0,120 and PND60, p=0,123) (Fig.6b).

Totally opposed was the situation for testicles weight, no differences were showed by Student's t-test at PND22 (p=1) and at PND30 (p=0,207), but in adult was observed a tissue weight significantly lower in GEN M than CON M (p=0,05) (Fig.6e).

Gonads histology

Examination of histologic uterus sections reveals some alterations in all animals. Severa edema in endometrium and hydrometra were observed in some treated animals at PND22 and PND30.

Histologically, occasionally presence of hyperplasia, pseudo-stratification and discontinuity of the endometrial lining epithelium was detected. Presence of inflammatory cells including a few neutrophils, macrophages and lymphocytes in the subepithelial layer and stratum compactum was shown. There was loosening of endometrial connective tissue stroma due to presence of oedematous fluid. Periglandular infiltration of lymphocytes and plasma cells along with atrophy and degeneration of endometrial glands were observed. One case of severe apoptosis in glandular and basal cells of epithelium was observed. Severe hemorrhage and hyperemia and congestion were also present.

In the ovaries atretic follicles and corpora lutea were identified in all treated animals at PND22. Atretic follicles were characterized by the presence of degenerating oocytes and/or apoptotic bodies among the granulosa cells. In contrast, the corpora lutea were identified by the presence of large pale-staining granulosa lutein cells. No morphometric measurement in the ovaries was performed; therefore microscopic observation did not allow us to define differences in treated animals compared to controls.

Degeneration, vacuolization, apoptosis or apoptotic bodies in testicles were recorded as following: absent (0), moderate (1), or severe (2) and reported in Table 8.

Spermatogenesis score, apoptosis and degeneration in male animals

	Control Male(C)	Spermato-genesis Score	Apoptosis	Degene-ration	Treated Male(T)	Spermato-genesis Score	Apoptosis	Degene-ration
PND12	**C1**	0	0	0	**T1**	1	0	0
	C2	1	0	0	**T2**	1	0	1
	C3	1	0	0	**T3**	1	0	0
	C4	1	0	0	**T4**	1	0	0
	C5	1	0	0	**T5**	1	0	0
	C6	1	0	0	**T6**	1	0	1
PND22	**C1**	2	0	1	**T1**	2	0	1
	C2	2	0	1	**T2**	2	0	1
	C3	2	0	1	**T3**	2	0	1
	C4	2	0	2	**T4**	2	0	2
	C5	2	0	1	**T5**	2	0	1
	C6	2	0	1	**T6**	2	0	1
PND30	**C1**	2	0	1	**T1**	2	2	1
	C2	2	2	0	**T2**	2	2	0
	C3	2	2	0	**T3**	2	1	0
	C4	3	2	1	**T4**	3	2	1
	C5	3	0	0	**T5**	3	0	0
	C6	3	1	1	**T6**	3	1	1
PND60	**C1**	4	0	0	**T1**	3	2	0
	C2	4	0	0	**T2**	3	2	1
	C3	3	1	0	**T3**	3	2	0
	C4	4	1	1	**T4**	4	0	1
	C5	3	1	0	**T5**	4	0	0
	C6	4	0	0	**T6**	3	1	0

Table 8: Spermatogenesis score, apoptosis and degeneration in testicles of control (C) and treated (T) male animals at PND12, PND22, PND30 and PND60 reported as absent (0), moderate (1), or severe (2)

Transverse section of testes from control mice showing spermatogenic cells at different stages of maturity and well developed interstitial cells. The seminiferous tubules are closely packed with clusters of Leydig cells and each tubule consists of intact tubular wall with the outer most basement membrane, spermatogonia and Sertoli cells resting on the membrane (Fig. 7). However, several histological changes were observed in the seminiferous tubules in mice exposed to genistein during

postnatal age. These changes included disorganized seminiferous tubules with severe epithelial vacuolation (Fig. 7e), and epithelial apoptosis. In any cases, testicular pathological changes included severe hypospermatogenesis and the absence of seminiferous epithelium and Sertoli cells in tubules (Fig. 7f).

Mammary gland analysis

Mammary gland length gradually increased with development with no significant differences between experimental groups. At PND22 gland length was 3.90±0.21 while in GEN treated animals was 3.03±0.39 (p=0.145). Mammary gland length tended to increase faster in PND30 control females, reaching 12.93±0.86 mm, compared to GEN treated females 10.55±0.19, although not significantly (p=0.068). However, we did not observe any difference in the length of mammary glands in adults (CON= 22.03±0.98, GEN= 21.65±2.51; p=0.896) (Fig. 8a).

We observed that the number of terminal end buds (TEBs) was not significantly affected by GEN treatment at any of the developmental stages we considered. TEB were present at PND22, although with a higher variability in control animals (CON= 7.00±4.36; GEN= 13.00±2.08; p=0.301, not shown). Their number was maintained in PND30 animals, with a decrease in the variability among animals in each group (CON= 10±1.91, GEN= 13.50±0.65; p=0.165; Fig. 8b). As expected (Macias and Hinck 2012), control mammary glands did not have TEBs. Similarly, no TEBs were observed in PND60 GEN treated females.

Moreover, the treatment did not affect the overall architecture of adult mammary gland. In fact the number of branches was similar in CON (6.67±1.44) and GEN treated females (7.17±1.47; p=0.408). However, the number of tertiary branches was higher in GEN treated females (Fig. 8c).

Hypothalamic systems

Kisspeptin system

We analyzed the effect postnatal administration of GEN during the development at four ages, PND12, PND22, PND30, PND60, on kiss system in three different hypothalamic nuclei: ARC, RP3V and PVN. The pattern of distribution of kiss-ir structures observed within the hypothalamic region of control animals was according to previous observations in rodents (Clarkson and Herbison 2006).

The quantitative analysis of CON and treated groups suggested that postnatal exposure to GEN

significantly altered the development of kiss peptide expression in the hypothalamic nuclei under study in a dimorphic manner. We measured the FA in ARC, where was difficult to distinguish body cells due to the high density of kiss-ir processes. Very interesting was the GEN effect at PND12, where two-way ANOVA revealed a significant interrelation between sex and treatment (F=155,539 and p<0,001). The analysis by Bonferroni test demonstrated that in GEN M was a significant increase compared to male controls (p<0,001) (Fig. 9a), while in GEN F a significant decrease (p<0,001) (Fig. 9b), reversing completely the basic dimorphism (Fig. 9a-b); in fact CON F showed an higher kiss-ir density than CON M (P<0,001), but with the treatment the FA was lower in GEN F respect to GEN M (p<0,001). No difference detected in males during the rest of development (at PND22 p=1; at PND30 p=0,179; at PND60 p=1), while the situation in female was different. At PND22 the FA in GEN F remained significantly lower than CON F (p=0,004), but after the puberty age at PND30 the kiss-ir signal increased in females treated (p<0,001). This peak in control animals was present in adult at PND60, where the FA in CON F resulted higher than GEN F (p<0,001). So there seems to be an anticipation of the system after GEN treatment in females at PND30 (Fig. 9b).

Kisspeptin-ir (Fractional Area) in ARC

	CON M	GEN M	CON F	GEN F	ANOVA 1 WAY	
	(MEAN±SEM)	(MEAN±SEM)	(MEAN±SEM)	(MEAN±SEM)	F	P
PND12	1,4±0,008	7,0±0,02	5,4±0,42	1,8±0,44	53,247	<0,001
PND22	1,9±0,179	2,4±0,32	12,0±0,89	8,7±0,42	64,367	<0,001
PND30	6,1±0,40	4,5±0,36	21,1±0,43	25,9±0,46	664,655	<0,001
PND60	9,2±1,33	8,9±0,31	25,2±1,53	12,9±0,51	58,391	<0,001

Table 9: Quantitative data for kisspeptin-ir (FA) in ARC, for different groups and different age of sacrifice of CD1 mice, are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

Within RP3V we analyzed the number of kiss cells, throughout development the system resulted dimorphic, as in other nuclei measured, in CON F the number of cells was higher than CON M. In males the two-by two comparison (Bonferroni test) demonstrated that no significant difference was present between treated and control animals (at PND12 p=0,429; at PND22 p=0,140; PND30 p=1; at PND60 p=1)(Fig. 9c). The effect of GEN was clearly showed in females, the number of cells was significantly increased in GEN F at PND12 (p=0,002), at PND22 (p=0,002) and PND30 (p=0,003). At PND60 the situation was completely reversed, in fact while in GEN F the number of kiss cells

remained medially stable (20,2±1,29 at PND30 and 19,6±0.93 at PND60), in control females increased significantly compared to treated animals (p<0,001) (Fig. 9d).

Kisspeptin positive cells in RP3V

	CON M	GEN M	CON F	GEN F	ANOVA 1 WAY	
	(MEAN±SEM)	(MEAN±SEM)	(MEAN±SEM)	(MEAN±SEM)	F	p
PND12	1,8±0,12	2,3±0,20	2,6±0,19	4±0,29	19,146	<0,001
PND22	2±0,20	3,5±0,18	7,5±0,46	10,5±0,69	73,769	<0,001
PND30	1,67±0,10	2,8±0,23	12,9±1,57	20,2±1,29	79,593	<0,001
PND60	1,6±0,30	2,2±0,42	31,7±2,23	19,6±0,93	149,712	<0,001

Table 10: Quantitative data for number of Kisspeptin cells in RP3V, for different groups and different age of sacrifice of CD1 mice, are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

Unlike other analyzed nuclei, where the kiss system was dimorphic throughout development, in PVN the sex difference was present from PND22; in fact, while in males the density of kiss-ir fibres was steady at all ages, in females increased strongly starting from PND22. The comparison by Bonferroni test between CON F and CON M confirmed the dimorphism (at PND12 p=0,493; at PND22, at PND30, PND60 p<0,001). No difference at any age was measured in males after postnatal GEN treatment (Fig. 10a left), while an anticipatory effect was present in females. The kiss-ir peak that in CON F was related at PND60 (p<0,001 CON F vs. GEN F), in GEN F the same density peak resulted at PND30 (p<0,001 GEN F vs. CON F) (Fig. 10b right).

Kisspeptin-ir fibers in PVN

	CON M	GEN M	CON F	GEN F	ANOVA 1 WAY	
	(MEAN±SEM)	(MEAN±SEM)	(MEAN±SEM)	(MEAN±SEM)	F	p
PND12	1,4±0,17	1,6±0,27	0,9±0,29	1,1±0,21	3,468	0,059
PND22	1,3±0,15	1,2±0,19	3,2±0,18	3,8±0,28	40,045	<0,001
PND30	1,1±0,14	1,1±0,23	3,7±0,19	6,7±0,31	134,996	<0,001
PND60	0,9±0,13	1,2±0,20	6,6±0,38	3,5±0,49	73,344	<0,001

Table 11: Quantitative data for kisspeptin-ir fibers (FA) in PVN, for different groups and different age of sacrifice of CD1 mice, are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

As demonstrated in our previous study, the distribution of PVN kiss-ir fibres appeared not homogeneous in PVN comparing the medial with the lateral part of the nucleus (Marraudino et al. 2017). For these we identified fourteen squares to divide in a more detailed way the nucleus in dorso-lateral, ventro-lateral, dorso-medial and ventro-medial part (Fig. 10b). In general, in male controls the highest kiss-ir signal was in ventro-medial part, but the differences were so low that they were not significant at any age and also with the GEN treatment no alteration was detected. Instead in females the GEN effect was well visible within dorso and ventral-medial parts, where the kiss-ir fibres were denser. At PND30 the FA in GEN F resulted in comparison with CON F higher in dorso-medial ($p<0,001$), but especially in ventro-medial part of PVN ($p<0,001$). In adult, at PND60 in control females the density of kiss fibres increased strongly only in ventral-medial part of the nucleus, where was present a significant difference between CON F vs. GEN F ($p<0,001$) (Fig. 10c).

Kisspeptin-ir fibres within subdivisions of PVN

		CON M	GEN M	CON F	GEN F	ANOVA 1 WAY	
		(MEAN±SEM)	(MEAN±SEM)	(MEAN±SEM)	(MEAN±SEM)	F	p
PND12	DL	1,2±0,27	1,1±0,20	0,4±0,12	0,3±0,09	6,31	0,011
	DM	1,4±0,13	1,6±0,20	0,9±0,26	1,2±0,16	1,78	0,214
	VL	1,1±0,21	1,1±0,12	0,7±0,16	0,5±0,10	3,731	0,049
	VM	1,6±0,10	2,1±0,15	1,3±0,47	1,5±0,09	1,411	0,296
PND22	DL	0,8±0,16	0,9±0,21	1,6±0,20	1,9±0,25	7,132	0,003
	DM	0,9±0,14	0,9±0,12	3,2±0,08	3,2±0,27	51,358	<0,001
	VL	1,0±0,13	1,1±0,26	1,7±0,25	2,1±0,26	4,737	0,014
	VM	2,1±0,26	1,7±0,21	4,7±0,33	6,0±0,50	33,322	<0,001
PND30	DL	0,5±0,13	0,5±0,18	1,4±0,14	2,6±0,17	39,361	<0,001
	DM	0,8±0,23	0,7±0,09	4,0±0,12	6,6±0,43	117,27	<0,001
	VL	0,4±0,07	0,7±0,23	1,8±0,33	2,5±0,71	5,385	0,014
	VM	1,7±0,12	1,9±0,42	5,7±0,46	10,9±0,36	142,079	<0,001
PND60	DL	0,3±0,09	0,4±0,08	1,2±0,32	0,5±0,11	9,864	0,007

DM	0,6±0,07	1,1±0,24	5,2±0,71	4,1±0,43	92,729	<0,001
VL	0,4±0,16	0,7±0,07	1,6±0,17	1,1±0,15	31,008	<0,001
VM	1,9±0,34	2,0±0,37	12,5±0,76	6,0±1,03	135,474	<0,001

Table 12: Quantitative data for kisspeptin-ir fibers (FA) within PVN divided in DorsoLateral (DL), DorsoMedial (DM), VentroLateral (VL) and VentroMedial (VM), for different groups and different age of sacrifice of CD1 mice, are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

POMC system

A qualitative inspection of control animals clearly evidenced a marked sexual dimorphism at different ages. In ARC at PND12 we observed a higher number of POMC-ir elements in male than female (FA, $p<0,001$; cells number, $p<0,001$; Bonferroni test), but at PDN30 the situation is opposed, only for the FA, POMC-ir in CON M decries significantly respect to CON F (FA, $p<0,001$; Bonferroni test); probably because in female estrogen levels rise considerably during the pubertal transition (PDN30) (Lopez and Tena-Sempere 2015).

Interesting, during the development, only significant variations due to GEN administration of POMC-ir analysed by comparison with Bonferroni test were at PND12 and PND30, deleting the dimorphism measured in the control mice. In fact, at PND12 in GEN M decreased significantly the number of cells (GEN M vs. CON M, $p=0,030$), while the female mice treated with GEN increased the cells number, but in a no significant manner (GEN F vs. CON F, $p=0,097$), with a final disappearance of dimorphism (GEN M vs. GEN F, $p=1$). This was present only for the number of POMC cells in ARC, but no for FA (GEN M vs. GEN F, $p=0,008$). At PND30, the dimorphism was measured only in fractional area, with GEN treatment the FA increased significantly in GEN M (vs. CON M, $p=0,024$), with a consequent lost of sexual dimorphism (GEN M vs. GEN F, $p=1$).

In adult (at PND60) the situation between male and female control was very similar. Qualitative observation suggested that postnatal treatment to GEN significantly affect the adult profile of POMC expression in ARC in a sexual manner, only females presented an alteration of POMC-ir elements. These observations were confirmed by the quantitative analysis. At PND60 the two-way ANOVA reported a significant effect of the GEN treatment for the interrelation between sex and treatment (FA, $F=7,371$; $p=0,015$; cells number, $F=13,710$; $p=0,002$). The following *post hoc* Bonferroni test showed statistically significant higher values in GEN F than CON F for the number of POMC cells ($p=0,047$), but no for FA ($p=0,081$) in ARC; while no differences were present in

males (p=0,657 and p=0,134, respectively for FA and cellular number) (see tables 13 and 14) (Fig.11).

