Chapter 3

# Endocannabinoids and Kisspeptins: Two Modulators in Fight for the Regulation of GnRH Activity

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Additional information is available at the end of the chapter

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## 1. Introduction

The master system in the control of reproductive functions is the communication into the hypothalamus-pituitary-gonadal axis (HPG), whose main actor is the hypothalamic gonadotropin releasing hormone (GnRH). Such a decapeptide triggers the release of pituitary gonadotropins [Follicular Stimulating Hormone (FSH) and Luteinizing Hormone (LH)] which in turn reach the gonads, induce the biosynthesis of steroids (mainly testosterone in males and estradiol/progesterone in females) and of other non steroidal substances (i.e. activin, follistastin, inhibin) modulating the gametogenesis in both sexes. In the last decades, a significant upsurge of studies aimed to define seveal actors and mechanisms supporting reproductive activity. Ultra short, short and long feedback in HPG communication finely modulate reproduction. Nevertheless, this picture is still puzzling and the complete knowledge of the full process has to be unravelled.

"*Only on the basis of an extensive comparative biology can authentic general biology emerge*" (Bern 1967). Besides the importance of evolutionary track in the research of adaptive phenomena, comparative approaches provide a deep insight into the physiological mechanism in building general models. At present, 25 GnRH molecular forms have been detected in metazoan, also in species lacking pituitary gland. Up to 15 molecular forms have been detected in vertebrates; fish, amphibians, reptiles, birds and also humans possess two GnRH molecular forms (GnRH1 and GnRH2, formerly known as mammalian GnRH and chicken 2 GnRH, respectively) as well as one GnRH receptor (GnRHR), at least. A third GnRH molecular form, GnRH3, is often detected in fish telencephalon and peripheral tissues (Pierantoni et al., 2002). In this respect, current hypothesis postulates that GnRH action progressively evolved from the control of simple basic functions in early metazoan to an indirect way to check gonadal activity in

vertebrates, through a sophisticated network of finely tuned neurons (Chianese et al., 2011a; Kah et al., 2007; Kavanaugh et al., 2008; Pierantoni et al., 2002; White et al., 1998). Despite both GnRH1 and GnRH2 share the ability to trigger gonadotropin discharge (Pierantoni et al., 2002), the coexistence of multiple forms of GnRHs in the brain let to hypothesize the division of functional roles. In this respect, GnRH2 activity is also involved in processes other than gonadotropin discharge such as the control of sexual behaviour, or local action at gonadal level. Food intake and energy balance, stress and many other environmental cues deeply affect reproductive success *via* GnRH2. Hence, in such a complex scenario emerged: 1) the need to integrate and convey all information to GnRH neurons, the major hierarchical elements of the HPG and 2) the need to discover possible intermediary neuronal populations in this chain of events (Fernandez-Fernandez et al., 2006; Herbison & Pape, 2001).

Therefore, in this review, we focus on endocannabinoid (ECB) and kisspeptin systems, two modulators of GnRH activity. ECBs are lipidic mediators capable to inhibit the release of hypothalamic GnRH (Scorticati et al., 2004), affecting, as a consequence, both steroroidogenesis and gonadal functions (Wang et al., 2004). While ECBs exert such a negative effect upon GnRH release, kisspeptins, the product of *kiss* gene, positively affect GnRH release. Impairment of kisspeptin system causes idiopathic hypogonadotropic hypogonadism and affects puberty onset (de Roux et al., 2003; Seminara et al., 2003). Thus, at hypothalamic level ECBs and kisspeptin modulate GnRH circuitry in opposite manner. Similarly to GnRH, ECBs and kisspeptin exert a direct effect upon gonadic activity, affecting steroidogenesis, spermatogenesis, spermatozoa functions, follicular development and oocyte maturation, suggesting the existence of a possible local crosstalk among these systems. Thus, in the next paragraphs the activity of ECBs and kisspeptin along the HPG will be properly discussed.

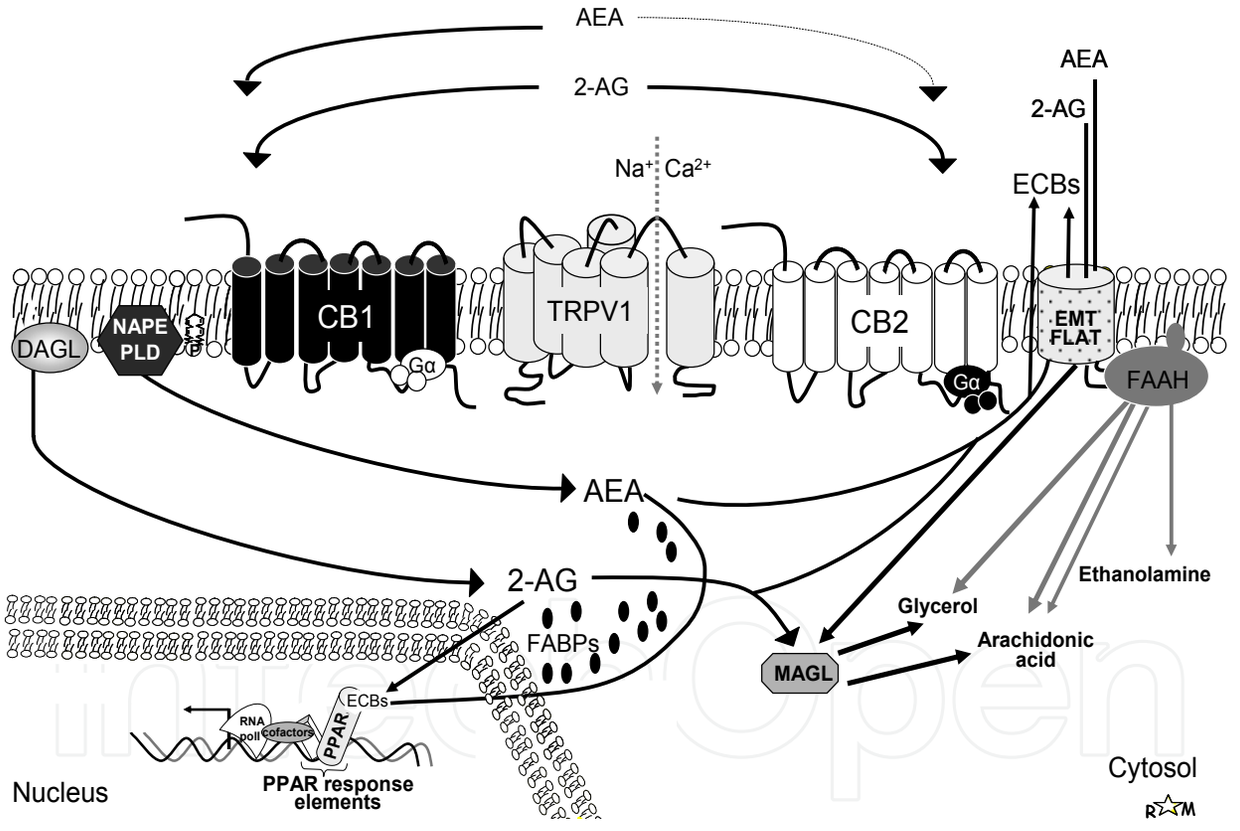
## 2. New modulators of GnRH/gonadotropin activity at central level

### 2.1. Endocannabinoid system

The endocannabinoid system (ECS) is an ancient, evolutionarily conserved system, well-characterized in mammalian and non-mammalian vertebrates (Buznikov et al., 2010; Fasano et al., 2009; McPartland et al., 2006). Such a system comprises ECBs, several ECB receptors (CBs), many enzymatic machineries responsible for ECB degradation and biosynthesis and ECB transporters (EMT) (Pierantoni et al., 2009). A schematic representation of ECS components is depicted in Figure 1.

