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Anandamide acts *via* kisspeptin in the regulation of testicular activity of the frog, *Pelophylax esculentus*

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ABSTRACT

In the frog *Pelophylax esculentus*, the endocannabinoid anandamide (AEA) modulates Gonadotropin Releasing Hormone (GnRH) system *in vitro* and down-regulates steroidogenic enzymes *in vivo*. Thus, male frogs were injected with AEA ± SR141716A, a cannabinoid receptor 1 (CB1) antagonist, to evaluate possible effects on GnRH and Kiss1/Gpr54 systems, gonadotropin receptors and steroid levels. In frog diencephalons, AEA negatively affected both GnRH and Kiss1/Gpr54 systems. In testis, AEA induced the expression of gonadotropin receptors, *cb1*, *gnrh2* and *gnrhr3* meanwhile reducing *gnrhr2* mRNA and Kiss1/Gpr54 proteins. Furthermore, aromatase (Cyp19) expression increased in parallel to testosterone decrease and estradiol increase. *In vitro* treatment of testis with AEA revealed direct effects on Cyp19 and induced the expression of the AEA-degrading enzyme Faah. Lastly, AEA effects on Faah were counteracted by the antiestrogen ICI182780, indicating estradiol mediated effect.

In conclusion, for the first time we show in a vertebrate that AEA regulates testicular activity through kisspeptin system.

1. Introduction

The release of hypothalamic Gonadotropin Releasing Hormone (GnRH) triggers the discharge of pituitary gonadotropins [Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH)] which in turn sustain the biosynthesis of sex steroids and the progression of gametogenesis. Long-, short- and ultra-short-loop feedbacks finely modulate the communication in hypothalamus pituitary gonadal (HPG) axis (Chianese et al., 2011a; Everett, 2006; Meccariello et al., 2014b; Pierantoni et al., 2002).

Kisspeptin system is one of the main gatekeeper of GnRH system in the brain (Oakley et al., 2009; Pinilla et al., 2012). Kisspeptins are a set of N-terminally truncated peptides belonging to the superfamily of RF-amide peptides that originate from the cleavage of a 145 amino acid precursor. *Via* the activation of the receptor Gpr54, kisspeptins have the ability to drive puberty onset in vertebrates and sex steroid dependent feedback in mammals (Clarkson and Herbison, 2009; Pinilla et al., 2012). Direct local effects of kisspeptins have been reported in non-mammalian and mammalian testis (Meccariello et al., 2014b), with functions related to the progression of germ cell differentiation (Chianese et al., 2015) as well as to the modulation of GnRH and estradiol signaling in amphibians (Chianese et al., 2013a, 2015), to Leydig cell activity in amphibians and mammals (Chianese et al., 2013a; Irfan et al., 2014; Salehi et al., 2015) and to sperm functions in mouse, rhesus monkey and humans (Hsu et al., 2014; Pinto et al., 2012).

By contrast, in vertebrates there is a general consensus concerning the inhibitory effect exerted by endocannabinoids on the reproductive physiology in both sexes (Battista et al., 2012; Bovolin et al., 2014; Meccariello et al., 2014a; Wang et al., 2006). Endocannabinoids, such as anandamide (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG), are lipid signaling molecules that mimic some of the effects of Δ^9-tetrahydrocannabinol (THC), the main active compound in marijuana plant. Endocannabinoids negatively modulate gonadotropin discharge and steroid biosynthesis in mammals (Battista et al., 2012; Cacciola et al., 2010; Meccariello et al., 2014a; Pierantoni et al., 2009; Wang et al., 2006),

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with mechanisms supposed to be cannabinoid receptor 1 (CB1) dependent (Wenger et al., 2001). In males, the most established consequences of LH suppression by endocannabinoids are the reduction of testosterone levels and the impairment of sperm quality and functions (Meccariello, 2014a, 2014b; Wang et al., 2006). However, upstream to gonadotropin regulation, the majority of endocannabinoid actions are directly or indirectly regulated by GnRH neurons within the hypothalamus (Maccarrone and Wenger, 2005) in that the activation of CB1 suppresses the release of GnRH in GT-1 cells and rat brain (Gammon et al., 2005; Scorticati et al., 2004) or modulates the expression of *gnrh* (Chianese et al., 2008, 2011b; Meccariello et al., 2008) and GnRH receptors (*gnrhrs*) in both diencephalon (Chianese et al., 2011b) and testis (Chianese et al., 2012, 2013b) in amphibians. Interestingly, the fine modulation of the endogenous tone of AEA by fatty acid amide hydrolase (FAAH) is the major checkpoint of endocannabinoid activity in biological systems (Giang and Cravatt, 1997) and the expression of FAAH is modulated by FSH and steroids in mouse testis (Grimaldi et al., 2012; Rossi et al., 2007).