POMC FA in ARC

	CON M (MEAN±SEM)	GEN M (MEAN±SEM)	CON F (MEAN±SEM)	GEN F (MEAN±SEM)	ANOVA 1 WAY F	ANOVA 1 WAY p
PND12	18,8±1,42	18,0±1,79	12,3±0,94	13,7±1,29	24,962	<0,001
PND22	19,8±1,82	20,7±2,14	18,8±0,91	18,1±1,35	1,287	0,327
PND30	11,41±0,54	14,7±0,84	15,8±0,42	14,5±0,92	8,673	0,002
PND60	8,66±1,53	6,65±1,31	7,44±0,79	12,4±1,17	4,138	0,024

Table 13: Quantitative data for POMC-ir structures (FA) in ARC, for different groups and different age of sacrifice of CD1 mice, are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

POMC positive cells in ARC

	CON M (MEAN±SEM)	GEN M (MEAN±SEM)	CON F (MEAN±SEM)	GEN F (MEAN±SEM)	ANOVA 1 WAY F	ANOVA 1 WAY p
PND12	56,3±3,72	50,5±1,51	46,6±2,02	51,2±2,77	13,957	0,002
PND22	40,9±2,44	51,22±3,68	41,9±5,34	39,6±4,32	1,617	0,242
PND30	29,5±2,32	33,0±1,68	36,4±1,19	38,0±2,61	2,212	0,132
PND60	33,33±2,88	23,9±2,96	30,2±2,78	43,2±3,94	7,708	0,002

Table 14: Quantitative data for number of POMC cells in ARC, for different groups and different age of sacrifice of CD1 mice, are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

Orexin system

The lateral hypothalamus, from most rostral to most caudal region, contained very large numbers of Orexin cells in both sexes of CD1 mice at all ages analyzed (Fig.12a). In general in LH only in adult the system resulted sexual dimorphic, the analysis by Bonferroni test showed that males had a significant higher number of Orexin cells (CON M vs. CON F; p<0,001). During the development, the cells number at PND30 was a significant increase in males (PND22 vs. PND30; p=0,008), but not in females (PND22 vs. PND30; p=0,145). At PND60 the situation was very interesting, in the male OX cells was perfectly identical at PND30, while in female the number decreased significantly (PND30 vs. PND60; p=0,002). The one-way ANOVA revealed a significant effect of treatment at PND22 (F=5,472; p=0,008), at PND 30 (F= 8,449; p=0,001) and at PND60 (F=8.904; p=0,001), but not at PND12 (F=2,346; p=0,111). Subsequent *post hoc* Bonferroni showed a statistically significant

decrease of OX-ir cell number in GEN-treated male mice in comparison to CON M both at PND30 ($p<0,001$) at PND60 ($p=0.041$) (Fig. 12b left), while at PND60 in females was present a statistical increase of cells in GEN F vs. CON F ($p<0,05$) (Fig. 12b right).

Orexin positive cells in LH

	CON M (MEAN±SEM)	GEN M (MEAN±SEM)	CON F (MEAN±SEM)	GEN F (MEAN±SEM)	ANOVA 1 WAY F	p
PND12	190,3±8,27	160,6±5,48	177,7±10,89	182,8±10,57	2,346	0,111
PND22	225,7±12,06	191,9±6,57	216,1±7,24	179,1±9,85	5,472	0,008
PND30	316,9±11,72	210,3±16,94	288,8±20,62	343,9±27,31	8,346	0,001
PND60	313,7±18,21	253,4±14,70	189,9±19,29	260,7±10,90	8,904	0,001

Table 14: Quantitative data for the number of Orexin cells in LH, for different groups and different age of sacrifice of CD1 mice, are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

The qualitative analysis of four levels under study (Fig. 13a) revealed that the distribution of orexin-cells within LH was different and changed in sexual different manner. In fact, males tended to have more OX-ir cells than females in the most rostral levels analyzed, but no in the most caudal level during all development. The quantitative analysis confirmed these observations, two-way ANOVA revealed a significant difference for level e for the interaction between level and sex (respectively at PND12 $F=184,148$ $p<0,001$; $F=56,054$ $p<0,001$; at PND22 $F=193,226$ $p<0,001$; $F=45,315$ $p<0,001$; at PND30 $F=45,315$ $p<0,001$; $F=14,624$ $p<0,001$; at PND60 $F=43,843$ $p<0,001$; $F=17,727$ $p<0,001$). The two-by two comparison (Bonferroni test) showed that GEN treatment decreased the number of OX cells in the early stages of life in males only in most posterior region of LH (level4, GEN M vs. CON M at PND12 $p=0,034$ and at PND22 $p<0,001$). On the other hand, in females, this significant decrease was present in the anterior region (level1 and level2, GEN F vs. CON F at PND22 $p=0,05$). After puberty, the alterations induced by GEN treatment were completely opposite. In GEN M a significant decrease interests the anterior region (GEN M vs. CON M at PND30, level1 $p=0,003$; level2 $p<0,001$), while in females the cells number increased in more caudal region of LH (GEN F vs. CON F at PND30, level3 $p=0,174$; at PND60 level2 $p=0,04$ and level3 $p=0,025$) (Fig. 13b).

		CON M	GEN M	CON F	GEN F	ANOVA 1 WAY	
		(MEAN±SEM)	(MEAN±SEM)	(MEAN±SEM)	(MEAN±SEM)	F	p
PND12	Level1	159,8±9,98	141,5±6,59	77,2±13,97	90,5±16,64	11,827	0,001
	Level2	254,6±9,45	234,8±7,73	215,6±14,39	195,75±12,53	4,851	0,014
	Level3	214,6±11,63	186,2±11,98	260±8,92	285,5±15,90	13,513	0,001
	Level4	132±13,45	79,8±3,50	158±18,15	159,5±9,37	10,307	0,001
PND22	Level1	136,8±13,40	154,6±14,18	153,2±7,51	108,5±10,90	4,12	0,022
	Level2	267,8±19,98	259±9,90	270±5,69	214,3±15,23	3,871	0,027
	Level3	315,2±26,35	248,5±21,43	320,4±14,43	299,5±17,46	2,83	0,068
	Level4	182,8±10,93	105,7±3,99	121±4,39	94,2±9,61	23,218	<0,001
PND30	Level1	281,4±20,10	167,2±23,43	235±14,57	249,8±15,8	6,558	0,004
	Level2	456,2±8,34	270,4±23,85	302,2±15,72	359±35,81	12,265	0,001
	Level3	330,8±30,58	224,4±32,00	372,8±35,14	480,4±38,26	9,633	0,001
	Level4	199,4±18,89	179,2±23,85	245,4±33,11	286,4±33,66	2,921	0,066
PND60	Level1	293,2±15,59	218,17±13,52	146,4±16,59	176,17±13,73	14,907	<0,001
	Level2	402,8±23,14	339,33±16,69	189,8±21,20	278,17±14,01	19,866	<0,001
	Level3	322,4±27,12	282±22,65	232±28,60	368,3±29,40	3,98	0,024
	Level4	236,6±14,93	174,17±27,11	191,4±21,67	220,17±19,42	1,652	0,213

Table 15: Quantitative data for the number of Orexin cells in four levels of LH in rostrocaudal sense, for different groups and different age of sacrifice of CD1 mice, are reported in the other columns (Mean±SEM). The results of the one-way ANOVA are reported at the right.

Discussion

Here we show that postnatal GEN administration (from PND1 to PND8) at a dose comparable to exposure of babies fed with soy-based formulas, deeply affects metabolism and reproduction. In fact, it altered the development of three different hypothalamic systems (POMC, OX and kiss) at central level, as well as a plethora of parameters associated to metabolism (leptin and T3 levels) and

reproduction (progesterone, testostone levels, VO, gonads, estrus cycle and mammary gland), at peripheral level.

Soy is acknowledged as a hormonally active food (Patisaul 2017). Moreover, isoflavones, as GEN, were found to induce numerous metabolic effects, which may contribute to alteration in body weight. However, the impact of GEN as an endocrine disruptor compound on Health is still debated. Contradictory evidences are available, underlining the importance of considering timing of exposure, dose/concentration and gender when establishing safety recommendations for dietary GEN intake, especially if in early-life. The same treatment may have a different outcome depending on the age of the animal. In fact, compared to dams on a soy-free diet, rats on soy-enriched diet gain less weight during pregnancy and, although they consume more food, they do not become heavier during lactation. Their offspring (both sexes), however, is significantly heavier (more pronounced in males), have higher food intake and females have an earlier pubertal onset (Cao et al. 2015). GEN treatment may induce sex specific effects as highlighted by the observation that postnatal oral administration (PND1 to PND22) of 50mg/kg GEN (the same dose of our study) in rat pups increases of fat/lean mass ratio, fat mass, adipocyte size and number, and decreased muscle fiber perimeter in females but not males (Strakovsky et al. 2014). Consistently, we demonstrated a sexual dimorphic obesogenic effect of postnatal GEN administrated but from PND1 to PND8 in CD1 mice. In fact, we observed an obesogenic effect from puberty until adulthood, limited to females. This increase the body weight was not correlated to alterations in food intake and daily feed efficiency, indicating a probable metabolic disruption. Concurrently, plasmatic levels of two important metabolic hormones, leptin and T3 were significantly decreased upon GEN treatment only in females.

Similar metabolic changes as well as alterations in the T3 and leptin may be induced by GEN through different pathways in other models (Szkudelska et al. 2007). GEN inhibits thyroid peroxidase (TPX) activity, binding to its active side. In the presence of iodide, the phytoestrogen is a substrate for TPX resulting in the formation of mono-, di- and triiodogenistein. The ability of GEN to affect thyroid function seems connected with high accumulation of the phytoestrogen in this gland (Szkudelska et al. 2007).

Furthermore, GEN treatment decreased leptin release from isolated adipocytes. This effect is mainly mediated by the inhibition of glucose metabolism downstream the formation of pyruvate. Since adipocytes are the main source of circulating leptin, the direct limitation of its secretion from fat

cells evoked by GEN may be the reason of reduced leptinemia in rats receiving soy or pure isoflavone (Szkudelska et al. 2007).

Leptin decrease may in turn impair the activation of hypothalamic POMC system. POMC neurons in the ARC have an anorexigenic action on food-intake control. These neurons co-express different neuropeptides, and a wide variety of receptors, such as leptin receptors (Cheung et al. 1997). Moreover, POMC system is more expressed in females (Nohara et al. 2011; Farinetti et al. 2017, submitted), and is sensitive to gonadal steroids (Xu et al. 2011). We recently demonstrated that this system could be a good target for EDCs action by using a chronic exposure to tributyltin (Farinetti et al. 2017, submitted). Interestingly, we observed that sexual dimorphism in POMC system changed along the development. At PND12 the expression was higher in males and at PND30 in females, probably this is possible to cyclicity of gonadal steroids during the development. Similarly, in the present study we demonstrated that GEN interfered with POMC system in ARC nucleus ad different ages of development as well as in adulthood. While displayed POMC-ir during the development was lower in GEN treated than control males, in adults GEN effect, an increase in the number of POMC in ARC, was limited to females. These suggest that the postnatal exposure of GEN determines long-term sex specific organizational effects on POMC system.

Another hypothalamic system involved in metabolic and food intake control is Orexin, at first identified as a regulator of arousal and wakefulness (Davis et al. 2011). OX discriminates physiological variation in glucose levels between meals modulating energy balance according to food intake (Messina et al. 2014). Moreover, OX signaling promotes obesity resistance enhancing spontaneous physical activity and regulating energy expenditure (Chieffi et al. 2017). We observed that postnatal GEN administration permanently affected this system. In fact, the number of OX neurons in LH was higher in males than females of the adult control groups, as expected (Brownell et al. 2010). It has been proposed that this dimorphism could be associated to sexual male maturation since in adult male rodents, Fos-ir in OX neurons increase markedly during copulation (Muschamp et al. 2007). Interestingly, we observed that this dimorphism was totally reverted in GEN mice: the cell number increased in GEN female and decreased in GEN male. GEN may interfere, thus, with OX system when it is more susceptible to dimorphic changes, hypothetically due to indirect control of gonadal steroids. In fact, OX neurons co-express few with estrogen (ER alpha) receptor for nothing with androgen (AR) receptor (Muschamp et al. 2007). Moreover, we demonstrated that different rostrocaudal levels of LH harbor OX subpopulations with specific

features. In fact, males had more OX-ir cells than females in the most rostral levels, while females presented a higher number of OX cells in the most caudal levels, particularly at PND30. Furthermore, those subpopulations displayed a sexually dimorphic response upon GEN postnatal treatment, with a decreased in the number of OX cells in adult males in rostral levels of LH, and an increase in more caudal levels of females.

POMC and OX neurons, project to PVN, the most important center of metabolic control. PVN modulates feeding behavior through the action of several pituitary hormones, including CRH and TRH, both indirectly via effects on energy expenditure, and directly through the HPA-axis (van Swieten et al. 2014). As demonstrated in chapter 6, in PVN kiss fibers reach TRH neurons, suggesting that a strong correlation between reproductive and metabolic control. In fact, PVN kiss fibers, essential which are also essential in HPG axis as they mediate GnRH release, form a sexually dimorphic network which is steroid hormone sensitive, particularly during development, and it express ERs. Moreover, many studies showed that the kisspeptin is an important target for EDCs action (as bisphenol A), but to date, very limited data, however, are available on the impact of phytoestrogens on kisspeptin signaling networks. In female rats, neonatal exposure to 10 mg/kg GEN, but not 1 mg/kg, resulted in a lower density of AVPV/PeN kisspeptin- ir fibers across the pubertal transition (Losa et al. 2011), an effect that persisted into adulthood and is indicative of masculinization. In the ARC, another region rich of kisspeptin, fiber density was unaltered by GEN but significantly decreased by developmental estrogen exposure (Bateman et al. 2008; Losa et al. 2011). Moreover, postnatal GEN exposure does not significantly affect kisspeptin-ir levels in adult males (Patisaul et al. 2009). Consistently, we observed that early postnatal exposure to GEN induced sexually dimorphic effects on kisspeptin system. No changes were observed in developing nor adult males, although a transient increase, the kiss-ir FA was present in RP3V at PND12 in GEN treated males. Similarly, as kisspeptin is under control of leptin, the GEN male at PND12 presented a significant peak of plasmatic leptin. The same correlation was present in females. A strong anticipation of kisspeptin system in ARC, in RP3V and in PVN correlated to a peak of plasmatic leptin was measured in PND 30 females treated with GEN. In adults, all kisspeptin system was lower in GEN female respect to control females.

The literature reporting GEN effects on estrous cyclicity, fecundity, ovulation and female reproductive behavior (Jefferson et al. 2006; Crain et al. 2008; Walshe et al. 2011). Thus, neonatal

treatment with genistein at environmentally relevant doses caused adverse consequences on female development that is manifested in adulthood (Jefferson, Padilla-Banks, and Newbold 2007). Effectively, the alterations present in kisspeptin system were reflected in the peripheral data with accelerated female puberty, expressed by VO and increase of uterus weight in pre-puberty time, and irregular estrous cycles, with a reduction of proestrus phase, probably correlated to increase of plasmatic progesterone and elongation of diestrous phase. Moreover, changes in uterine morphology could delay or inhibit the transformation of the endometrium into a receptive state, resulting in failure of embryo to implant (Giudice 2006). Currently, only few studies have reported on the changes in the uterine morphology that include alteration in the thickness of the endometrium and epithelial heights following high doses of genistein treatment (Diel et al. 2006). Our results show that genistein could stimulate uterine fluid secretion and excessive fluid accumulation in the uterine lumen. Additionally, could also induce hyperplastic and hypertrophic changes in the uterus. These effects show significance towards normal fertility where disturbances in the normal volume of the uterine fluid and morphology of the uterus could potentially cause infertility.

Notably, orexin also exert their endocrine effects at the testicular level, as OX directly stimulates testosterone secretion in rat testis. In our animals, we showed a decrease of OX neurons in LH of adult males treated with GEN and a correlated decrease of fecal testosterone and weight of testicles in the same age.

In addition, we analyzed also the mammary gland, which seem not directly affected by postnatal Gen treatment. This was not surprising since mammary gland early development and sexual differentiation occur before birth and are independent of sexual steroids (Macias and Hinck 2012) although gestational exposure to GEN (Rudel et al. 2011) or other EDCs as BPA (Muñoz-de-Toro et al. 2005) affected mammary gland development. However, mammary glands respond to endocrine stimulation from puberty (Macias and Hinck 2012). The differences observed are likely to be mediated by differences in hormonal levels. Namely, we observed that the number of animals displaying TEB at PND22 was higher in GEN treated group than in controls. Since branching morphogenesis initiate at puberty (Macias and Hinck 2012), these data confirm that puberty is premature in GEN treated animals. Advanced puberty was observed also with post weaning GEN treatment, although this treatment had a more apparent effect on the mammary gland anatomy (Li et al. 2014). At this stage, pubertal development of mammary gland is driven by GH (through IGF1) and estrogen (through ERα; Macias & Hinck 2012). As most of the females in each group reach

puberty, at PND30, no differences were observed in the P4, nor in the testosterone (or E2) levels. Short tertiary branches develop under the influence of P4 during diestrus (Macias and Hinck 2012). Accordingly we observed that GEN treated females displayed a higher number of tertiary branches and higher levels of P4, possibly due to the longer length of diestrous phase that we observed. Similarly, disruption of the estrous cycle with an increase of both the estrous and diestrous phases was observed in post-weaning genistein treatment (Li et al. 2014).

In conclusion, the early postnatal exposure of CD1 mice to GEN determines long-term sex specific organizational effects on neural circuits controlling food intake, energy metabolism and reproduction. The increase of body weight, the decrease of T3 and leptin only on GEN female but not of food intake and daily feed efficiency, as well as the morphological alterations on the circuits expressing Orexin, POMC, or Kisspeptin suggest that the obesogenic GEN effect is sexually dimorphic and is due, at least partly, to alteration of metabolic regulation, thus GEN may be classified as a Metabolism disrupting chemical (MDC). MDC is any EDC that is able to promote metabolic changes that can result in obesity, T2D or fattyliver in animals including humans (Heindel et al. 2017). Evidences indicate that MDCs might alter food intake, with different effects based on dose and duration of exposure (Angle et al. 2013; Mackay et al. 2013; Bo et al. 2016), develop obesity and T2DM during adulthood after perinatal exposure (Garcia-Arevalo et al. 2016).

References

Cao, J., R. Echelberger, M. Liu, E. Sluzas, K. McCaffrey, B. Buckley, and H. B. Patisaul. 2015. 'Soy but not bisphenol A (BPA) or the phytoestrogen genistin alters developmental weight gain and food intake in pregnant rats and their offspring', *Reprod Toxicol*, 58: 282-94.

Castellano, J. M., and M. Tena-Sempere. 2016. 'Metabolic control of female puberty: potential therapeutic targets', *Expert Opin Ther Targets*, 20: 1181-93.