In general, ECBs are amides, esters and ethers of long-chain polyunsaturated fatty acid, isolated from brain, peripheral tissues and reproductive fluids (Devane et al., 1992; Sugiura et al., 1995; Schuel et al., 2002); they mimic the effects of the phytocannabinoid $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), the psychoactive constituent of marijuana plant, *Cannabis sativa*. The main ECBs are the N-arachidonoyl-ethanolamine (AEA, anandamide), the first ECB discovered in porcine brain (Devane et al., 1992), and 2-arachidonoylglycerol (2-AG) (Sugiura et al., 1995). ECBs have the ability to activate a wide range of CBs: the most studied are CB1 and CB2, classical seven transmembrane spanning G coupled receptors (Matsuda et

al., 1990; Munro et al., 1993) widely distributed in both brain and peripheral tissues, gonads included (Galiegue et al., 1995, Shire et al., 1995, Brown et al., 2002). The orphan G coupled receptor GPR55 is currently accounted as the third CB (Lauckner et al., 2008). AEA, but not 2-AG, selectively acts as an intracellular ligand of the transient potential type1 vanilloid receptor (TRPV1) channel, a six transmembrane spanning receptor whose structure forms a ligand gated non selective cationic channel activated by capsaicin, one of red chilli pepper component (van der Stelt & Di Marzo, 2004, 2005). Lastly, direct nuclear action of ECBs has been postulated since many ECBs [for instance AEA, 2-AG, N-oleoyl-ethanolamine (OEA), N-palmitoyl-ethanolamine (PEA), noladin ether and virodhamine], the phytocannabinoid $\Delta^9$-THC, CB agonists (HU210, WIN55121-2) as well as cannabinoid metabolites have the ability to activate also PPAR (peroxisome-proliferator-activated receptor) family of nuclear receptors (O'Sullivan, 2007; Sun & Bennett, 2007). In order to activate PPAR receptors, cytoplasmic-nuclear translocation of ECBs requires the fatty acid binding proteins (FABPs) as intracellular carriers (Kaczocha et al., 2012).




ECS comprises ECBs, CBs, ECB biosynthetic and hydrolizing enzymes, as well as membrane and intracellular carriers (See text for details). Figure modified from: Pierantoni et al., 2009.

**Figure 1.** Schematic representation of ECS components.

ECBs activity strongly depends on the balance between their biosynthetic and hydrolyzing pathways. AEA and 2-AG are usually released from membrane phospholipid precursors through the activation of N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), respectively (Bisogno et al., 2003; Okamoto et al., 2004).

Two fatty acid amide hydrolases (FAAH and FAAH-2) (Cravatt et al., 1996; Wei et al., 2006) as well as *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) (Tsuboi et al., 2005; Ueda et al., 2010) release arachidonic acid and ethanolamine from AEA. Besides FAAH, 2-AG is cleaved into arachidonic acid and glycerol by a specific monoacylglycerol lipase (MAGL) (Dinh et al., 2002; Ho et al., 2002). Despite of their lipidic nature, data concerning the existence of a membrane carrier able to mediate ECBs transport is discussed. Recently, in neuronal cells a FAAH-like AEA transporter (FLAT) has been identified. Such a molecule is encoded by a splicing variant of *FAAH-1*, lacks the catalytic activity of FAAH but has the ability to bind AEA (Fu et al., 2011).

Nowadays, ECS elements have been identified in the central and peripheral nervous system as well as in gonads and gametes, demonstrating a deep involvement of the system in the control of reproductive functions, both at central and local level (Battista et al., 2012).

In marijuana smokers as well as in animal models, cannabinoids and ECBs interfere in the neuroendocrine control of reproductive function impairing GnRH and LH production, gonadic steroid production, spermatogenesis, ovulation, embryo development and implantation, as well as sexual behaviour (Murphy et al., 1998; Pagotto et al., 2006; Wang et al., 2006). In the brain, $\Delta^9$-THC and ECBs are well known retrograde signals that act at presynaptic level in order to inhibit the release of specific neurotransmittes [i.e. $\gamma$-aminobutyric acid (GABA)]. Current opinion postulates that ECB mediated LH, but not FSH, inhibition is the result of hypothalamic ECB activity. In fact, ECBs are well known inhibitors of GnRH release (Scorticati et al., 2004) and GnRH transcription (Chianese et al., 2011b; Meccariello et al., 2008). By contrast, direct or indirect action of ECBs upon GnRH secreting neurons is still controversial and under investigation. ECBs inhibit several neuronal systems, positively involved in GnRH circuitry (i.e. norepinephrine and glutamate); by contrast, they activate well known inhibitors of GnRH activity [i.e. dopamine, endogenous opioid peptides and corticotrophin-releasing hormone (Murphy et al., 1998)]. Current hypothesis postulates that ECBs interfere in the well known regulation of GnRH neurons by long loop gonadal steroid feedback trough steroids receptor expressing afferents such as GABAergic neurons. For instance, there is a growing consensus that GABA can act through the GABAA receptor to exert both depolarizing and hyperpolarizing effects on GnRH neurons (Herbison & Moenter, 2011). In male mice, GnRH-secreting neurons tonically release 2-AG in presynaptic fissure, which in turn activates CB1 located on GABAergic afferents, in tight relationship with GnRH neurons. The activation of CB1 inhibits the spontaneous release of GABA (Figure 2). As a consequence, postsynaptic GABA receptors (GABAA and GABAB), located on GnRH-secreting neurons, are not activated and GnRH is not released (Farkas et al., 2010). ECB biosynthesis in GnRH secreting neurons might be induced by the activation of metabotropic glutamate receptor (mGluR) located on astrocytes. In fact, in female mice, a complementary hypothesis suggests that local GnRH-GABA circuits uses just the glia derived prostaglandins and/or ECBs in a steroid dependent fashion (Glanowska & Moenter, 2011). GnRH neurons interact with their afferent neurons using several mechanisms and these local circuits can be modified by both sex and steroid feedback. ECBs tone is certainly a key factor in ECBs activity. The inhibitory effect of AEA on GnRH-secreting neurons is reversed by estrogens (Scorticati et al., 2004), through the

inhibition of astrocyte mGluR. Such a process inhibits prostaglandin mediated release of ECBs from GnRH secreting neurons (Glanowska & Moenter, 2011). Alternatively, estradiol might directly prevent the ECB mediated inhibition of GABA neurons (Glanowska & Moenter, 2011). In the brain, functional relationships between CB1 and FAAH emerged, since they have a quite overlapping localization (Egertovà et al., 1998). Since estradiol modulates the transcription of FAAH hydrolase, whose promoter contains an ERE element (Waleh et al., 2002), it is not excluded that estradiol might reverse the adverse activity of AEA on GnRH neurons, by means of FAAH upregulation and AEA degradation.

Conversely, it is not excluded that neuronal systems other than GABAergic, as for example kisspeptin neurons described in the next paragraphs, might modulate GnRH-secreting neurons activity *via* ECBs in an estradiol dependent fashion. A model for proposed circuits and possible mechanisms of GnRH neurons activity in males and females are, respectively, reported in Figures 2 and 3. The use of CB1 knockout mice (*CB1*$^{-/-}$) also contributed to elucidate the mechanism of ECB mediated LH inhibition. AEA decreases both LH and prolactin (PRL) in *CB1*$^{-/-}$ mice whereas 2-AG is able to suppress LH in wild-type, but not in *CB1*$^{-/-}$ mice (Olàh et al., 2008). Thus, receptors other than CB1 might be involved in such a signalling. In such a context, the main candidate is TRPV1 (Olàh et al., 2008), whose expression has been reported in the hypothalamus but not in pituitary gland.