At present, there is no information about a functional crosstalk between endocannabinoids and kisspeptins in the regulation of testicular activity. Thus, we choose to elucidate this aspect in the frog, *Pelophylax esculentus* (previously known as *Rana esculenta*), an experimental animal model that has provided recognized deep insights in the elucidation of evolutionarily conserved mechanisms in comparative endocrinology (Chianese et al., 2011a). In this seasonal breeder, *gpr54* has been cloned and localized in testis (Chianese et al., 2013a), whereas the administration of kisspeptin 10 (Kp10) at the onset of a new spermatogenic wave directly induces the progression of spermatogenesis and modulates GnRH testicular system (Chianese et al., 2015). An interesting interplay between estradiol and Kp10 has also recently emerged. Indeed, estradiol induces the expression of *gpr54* and Kp10, in turn, induces the expression of estrogen receptors *erα* and *erβ* (Chianese et al., 2013a, 2015). Finally, a complete endocannabinoid system has been characterized in frogs (Chianese et al., 2012; Cobellis et al., 2006; Meccariello et al., 2006, 2007) and we have recently demonstrated that *in vivo*, but not *in vitro*, the endocannabinoid AEA has the ability to decrease the expression of *cytochrome P450 17alpha-hydroxylase/17,20 lyase* (*cyp17*) and *3β-hydroxysteroid dehydrogenase/D-5-4 isomerase* (*3β-hsd*), key enzymes of steroidogenesis (Chianese et al., 2014).

Here, in both diencephalon and testis, we have analyzed *in vivo* the effects of AEA on the expression of: i) GnRH system, composed of two ligands (GnRH1 and GnRH2) and three receptors (GnRHR1, R2, R3) (Chianese et al., 2011b); ii) Gpr54 and kisspeptin, well-known modulators of GnRH activity. We have also evaluated in testis the expression of aromatase Cyp19 and measured intratesticular levels of testosterone and estradiol. Direct effects of AEA on Cyp19 were studied by *in vitro* treatment and the possible crosstalk between AEA and estradiol to regulate Faah expression was assayed in testis. For the first time in a vertebrate species we provide evidence that AEA modulates testis physiology *via* kisspeptin and estradiol activity.

2. Materials and methods

Experiments were performed under the guidelines established in the National Institute of Health *Guide for Care and Use of Laboratory Animals* and approved by Ministry of Education, University and Research, Italy. The capture of frog from the wild and the following treatments were approved by the Ministry of Health, Directorate General of Veterinary Health and Food Safety.

2.1. Experimental procedures

2.1.1. Animals and tissue collection

P. esculentus male frogs were captured in February in the neighborhood of Naples (Italy) and immediately sacrificed after capture to gain testis for *in vitro* experiments or treated *in vivo* as reported below. The animals were anaesthetized with ethyl-3-aminobenzoate methanesulfonate salt (MS222, Sigma–Aldrich, Milan, Italy) and euthanized by decapitation. Brains were dissected as previously reported (Chianese et al., 2011b), diencephalons and testes were quickly removed, flash frozen in liquid nitrogen and stored at −80 °C until used for total RNA, protein or steroid extraction.