Chieffi, S., M. Carotenuto, V. Monda, A. Valenzano, I. Villano, F. Precenzano, D. Tafuri, M. Salerno, N. Filippi, F. Nuccio, M. Ruberto, V. De Luca, L. Cipolloni, G. Cibelli, M. P. Mollica, D. Iacono, E. Nigro, M. Monda, G. Messina, and A. Messina. 2017. 'Orexin System: The Key for a Healthy Life', *Front Physiol*, 8: 357.

Cimafranca, M. A., J. Davila, G. C. Ekman, R. N. Andrews, S. L. Neese, J. Peretz, K. A. Woodling, W. G. Helferich, J. Sarkar, J. A. Flaws, S. L. Schantz, D. R. Doerge, and P. S. Cooke. 2010. 'Acute and chronic effects of oral genistein administration in neonatal mice', *Biol Reprod*, 83: 114-21.

Clarkson, J., and A. E. Herbison. 2006. 'Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons', *Endocrinology*, 147: 5817-25.

Cowley, M. A., J. L. Smart, M. Rubinstein, M. G. Cerdan, S. Diano, T. L. Horvath, R. D. Cone, and M. J. Low. 2001. 'Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus', *Nature*, 411: 480-4.

D'Aloisio, A. A., D. D. Baird, L. A. DeRoo, and D. P. Sandler. 2010. 'Association of intrauterine and early-life exposures with diagnosis of uterine leiomyomata by 35 years of age in the Sister Study', *Environ Health Perspect*, 118: 375-81.

Diel, P., T. Hertrampf, J. Seibel, U. Laudenbach-Leschowsky, S. Kolba, and G. Vollmer. 2006. 'Combinatorial effects of the phytoestrogen genistein and of estradiol in uterus and liver of female Wistar rats', *J Steroid Biochem Mol Biol*, 102: 60-70.

Dixon, R. A., and D. Ferreira. 2002. 'Genistein', *Phytochemistry*, 60: 205-11.

Elias, C. F., C. Aschkenasi, C. Lee, J. Kelly, R. S. Ahima, C. Bjorbaek, J. S. Flier, C. B. Saper, and J. K. Elmquist. 1999. 'Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area', *Neuron*, 23: 775-86.

Franke, A. A., L. J. Custer, and Y. Tanaka. 1998. 'Isoflavones in human breast milk and other biological fluids', *Am J Clin Nutr*, 68: 1466S-73S.

Giudice, L. C. 2006. 'Endometrium in PCOS: Implantation and predisposition to endocrine CA', *Best Pract Res Clin Endocrinol Metab*, 20: 235-44.

Heine, P. A., J. A. Taylor, G. A. Iwamoto, D. B. Lubahn, and P. S. Cooke. 2000. 'Increased adipose tissue in male and female estrogen receptor-alpha knockout mice', *Proc Natl Acad Sci U S A*, 97: 12729-34.

Holstein, A. F., W. Schulze, and M. Davidoff. 2003. 'Understanding spermatogenesis is a prerequisite for treatment', *Reprod Biol Endocrinol*, 1: 107.

Jefferson, W. N., E. Padilla-Banks, and R. R. Newbold. 2007. 'Disruption of the developing female reproductive system by phytoestrogens: genistein as an example', *Mol Nutr Food Res*, 51: 832-44.

Johnsen, S. G. 1970. 'Testicular biopsy score count--a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males', *Hormones*, 1: 2-25.

Jungbauer, A., and S. Medjakovic. 2014. 'Phytoestrogens and the metabolic syndrome', *J Steroid Biochem Mol Biol*, 139: 277-89.

Li, R., F. Zhao, H. Diao, S. Xiao, and X. Ye. 2014. 'Postweaning dietary genistein exposure advances puberty without significantly affecting early pregnancy in C57BL/6J female mice', *Reprod Toxicol*, 44: 85-92.

Lopez, M., and M. Tena-Sempere. 2015. 'Estrogens and the control of energy homeostasis: a brain perspective', *Trends Endocrinol Metab*, 26: 411-21.

Macchi, E., A. S. Cucuzza, P. Badino, R. Odore, F. Re, L. Bevilacqua, and A. Malfatti. 2010. 'Seasonality of reproduction in wild boar (Sus scrofa) assessed by fecal and plasmatic steroids', *Theriogenology*, 73: 1230-7.

Macias, H., and L. Hinck. 2012. 'Mammary Gland Development', *Wiley Interdiscip Rev Dev Biol*, 1: 533-57.

Marraudino, M., D. Miceli, A. Farinetti, G. Ponti, G. Panzica, and S. Gotti. 2017. 'Kisspeptin innervation of the hypothalamic paraventricular nucleus: sexual dimorphism and effect of estrous cycle in female mice', *J Anat*, 230: 775-86.

Messina, G., C. Dalia, D. Tafuri, V. Monda, F. Palmieri, A. Dato, A. Russo, S. De Blasio, A. Messina, V. De Luca, S. Chieffi, and M. Monda. 2014. 'Orexin-A controls sympathetic activity and eating behavior', *Front Psychol*, 5: 997.

Morrison, C. D., G. J. Morton, K. D. Niswender, R. W. Gelling, and M. W. Schwartz. 2005. 'Leptin inhibits hypothalamic Npy and Agrp gene expression via a mechanism that requires phosphatidylinositol 3-OH-kinase signaling', *Am J Physiol Endocrinol Metab*, 289: E1051-7.

Muñoz-de-Toro, M., C. M. Markey, P. R. Wadia, E. H. Luque, B. S. Rubin, C. Sonnenschein, and A. M. Soto. 2005. 'Perinatal Exposure to Bisphenol-A Alters Peripubertal Mammary Gland Development in Mice', *Endocrinology*, 146: 4138-47.

Newbold, R. R. 2010. 'Impact of environmental endocrine disrupting chemicals on the development of obesity', *Hormones (Athens)*, 9: 206-17.

Nistal, M., and R. Paniagua. 1999. 'Testicular biopsy. Contemporary interpretation', *Urol Clin North Am*, 26: 555-93, vi.

Patisaul, Heather B., and Scott M. Belcher. 2017. *Endocrine disruptors, brain, and behaviors* (Oxford University Press: New York, NY).

Ponti, G., A. Rodriguez-Gomez, A. Farinetti, M. Marraudino, F. Filice, B. Foglio, G. Sciacca, G. C. Panzica, and S. Gotti. 2017. 'Early postnatal genistein administration permanently affects nitrergic and vasopressinergic systems in a sex-specific way', *Neuroscience*, 346: 203-15.

Rudel, R. A., S. E. Fenton, J. M. Ackerman, S. Y. Euling, and S. L. Makris. 2011. 'Environmental Exposures and Mammary Gland Development: State of the Science, Public Health Implications, and Research Recommendations.' in, *Environ Health Perspect*.

Ruhlen, R. L., K. L. Howdeshell, J. Mao, J. A. Taylor, F. H. Bronson, R. R. Newbold, W. V. Welshons, and F. S. vom Saal. 2008. 'Low phytoestrogen levels in feed increase fetal serum estradiol resulting in the "fetal estrogenization syndrome" and obesity in CD-1 mice', *Environ Health Perspect*, 116: 322-8.

Setchell, K. D., L. Zimmer-Nechemias, J. Cai, and J. E. Heubi. 1997. 'Exposure of infants to phyto-oestrogens from soy-based infant formula', *Lancet*, 350: 23-7.

Silber, S. J., Z. Nagy, P. Devroey, H. Tournaye, and A. C. Van Steirteghem. 1997. 'Distribution of spermatogenesis in the testicles of azoospermic men: the presence or absence of spermatids in the testes of men with germinal failure', *Hum Reprod*, 12: 2422-8.

Slikker, W., Jr., A. C. Scallet, D. R. Doerge, and S. A. Ferguson. 2001. 'Gender-based differences in rats after chronic dietary exposure to genistein', *Int J Toxicol*, 20: 175-9.

Strom, B. L., R. Schinnar, E. E. Ziegler, K. T. Barnhart, M. D. Sammel, G. A. Macones, V. A. Stallings, J. M. Drulis, S. E. Nelson, and S. A. Hanson. 2001. 'Exposure to soy-based formula in infancy and endocrinological and reproductive outcomes in young adulthood', *JAMA*, 286: 807-14.

Viglietti-Panzica, C., N. Aste, J. Balthazart, and G. C. Panzica. 1994. 'Vasotocinergic innervation of sexually dimorphic medial preoptic nucleus of the male Japanese quail: influence of testosterone', *Brain Res*, 657: 171-84.

Watson, R. E., S. J. Wiegand, R. W. Clough, and G. E. Hoffman. 1986. 'Use of Cryoprotectant to Maintain Long-Term Peptide Immunoreactivity and Tissue Morphology', *Peptides*, 7: 155-59.

Zammaretti, F., G. Panzica, and C. Eva. 2007. 'Sex-dependent regulation of hypothalamic neuropeptide Y-Y1 receptor gene expression in moderate/high fat, high-energy diet-fed mice', *J Physiol*, 583: 445-54.

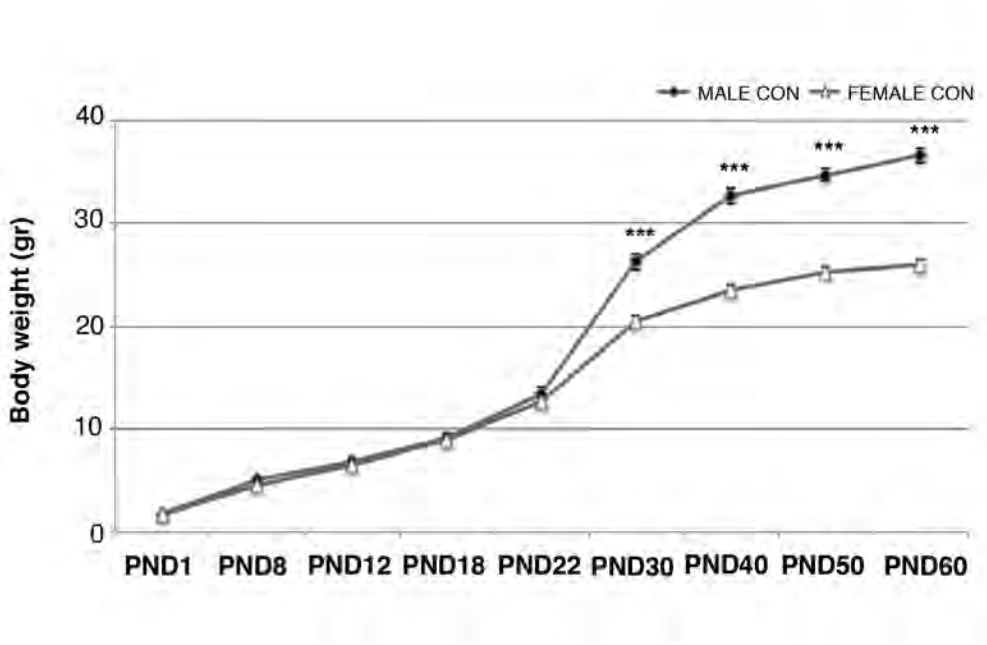

Figure 2. Histogram represents body weight gain (expressed as gram; mean±SEM) during the development of male and female control (CON) CD1 mice. ***$p \leq 0.001$ (Tukey test).

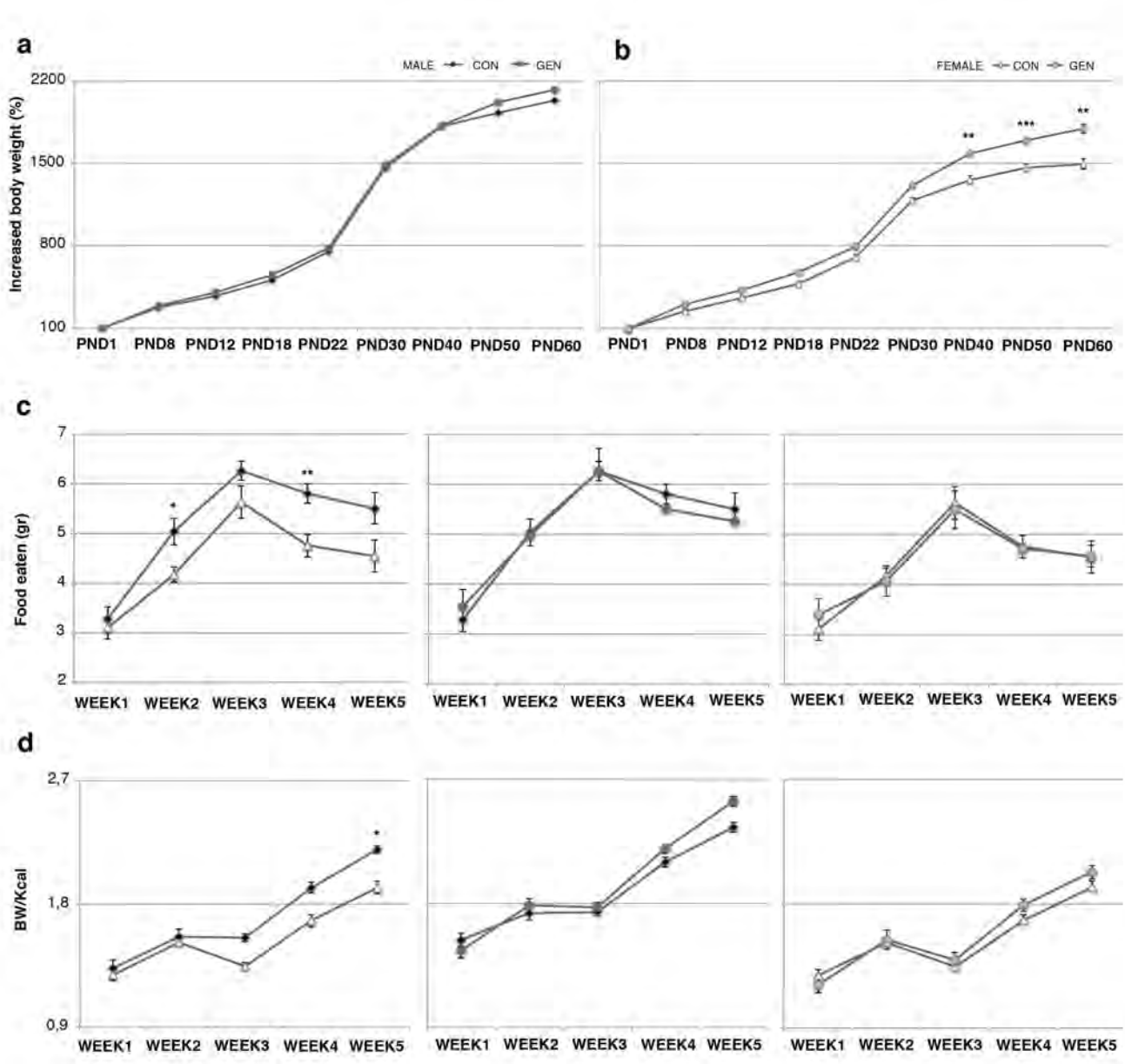

Figure 3. Histograms represent body weight gain (expressed as percentage; mean±SEM) during the development of male CON vs. male GEN (**a**) and female CON vs. female GEN (**b**) CD1 mice. (**c**) The lines represent variations of the amount (in grams; mean±SEM) of food intake during five weeks after weaning in control male and female, male CON vs. male GEN, and female CON vs. female GEN of CD1 mice. (**d**) The lines represent variations of the daily feed efficiency (expressed as index of body weight/kcal introduced; mean±SEM) calculated during five weeks after weaning in control male and female, male CON vs. male GEN, and female CON vs. female GEN of CD1 mice. *$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ (Tukey test).

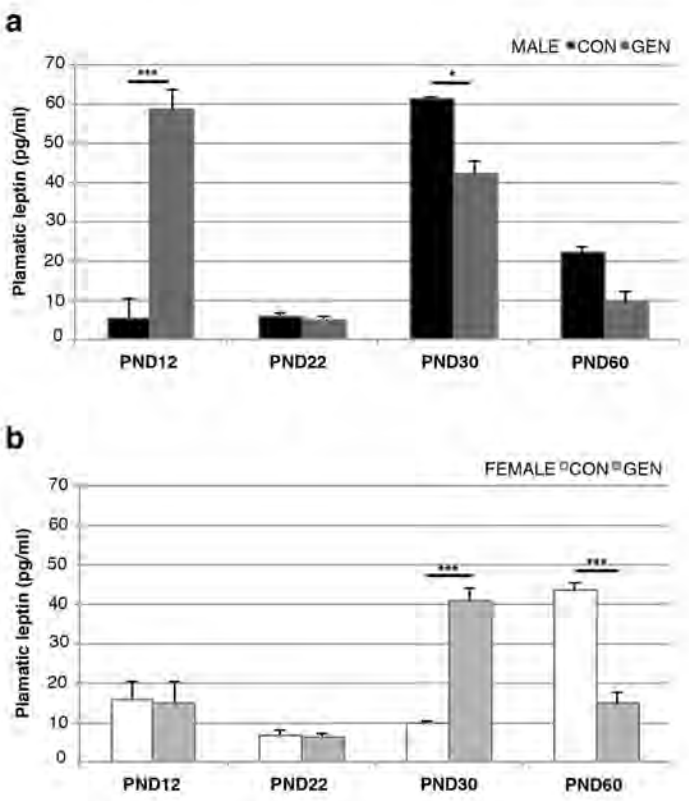

Figure 4. Histograms represent circulating leptin levels (expressed in pg/ml; mean±SEM) in males, control (CON) and treated (GEN) (**a**), and in females, CON and GEN (**b**) during the development at postnatal day (PND) 12, PND22, PND30, and PND60. *$p < 0.05$; ***$p \leq 0.001$ (Tukey test).