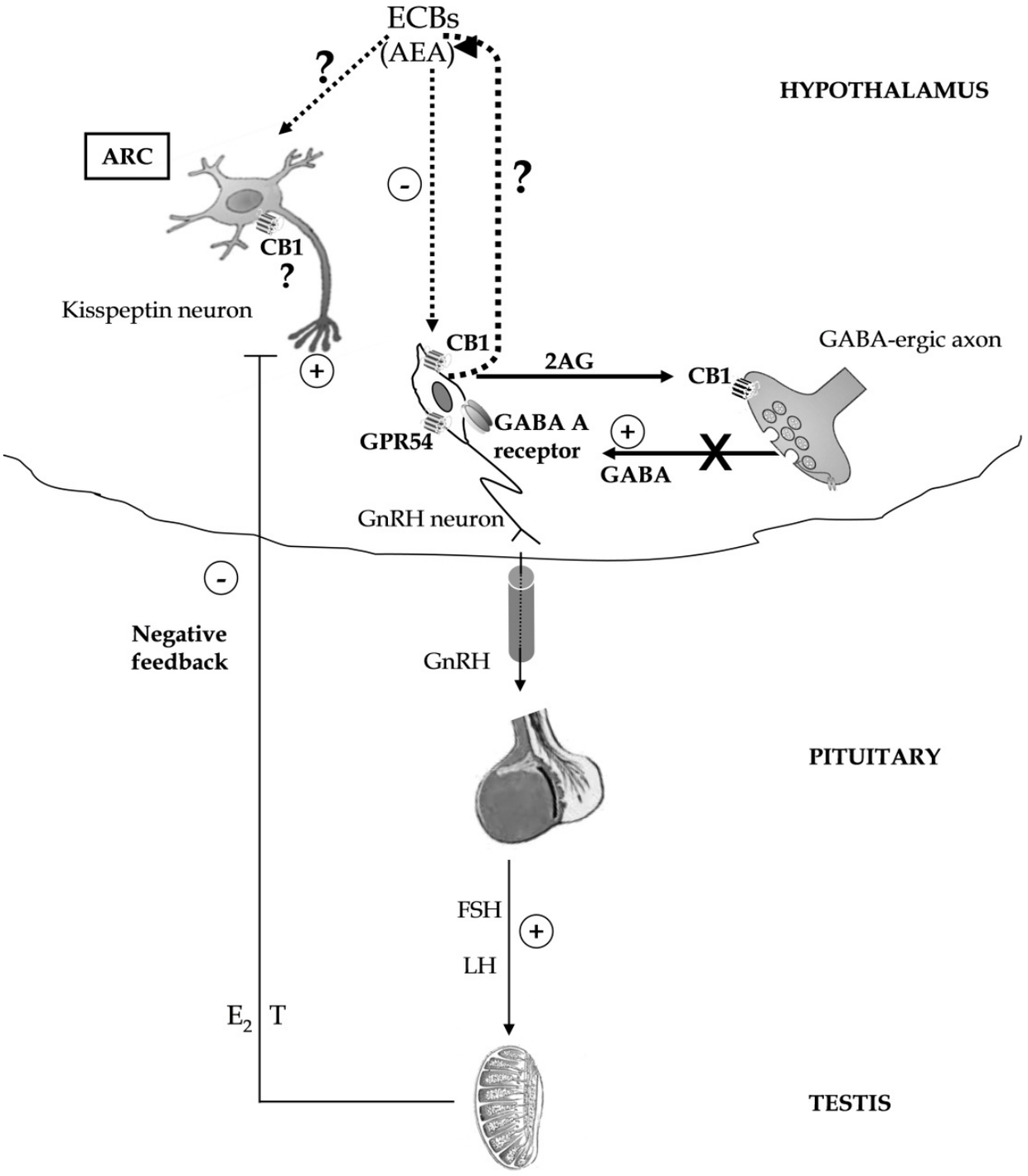
The basal crosstalk between ECS and GnRH is evolutionarily conserved, since it has been described also in lower vertebrates (Chianese et al., 2008, 2011b; Cottone et al., 2008, Meccariello et al., 2008). In both amphibians and teleost fish, CB1 was detected in the forebrain, the encephalic macro-area containing the anterior preoptic area, the encephalic region mainly involved in GnRH activity and in the control of gonadotropin discharge (Cottone et al., 2003, 2005; Lam et al., 2006; Migliarini et al., 2006; Meccariello et al., 2008; Valenti et al., 2005). Besides an involvement in food intake, also in lower vertebrates ECS negatively modulates neuroendocrine machinery and reproduction. In fish forebrain, CB1 colocalizes with GnRH3, the GnRH molecular form mainly detected in the telencephalon of fish (Cottone et al., 2008). A CB1 mediated self modulation of GnRH secreting neurons emerged in the anuran amphibian *Rana esculenta* (Meccariello et al., 2008). In male frogs, GnRH1 and CB1 share the localization inside the basal telencephalon and septum (Cottone et al., 2008; Meccariello et al., 2008); most GnRH1 secreting neurons are close to CB1 expressing neurons whereas a subpopulation of GnRH1 secreting neurons - in the approximate order of 20% - coexpresses CB1 (Meccariello et al., 2008). Such a neuroanatomical observation finds a possible functional explanation at molecular level. In fact, mRNA and protein profiles of CB1 and GnRH1 are opposite in frog diencephalon during the annual sexual cycle (Chianese et al., 2008; Meccariello et al., 2008). Treatments of male diencephalons with buserelin, a long acting GnRH analogue, inhibit *GnRH1* transcription and upregulate *CB1* transcription; conversely, AEA treatments downregulate *GnRH1* expression. In this respect, GnRH secreting neurons might produce ECBs in order to properly suppress GnRH secreting activity (ultrashort feedback). This saga is becoming more intricate since in this amphibian a second GnRH molecular form - GnRH2, with a suggested hypophisiotropic role (Pierantoni et al., 2002) - and three GnRHRs (GnRHR1,

GnRHR2, GnRHR3) have been cloned (Chianese et al., 2011b). In different periods of the annual reproductive cycle, AEA also inhibits *GnRH2* expression and upregulates the expression of *GnRHR1*, *GnRHR2*, but not of *GnRHR3* (Chianese et al., 2011b). Also immortalized neuronal cell lines (GT1) are both target and source of ECBs; *in vitro* they have the ability to produce and secrete ECBs (2-AG and AEA), to uptake and degrade ECBs, and possess CBs (both CB1 and CB2); the activation of CBs inhibits the pulsatile release of GnRH (Gammon et al., 2005). Nevertheless, such observations did not found any confirmation *in vivo*, although GnRH secreting neurons are close to cannabinergic fibres and scantly express CB1 (Gammon et al., 2005). By contrast, micro-array analysis revealed CB2 expression in a subpopulation of GnRH secreting neurons (Todman et al., 2005).

ECS interferes in GnRH circuitry also modulating the activity of neuronal populations that usually converge environmental, stressors, metabolic and photoperiodic cues at different levels of HPG. Stress and food intake, well-known processes under ECBs control (Pagotto et al., 2006), interfere in GnRH secretion. It is interesting to include in this scenario the gonadotropin-inhibitory hormone (GnIH). GnIH belongs to the super-family of RFamide neuropeptides, but its role in the control of gonadotropin secretion is negative, thus proposing the existence of a balance between stimulatory and inhibitory systems in the control of reproduction. Interestingly, this concept does not represent a rule; in fact, in male Syrian hamster, the mammalian ortholog of avian GnIH, the RFRP-3, works on the gonadotropic axis as a stimulator, inducing LH, FSH and testosterone secretion, *via* GnRH neurons activation. Furthermore, this effect might not only vary across species, but also include sex-specific differences in the same species, due to the loss of RFRP-3 mediated stimulation in females (Ancel et al., 2012).