2.1.2. In vivo AEA treatment

Male frogs (n = 10/treatment) were divided in three experimental groups and injected in the dorsal sac with Krebs Ringer bicarbonate buffer for amphibians (KRB) alone, (control group, C), 10^{-8} M AEA (purchased from Sigma–Aldrich Corp., Milan, Italy) in KRB (treatment group, AEA) and 10^{-8} M AEA + 10^{-7} M SR141716A (SR, produced by Sanofi Research, Montpellier, France) – a selective antagonist of CB1 also known as Rimonabant (Rinaldi-Carmona et al., 1994) (combined treatment, AS). In the last treatment, frogs were preliminarily injected with 10^{-7} M SR/KRB and after 30 min animals were injected with 10^{-8} M AEA/KRB. Two h after injections, diencephalons and testes were removed and stored at −80 °C. AEA doses and incubation times were chosen on the basis of previous experiments carried out in both rat (Scorticati et al., 2004) and frog (Chianese et al., 2011b, 2012; Meccariello et al., 2008). Testes collected from the animals used in this *in vivo* experiments were previously used to assess the expression of *cyp17* and *3β-hsd* (Chianese et al., 2014).

2.1.3. In vitro incubations of frog testis with AEA and 17β-estradiol (E_2)

Testes of male frogs (n = 5 animals/treatment) were quickly removed, minced and treated for 1 h as follows: Krebs–Ringer bicarbonate buffer for amphibians (KRB) alone, control group, C; KRB/10^{-9} M AEA, treatment group, AEA; KRB/10^{-8} M SR for 30 min and then KRB/10^{-9} M AEA and 10^{-8} M SR for 1 h, treatment group, AS; KRB/10^{-8} M SR for 1 h, treatment group, SR; KRB/10^{-6} M E_2, treatment group, E_2; KRB/10^{-5} M ICI182780, (ICI, Zeneca Pharmaceuticals, Macclesfield, UK), a high affinity estrogen receptor antagonist, for 30 min and then KRB/10^{-6} M E_2 and 10^{-5} M ICI, treatment group, EI; KRB/10^{-5} M ICI for 30 min and then KRB/10^{-9} M AEA and 10^{-5} M ICI, treatment group, AI.

E_2 and ICI doses and incubation times were chosen on the basis of previous studies (Cobellis et al., 1999). After the treatments, testes were stored at −80 °C and then processed for RNA extraction/qPCR and protein extraction/Western blot analyses.

2.2. Preparation of complementary DNA (cDNA) and PCR analysis

2.2.1. Total RNA preparation

The extraction of total RNA from diencephalons (n = 5) and testes (n = 5) was performed using Trizol Reagent (1 ml/50–100 mg tissue) according to the manufacturer's instructions (Life Technologies, Paisley, UK). To eliminate any genomic DNA contaminations, 10 μg RNA samples were treated with 1 μl Deoxyribonuclease (DNaseI 10 U/μl) (Amersham Pharmacia Biotech) at 37 °C for 30 min. Total RNA purity and integrity were determined by spectrophotometer analyses at 260/280 nm and by agarose gel electrophoresis.

2.2.2. cDNA synthesis

The reverse transcription of total RNA in cDNA was carried out using 5 μg of total RNA, 0.5 μg of oligo $dT_{(18)}$, 0.5 mM dNTP mix, 5 mM DTT, 1× first-strand buffer, 40U RNase Out and 200U SuperScript III RNaseH⁻ Reverse Transcriptase in a final volume of 20 μl, following the manufacturer's instructions (Life Technologies). Total RNA not treated with reverse transcriptase was used as negative control.

2.2.3. Cloning of lh receptor (lhr), fsh receptor (fshr) and cyp19 from P. esculentus testis

To clone *lhr*, *fshr* and *cyp19*, 1 μl of diluted (1:5) cDNA was used for standard PCR analysis in combination with 10 pmol of oligonucleotide primers designed upon *Rana rugosa* nucleotide sequences (Maruo et al., 2008; Suda et al., 2011). The sizes of the predicted amplificates were 254, 255 and 308 bp, for *lhr*, *fshr* and *cyp19*, respectively. PCR conditions were: 94 °C, 5 min, 1 cycle; 94 °C, 30 s, 58 °C (or 65 °C), 30 s for *cyp19* (or *lhr*/*fshr*), 72 °C, 30 s, 30 cycles; 72 °C, 7 min. PCR products were cloned in pGEM-T Easy Vector (Promega Corp., Madison, WI), transformed into DH5α high efficiency competent cells and recombinant colonies were identified by blue/white color screening. Recombinant plasmids containing cDNA fragments of the predicted sizes were extracted by QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and the inserts were sequenced on both strands by Primm Sequence Service (Primm srl, Naples Italy).