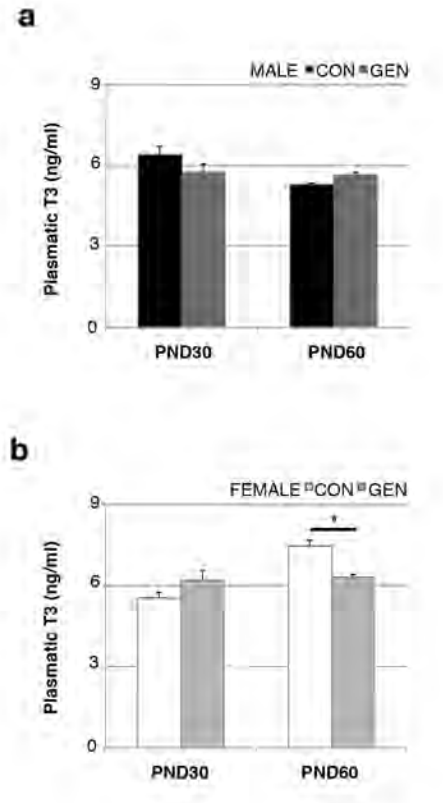

Figure 5. Histograms represent circulating triiodothyronine (T3) levels (expressed in ng/ml; mean±SEM) in males, control (CON) and treated (GEN) (**a**), and in females, CON and GEN (**b**) during the development at postnatal day (PND) 12, PND22, PND30, and PND60. *$p < 0.05$ (Tukey test).

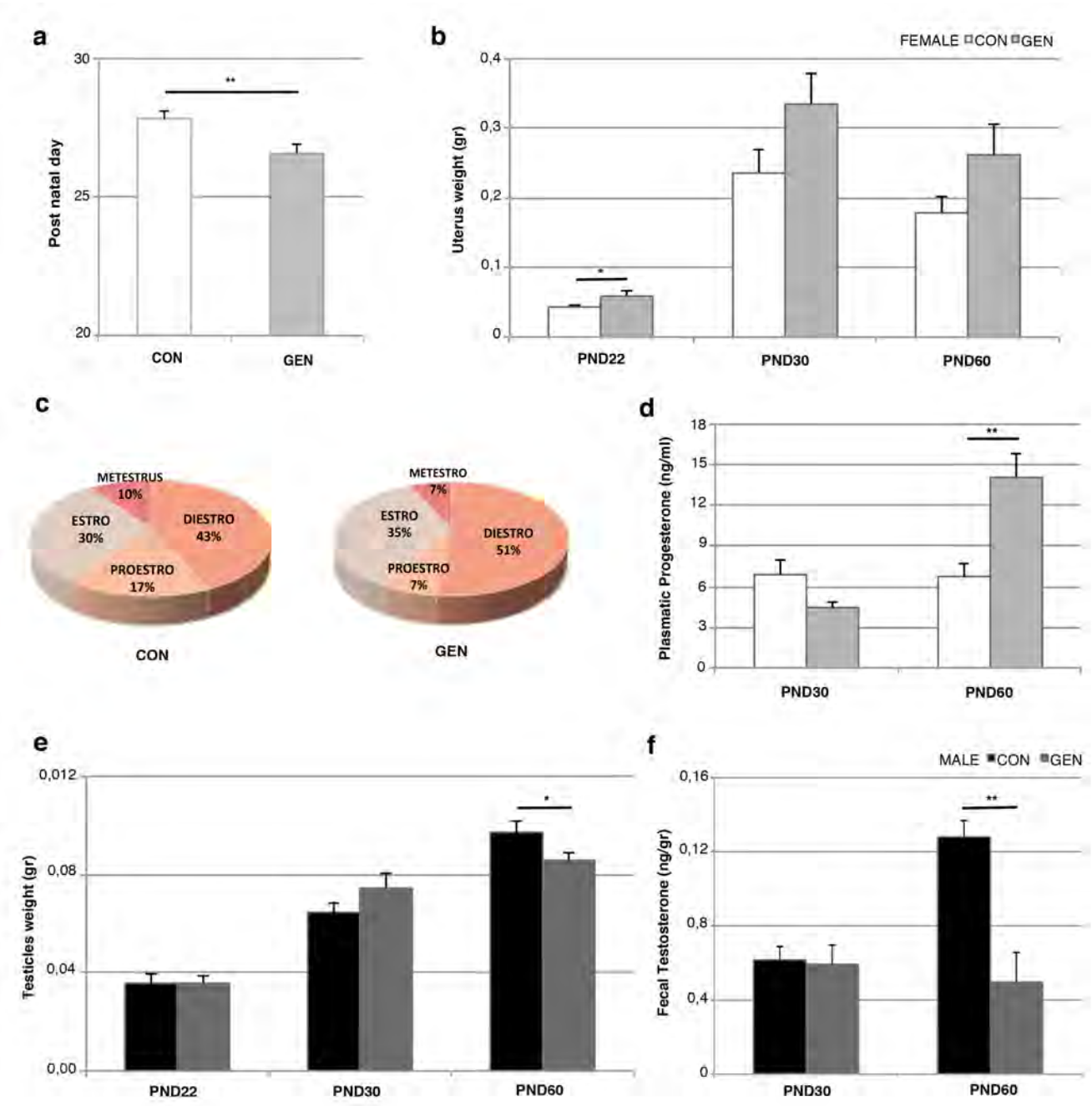

Figure 6. (**a**) Histogram represents the evaluation of the day of vaginal opening (VO) in female control (CON) and treated (GEN) CD1 mice (expressed in mean±SEM). (**b**) Histogram represents uterus weight gain (expressed in gram; mean±SEM) measured during the development at postnatal day (PND) 22, PND30 and PND60 of females CON and GEN. (**c**) Pie charts rapresent the time spent (expressed as percentage) in each phases of estrus cycle (estrus, metestrus, diestrus and proestrus). (**d**) Histogram represents circulating progesterone levels (expressed in ng/ml, mean±SEM) in females, CON and GEN, during the development at PND30, and PND60. (**e**) Histogram represents testicles weight gain (expressed in gram; mean±SEM) measured during the

development at postnatal day (PND) 22, PND30 and PND60 of males CON and GEN. (**f**) Histogram represents fecal testosterone levels (expressed in ng/ml) in males, CON and GEN, during the development at PND30, and PND60. *$p < 0.05$; **$p \leq 0.01$. VO, uterus and testicles weight, and time spent in each phases of estrus cycle were compared by Student's t-test, while the hormones, progesterone and testosterone, by Tukey test.

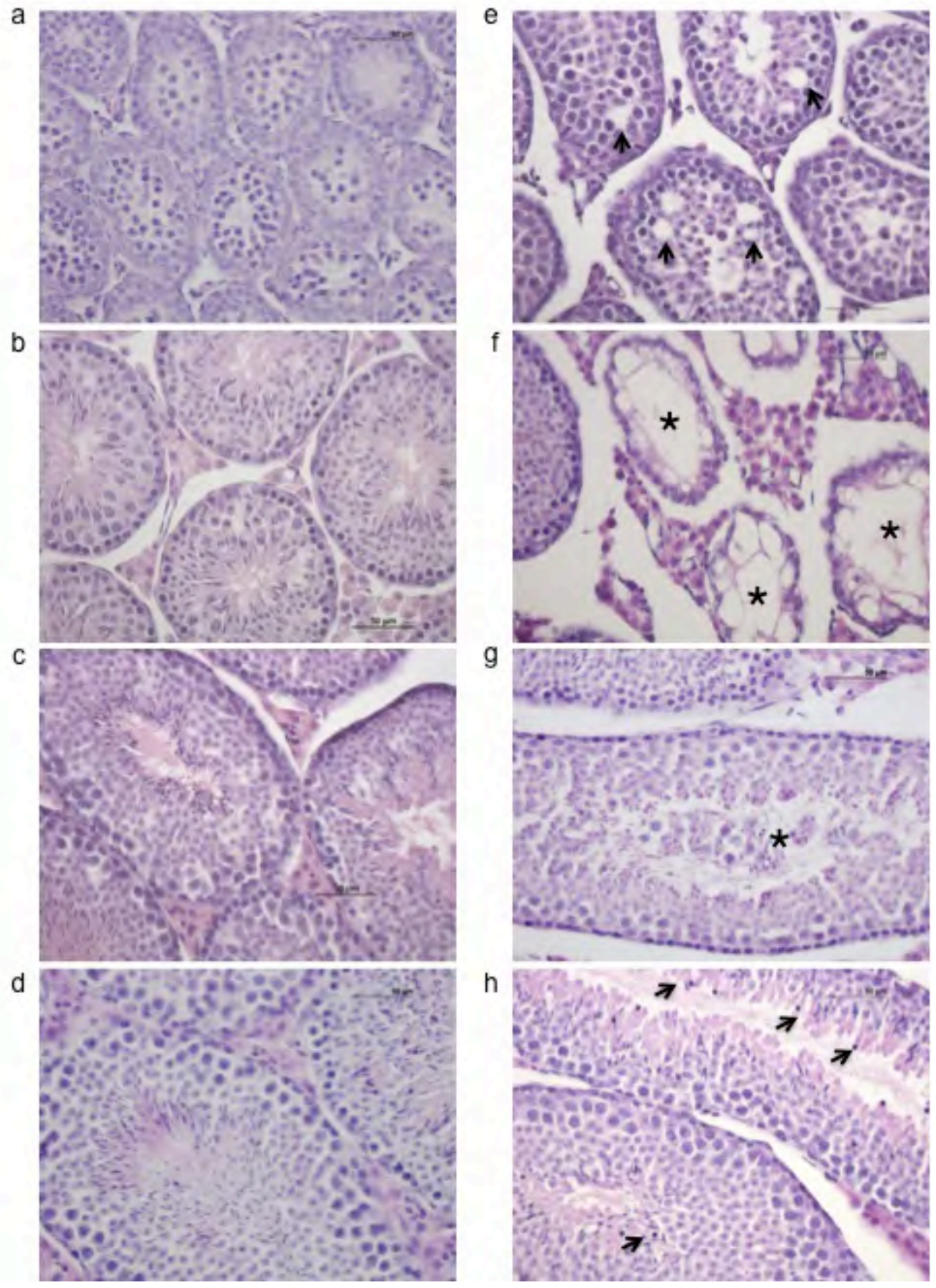
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Figure 7. (a; b; c; d) Transverse section of testes from control mice showing the presence of normal tubular structure with spermatogenic cells at different stages of development, respectively at PND12, PND22, PND30, PND60. (e: f; g; h) Transverse sections of testes of mice exposed to GEN (50 mg/Kg body weight), respectively, showing different arrest of spermatogenesis with few spermatogonia, enlarged inter-tubular space, ruptured epithelium, vacuolization and lumen with reduced number of sperm tails. In particular, seminiferous epithelial vacuolation (e, arrows) such as hypospermatogenesis (f, asterisks) with luminal cell debris (g, asterisks) and apoptosis (h, arrows) are visible in the tubules. H&E stain, 400x

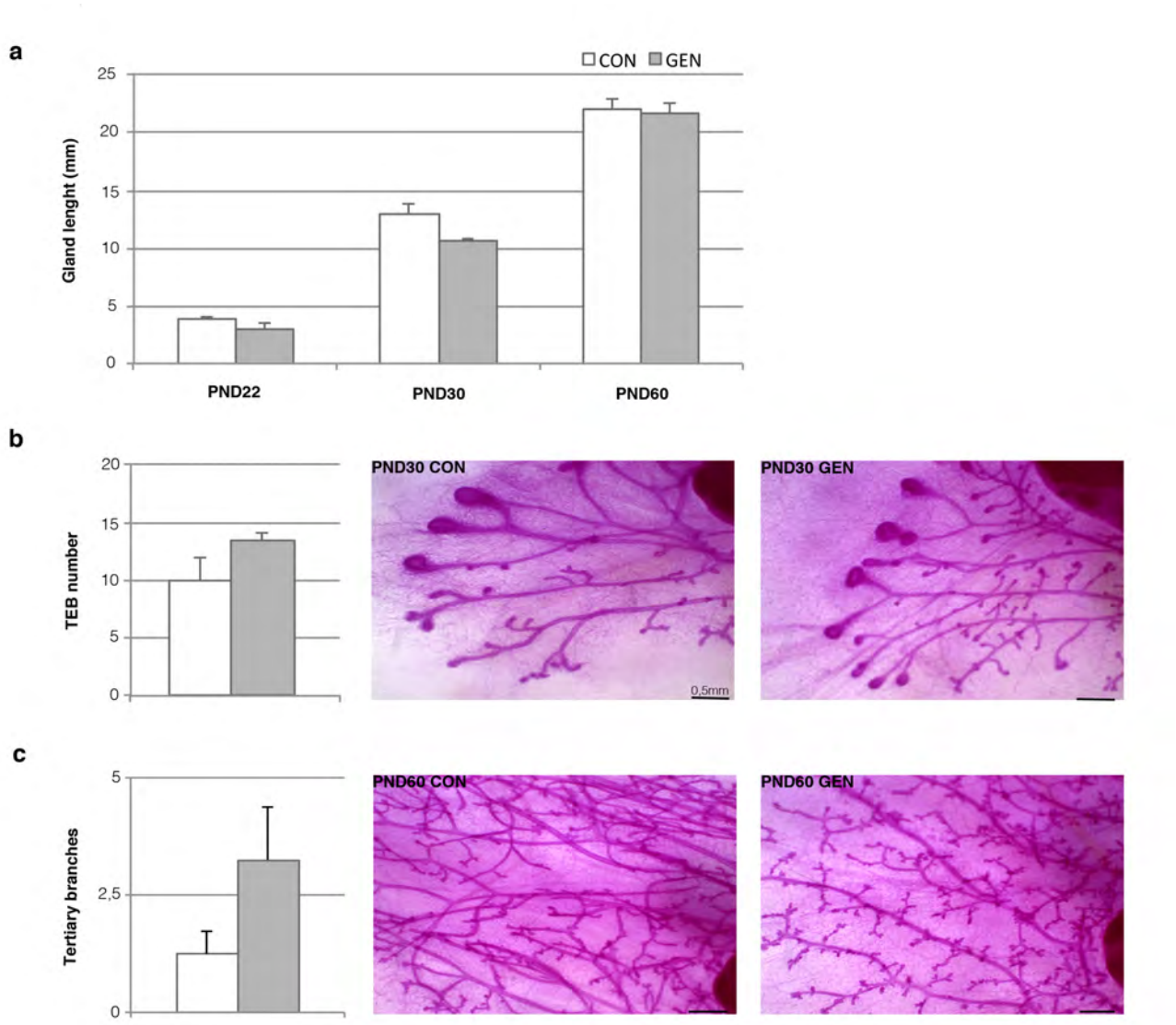

Figure 8. (**a**) Histogram represents mammary gland length (expressed as millimeters; mean±SEM) of female control (CON) and treated (GEN) CD1 mice during the development at postnatal day (PND) 22, PND30, and PND60. (**b**) Histograms (left) and photomicrographs (right) represent number of terminal end buds (TEBs) of mammary gland in female control (CON) and treated (GEN) CD1 mice at PND30. (**c**) Histograms (left) and photomicrographs (right) represent number of tertiary branches of mammary gland in female control (CON) and treated (GEN) CD1 mice at PND30 (expressed as mean±SEM). Scale bar = 0, 5 mm

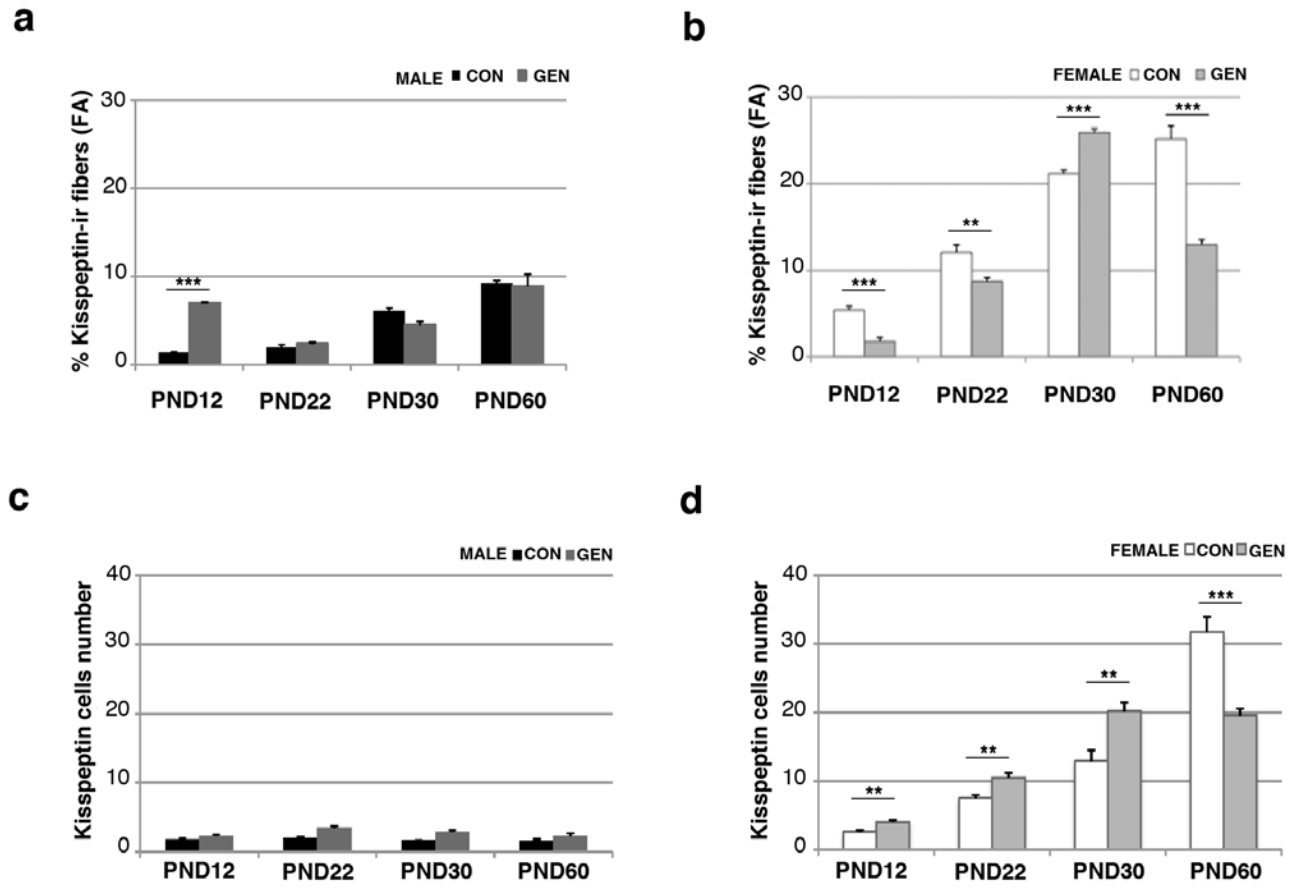

Figure 9. (**a-b**) Histograms represent percentage of area (FA; mean±SEM) covered by kisspeptin immuno positive structures in ARC of males control (CON) and treated (GEN) (**a**) and in females CON and GEN (**b**) at different ages of sacrifice (postnatal day, PND12, PND22, PND30, and PND60). (**c-d**) Histograms represent the number of cell kisspeptin-ir (mean±SEM) in RP3V of males control (CON) and treated (GEN) (**c**) and in females CON and GEN (**d**) at different ages of sacrifice (postnatal day, PND12, PND22, PND30, and PND60). **p ≤ 0.01; ***p ≤ 0.001 (Bonferroni test).