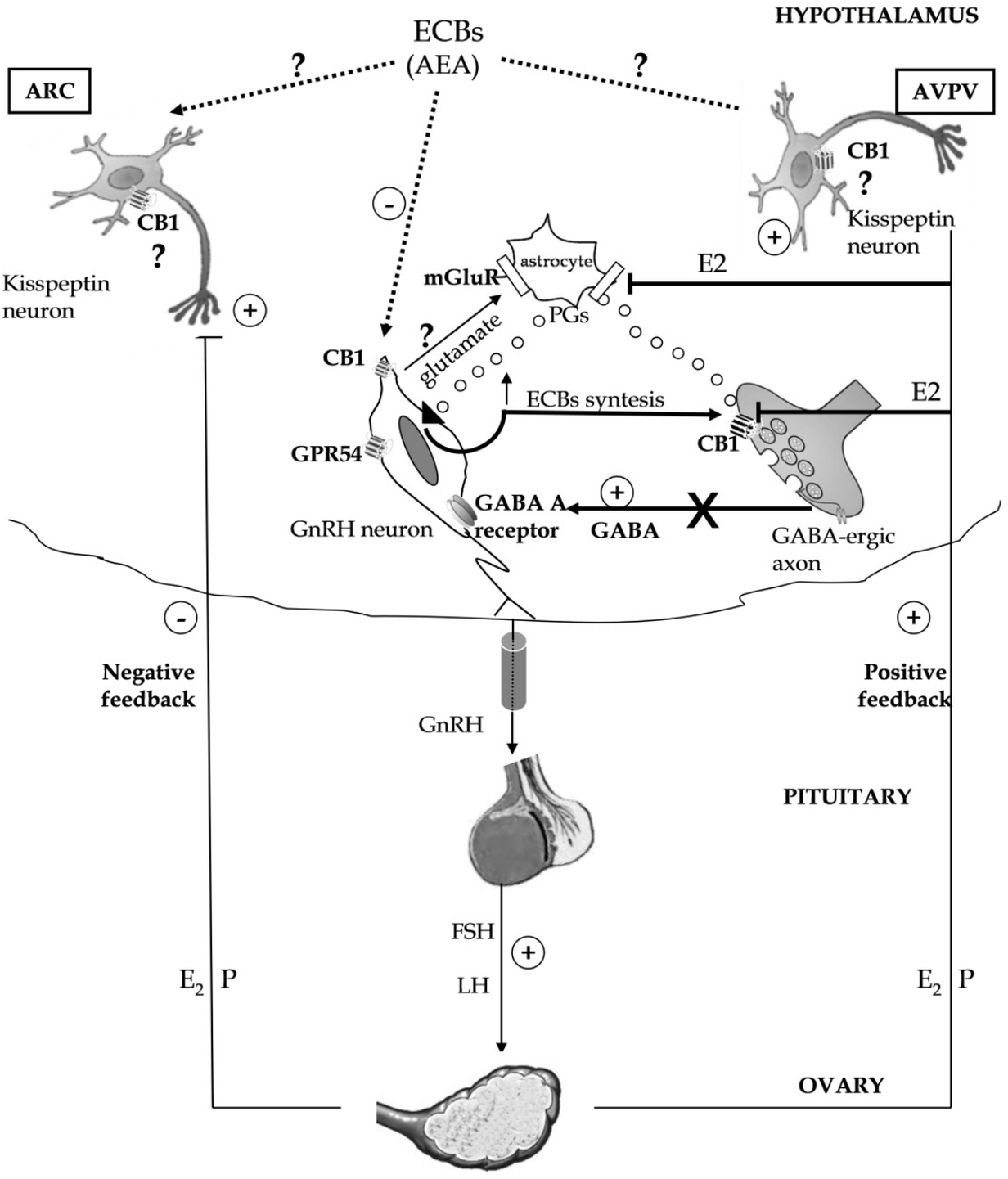
Besides the effect in the hypothalamus, still controversial is the direct activity of ECBs on the pituitary. ECB binding sites, as well as the expression of CBs, biosynthetic and hydrolyzing enzymes have been reported in pituitary *pars distalis* and in pituitary cell cultures (Gonzales et al., 1999, 2000; Lynn & Herkenham, 1994; Murphy et al., 1998; Wenger et al., 1999).

Nevertheless, the localization of CBs in the gonadotropes is confirmed in amphibians and mammals, but not in humans (Cesa et al., 2002; Wenger et al., 1999; Yasuo et al., 2010a). For instance, in rats, AEA exhibits differential effects on the *in vitro* secretion of LH, and pituitary hormones other than gonadotropins [i.e. PRL, corticotrophin (ACTH) and growth hormone (GH)] (Wenger et al., 2000). In a sex steroid dependent fashion, rats express *CB1* in pituitary gland. In females, CB1 expression and AEA content depende on the phase of the ovarian cycle and, in general, pituitary AEA content is opposite to that observed in the hypothalamus (Gonzales et al., 2000). Recently, ECBs have been included among tuberalins, the messengers supposed to be secreted from the *pars tuberalis* - a brain area located between the median eminence, the pituitary portal vessels and the pituitary *pars distalis* - to target the pituitary *pars distalis* (Yasuo et al., 2010b). In both hamsters and humans, *pars tuberalis* produces high levels of 2-AG and low levels of other ECBs (AEA, PEA and OEA), while the pituitary *pars distalis* possesses CB1 (Yasuo et al., 2010a, 2010b). Such a crosstalk might be involved in gonadal response to photoperiodic changes.




GnRH neuron, through GPR54 receptor, directly receives ARC kisspeptin input and conveys it to pituitary and testis. Gonadal steroids inhibit GnRH secretion through ARC kisspeptin neurons. 2-AG synthesized from GnRH neuron - by means of the CB1 activation - blocks GABA release toward GnRH neuron. ECBs directly act upon GnRH neuron since it express CB1; possibly, ECBs may have a further action on kisspeptin neuron. Anyway, an autocrine regulation of GnRH neuron - through ECBs - may exist.

**Figure 2.** Model for possible regulatory network along the male HPG.




Gonadal steroids exert both inhibitory and stimulatory effects on GnRH neurons, through ARC and AVPV kisspeptin neurons, respectively. While CB1 expression and so ECBs action on GnRH neuron are sure, it remains doubtful the mediation of kisspeptin neurons in the inhibitory regulation of GnRH neuron from ECBs. GnRH neuron is able to produce ECBs; these, in turn, bind CB1 on the GABA-ergic afferent, thus to inhibit GABA release. A possible alternative mechanism for this regulation supposes possible glutamate release from GnRH neuron. Glutamate - through metabotropic glutamate receptor (mGluR) activation - stimulates astrocytes to produce prostaglandins (PGs). These in turn can stimulate GnRH neuron to produce ECBs or regulate CB1 trafficking on GABA-ergic axon. Estradiol blocks this circuit inhibiting mGluR or CB1 activity.

**Figure 3.** Model for possible regulatory network along the female HPG.

## 2.2. Kisspeptin system

Kisspeptins belong to the family of RFamide peptides, encoded by the *kiss1* gene, originally detected as a metastasis-suppressor gene in several malignancies (Lee et al., 1996). Kiss name just derives from its role as a suppressor sequence (ss); the letters "Ki" were added after in homage to the location of its discovery, Hershey, Pennsylvania, home of the famous "Hershey Chocolate Kiss". The major kisspeptin product - known as kisspeptin-54 - is a 54 amino acid peptide (Ohtaki et al., 2001); for its instability, it is proteolitically cleaved into shorter peptides (kisspeptin-10, -13 and -14) (Kotani et al., 2001). In 2001, four independent groups showed that all kisspeptin forms bind and activate with similar affinity the orphan G protein-coupled membrane receptor, GPR54 (Clements et al., 2001; Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001).

Hypogonadotropic hypogonadism has been described in mice lacking a functional *kiss* gene or in human and mice with mutations/targeted deletions of *GPR54* genes (Oakley et al., 2009).

Multiple and intricate are the molecular pathways activated by the kisspeptin/GPR54 system to exert its functions in a cell specific way. Starting from G-protein $G_{q/11}$ stimulation, kisspeptins induce phospholipase C (PLC) activation and intracellular calcium mobilization; kisspeptins are also able to induce a strong, sustained stimulation of phosphorylation of the MAP kinases extracellular signal regulated kinases ERK1 and ERK2 and a weak stimulation of p38 MAPK phosphorylation, whereas no activation was observed for stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) (Castaño et al., 2009 and references therein).