Alignments, conducted by lAlign and ClustalW multiple alignments, revealed nucleotide identity of 93%, 98% and 95% against *R. rugosa lhr*, *fshr* and *cyp19* sequences, respectively. At protein level amino acid identity was: 94% for Lhr, 99% for Fshr and 93% for Cyp19 against *R. rugosa* sequences.

2.2.4. Quantitative real time RT PCR (qPCR)

All qRT-PCR were prepared in a final volume of 20 μl containing 1 μl of 1:5 diluted in water cDNA, 0.5 μM primers and 10 μl of SSo Fast EvaGreen supermix (Bio-Rad). Assays were run twice in duplicates using the Mastercycler CFX-96 (Bio-Rad); a negative control in which cDNA was replaced by water was also included. Relative expression of *cb1*, *gnrh1*, *gnrh2*, *gnrhr1*, *gnrhr2*, *gnrhr3*, *lhr*, *fshr*, *gpr54* and *cyp19*, corrected for PCR efficiency [*lhr*, E: 105.4%, R^2: 0.997, slope: −3.199; *fshr*, E: 108.8%, R^2: 0.999, slope: −3.127; *cyp19* E: 109.3%, R^2: 0.998, slope: −3.118; references (Chianese et al., 2011b, 2012, 2013a) for the others] was normalized toward the reference gene *fp1*, accordingly to previous reports (Chianese et al., 2011b, 2012) and quantified using the comparative Cq method with the formula $2^{-\Delta\Delta Cq}$. Assay included a melting curve analysis for which all samples displayed single peaks for each primer pair.

2.3. Protein analysis

2.3.1. Protein extraction

Protein extracts were prepared from frog diencephalons (n = 5) and testes (n = 5). Tissues were homogenized in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors [0.5 mM phenylmethylsulfonylfluoride (PMSF), 4 μg/ml leupeptine, 4 μg/ml chymostatin, 4 μg/ml pepstatin A, and 5 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)]. The lysate was incubated on ice for 30 min. Supernatant containing the cleared protein extract was collected after centrifugation at 10,000× *g* for 15 min at 4 °C. Protein concentration was determined using the Lowry assay (Lowry et al., 1951).

2.3.2. Western blot analysis

Proteins (50 μg) were separated using the Mini-Protean precast gels 4–20% (Bio-Rad) and transferred to polyvinylidene fluoride filters by TransBlot Turbo Transfer System (Bio-Rad). To prevent nonspecific binding, membranes were treated for 2 h with blocking solution [5% non-fat powdered milk, 0.25% polyxyethylene sorbitan monolaurate (Tween20)] in Phosphate Buffered Saline [(PBS), 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.6] and then incubated with the primary antibody [rabbit polyclonal anti-Gpr54 raised against amino acids 141–342 mapping within an internal region of Gpr54 of human origin, diluted 1:500 (sc-134499); goat polyclonal anti-Cyp19 raised against a peptide mapping within an internal region of Cyp19 of human origin, diluted 1:200 (sc-14244); goat polyclonal anti-Kiss1 raised against a peptide mapping at the C-terminus of Kiss1 of human origin, diluted 1:500 (sc-18134); Santa Cruz Biotechnology, Inc., Milan, Italy. Rabbit polyclonal anti-Faah raised against amino acids 561–579 of Faah of rat origin, diluted 1:500 (no. 101600); Cayman Chemical, Ann Arbor, MI, USA] in PBS 3% non-fat powdered milk solution overnight at 4 °C on an orbital shaker. After washing three times in TBS/0.25% Tween20, filters were incubated with 1:2000 horseradish peroxidase-conjugated IgG (Dako Corp., Milan, Italy) in TBS 1% normal serum (NSS; Dako Corp.) and then washed again. The immune complexes were detected using the enhanced chemiluminescence Western blotting detection system (ECL; Amersham Pharmacia Biotech) following the manufacturer's instructions. In order to quantify protein amount, the membranes, stripped at 60 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM TrisHCL, pH 7.6), were reprobed with Erk2 (Extracellular regulated kinase 2) antibody (rabbit polyclonal anti-Erk2 diluted 1:1000; sc-154; Santa Cruz Biotechnology).