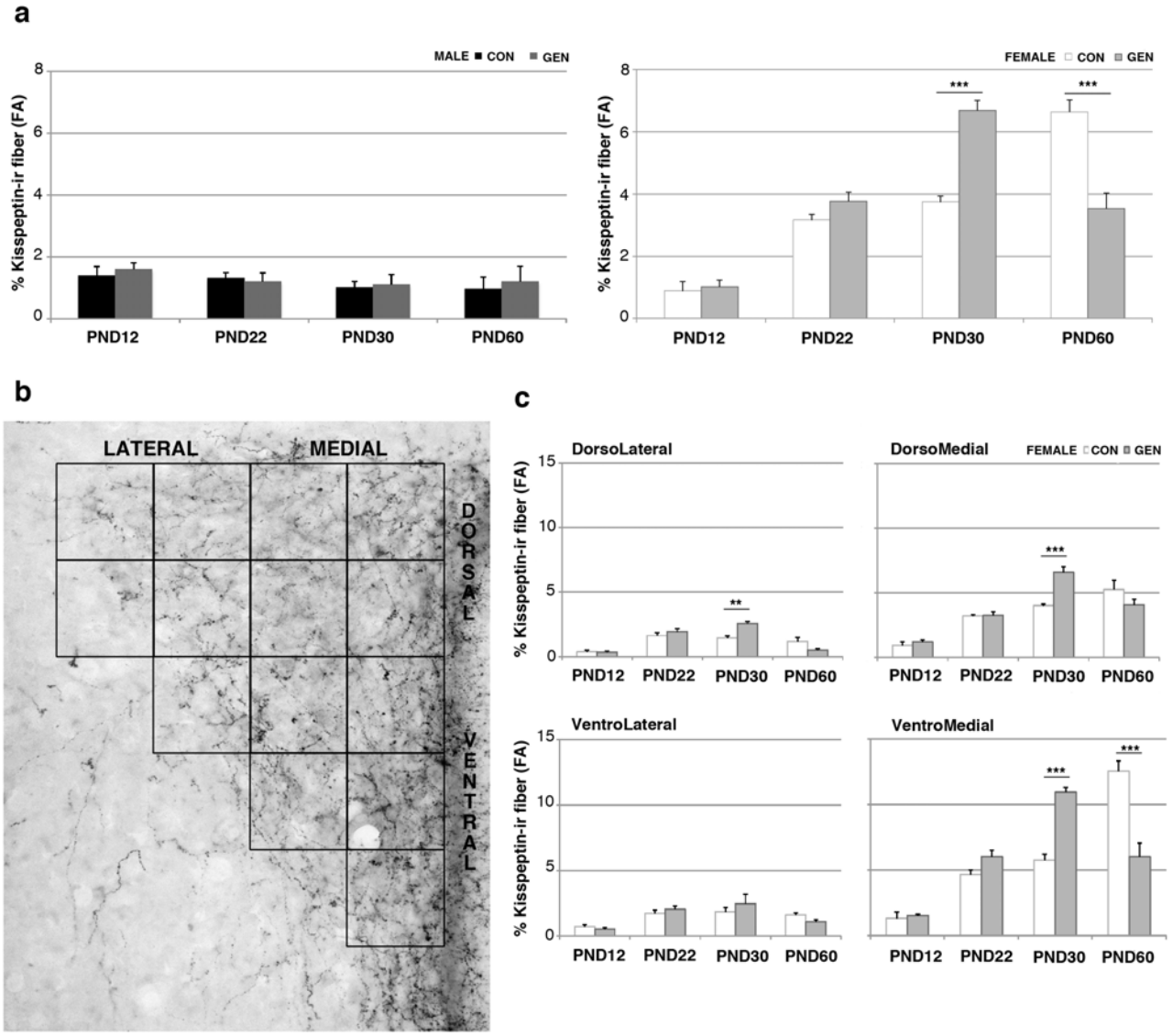

Figure 10. (**a**) Histograms represent the fractional area (FA; mean±SEM) covered by kiss-ir fibers in the PVN (left) in males, control (CON) and treated (GEN), and (right) in females, CON and GEN, at different ages of sacrifice (postnatal day, PND12, PND22, PND30, and PND60). (**b**) The representative subdivision of PVN in fourteen quadrants to identify the four parts of nucleus (DM, dorso-medial; DL, dorsolateral; VM, ventro-medial; VL, ventro-lateral). (**c**) Histograms represent the fractional area (FA) covered by kiss-ir fibers in the PVN (DM, DL, VM, VL) in female CON and GEN CD1 mice at different ages of sacrifice (PND12, PND22, PND30, and PND60). ***$p \leq 0.001$ (Bonferroni test).

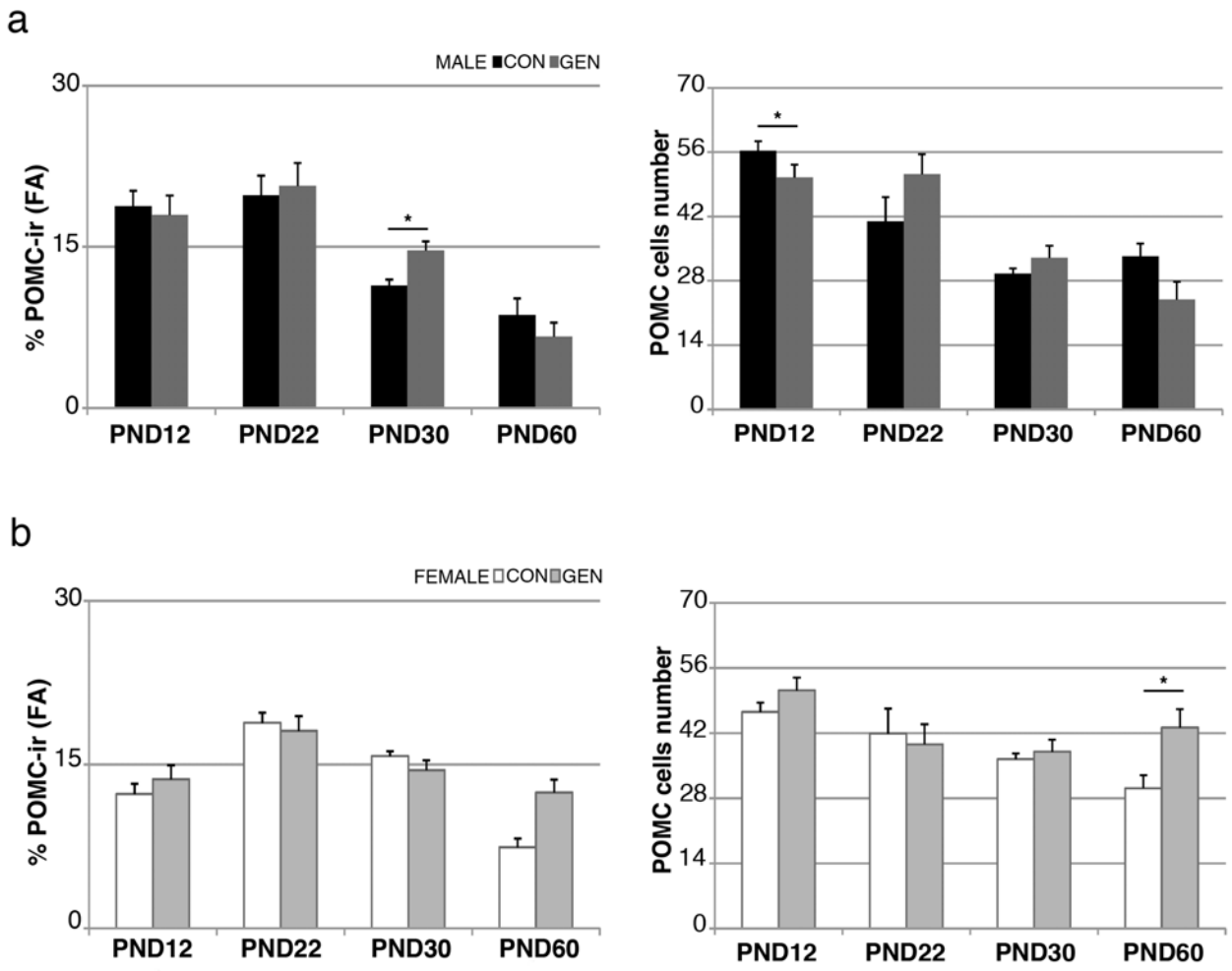

Figure 11. (**a-b**) Histograms represent percentage of area (FA: mean±SEM) covered by POMC immuno positive structures (left) and cell bodies (right) in ARC of males control (CON) and treated (GEN) (**a**) and in females CON and GEN (**b**) at different ages of sacrifice (postnatal day, PND12, PND22, PND30, and PND60). . *$p < 0.05$; **$p \leq 0.01$ (Bonferroni test).

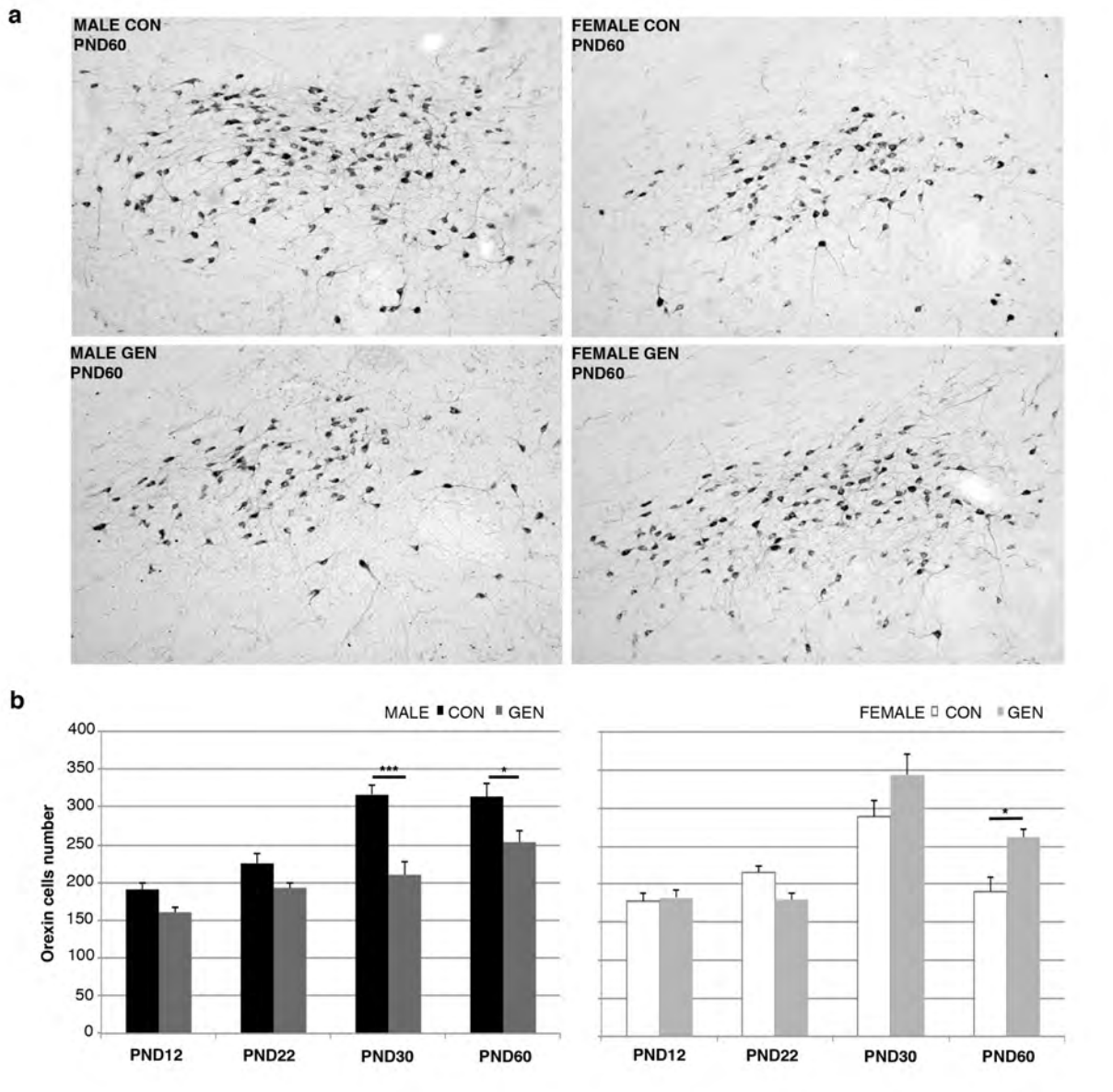

Figure 12. (**a**) Photomicrographs show the Orexin cells in LH in adult CD1 mice of different groups analyzed. (**b**) Histograms represent the Orexin cells (mean±SEM) in LH of males control (CON) and treated (GEN) (left) and in females CON and GEN (right) at different ages of sacrifice (postnatal day, PND12, PND22, PND30, and PND60). . *$p < 0.05$; ***$p \leq 0.001$ (Bonferroni test).