The "reproductive" facet of kisspeptins got its disclosure in 2003 when mutations in *kiss1* or *GPR54* genes were associated with idiopathic hypogonadotropic hypogonadism and impaired pubertal maturation (de Roux et al., 2003; Seminara et al., 2003).

Since, the demonstration that kisspeptins are able to stimulate LH and, to a lesser extent, FSH secretion in several species (Roa et al., 2009 and references therein). However, a more consistent hypogonadotropic phenotype characterizes *GPR54* knock-out (KO) mice, compared to *kiss*-KO, suggesting the existence of other possible endogenous ligands for the receptor.

By contrast, GPR54 seems to be the only receptor responsible for the effects of kisspeptins (Lapatto et al., 2007). The main experimental evidences supporting the involvement of kisspeptin in the control of GnRH secreting neuron activity and gonadotropin discharge are summarized in Table 1. The activation of GnRH neurons appears under the control of a kisspeptin tone, but also involves an increase of the kisspeptin neuron projections to GnRH neurons (Roa et al., 2008). Accordingly, in rodents, the detection of kisspeptin-immunoreactive fibres around GnRH neuron cell bodies only starts from postnatal day 25 onwards, and the number of kisspeptin fibres increases at puberty (Clarkson & Herbison, 2006), suggesting that GnRH neurons become more sensitive to kisspeptin just before the onset of puberty. Surprisingly, at least in rodents, electrophysiological studies have demonstrated that a subset of GnRH neurons - responsive to group I metabotropic glutamate receptor agonists - is insensitive to kisspeptins (Dumalska et al., 2008).

In rodents, two major kisspeptin neuronal populations are located at the arcuate nucleus (ARC) and the preoptic area (POA) or the anteroventral periventricular nucleus (AVPV) of the hypothalamus (Mikkelsen et al., 2009). Although in these encephalic districts the kisspeptin-immunoreactive neuron distribution is highly similar in both male and female, the number of cell bodies detected in the AVPV shows a substantial sexual dimorphism being higher in female than in male, at least in rodents (Clarkson & Herbison, 2006). In contrast, in sheep and primates, hypothalamic kisspeptin neurons are especially located in the ARC/infundibular region (Estrada et al., 2006). In this neuro-anatomical picture, although GPR54 and kisspeptin immunoreactivities are rather overlapping, there are some examples of receptor-ligand mismatch. In particular, the ARC is rich in kisspeptin fibres and devoid of GPR54 (Herbison et al., 2010). Over again, the existence of unidentified receptors or ligands appears an intriguing interpretation of this phenomenon.

Key aspect of kisspeptin populations is the high degree of anatomical heterogeneity that may underlie a functional discernable network in the control of GnRH secretion. In this view, in the ARC - but not in the POA/AVPV - kisspeptin neurons co-localize two other neuropeptides, neurokinin B (NKB) and dynorphin (DYN), in several species analyzed to date. For convenience, these cells have been termed KNDy (*K*isspeptin, *N*eurokinin B, *D*ynorphin) (Cheng et al., 2010). They constitute a central node in the control of GnRH secretion, given also the recent observation that genetic inactivation of *TAC3* or *TACR3*, which encode NKB peptide and its receptor, respectively, causes hypogonadotropic hypogonadism (Topaloglu et al., 2009). KNDy cells establish an interconnected network, through extensive reciprocal connections, which might serve to promote auto-regulatory mechanisms; in addition, they have a suggested dual peptidergic as well as glutamatergic phenotype (Lehman et al., 2010a). Conversely, in the AVPV kisspeptin neurons co-localize galanin, a neuropeptide involved in female reproductive functions (Vida et al., 2009), and tyrosine hydroxylase, a marker for dopaminergic neurons (Kauffman et al., 2007). Starting from ARC and AVPV nuclei, both trans-synaptic (indirect) and direct kisspeptin appositions respectively reach GnRH neurons (Clarkson & Herbison, 2006). Evidence for potential inputs at GnRH terminals in the median eminence has also been reported (Lehman et al., 2010a). Anyway, the presence of GPR54 at GnRH nerve terminal level has yet to be demonstrated.

Most work has emphasized the ability of kisspeptin neurons to mediate gonadal steroid feedback on GnRH release. Indistinctly from the specific kisspeptin population observed, all neurons co-localize estradiol (ER), progesterone (PR) and androgen (AR) receptors; particular attention concerns ER, since the greatest percentage of both ARC and AVPV kisspeptin neurons expresses the isoform ER$\alpha$, in comparison with GnRH neurons that express the isoform ß (Lehman et al., 2010b and references therein). In female, throughout most of the cycle, GnRH/LH secretion is under a negative feedback from ovarian steroids, with estradiol that controls pulse amplitude and progesterone pulse frequency. The feedback becomes positive at the end of the follicular phase to sustain pre-ovulatory GnRH/LH surge and the ovulation (Karsch, 1987). This sexually dimorphic event - only females of most species exhibit it – specifically depends on ER$\alpha$ and occurs *via* a classical

mechanism involving transcriptional regulation of gene expression. Of important note, AVPV kisspeptin neurons mediate this positive feedback triggering ovulation; in this respect, kisspeptin signalling might have potential therapeutic roles in the control of ovulation (Clarkson & Herbison, 2009). On the other hand, ARC KNDy neurons convey to GnRH neurons the estrogen negative feedback, at least in rodents, sheep and primates (Roa et al., 2008). Differently, in ewes, KNDy neurons alone govern both inhibitory and stimulatory estrogen effects on GnRH secretion, leaving open the question of how the same sub-population of neurons is able to discern these inputs; a possible explanation could lie in DYN that has the well known ability to mediate the inhibitory feedback of progesterone on GnRH secretion (Goodman et al., 2004). Although some aspects of ARC KNDy neurons have been elucidated, their physiology and their ability to integrate inputs in the control of reproduction are still partially unknown. The use of experimental approach such as cell ablation, that consists in the complete elimination of a whole cell type, can result in quite different phenotypes from complementary gene KO approach. This is the case of kisspeptin/GPR54 neuron ablation (Mayer & Boehm, 2011). The major upsetting observations coming from this study concern the loss of effect of kisspeptin/GPR54 ablation on the timing of puberty onset in female mice, suggesting a regulatory circuit upstream GnRH pulse, independent from kisspeptin signalling. Despite the formation of smaller ovaries, these animals are fertile and, even if GPR54 neuron ablation notably reduces the number of GnRH neurons to almost 10%, this limited percentage seems to be sufficient for a normal reproductive development. Only in the case of acute kisspeptin neuron ablation, animals show acyclicity and infertility, suggesting an essential role for kisspeptin neurons themselves, but not for kisspeptin signalling in ovulatory cyclicity (Mayer & Boehm, 2011).

Reproductive success strongly depends on energy balance, environmental and stressor cues (Bouret et al., 2004). In this perspective, ARC KNDy neurons have been suggested to convey metabolic and stressor information on HPG by means of the expression of glucocorticoid and leptin receptor - where leptin is a hormone secreted by white adipose tissue in proportion to the amount of body energy stores (Bouret et al., 2004, Kinsey-Jones et al., 2009). Conditions of hypoleptinemia have been linked to decreased hypothalamic expression of *kiss1* mRNA and to the inhibition of reproductive functions (Smith et al., 2006). Since GnRH neurons do not directly respond to nutritional conditions (Louis et al., 2011), current hypothesis is that kisspeptin neurons represent intermediate obligate pathways to transmit leptin actions to GnRH neurons that do not express leptin receptor (Castellano et al., 2009, Smith et al., 2006, Tena-Sempere, 2010).