2.3.3. Antisera specificity

Specificity of Cyp19, Kiss1 and Faah antiserum was tested through competition studies using the antibody previously preabsorbed for 18 h at 4 °C on an orbital shaker with a five-fold excess of the corresponding blocking peptide [sc-14244-P, sc-18134 P and no. 301600, respectively, in 500 μl of PBS, accordingly to manufacturer's instructions (Santa Cruz Biotechnology for Cyp19 and Kiss1, Cayman Chemical for Faah)].

Instead, since no blocking peptide was available to check the specificity of Gpr54 antiserum, Western blot analysis was carried out by omitting primary antibody.

2.4. Steroid detection

2.4.1. Steroid extraction from testis

Testes (n = 5) were homogenized in 70% methanol and extracted with 2 × 7 ml diethyl ether. After drying, each extract was dissolved in phosphate buffered saline (PBS 0.1 M, pH 7), 0.2% gelatine for hormone determination (Pierantoni et al., 1984).

2.4.2. EIA assay

Intra-testicular 17β-estradiol and testosterone levels were quantified using commercially available EIA kits according to the directions provided by the manufacturer (Cayman, Florence, Italy and DRG diagnostics GmbH, Germany, respectively). Kits were competitive assays recommended for quantification of hormones from tissue with a detection limit of approximately 20 pg/ml and 83 pg/ml, for 17β-estradiol and testosterone, respectively.

The intra- and inter-assay coefficients of variation were determined at multiple points to be 7.7% and 6.6%, 5.9% and 1.7%, for 17β-estradiol and testosterone, respectively. All determinations were made in triplicate from each sample.

2.5. Statistics

Quantitative PCR data were reported as normalized mean fold expression ± s.e.m. over value 1 arbitrarily assigned to control group (reference sample). Western blot signals were scanned and protein levels were plotted as quantitative densitometry analysis of signals. Data were expressed as mean fold increase ± s.e.m. over the value 1 arbitrarily assigned to the sample that exhibits the lowest protein/Erk2 ratio and are representative of three independent experimental procedures.

ANOVA followed by the Duncan's test for multi-group comparison was carried out to assess the significance of differences.

3. Results

3.1. AEA effects on cb1 and gnrh system in frog diencephalon

AEA treatment significantly stimulated *cb1* expression ($P < 0.01$ *vs.* control group) (Fig. 1A) and inhibited those of *gnrhs* (Fig. 1B, C) and *gnrhrs* in frog diencephalon (Fig. 1D–F) ($P < 0.01$ *vs.* control group). Pre-treatment with SR141716A, a specific antagonist of CB1, counteracted all the reported effects on gene expression.

3.2. AEA effects on Gpr54 and Kiss1 in frog diencephalon

Gpr54 mRNA decreased in AEA treated frogs ($P < 0.01$ *vs.* control group) (Fig. 2A). Interestingly, the profile of Gpr54 protein expression was comparable to that of mRNA ($P < 0.01$ *vs.* control group) (Fig. 2B). The pre-treatment with SR141716A returned both Gpr54 mRNA and protein levels to that of control group (Fig. 2A, B). In order to analyze Gpr54 antiserum specificity, a Western blot analysis was carried out by omitting primary antibody. The previously observed signal completely disappeared (data not shown).

To validate a possible crosstalk between endocannabinoids and kisspeptin system, we analyzed the effects of AEA treatment on Kiss1 protein levels, as well. Remarkably, such a treatment significantly decreased Kiss1 expression in frog diencephalon ($P < 0.01$ *vs.* control group) (Fig. 2C). The specificity of Western blot signal was determined by preabsorption of the antibody with a large excess (five-fold excess) of the blocking peptide. Disappearance of the predicted band of approximately 15 kDa band was used as criterion of signal specificity to identify Kiss1 protein (Fig. 2C).

3.3. AEA effects on cb1, gnrh and kisspeptin systems in frog testis

As in the diencephalon, also in testis we observed the AEA dependent increase of *cb1* ($P < 0.01$ *vs.* control group) (Fig. 3A).