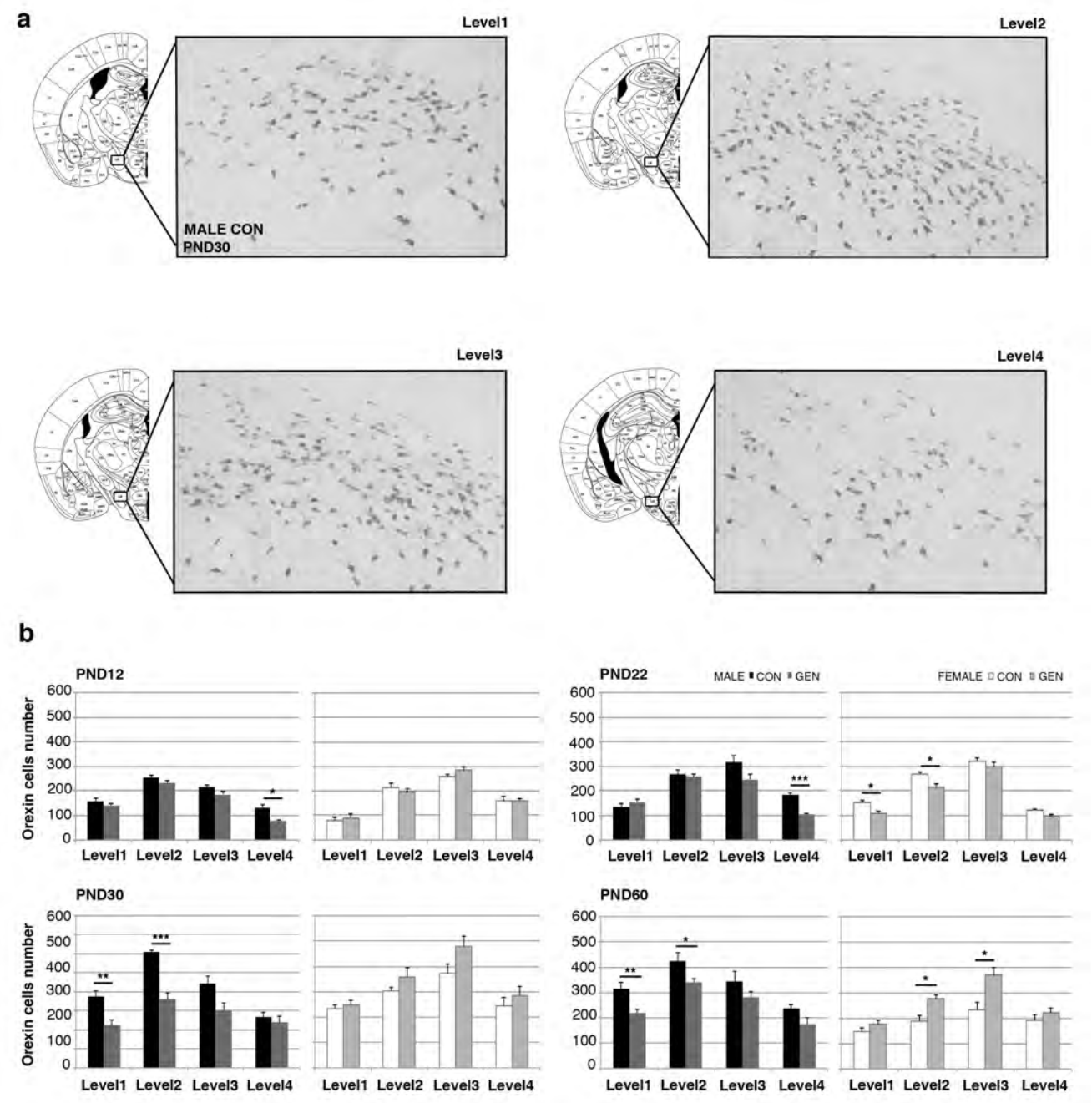

Figure 13. (**a**) Photomicrographs show the Orexin cells in for different levels of LH in male control CD1 mice at postnatal day (PND) 30. (**b**) Histograms represent the Orexin cells (mean±SEM) in LH of males control (CON) and treated (GEN) (left) and in females CON and GEN (right) at different ages of sacrifice (postnatal day, PND12, PND22, PND30, and PND60). *$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ (Bonferroni test).

CHAPTER 8

Experiment 6

'Sexually dimorphic effect of genistein on hypothalamic neuronal differentiation in vitro'

Sexually dimorphic effect of genistein on hypothalamic neuronal differentiation in vitro

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Abstract

The developmental actions of estradiol have been well characterized in the hypothalamus, where this hormone generates sex differences in neuronal circuits controlling neuroendocrine events, feeding, growth, reproduction and behavior. *In vitro*, estradiol promotes neuritic processes' outgrowth of different neuronal types. The phytoestrogen genistein has, *in vivo*, a role in alteration, in a permanent and sexually different way, of some behavior and neural circuits, but it still remains elusive through which type of estrogen receptor, α, β or G-protein coupled estrogen receptor (GPER), genistein exerts its effect. In this work we studied if genistein, like estradiol, could affect neuritic outgrowth (neuritogenesis) in hypothalamic neurons in culture and what estrogen receptor is likely to be implicated.

Hypothalamic male and female cultures, obtained from embryonic day 14 (E14) CD1 mice, were treated with genistein (0.5 μM) or vehicle. The analysis of the processes' arborization shows that the controls have a significant sex dimorphism: female neurons have more intersections than male. The treatment with genistein induces a significant increase only in female and neurons of genistein-treated female cultures have longer processes.

In a second experiment, female cultures were treated whit vehicle or genistein (0.5 μM) associated with selective antagonists of estrogen receptors. Both the antagonists for estrogen receptor α, β and GPER blocked the neuritogenic effect of genistein in female cultures.

In conclusion, the effect of genistein on hypothalamic neuronal cultures is sexually dimorphic and is possibly mediated by estrogen receptor α, β and GPER.

Key words: phytoestrogen, sex difference, hypothalamic neurons, neuritogenesis, estrogen receptors.

Introduction

Some natural non-steroidal molecules, contained in many vegetal species used also for human and animal food (i.e. leguminous including the soy), show a weak estrogenic activity and are therefore called phytoestrogens (Korach et al. 1994). These compounds have a weak to moderate affinity to estrogen receptors (ERs) and are therefore considered xeno-estrogens and included in the list of endocrine disrupting chemicals (EDCs)(Nie et al. 2017). The more diffuse phytoestrogens are the flavonoids including cumestrol, daidzein and genistein (GEN), which is probably the most abundant one (Dixon and Ferreira 2002). Due to its ability of binding ERs, GEN is generally considered as a beneficial molecule and is used as a natural substitute for endogenous hormones in some physiological situations (i.e. menopause).

However, some recent studies demonstrated that GEN may have, *in vivo*, a role in alteration, in a permanent and sexually differentiated way, of some behavior and in some neural circuits (Ponti et al. 2017; Rodriguez-Gomez et al. 2014). Moreover a study *in vitro* showed that this phytoestrogen improved hippocampus neuronal cell viability and proliferation (Pan et al. 2012).

Estrogens play very important roles for the growth, differentiation and functioning of many target tissues including the central nervous system (Couse and Korach 1999). Many studies have shown that estradiol (E_2) enhances neurite outgrowth and elongation in living hypothalamic neurons with sex related differences (Diaz et al. 1992), also in embryonic culture before the critical period of brain masculinization (Scerbo et al. 2014).

Therefore phytoestrogens and in particular GEN, that is able to interact with the estrogen-dependent neural pathways in a complex and multidirectional ways, may interfere with the endocrine system and lead to permanent alterations of estrogen sensitive circuits (Frank, Brown, and Clegg 2014). GEN actions have been investigated in several studies but it still remains elusive through which type of estrogen receptor (ERα, ERβ or GPER) GEN exerts its effects. Kuiper et al. demonstrated that the phytoestrogens have only agonistic activity on estrogen receptors (ERs), measured in the solid-phase ligand-binding system; in particular, seems that GEN has a higher affinity as an agonist for ERβ (Kuiper et al. 1998).

In this study we explored if GEN, like estradiol, could affect processes' outgrowth (neuritogenesis) in hypothalamic neurons in culture, to determine if this action could be sexually differentiated and what estrogen receptor (ER) is likely to be implicated.

Materials and methods

Animals

The embryos used for this study were obtained from CD1 mice raised in the Cajal Institute (Madrid, Spain). The day of vaginal plug was defined as E0. All procedures for handling and killing the animals used in this study were in accordance with the European Commission guidelines (86/609/CEE and 2010/63/UE) and the Spanish Government Directive (R.D. 1201/2005). The Cajal Institute Ethic Committee of Animal Experimentation approved experimental procedures.

Hypothalamic neuronal cultures and cell treatments

Hypothalamic neurons were obtained from male and female mouse embryos at embryonic day 14 (E14), when neuronal development is independent of gonadal hormones, in fact in mice the peak of testosterone production by fetal testis is at E17-18 (O'Shaughnessy et al. 1998; O'Shaughnessy, Baker, and Johnston 2006). Cells were cultured separately according to the sex and/or genotype of fetal donors. Male fetuses were identified under a dissecting microscope by the presence of the spermatic artery on the developing gonad (Scerbo et al. 2014). The brain was dissected out and the meninges were removed. Then, the ventromedial hypothalamic region, delimited by the optic chiasm, the lateral hypothalamic sulcus and the mammillary bodies, was dissected out from the diencephalon. The soft block of tissue was dissociated to single cells after digestion for 15 min at 37◦C with 0.5% trypsin (Worthington Biochemicals, Freehold, NJ, USA) and DNaseI (Sigma-AldrichCo. St. Louis, MO, USA) and washed in Ca2+/Mg2+-free Hank's Buffered Salt Solution. Neurons were counted and plated on glass coverslips coated with poly-L-lysine (Sigma-Aldrich) at a density of 200 cells/mm^2, were cultured in phenol red free Neurobasal supplemented with B-27 and GlutaMAXI (Invitrogen, Crewe, UK). Under these conditions the cells were maintained *in vitro* for 3 days (DIV).

For the treatment, male and female cultures were incubated for 1 DIV with 0.5 μM genistein (GEN, Sigma-Aldrich) or vehicle; others female cultures were always treated for 1 DIV but in combination 0.5 μM GEN with the selective ERα antagonist 1,3-bis (4-hydroxyphenyl)-4-methyl-5- [4-(2-piperidinyl-ethoxy) phenol]-1H –pyrazole dihydrochloride (MPP; 10^{-8}M); the selective ERβ antagonist 4-[2-phenyl-5,7-bis (trifluoromethyl) pyrazolo [1,5-a]pyrimidin-3-yl]phenol (PHTPP;10^{-8}M) and the selective GPER antagonist G15 (10^{-8}M) dissolved in Neurobasal medium.

Immunocytochemistry

Cells were fixed for 20 min at room temperature in 4% paraformaldehyde and permeabilized for 4 min with 0.12% Triton-X plus 0.12% gelatin in phosphate buffered saline (PBS). Cells were then washed with PBS/gelatin and incubated for 1 h with anti-microtubule associated protein-2 (MAP-2) chicken polyclonal antibody (diluted 1:100 in PBS/gelatin; Sigma-Aldrich). Finally, the cells were incubated for 45 minutes with secondary antibodies, Alexa Fluor® 488 (492/520 nm) made in donkey anti-chicken (1:500 in PBS/gelatin). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

Analysis of neuronal processes

Neuronal processes' number and branching was assessed with the method of Sholl (Sholl 1953), using a grid of 6 concentric circles with increasing radius of 20 μm. The grid was superimposed to the images of MAP-2-immunoreactive neurons at a magnification of X 400, placing the innermost circle over the soma. The number of the intersections of the processes with the lines of the grid was counted in 30 cells per each experimental condition.

Statistical analysis

The obtained 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 one way ANOVA and with *post hoc* Bonferroni test; values of $p \leq 0.05$ were considered significant.

Results

GEN effect is sexually dimorphic

The number of intersections of neuronal processes with the lines of the grid used for Sholl analysis was determined in male and female neurons immunostained for microtubule associated protein-2 (MAP2). The results showed a significant sex dimorphism (p=0.007) (Fig.1B). The female hypothalamic neurons had an increased neuritic arborization than male neurons. (Fig.1A). We treated male and female hypothalamic neurons with vehicle (Control) or 0.5 μM GEN for one day. The morphological analysis showed a significant increase in the number of intersections of the cell processes of female neurons treated with GEN. This effect was not detected in male neurons (p=0.001 female GEN vs. female Control, male Control and male GEN) (Fig.1B). GEN did not affect the mean number of primary processes, neither in male or female neurons

(F=2,287; p=0.133). However, female neurons treated with GEN had a higher number of intersections with the more external circles, indicating that in this group of neurons have longer processes (Fig.1C).

GEN effect is mediated by ERα, ERβ and GPER

After the results of the first experiment, showing that GEN has a significant effect only in female neurons, we exposed female cell cultures to vehicle or to 0.5 μM GEN associated with MPP or PHTPP or G15 (selective antagonists of ERα, ERβ and GPER, respectively). The number of intersections of neuronal processes in the Sholl analysis showed that the GEN effect on female cultures is mediated by ERα, ERβ and GPER. In fact, the data show a significant increase in neurite arborization of female neurons treated with GEN compared to control female neurons (p=0.001), and a significant decrease in female neurons treated with GEN and the ERα antagonist MPP (p=0.001), the ERβ antagonist PHTPP (p=0.015) or the GPER antagonist G15 (p=0.002) in comparison to neurons treated with GEN alone (Fig.2B).

Discussion

In this study we analyzed for first time the effect of GEN on hypothalamic neurons in culture. The results confirm previous data on the existence of sex differences in neuritogenesis of hypothalamic neurons obtained from E14 mice embryos, before the critical period of brain masculinization (Scerbo et al., 2014). We now also show a sex difference in the response to GEN treatment, in fact, only in female neurons GEN induced a significant increase in the arborization of neuronal processes, assessed by Sholl analysis and we demonstrate that this GEN effect is mediated by ERα, ERβ and GPER.

Male and female hypothalamic cultures showed a different neuritogenesis, female neurons showed an increased processes' arborization compared to female neurons. In a previous study, Scerbo et al. analyzed the development of CD1 mouse hypothalamic male and female neurons obtained from E14 embryos. They found that at 1 day in vitro (DIV), 18% of male neurons showed neurites (Stage II), while 48% of female neurons had already reached this stage. They also show that the proportion of neurons with branched processes at 1 DIV was higher in female cultures (Scerbo et al. 2014). The biological consequences of this neural sex difference are unknown; especially because we used E14 mouse embryos, when neuronal development should be still independent by gonadal hormones. In mice, the peak of testosterone, the primary gonadal steroid in males, is produced by fetal testis at E17-18 (O'Shaughnessy et al. 1998;

O'Shaughnessy, Baker, and Johnston 2006). Testosterone can be locally aromatized to estradiol by the enzyme aromatase cytochrome P450, that is present in the hypothalamus (Somponpun and Sladek 2004).

In vivo, the phytoestrogen GEN exerts alterations of some behaviors and neuronal circuits in a permanent and sexually different manner. In fact, prenatal or postnatal administration of GEN in rodents interferes with anxiety-related behaviors (Rodriguez-Gomez et al. 2014) and with neuronal Nitric Oxide Synthase (nNOS), Vasopressin (AVP) and Kisspeptin (Kiss) pathways in the adult (Ponti et al. 2017); Losa et al., 2011). *In vitro,* we have shown by the present morphological analysis that GEN treatment had a sexually different effect with an increase in neuritogenesis only in female neurons.

Previous *in vivo* and *in vitro* data underlined the expression of ERα, ERβ and GPER in the hypothalamus and the their potential roles in GEN effects on different hypothalamic systems (Ponti et al. 2017; Rodriguez-Gomez et al. 2014; Somponpun and Sladek 2004). In older studies, Kuiper et al. demonstrated that GEN acts as an agonist on ERα and ERβ, but has a higher affinity for ERβ, based on solubilized receptor-binding assay (Kuiper et al. 1998). In addition, the ability of GEN to mimic the effect of estradiol on NMDA stimulated AVP and oxytocin release from hypothalamus suggests that the GEN effect is mediated by ERβ (Somponpun and Sladek 2004). These studies did not considered the effect of GEN on the two sexes, whereas, here we demonstrate that GEN has a neuritogenic effect only in female hypothalamic neurons and its action is mediated by ERα, ERβ and GPER. This could probably explain why, *in vivo,* the GEN effect is different according to various neuronal populations and hypothalamic nuclei examined so far.

Acknowledgements

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Author contributions

MM performed experiments, analyzed data and wrote the paper. MAA performed experiments and analyzed data. SG, GCP and LMGS designed experiments, wrote and supervised the paper.

References

Couse, J. F., and K. S. Korach. 1999. 'Estrogen receptor null mice: what have we learned and where will they lead us?', *Endocr Rev*, 20: 358-417.

Diaz, H., A. Lorenzo, H. F. Carrer, and A. Caceres. 1992. 'Time lapse study of neurite growth in hypothalamic dissociated neurons in culture: sex differences and estrogen effects', *J Neurosci Res*, 33: 266-81.

Dixon, R. A., and D. Ferreira. 2002. 'Genistein', *Phytochemistry*, 60: 205-11.

Frank, A., L. M. Brown, and D. J. Clegg. 2014. 'The role of hypothalamic estrogen receptors in metabolic regulation', *Front Neuroendocrinol*, 35: 550-7.

Kuiper, G. G., J. G. Lemmen, B. Carlsson, J. C. Corton, S. H. Safe, P. T. van der Saag, B. van der Burg, and J. A. Gustafsson. 1998. 'Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta', *Endocrinology*, 139: 4252-63.

Nie, Q., M. Xing, J. Hu, X. Hu, S. Nie, and M. Xie. 2017. 'Metabolism and health effects of phyto-estrogens', *Crit Rev Food Sci Nutr*, 57: 2432-54.

O'Shaughnessy, P. J., P. J. Baker, and H. Johnston. 2006. 'The foetal Leydig cell--differentiation, function and regulation', *Int J Androl*, 29: 90-5; discussion 105-8.

O'Shaughnessy, P. J., P. Baker, U. Sohnius, A. M. Haavisto, H. M. Charlton, and I. Huhtaniemi. 1998. 'Fetal development of Leydig cell activity in the mouse is independent of pituitary gonadotroph function', *Endocrinology*, 139: 1141-6.

Pan, M., H. Han, C. Zhong, and Q. Geng. 2012. 'Effects of genistein and daidzein on hippocampus neuronal cell proliferation and BDNF expression in H19-7 neural cell line', *J Nutr Health Aging*, 16: 389-94.

Ponti, G., A. Rodriguez-Gomez, A. Farinetti, M. Marraudino, F. Filice, B. Foglio, G. Sciacca, G. C. Panzica, and S. Gotti. 2017. 'Early postnatal genistein administration permanently affects nitrergic and vasopressinergic systems in a sex-specific way', *Neuroscience*, 346: 203-15.

Rodriguez-Gomez, A., F. Filice, S. Gotti, and G. Panzica. 2014. 'Perinatal exposure to genistein affects the normal development of anxiety and aggressive behaviors and nitric oxide system in CD1 male mice', *Physiol Behav*, 133: 107-14.

Scerbo, M. J., A. Freire-Regatillo, C. D. Cisternas, M. Brunotto, M. A. Arevalo, L. M. Garcia-Segura, and M. J. Cambiasso. 2014. 'Neurogenin 3 mediates sex chromosome effects on the generation of sex differences in hypothalamic neuronal development', *Front Cell Neurosci*, 8: 188.