Thus, not only kisspeptins represent a new well consolidated class of modulators of GnRH neuron activity, but also there is an intricate network of regulators that operate up-stream kisspeptin neurons making the study of their physiology much more complicate. Therefore, it is interesting to include in this scenario also the GnIH; it is remarkable that GnIH - through its ability to induce an increase of *kiss1* expression in the the ARC - could act up-stream and not always opposite to kisspeptin neurons, thus to completely upset the idea of the balance kisspeptin/GnIH conceived to date (Ancel et al., 2012).

| *Evidence* | *Reference* |
|---|---|
| *- Hypothalamus* | |
| GnRH antagonists completely abrogate kisspeptin releasing effects on LH and FSH | Matsui et al., 2004; Navarro et al., 2005a; Navarro et al., 2005b |
| GnRH neurons in the rat forebrain express *GPR54* gene | Irwig et al., 2004 |
| Kisspeptin induces *c-fos* expression in rodent GnRH neurons | Irwig et al., 2004 |
| Kisspeptin induces long-lasting depolarization responses of GnRH neurons | Han et al., 2005 |
| Kisspeptin loses its stimulatory effect upon LH secretion in GnRH-deficient *hpg* mice | Roa et al., 2009 |
| *- Hypothalamus cell lines* | |
| GT1-7 cell lines - parentally related to GnRH neurons - express *GPR54* and respond to kisspeptin stimulation | Quaynor et al., 2007 |
| *- Pituitary* | |
| *Kiss1* and *GPR54* expression is differentially regulated by steroids | Richard et al., 2008 |
| *- Pituitary cell lines* | |
| GPR54 is expressed in fractions of ovine pituitary cells enriched for gonadotropes, somatotropes and lactotropes | Smith et al., 2008b |
| Gonadotrope and somatotrope functions are regulated by kisspeptins in nonhuman primate pituitary | Luque et al., 2011 |
| Kisspeptin inhibits LH expression in eel | Pasquier et al., 2011 |

This table provides a list of references in which the involvement of kisspeptins in the control of GnRH neuron activity as well as of gonadotropin discharge has been demonstrated.

**Table 1.** Experimental evidences supporting the mechanisms of action of kisspeptins.

As puberty, physiological conditions as pregnancy, lactation and aging suppose a strong contribution of kisspeptins in the regulation of gonadotropin secretion.

During pregnancy, in particular, hypothalamic *kiss1* expression increases and global state of hyper-responsiveness to kisspeptin, probably due to elevated levels of estradiol and progesterone, emerges. Moreover, circulating levels of kisspeptin - mainly derived from the placenta - dramatically increase, suggesting a possible involvement of this signalling in the regulation of trophoblast invasion during the first trimester (Horikoshi et al., 2003). Instead, the suckling stimulus during lactation might be responsible for the suppression of *kiss1* expression in the ARC and so for LH reduction (Yamada et al., 2007). Lastly, aging causes a high degree of reproductive system disruption, most obviously in females; this defect mainly concerns AVPV nucleus. Of note, during menopause, kisspeptin neurons are subjected to a high degree of hypertrophy (Downs & Wise, 2009).

Besides mammals, some important evidences concerning kisspeptin system have also been obtained in non-mammalian species. Recent advances have let to the identification of multiple isoforms of both *kiss* and *GPR54* in non-mammalian vertebrates, contrarily to mammals where only one gene coding for both the ligand and the receptor is present. This complication has been suggested as a consequence of genome duplication events (Tena-Sempere et al., 2012; Um et al., 2010). In this light, fish have two forms of kisspeptin (*kiss1* and *kiss2*) and GPR54 (*GPR54-1* and *GPR54-2*), whereas in amphibians a second round of gene duplication may have contributed to the generation of subtypes for both ligand and receptor (*kiss1a*, *-1b*, *-2*; *GPR54-1a*, *-1b*, *-2*) (Tena-Sempere et al., 2012; Um et al., 2010). As previously suggested for GnRH, the presence of two kisspeptin systems might indicate a diversity of roles. Accordingly, zebrafish possess two independent kisspeptin systems, with kiss2 mostly involved in the control of reproductive functions, through interactions with GnRH neurons, whereas kiss1 is supposed to be implicated in the perception of environmental and metabolic signals (Servili et al., 2011). A possible relationship between food intake and kisspeptin signalling has been proposed in Senegalese sole, as well; this function has been attributed to kiss2, being the only isoform found in this specie, in contrast to the evident situation in most fish (Mechaly et al., 2011).

Lower vertebrates, as in particular fish models, strongly respond to environmental conditions, mostly thermal and light cues. In tilapia brain, GPR54 co-localizes with neurons expressing GnRH1, GnRH2 and GnRH3, suggesting an involvement of kisspeptin system in the regulation of GnRH system expression as in mammals (Parhar et al., 2004). Higher levels of *GPR54* expression in the brain have been discovered in mature than in immature animals, thus to hypothesize a link between gonadal development and encephalic *GPR54* expression (Parhar et al., 2004). Similarly to mammals, *kiss1* gene has been identified in two distinct neuronal populations that exhibited a differential response to steroid milieu (Kanda et al., 2008). Furthermore, in fish, light has been shown to have a strong impact on kisspeptin system; in particular, long day - a typical permissive light condition - induces an increase in kisspeptin neuron number, supporting a role for this system in the mediation of light response and reproduction. Such a mediation has been evaluated in mammals, as well. In particular, *kiss1* mRNA and protein expression in ARC of Syrian hamsters decreases in short day condition, in correlation with decreased reproductive activity (Revel et al., 2006). Taken together these findings assign to kisspeptins an important role in the photoperiodic control of reproduction, processes under control of melatonin signals, whose actions on GnRH neurons are not direct. Currently, another wedge has been added to the picture, just in Syrian hamster with the demonstration that RFRP-3 is able to reactivate the reproductive axis blocked under photoinhibitory short-day conditions, thus suggesting that RFRP-3 could be the missing link between melatonin and kisspeptins (Ancel et al., 2012). In sheep, reproductive function is activated by short-day and inhibited by long-day photoperiods; during the breeding season, ARC shows an increase of *Kiss1* and a decrease of *RFRP* expression. Furthermore, the number of kisspeptin fibres onto GnRH neurons increases in comparison to that of RFRP, thus suggesting that these two peptides act in concert, with opposing effects, to regulate GnRH neuron activity (Smith et al., 2008a).

In goldfish, *in vivo* administration of kisspeptin induces LH release, indicating a conserved role from fish to mammals (Li et al., 2009). Anyway, the physiology of kisspeptin system seems to be rather controversial even among piscine species. This is the case of the eel, *Anguilla anguilla*, where kisspeptin - through a direct activity on the pituitary has an inhibitory - and not a stimulatory - effect on *LHβ* expression; this effect is also specific, since no action on other pituitary glycoprotein hormone subunits has been shown (Pasquier et al., 2011).

In amphibian model, the presence of three isoforms for both ligand and receptor makes the evaluation of possible physiological roles for each component likely complex. As in fish, in amphibian all isoforms of kisspeptin and GPR54 are highly expressed in the brain, notably in the hypothalamus, allowing to hypothesize a conserved neuroendocrine and neuromodulatory role in the control of puberty onset and reproduction. Moreover, the expression of *GPR54-1a* and *-2* in the pituitary would support a direct neuroendocrine action at pituitary level (Lee et al., 2009). In *Rana catesbeiana*, in particular, GPR54 is primarily expressed in the hypothalamus and pituitary, and weakly expressed in the testis. Of note, neither a faint signal has been detected in other peripheral tissues, such as heart, spleen, liver, supporting an exclusive role for kisspeptin system in the control of central functions (Moon et al., 2009).