AEA treatment failed to have any effect on *gnrh1*, but induced the expression of *gnrh2* ($P < 0.01$ *vs.* control group) (Fig. 3B, C). No effect was observed on *gnrhr1* (Fig. 3D), whereas the expression levels of *gnrhr2* (Fig. 3E) and *gnrhr3* (Fig. 3F) were significantly lower and higher than those in control group, respectively

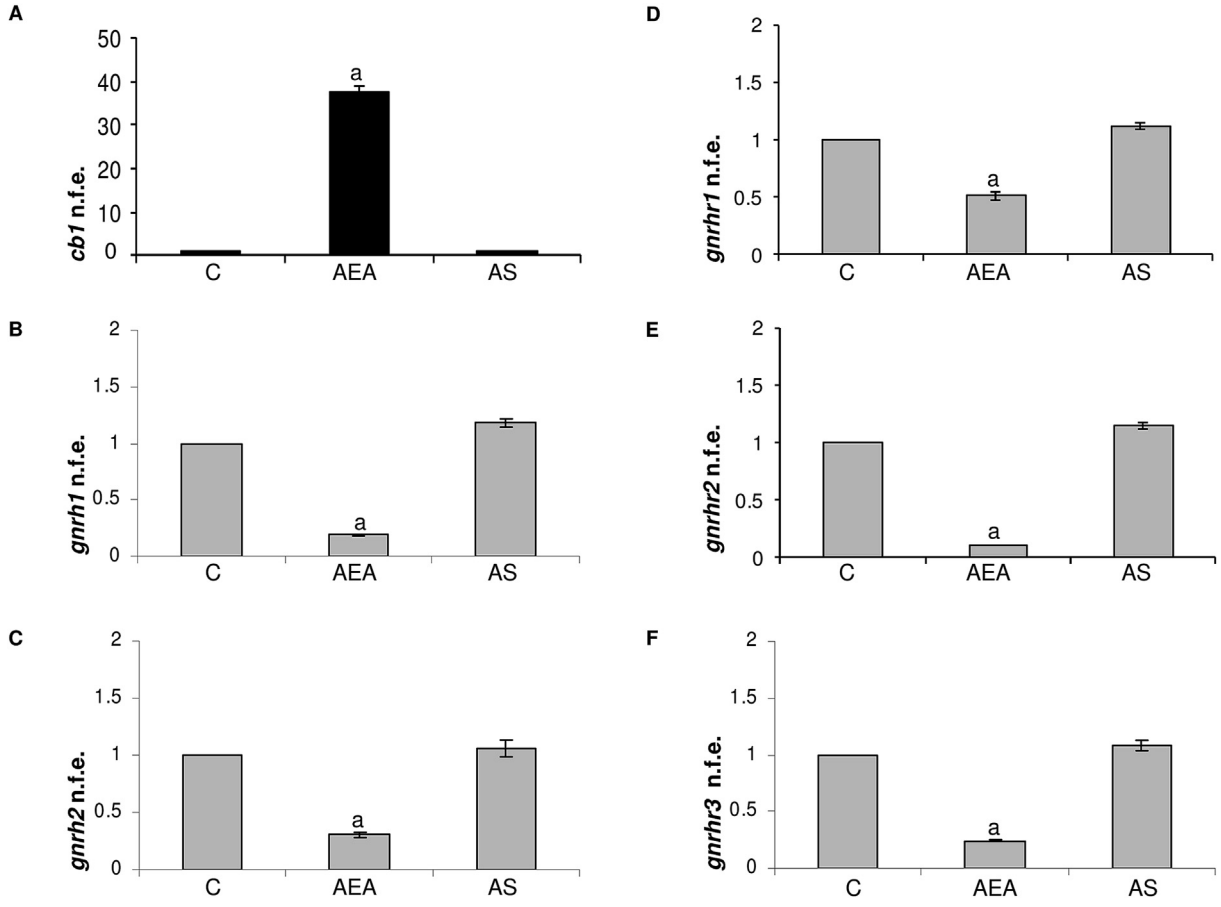

Fig. 1. Effects of AEA treatment on the expression of *cb1*, *gnrhs* and *gnrhrs* in frog diencephalon. Quantitative PCR for *cb1* (A), *gnrh1* (B), *gnrh2* (C), *gnrhr1* (D), *gnrhr2* (E) and *gnrhr3* (F). AEA was injected in the dorsal sac of frogs collected in February. C, control group, diencephalons collected from frogs injected with KRB alone; AEA, diencephalons collected from frogs injected with 10^{-8} M AEA; AS, diencephalons collected from frogs injected with both 10^{-7} M SR141716A and 10^{-8} M AEA. Data are reported as normalized mean fold expression (n.f.e.) ± s.e.m. and are representative of three separate experiments at least. "a" indicates statistically significant differences among samples ($P < 0.01$).