Sholl, D. A. 1953. 'Dendritic organization in the neurons of the visual and motor cortices of the cat', *J Anat*, 87: 387-406.

Somponpun, S. J., and C. D. Sladek. 2004. 'Depletion of oestrogen receptor-beta expression in magnocellular arginine vasopressin neurones by hypovolaemia and dehydration', *J Neuroendocrinol*, 16: 544-9.

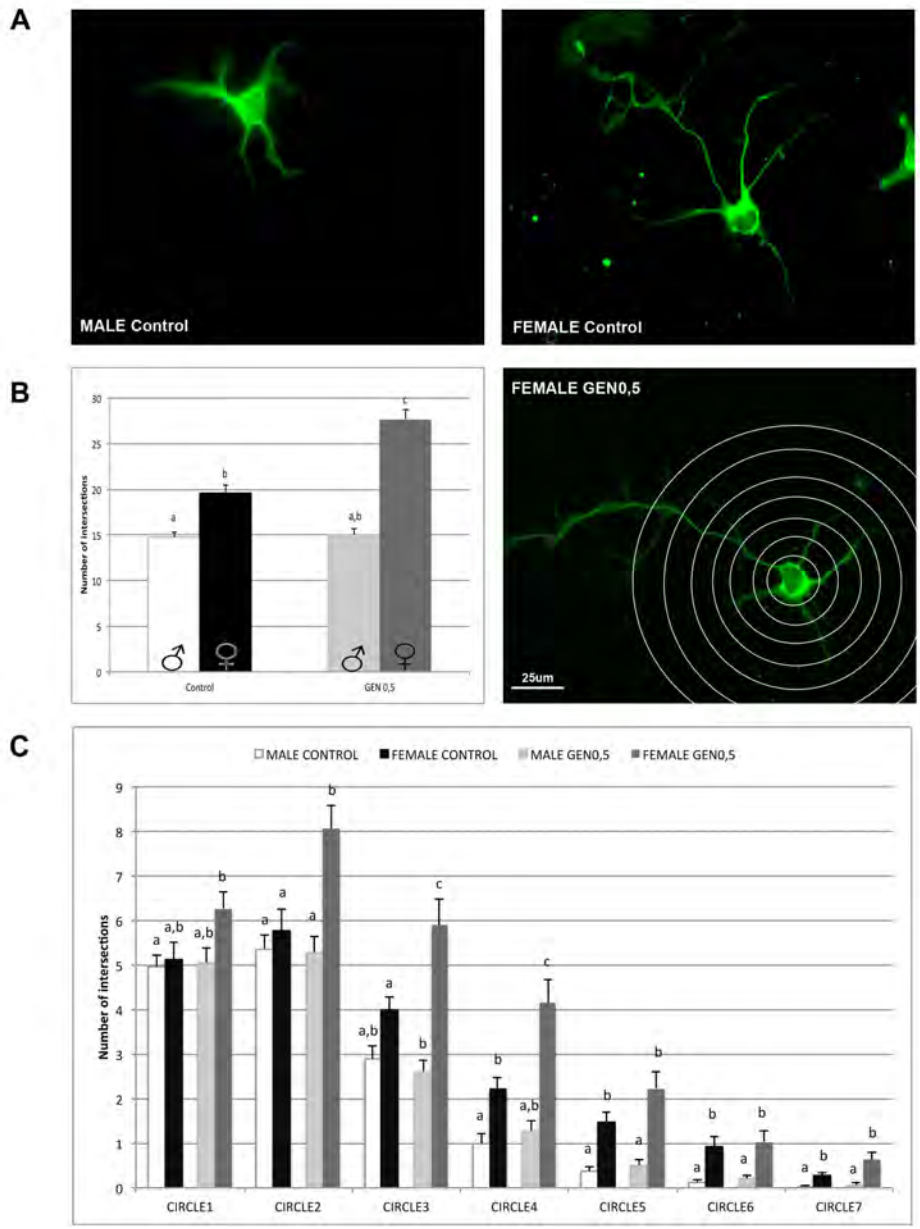

Figure 1. **Neuritogenic effects of genistein (GEN, 0.5 µM) on** primary **male and female hypothalamic neurons.**

A, Representative examples of control male and female primary hypothalamic neurons immunostained for microtubule associated protein-2 (MAP2).

B, Number of intersection of neuronal processes in the Sholl analysis under control conditions and after GEN treatment (left panel). Control neurons show a significant sex dimorphism (p=0.007). The treatment with GEN induces a significant increase only in female neurons (p=0.0001 vs female Control; male Control and male GEN). Right panel, example of a female neuron treated with GEN showing the grid used for Sholl analysis.

C, N number of intersections of the processes of female neurons with the different concentric circles of the Sholl grid. Neurons treated with GEN had a higher number of intersections with the more external circles, indicating that in this group, neurons have longer processes.

Scale bar = 25 μm. Data are the mean ± SEM of 30 hypothalamic neurons per sex.

Bars with different letters are significantly different between male and female for all analyzed cultures ($p<0.05$; *post hoc* Bonferroni test).

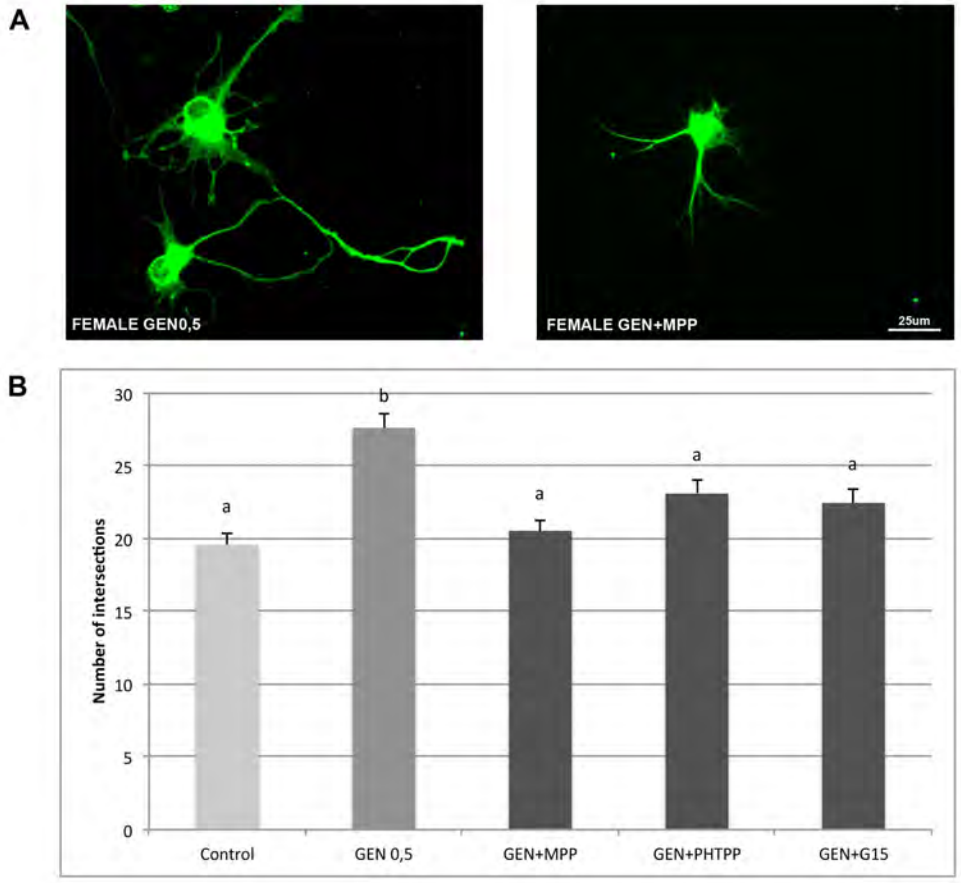

Figure 2. **Neuritogenic effects of genistein (GEN, 0.5 µM) and selective antagonist of estrogen receptors in female primary hypothalamic neurons.**

A, Representative examples of female neurons treated with GEN (left panel) or GEN and the ERα antagonist MPP (right panel). Neurons are immunostained for microtubule-associated protein-2 (MAP2).

B, The bar graph represents the number of intersections of neurites in the Sholl analysis. The statistical analysis indicate a significant effect in the effect of GEN compared to control (p=0.0001) and significant effects of the treatment with MPP (p=0.0001), PHTPP (p=0.015) or G15 (p=0.002) and GEN compared to GEN alone.

Scale bar = 25 µm. Data are the mean ± SEM of 30 hypothalamic neurons.

Bars with different letters are significantly different between different treatments for all analyzed cultures ($p<0.05$; *post hoc* Bonferroni test).

CHAPTER 9

General Conclusions

The Paraventricular nucleus (PVN) is a small nucleus localized within the anterior region of the hypothalamus. In rats, it consists of approximately 100,000 neurons in a volume of about 0.5 mm^3 and it is arranged in a wing shape fashion along the dorsal portion of the third ventricle. This small nucleus is one of the most important autonomic control centers in the brain. Its neurons play important roles in controlling stress, growth, reproduction, immune and other more traditional autonomic functions (gastrointestinal, renal and cardiovascular), but it is also important for the regulation of the energetic metabolism and food intake (Ferguson, Latchford, and Samson 2008). For this reason, I chose to study in my thesis the PVN innervation of the kisspeptin system that, as reported in the introduction, is a bridge between the control of metabolism and that of reproduction. In the literature the presence of kisspeptin fibers within the PVN was always mentioned but never in deeply investigated. In fact, many researches studied the kisspeptin system limited to two hypothalamic regions where cell bodies are located [the rostral periventricular region (RP3V), and the arcuate nucleus (ARC)] and their projections to the gonadotropin-releasing hormone (GnRH) neurons. Actually, the primary role of kisspeptin is to regulate the GnRH neurons for the control of puberty and fertility. Only in the last few years, some studies analyzed the importance of metabolic conditions and of the amount of energy reserves of an organism for the modulation of pubertal timing, suggesting a new role for

kisspeptin: to integrate the different peripheral and central signals and to transmit metabolic information on reproductive centers (Castellano and Tena-Sempere 2016).

In my first experiment (Chapter 3, Marraudino et al., 2017), performed, as all other experiments of this thesis, in CD1 mice, I confirmed previous data of the literature (Clarkson and Herbison 2006; Kauffman et al. 2007) indicating that the two groups of kisspeptin neurons clustered in RP3V and ARC are strongly sexually dimorphic, with more cells and fibers in females than in males. Moreover, I studied in detail the kisspeptin fibers' distribution within the PVN by a particular method (see Chapter 1, Microscopy technique: a new method of seeing the PVN) to better visualize the kisspeptin fibers within the PVN that briefly consist in recording at higher magnification (40 x objective) the PVN images subdividing the nucleus into two regions, dorsal and ventral. Each single field was acquired with four different planes of focus. The final image was a more defined image of the whole monolateral PVN. To measure the kisspeptin fibers within PVN, I divided the nucleus into four squares to cover its full extension (see Chapter 3; Chapter 4 and Chapter 5, Materials and Methods). 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, ventromedial and ventro-lateral. However, most of the ventro-lateral subdivision lays outside the PVN; for this reason in the last experiment, where I analyzed the kisspeptin within PVN (Chapter 7), I used a major number of smaller squares, fourteen. In this way, the squares cover the whole PVN in a better detail.
Previous studies (Clarkson et al. 2009; Clarkson and Herbison 2006; Lehman, Hileman, and Goodman 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 RP3V (Yeo and Herbison 2011; Yip et al. 2015). I showed, for the first time, that the kisspeptin innervation (a) 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), (b) is sexually dimorphic (with females having a denser innervation than males), (c) is not homogenously distributed within the nucleus (the density of kisspeptin fibers is higher in the medial than lateral PVN).

After the study in adult mice, in the second experiment (Chapter 4) I studied the post-natal development of the female kisspeptin system, focusing my attention on the development of the kisspeptin innervations of the PVN. The choice of using females and an interval between

postnatal day (PND) 12 and 30 is based on the previous literature. In fact, the expression of kisspeptin is dimorphic, higher in females than in males, and *in situ* hybridization and IHC studies have demonstrated a significant modification of the kisspeptin system during pre-pubertal period, particularly in RP3V (Clarkson and Herbison 2006; Clarkson et al. 2009). Moreover, in mice AVPV Kiss1 mRNA and kisspeptin protein are undetectable prior to PND10-PND15, and then they increase from PND 15 to adulthood (Clarkson and Herbison 2006; Clarkson et al. 2009), while data regarding peri-pubertal changes in ARC Kiss1 gene expression are either lacking, incomplete, or conflicting. In my study I found a moderate increase of kisspeptin immunoreactivity in the RP3V at PND15, which became stronger at PND18 and continued with a gradual increase up to PND30. In ARC I showed a strong increase of kiss-ir at PND18. After this peak, the signal was slightly decreasing until PND30. Thus, my data demonstrated the presence of a strong increase of kisspeptin signal before the puberty in both nuclei. The analysis of the kisspeptin innervation of the PVN during the development, showed changes of fibers' density very similar to those observed in the RP3V and ARC. Interestingly, the heterogeneity of kiss distribution within the PVN was already present during the development. In fact, I demonstrated that the medial part of the PVN showed a higher density of kisspeptin fibers in comparison to the lateral part along the entire developmental period that I observed. Moreover, the changes in the medial part are similar to those observed in the RP3V and ARC, while the innervation of the lateral part is always lower and we have not observed any strongly significant change in this part of the PVN during the development. The temporal correlation between the development of kisspeptin fibers in PVN and the changes in the immunoreactivity in the AVP and ARC, suggests the hypothesis that both nuclei may contribute to the PVN innervation (see Chapter 4, Discussion).

Several studies had already demonstrated that the kisspeptin system changes during the estrous cycle, showing the highest expression in RP3V during estrus (*positive feedback*), and in ARC during diestrus (*negative feedback*) (Smith et al. 2006). As reported in Chapter 3 (Marraudino et al., 2017), this is true also for the innervation of the PVN. Kisspeptin neurons express estrogen receptor alpha (ERα) (~90%) (Franceschini et al. 2006; Smith et al. 2005), androgen receptor (~65%) (Smith et al. 2005), and also the progesterone receptor (~86%) (Smith et al. 2007). In the Chapter 5, I investigated the role played by estradiol (E_2) or progesterone (P), alone and together, in the regulation of the female kisspeptin system. I used an experimental paradigm mimicking the hormonal situation typical of the late proestrus (E_2+P), the early proestrus (only E_2) and the diestrus (only P) in ovarectomized female mice. According to my results, P is playing an

important role to activate ARC kiss neurons, whereas E_2 (alone or together at P) activates RP3V kisspeptin neurons. In the PVN, both E_2 and P induced an increase of the kisspeptin fibers in comparison to the ovarictomized females, indicating that both nuclei contribute to the PVN innervation, thus confirming previous data collected during the post-pubertal development (see Chapter 5, Discussion).

In all experiments (Chapter 3, 4 and 5) emerged that the Kisspeptin innervation within the PVN is heterogeneous, with a higher density of fibers in the medial than in the lateral part of nucleus. Besides, in the third experiment (Chapter 5), I showed that the kisspeptin innervation of the medial part of the PVN is under the influence of both E_2 and P (alone or together), whereas the lateral part shows very limited changes. This suggests that there is an estrogen-insensitive kisspeptin innervation of the PVN that arrive from both rostral (RP3V) and caudal (ARC) kisspeptin cell groups. When E_2 increases, there is a consequent activation of kisspeptin system in RP3V that will increase the fibers' immunoreactivity within the medial part of PVN. These data confirmed the results obtained in the first experiment, in which analyzing the changes in estrus cycle of kiss density in PVN in line with RP3V (a higher density of positive fibers during estrus) and showing the absence of co-expressed neurokinin B (NkB) and kisspeptin in PVN, strongly present in ARC (Navarro et al. 2009), I hypothesized that kisspeptin fibers observed in the PVN should arrive mostly from RP3V.
In summary, these experiments demonstrated the presence of a large innervation of the PVN by both RP3V and ARC nuclei, with a marked difference among the medial (highly innervated) and the lateral part of the PVN. The density of this innervation fluctuates according to the changes in the RP3V and ARC during the estrous cycle, and it shows an increase before the onset of puberty. The main question is the target of these fibers. In former studies, it was denied the presence of the kisspeptin receptor (Kiss1r) in the PVN (Herbison et al. 2010), therefore it was discussed the possibility that the kisspeptin in the PVN may interact with some other receptors (as for example the RF-amide receptors GPR74 and 147; Oishi et al. 2011). However, a recent in situ hybridization study demonstrated that in the PVN Kiss1r-expressing neurons were sparsely distributed, mainly in the medial part of the nucleus (Higo et al. 2016), and this data is perfectly in line with my results.