Peripheral administration of kisspeptin also stimulates LH as well as GH and PRL secretion (Kadokawa et al., 2008; Yang et al., 2010). Therefore, an additional target of kisspeptin might be outside the blood-brain barrier (Matsui et al., 2004) and some evidences suggest autocrine/paracrine actions of kisspeptins at the pituitary level. Hence, indicative results as the presence of a functional kisspeptin receptor in the pituitary, combined with the finding that kisspeptin is released in hypophyseal portal blood, reinforce the idea that kisspeptin could be able of a dual action at both the hypothalamus and pituitary level. In support of this hypothesis, both *kiss* and *GPR54* are expressed in the pituitary of several species investigated to date (Richard et al., 2009); GPR54 has also been detected in ovine cellular fractions enriched of gonadotropes, somatotropes and lactotropes (Smith et al., 2008b). Once again, this duality suggested in many species is not the rule, since in a sheep model of hypothalamo-pituitary disconnection, kisspeptin loses its stimulatory action on LH secretion, thus assuming an exclusive effect upstream of the pituitary (Smith et al. 2008b). Anyway, this debate is still open and surely warrants further investigations.

## 3. Gonadic action of GnRH molecular forms

### 3.1. GnRH molecular forms and GnRHRs expression and activity in male and female gonads

An intriguing question is the synthesis of GnRH at gonadal level. Despite in hypophysectomised animals GnRH agonist administration induces steroidogenesis and GnRH like molecules have been detected in the main circulation of elasmobranches, in tetrapods the peptide has never been detected in plasma (King et al., 1992). Extra brain synthesis and function of GnRH - expecially GnRH2 - and GnRHRs (both mRNA and

protein) have been detected in vertebrate gonads, humans included, endometrium, placenta, and endometrial cancer cells (Pierantoni et al., 2002; Ramakrishnappa et al., 2005; Singh et al., 2007; Wu et al., 2009).

In males, GnRH involvement in paracrine Sertoli/Leydig cell communication has been postulated in both mammals and lower vertebrates (Pierantoni et al., 2002; Sharpe 1986). In rodent and human testis, the main source of testicular GnRH are Sertoli cells, as well as spermatogenetic cells whereas GnRHR has been mainly located in interstitial Leydig cells (Bahk et al., 1995). Thus, GnRH likely acts as paracrine mediator for steroidogenesis and spermatogenesis progression. GnRH involvement in sperm release has also been reported since GnRH agonist/antagonist, induces/suppresses the spermiation in amphibians and lampreys (Deragon & Sower 1994; Pierantoni et al., 1984a, 1984b). Despite the presence of a functional GnRHR in spermatozoa is questionable, GnRH involvement in sperm function at fertilization has also been proposed, since GnRH antagonists inhibit *in vivo* and *in vitro* fertilization in rodents (Morales et al., 2002a). Most information concerning the local activity of GnRH derived from studies carried out in lower vertebrates, where, as in humans, GnRH2 is the main form detected at peripheral level (Pierantoni et al., 2002; White et al., 1998). However, it is not excluded that the existence of multiple forms of GnRH might be linked to the development of specific functions. For instance, in the amphibian, *Rana esculenta,* we have just cloned and characterized two GnRH molecular forms (GnRH1 and GnRH2) and three GnRHRs, with a specific expression pattern in testis, a specific testicular localization and probably, a specific function during the spermatogenesis (Chianese et al., unpublished). In the frog *Rana esculenta*, GnRH cooperates with estradiol in order to gain spermatogonial proliferation in a mechanism involving the protooncogene *c-fos* (Cobellis et al., 2002). During the frog annual sexual cycle, FOS protein appeared inside the cytoplasm of spermatogonia before the proliferative period, whereas it appeared inside the nucleus as soon as spermatogonia proliferation resumes (Cobellis et al., 2002). Estradiol, produced by Leydig cells, induces the transcription of *c-fos* inside the spermatogonia and the protein is stored in cytoplasmic compartment (Cobellis et al., 2002); then, at the end of the winter stasis, GnRH, produced by Sertoli cells, induces FOS activity by means of FOS protein traslocation from cytoplasmic to nuclear compartment (Cobellis et a., 2003) with a consequent increase of spermatogonial mitotic index. The involvement of *c-fos* as well as of estradiol in spermatogonial proliferation has recently been confirmed also in cell lines (He et al., 2008; Sirianni et al., 2008). However, GnRH role in spermatogonial proliferation is an evolutionarily conserved mechanism since it has been demonstrated also in bivalve mollusc (Treen et al., 2012).

As for testis, also in the ovary of mammalian and non-mammalian vertebrates GnRH binding sites as well as at least one GnRH molecular form and one GnRHR have been detected (Pierantoni et al., 2002). Besides testis, in the ovary GnRH activity highly depends on the state of maturation (Guilgur et al., 2009; Pierantoni et al., 2002; Uzbekova et al., 2002; Wu et al., 2009). In lamprey, GnRH induces both steroidogenesis and ovulation, whereas in teleost fish different GnRH molecular forms differentially modulate both meiosis resumption and steroidogenesis (Nabissi et al. 2000; Pati & Habibi, 2000). In rodents,

GnRHR expression rate and localization change during the reproductive cycle, with high expression levels observed in granulosa cells of atretic follicles as well as in mural granulosa cells of Graffian and preovulatory follicles (Bauer-Dantoin & Jameson, 1995; Kogo et al., 1995). Thus, GnRH seems to have a direct role in follicular atresia, a well known phenomenon of cell death. In *in vitro* cultures, GnRH inhibits DNA synthesis (Saragueta et al., 1997) or induces apoptosis in rat granulosa cells (Billig et al., 1994). Studies have shown the evidence for GnRH-induced remodelling of the extra cellular matrix by inducing structural luteolysis in superovulated rats through stimulation of specific matrix metalloproteinase (Goto et al., 1999). In human ovary, GnRH acts as an autocrine modulator of granulosa cells and has the ability to inhibit progesterone biosynthesis (Peng et al, 1994). Lastly, in addition to endocrine regulation, GnRH is also known to act in an autocrine and paracrine manner in order to suppress cell proliferation and to activate apoptosis in the endometrium and endometrial cancer cells through several mechanisms (Wu et al., 2009). In human ovary cell lines, such a mechanism involves GnRH2 and not GnRH1, and opens question of a functional GnRHR2 in humans (Leung et al., 2003). Hypothesis of remnant *GnRHR2* genes in human, mouse and rat genomes is reported in literature (Pawson et al., 2003); at present, it remains unknown whether or not GnRHR2 is expressed as a full-length, properly processed and functional transcript in humans.

However, both GnRH1 and GnRH2 exhibit regulatory roles in tissue remodelling during embryo implantation and placentation, which suggests that these hormones may have important roles in embryo implantation and early pregnancy (Wu et al., 2009).

### 3.2. Are ECBs and kisspeptin putative local modulators of GnRH/gonadotropin activity?

As for GnRH, also ECBs and kisspeptin biosynthesis and activity have been reported at gonadal levels, thus opening new questions in their possible local crosstalk. Besides the well known suppression of LH in both marijuana smokers and animal model, events due to hypothalamic GnRH suppression, ECBs deeply affect male and female reproductive functions (Wang et al., 2006; Wenger et al., 2001) and they have been detected in reproductive fluids (Schuel et al., 2002; Wang et al., 2006). In males, ECS modulates the progression of spermatogenesis, spermatozoa functions and the activity of testicular somatic cells in mammalian, non-mammalian vertebrates as well as in invertebrates. (Battista et al., 2012; Cacciola et al 2008; Cobellis et al., 2006; Cottone et al., 2008; Grimaldi et al., 2009; Maccarrone et al., 2003, 2005; Pierantoni et al., 2009, Schuel et al., 1991; Wang et al., 2006). In females, ECBs represent fertility signals in folliculogenesis, follicle maturation, oocyte maturation and ovulation (El-Talatini et al., 2009). Then, ECBs and CBs drive embryo transport, survival, implantation, development and growth, placentation and labour (Battista et al., 2012). Consistently, marijuana smokers exhibit several reproductive dysfunctions such as decreased LH levels in both sexes, decreased testosterone level, decreased sperm quality (oligospermia), sperm abnormality and block of acrosome reaction in males, whereas menstrual cycle disorders, reduced birth rates, preterm birth, low foetal birth weight have been described in women (Bari et al., 2011; Wang et al., 2006).

As for kisspeptin concerns, at present, the physiological significance of kisspeptin signalling at gonadal level is under investigation. Besides hypothalamic kisspeptin signalling is critical for puberty onset (Mayer & Boehm, 2011), human and rodent gonads express both *GPR54* and *kiss1* genes (Funes et al., 2003; Terao et al., 2004). With respect to GnRH and ECS, in rat ovary, in particular, *kiss1* mRNA expression shows a fluctuation dependent on the phase of the cycle, with a strong increase before ovulation and a dramatic decrease when ovary is at an immature state (Roa et al., 2007). Also in zebrafish females, *GPR54* expression follows the ovarian development with a decline of expression going toward the reproductive maturity; conversely, an increase of *kiss1* expression coincides with the appearance of mature oocytes (Biran et al., 2008; Filby et al., 2008).

At 7 weeks of age, *GPR54* KO mice display a reduced size of the internal and external reproductive organs with hypoplasia of seminiferous tubules, interstitial Leydig cells, uterine horns and mammary glands; these results let to hypothesize an involvement of GPR54 in cell proliferation and differentiation that are properly necessary for gonadal development (Funes et al., 2003). Interestingly, in zebrafish male, high levels of both ligand and receptor expression have been observed during the first stages of spermatogenesis, when testis is mainly populated by type A spermatogonia to decrease after puberty (Biran et al., 2008; Filby et al., 2008). Kisspeptin expression is modulated by estradiol (Clarkson & Herbison, 2009) and estradiol cooperates with GnRH in order to induce proliferation of spermatogonia (Cobellis et al., 2003), thus raising the possibility of kisspeptin involvement in such a process. The presence of CB2 protein in mouse differentiating spermatogonia (Grimaldi et al., 2009) makes such an item an interesting issue for future investigations.

In disagreement with the general idea that kisspeptin signalling might have a positive impact on reproductive functions, also at a local level, a degenerative effect of kisspeptin administration on maturing rat testes has been reported (Ramzan & Qureshi, 2011). In particular, LH and testosterone suppression and severe degeneration of spermatogenesis in prepubertal testes in a dose-dependent manner have been reported. Such a disruption consists in: Sertoli cells impairment, meiosis inhibition concomitantly to increased spermatogonial proliferation. Already at low doses of kisspeptins, seminiferous tubules show intraepithelial vacuolizations that could be the cause of their massive degeneration, germ cells undergo necrosis, round and elongated spermatids have abnormal acrosome and the interstitial compartment is enlarged. Anyway, testicular degeneration observed after kisspeptin treatment has been suggested to be centrally mediated, and specifically due to an acute hyper-stimulation of the HPG axis (Thompson et al., 2009).

Worth mentioning, the negative effect of kisspeptin on testosterone secretion just reported in rats is in total disagreement with many other evidences provided in both rodents and humans where kisspeptin administration increases testosterone levels (Dhillo et al., 2005; Patterson et al., 2006). Such a point might represent a key switch in autocrine-paracrine communications in Leydig-Sertoli cells - germ cells circuitry involving also GnRH and ECS. AEA administration suppresses testosterone levels (Wenger et al., 2001). Such an issue is surely a consequence of hypothalamic GnRH inhibition, but it is not excluded a direct action

at testicular level. Leydig cells are the main source of testicular steroids and express both *GnRHR* and *CB1* (Bahk et al., 1995; Wenger et al 2001); Sertoli cells are the suggested source of GnRH and, in a FSH dependent fashion, modulate ECB tone and aromatase activity (Bahk et al., 1995; Rossi et al., 2007); germ cells are a suggested source of ECBs and have the ability to respond to ECBs and GnRH (Grimaldi et al., 2009). In such a story, the localization of kisspeptin/GPR54 inside the testis, to our knowledge, is completely lacking.

Really interesting might be the sequential activation of ECS, kisspeptin and GnRH in the diachronic process of epididymal sperm motility acquisition, post-ejaculatory events (capacitation and hyperactivation), and the capacity to recognize and to bind to the oocyte investments and egg plasma membrane. Mammalian spermatozoa acquire the ability to swim during their transit from the testis to the oviduct under the control of several external and intracellular factors. In vertebrates, spermatozoa possess a complete ECS and, at least in humans, evidence of kisspeptin system activity has been provided (Pinto et al., 2012; Wang et al., 2006). ECBs, *via* CB1, operate into the epididymis to regulate sperm motility acquisition and to prevent premature acrosome reaction (Cobellis et al., 2010; Ricci et al., 2007; Wang et al., 2006). To date, in species with external fertilization ECBs control the number of motile spermatozoa keeping sperm motility quiescent until their release in aquatic environment (Cobellis et al., 2006). *In vitro*, kisspeptin stimulates an irregular flagellar beating that is typical of a hyperactivation state, a condition critical for fertilization (Pinto et al., 2012). Then, it is intriguing to note that CB1, but not kisspeptin, controls the zona pellucida induced acrosome reaction (Wang et al., 2006) and GnRH increases sperm-zona pellucida binding in humans (Morales et al., 2002b).

## 4. Closing remarks

Astonishing progress has been accomplished the understanding of how intricate is the scenario that sustains the functionality of the reproductive axis. However, numerous new regulators are still emerging thus suggesting that many other key aspects have to be unravelled. A deep involvement of ECS and kisspeptin system in the modulation of GnRH activity clearly emerged at hypothalamic level. Nevertheless, a functional crosstalk between kisspeptin system, ECS and GnRH has never been investigated so far, neither in the brain nor in male and female gonads. At central level, ECBs and kisspeptins have opposite effects upon GnRH secreting neurons and glutamatergic glial cell surrounding GnRH neurons. In this respect, the balance between AEA and kisspeptin tone might represent a cooperative switch on/off signal for the activity of HPG axis. This aspect becomes more intriguing whether related to the existence of a complicate and multifunctional hypothalamic/gonadal GnRH system in non- mammalian vertebrates and humans.

Most work has attempted to rapidly decipher molecular mechanisms that control kisspeptin, ECBs and GnRH activity at peripheral level, but at moment many aspects of this debate are far from being fully elucidated and warrant further investigation.

Thus, altogether, the putative crosstalk among ECS, kisspeptin system and GnRH might provide a deep insight into the complex field of reproductive biology, opening the avenue to novel therapeutic approaches able to cure and prevent human infertility.

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## Abbreviations

AEA, anandamide; 2-AG, 2-arachidonoylglycerol; AR, androgen receptor; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; CBs, ECB receptors; ER, estradiol receptor; DAGL, diacylglycerol lipase; $\Delta^9$-THC, $\Delta^9$-tetrahydrocannabinol; DYN, dynorphin; ECBs, endocannabinoids; ECS, endocannabinoid system; EMT, ECB transporters; FABPs, fatty acid binding proteins; FAAH, fatty acid amide hydrolase; FLAT, FAAH-like AEA transporter; FSH, follicular Stimulating Hormone; GH, growth hormone; GnIH; gonadotropin-inhibitory hormone; GnRH, gonadotropin releasing hormone; HPG, hypothalamus-pituitary-gonadal axis; KNDy, *K*isspeptin, *N*eurokinin B, *D*ynorphin cells; LH, Luteinizing Hormone; MAGL, monoacylglycerol lipase; NAAA, *N*-acylethanolamine-hydrolyzing acid amidase; NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D; NKB, neurokinin B; POA, preoptic area; PPAR, peroxisome-proliferator-activated receptor; PR, progesterone receptor; PRL, prolactin; RFRP-3, RFamide-related peptide.

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