The next question was to identify the neurons receiving the kisspeptin innervation within the PVN. The PVN is characterized by a very complex cyto-architecture and different types of neurons. The lateral part contains magnocellular neurons chiefly projecting to the posterior

pituitary (where they release oxitocin, OT, and vasopressin, AVP, into the blood), the medial part of the 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. CRH; (Wang et al. 2011); TRH; (Kadar et al. 2010); TH; (Ruggiero et al. 1984); nNOS; (Gotti et al. 2004; Gotti et al. 2005); AVP; (Caldwell et al. 2008); somatostatin; (Tan et al. 2013). In my first study (Chapter 3) I compared by double-immunofluorescence the distribution of some of these neuronal populations and kisspeptin fibers within the PVN, and showed that AVP and nNOS neurons were not related to the kisspeptin system, while the presence of several OT and TH neurons in the medial PVN, where the concentration of kiss-ir fibers is massive, suggested a possible interrelation between these systems (see Chapter 3, Discussion). In the fourth experiment (Chapter 6) I analyzed the relationships between the kisspeptin and another hypothalamic neuronal population, the TRH producing neurons. TRH has an important role in the regulation of energy homeostasis not only for the control of thyroid function, but also through effects on feeding behavior, and on energy consuming activities as thermogenesis or activation of locomotion (Lechan and Fekete 2006). By double-immunofluorescence I demonstrated that in male and female adult CD1 mice there are close relationships between kisspeptin fibers and TRH neurons within PVN. This suggests a correlation among the gonadal steroids hormones, kisspeptin projections, and metabolism-controlling TRH neurons. Previous data suggested a possible correlation between kisspeptin and the regulation of body metabolism: the kisspeptin expression is altered in conditions of reproductive impairment linked to metabolic stress and the normalization of the kiss contributes to improving the reproductive phenotype despite unfavorable metabolic conditions (Castellano et al. 2011; Castellano and Tena-Sempere 2016; Roa et al. 2009). Moreover, studies on transgenic mice demonstrated that the leptin receptor is present in about 10% of kisspeptin neurons in the ARC nucleus, but it coexists in a few scattered elements of the RP3V population (Cravo et al. 2011). My results demonstrated that there is a strong neuroanatomical correlation between reproductive and metabolic control in PVN due to kisspeptin projections on TRH neurons.

Kisspeptin system, as broadly discussed, is susceptible to gonadal steroids, and for this reasons it is a sensible target for Endocrine disrupting chemicals (EDCs). In rodents, developmental exposure to EDCs including PCBs, atrazine, BPA and GEN can perturb aspects of the sex-specific GnRH feedback system resulting in accelerated female puberty, irregular estrous cycles, subfertility, and premature anestrous (Patisaul and Belcher 2017). The first studies on vulnerability of kisspeptin to EDCs were reported in 2009, using BPA and, immediately after,

other works detected alterations of this system using PCBs (reviewed in Patisaul 2017). While, about the impact of phytoestrogens on kisspeptin signaling networks, the data present in literature are very limited. Developmental exposure to GEN has a masculinizing influence on the rat female kisspeptin system, advances female puberty, and produces estrous cycle irregularities (Bateman and Patisaul 2008). GEN or other soy phytoestrogens could have an obesogenic effect (Newbold 2010) or might counteract aspects of metabolic syndrome (Jungbauer and Medjakovic 2014). In fact, in rats GEN treatment changes body weight, but the effect depends on sex, age, and hormonal status (Cao et al. 2015; Ruhlen et al. 2008; Slikker et al. 2001). For these reasons, in the experiment reported in Chapter 7, I investigated the effects of GEN on kisspeptin system (as a paradigm for the metabolic and reproductive control) and also on two other hypothalamic systems related to food intake and metabolic control, pro-opio-melanocortin (POMC) and Orexin. In this experiment, male and female mice were exposed to GEN, in a dose comparable to that of babies fed with soy-based formulas, during the post-natal week (critical period for the sexual differentiation of the hypothalamus). Confirming previous studies showing that GEN exposure during the early postnatal period favors the development of obesity in female, but not male rats (Strakovsky et al. 2014); in my experiment I found that the early postnatal exposure to GEN determines long-term sex-specific organizational effects on different aspects of network controlling food intake, energy metabolism and reproduction. The early GEN treatment induced a sexually dimorphic obesogenic effect (adult females increased the body weight), coupled to some alterations of metabolic regulation: (a) decrease of T3 and leptin only in female; (b) no alterations of food intake or daily feed efficiency; (c) morphological alterations of the circuits expressing Orexin, POMC, and kisspeptin. I also observed strong reproductive alterations: (a) anticipation of puberty in GEN female, increase of uterus weight in pre-puberty time, and higher number of tertiary branches (TEB) in mammary gland at PND22; (b) irregular estrous cycles, with a reduction of proestrus phase; (c) higher number of TEB in mammary gland of adult GEN female; (d) decrease of fecal testosterone and weight of testicles in adult males (for more details see Chapter 7, Discussion).

In summary, this experiment in mice, as well other experiments performed in rats, demonstrated that hypothalamic circuits are primary targets for the action of GEN, and that this action is sexually dimorphic. However, it is not clear the mechanism(s) through which GEN is influencing the brain circuits' development. It has been suggested that ERα is primarily responsible for masculinization while ERβ could be more important for defeminization (Kudwa et al. 2006). In my last experiment (Chapter 8), I tested if GEN has sexually dimorphic effects also on processes' outgrowth (neuritogenesis) of embryonic hypothalamic neurons *in vitro* and

what estrogen receptor (ER) is likely to be implicated. The results confirmed the hypothesis showing the existence of sex differences in neuritogenesis of hypothalamic neurons before the critical period of brain masculinization, after the GEN treatment there was a significant increase of the processes' arborization only in female cell culture. GEN is a natural occurring ERβ selective agonist (Jefferson, Patisaul, and Williams 2012), besides there are evidences that GEN can bind with the non nuclear estrogen receptor GPR30 (Thomas and Dong 2006). In this last experiment, I demonstrated that the GEN effect on the outgrowth of processes is mediated by ERα, ERβ and GPR30 (see Chapter 8, Discussion). This probably could explain why the GEN effect *in vivo* is different for each neuronal populations and examined hypothalamic nuclei .

In conclusion, the experiments presented in this thesis support the idea that kisspeptin is not only the main player for the control of reproductive functions, but that it is also involved in the control of metabolism. The link among the two systems is probably the PVN, where kisspeptin fibers release in a estrogen-depending way the neuropeptide to act on some of the peptidergic circuits of the PVN that are involved in the control of energy metabolism and food intake. The kisspeptin system is particularly vulnerable to EDCs, as demonstrated by our study on the effects of GEN on both reproduction and metabolism.

References

Bateman, H. L., and H. B. Patisaul. 2008. 'Disrupted female reproductive physiology following neonatal exposure to phytoestrogens or estrogen specific ligands is associated with decreased GnRH activation and kisspeptin fiber density in the hypothalamus', *Neurotoxicology*, 29: 988-97.

Caldwell, H. K., H. J. Lee, A. H. Macbeth, and W. S. Young, 3rd. 2008. 'Vasopressin: behavioral roles of an "original" neuropeptide', *Prog Neurobiol*, 84: 1-24.

Cao, J., R. Echelberger, M. Liu, E. Sluzas, K. McCaffrey, B. Buckley, and H. B. Patisaul. 2015. 'Soy but not bisphenol A (BPA) or the phytoestrogen genistin alters developmental weight gain and food intake in pregnant rats and their offspring', *Reprod Toxicol*, 58: 282-94.

Castellano, J. M., A. H. Bentsen, M. A. Sanchez-Garrido, F. Ruiz-Pino, M. Romero, D. Garcia-Galiano, E. Aguilar, L. Pinilla, C. Dieguez, J. D. Mikkelsen, and M. Tena-Sempere. 2011. 'Early metabolic programming of puberty onset: impact of changes in postnatal feeding and rearing conditions on the timing of puberty and development of the hypothalamic kisspeptin system', *Endocrinology*, 152: 3396-408.

Castellano, J. M., and M. Tena-Sempere. 2016. 'Metabolic control of female puberty: potential therapeutic targets', *Expert Opin Ther Targets*, 20: 1181-93.

Clarkson, J., W. C. Boon, E. R. Simpson, and A. E. Herbison. 2009. 'Postnatal development of an estradiol-kisspeptin positive feedback mechanism implicated in puberty onset', *Endocrinology*, 150: 3214-20.

Clarkson, J., and A. E. Herbison. 2006. 'Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons', *Endocrinology*, 147: 5817-25.

Cravo, R. M., L. O. Margatho, S. Osborne-Lawrence, J. Donato, Jr., S. Atkin, A. L. Bookout, S. Rovinsky, R. Frazao, C. E. Lee, L. Gautron, J. M. Zigman, and C. F. Elias. 2011. 'Characterization of Kiss1 neurons using transgenic mouse models', *Neuroscience*, 173: 37-56.

Ferguson, A. V., K. J. Latchford, and W. K. Samson. 2008. 'The paraventricular nucleus of the hypothalamus - a potential target for integrative treatment of autonomic dysfunction', *Expert Opin Ther Targets*, 12: 717-27.

Franceschini, I., D. Lomet, M. Cateau, G. Delsol, Y. Tillet, and A. Caraty. 2006. 'Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor alpha', *Neurosci Lett*, 401: 225-30.

Gotti, S., S. Chiavegatto, M. Sica, C. Viglietti-Panzica, R. J. Nelson, and G. Panzica. 2004. 'Alteration of NO-producing system in the basal forebrain and hypothalamus of Ts65Dn mice: an immunohistochemical and histochemical study of a murine model for Down syndrome', *Neurobiol Dis*, 16: 563-71.

Gotti, S., M. Sica, C. Viglietti-Panzica, and G. Panzica. 2005. 'Distribution of nitric oxide synthase immunoreactivity in the mouse brain', *Microsc Res Tech*, 68: 13-35.

Higo, S., S. Honda, N. Iijima, and H. Ozawa. 2016. 'Mapping of Kisspeptin Receptor mRNA in the Whole Rat Brain and its Co-Localisation with Oxytocin in the Paraventricular Nucleus', *J Neuroendocrinol*, 28.

Jefferson, W. N., H. B. Patisaul, and C. J. Williams. 2012. 'Reproductive consequences of developmental phytoestrogen exposure', *Reproduction*, 143: 247-60.

Jungbauer, A., and S. Medjakovic. 2014. 'Phytoestrogens and the metabolic syndrome', *J Steroid Biochem Mol Biol*, 139: 277-89.

Kadar, A., E. Sanchez, G. Wittmann, P. S. Singru, T. Fuzesi, A. Marsili, P. R. Larsen, Z. Liposits, R. M. Lechan, and C. Fekete. 2010. 'Distribution of hypophysiotropic thyrotropin-releasing hormone (TRH)-synthesizing neurons in the hypothalamic paraventricular nucleus of the mouse', *J Comp Neurol*, 518: 3948-61.

Kauffman, A. S., M. L. Gottsch, J. Roa, A. C. Byquist, A. Crown, D. K. Clifton, G. E. Hoffman, R. A. Steiner, and M. Tena-Sempere. 2007. 'Sexual differentiation of Kiss1 gene expression in the brain of the rat', *Endocrinology*, 148: 1774-83.

Kudwa, A. E., V. Michopoulos, J. D. Gatewood, and E. F. Rissman. 2006. 'Roles of estrogen receptors alpha and beta in differentiation of mouse sexual behavior', *Neuroscience*, 138: 921-8.

Lechan, R. M., and C. Fekete. 2006. 'The TRH neuron: a hypothalamic integrator of energy metabolism', *Prog Brain Res*, 153: 209-35.

Lehman, M. N., S. M. Hileman, and R. L. Goodman. 2013. 'Neuroanatomy of the kisspeptin signaling system in mammals: comparative and developmental aspects', *Adv Exp Med Biol*, 784: 27-62.

Navarro, V. M., M. A. Sanchez-Garrido, J. M. Castellano, J. Roa, D. Garcia-Galiano, R. Pineda, E. Aguilar, L. Pinilla, and M. Tena-Sempere. 2009. 'Persistent impairment of hypothalamic KiSS-1 system after exposures to estrogenic compounds at critical periods of brain sex differentiation', *Endocrinology*, 150: 2359-67.

Newbold, R. R. 2010. 'Impact of environmental endocrine disrupting chemicals on the development of obesity', *Hormones (Athens)*, 9: 206-17.

Patisaul, Heather B., and Scott M. Belcher. 2017. *Endocrine disruptors, brain, and behaviors* (Oxford University Press: New York, NY).

Roa, J., D. Garcia-Galiano, L. Varela, M. A. Sanchez-Garrido, R. Pineda, J. M. Castellano, F. Ruiz-Pino, M. Romero, E. Aguilar, M. Lopez, F. Gaytan, C. Dieguez, L. Pinilla, and M. Tena-Sempere. 2009. 'The mammalian target of rapamycin as novel central regulator of puberty onset via modulation of hypothalamic Kiss1 system', *Endocrinology*, 150: 5016-26.

Ruggiero, D. A., H. Baker, T. H. Joh, and D. J. Reis. 1984. 'Distribution of catecholamine neurons in the hypothalamus and preoptic region of mouse', *J Comp Neurol*, 223: 556-82.

Ruhlen, R. L., K. L. Howdeshell, J. Mao, J. A. Taylor, F. H. Bronson, R. R. Newbold, W. V. Welshons, and F. S. vom Saal. 2008. 'Low phytoestrogen levels in feed increase fetal serum estradiol resulting in the "fetal estrogenization syndrome" and obesity in CD-1 mice', *Environ Health Perspect*, 116: 322-8.

Slikker, W., Jr., A. C. Scallet, D. R. Doerge, and S. A. Ferguson. 2001. 'Gender-based differences in rats after chronic dietary exposure to genistein', *Int J Toxicol*, 20: 175-9.

Smith, J. T., B. V. Acohido, D. K. Clifton, and R. A. Steiner. 2006. 'KiSS-1 neurones are direct targets for leptin in the ob/ob mouse', *J Neuroendocrinol*, 18: 298-303.

Smith, J. T., C. M. Clay, A. Caraty, and I. J. Clarke. 2007. 'KiSS-1 messenger ribonucleic acid expression in the hypothalamus of the ewe is regulated by sex steroids and season', *Endocrinology*, 148: 1150-7.

Smith, J. T., H. M. Dungan, E. A. Stoll, M. L. Gottsch, R. E. Braun, S. M. Eacker, D. K. Clifton, and R. A. Steiner. 2005. 'Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse', *Endocrinology*, 146: 2976-84.

Tan, H. Y., L. Huang, D. Simmons, J. D. Veldhuis, F. J. Steyn, and C. Chen. 2013. 'Hypothalamic distribution of somatostatin mRNA expressing neurones relative to pubertal and adult changes in pulsatile growth hormone secretion in mice', *J Neuroendocrinol*, 25: 910-9.

Thomas, P., and J. Dong. 2006. 'Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption', *J Steroid Biochem Mol Biol*, 102: 175-9.

Wang, L., M. Goebel-Stengel, A. Stengel, S. V. Wu, G. Ohning, and Y. Tache. 2011. 'Comparison of CRF-immunoreactive neurons distribution in mouse and rat brains and selective induction of Fos in rat hypothalamic CRF neurons by abdominal surgery', *Brain Res*, 1415: 34-46.

Yeo, S. H., and A. E. Herbison. 2011. 'Projections of arcuate nucleus and rostral periventricular kisspeptin neurons in the adult female mouse brain', *Endocrinology*, 152: 2387-99.

Yip, S. H., U. Boehm, A. E. Herbison, and R. E. Campbell. 2015. 'Conditional Viral Tract Tracing Delineates the Projections of the Distinct Kisspeptin Neuron Populations to Gonadotropin-Releasing Hormone (GnRH) Neurons in the Mouse', *Endocrinology*, 156: 2582-94.

List of papers

M. Marraudino, D. Miceli, A. Farinetti, G.C. Panzica, S. Gotti.
The Kisspeptin Innervation of the Hypothalamic Paraventricular Nucleus: Sexual Dimorphism and Effect of Estrous Cycle in Female Mice. J. Anatomy. **2017** Jun;230(6):775-786. doi:10.1111/joa.12603.

M. Marraudino, M. Martini, S. Trova, A. Farinetti, G. Ponti, S. Gotti, G.C. Panzica.
Effects of estradiol and progesterone on regulation of the Kisspeptin system in ovariectomized CD1 mice. Submitted to Brain Research.

G. Ponti, A. Rodriguez-Gomez, A. Farinetti, **M. Marraudino**, F. Filice, B. Foglio, G. Sciacca, G.C.Panzica, S.Gotti.
Early postnatal genistein administration permanently affects nitrergic and vasopressinergic systems in a sex-specific way. Neuroscience. **2017** Mar 27;346:203-215. doi:10.1016/j.neuroscience.2017.01.024.

E. Bo, A. Farinetti, **M. Marraudino**, D. Sterchele, C. Eva, S. Gotti, G.C. Panzica.
Adult exposure to tributyltin affects hypothalamic neuropeptide Y, Y1 receptor distribution, and circulating leptin in mice. Andrology**. 2016** Jul;4(4):723-34. doi: 10.1111/andr.12222.

L. Oboti, S. Trova, R. Schellino, **M. Marraudino**, N.R Harris, O.M Abiona, M. Stampar, W. Lin, P. Peretto.
Activity Dependent Modulation of Granule Cell Survival in the Accessory Olfactory Bulb at Puberty. Frontiers Neuroanatomy. **2017** May 23;11:44. doi: 10.3389/fnana.2017.00044.

A.Farinetti, **M. Marraudino**, G. Ponti, G.C. Panzica, S.Gotti.
Chronic treatment with tributyltin induces sexually dimorphic alterations of POMC system and activated leptin receptor in the hypothalamic Arcuate nucleus of adult mice. Submitted to Cell and Tissue Research.

G. Ponti, A. Farinetti, **M. Marraudino**, G.C. Panzica, Stefano Gotti.
Sex steroids and adult neurogenesis in the ventricular-subventricular zone. Submitted to Frontiers in Endocrinology.