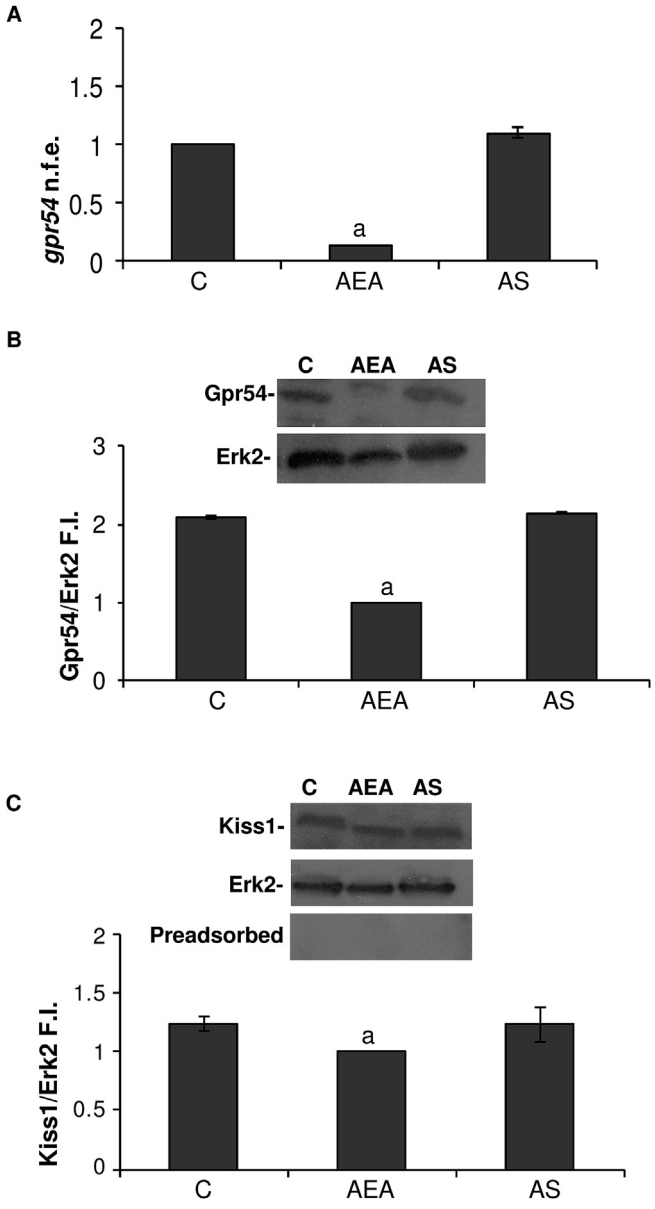

Fig. 2. Effects of AEA treatment on the expression of Gpr54 and Kiss1 in frog diencephalon. Quantitative PCR for *gpr54* (A); Western blot for Gpr54 in AEA treated frogs (B); Western blot for Kiss1 in AEA treated frogs with the corresponding analysis of signal specificity by reprobing the same filter with the Kiss1 antiserum preabsorbed with a five-fold excess of the blocking peptide (C). Western blot signals were normalized against Erk2. C, control group, diencephalons collected from frogs injected with KRB alone; AEA, diencephalons collected from frogs injected with 10^{-8} M AEA; AS, diencephalons collected from frogs injected with both 10^{-7} M SR141716A and 10^{-8} M AEA. Quantitative PCR data are reported as normalized mean fold expression (n.f.e.) ± s.e.m.; Western blot data are reported as mean fold increase (F.I.) ± s.e.m. Data are representative of three separate experiments at least. "a" indicates statistically significant differences among samples ($P < 0.01$).

($P < 0.01$).

The expression of Gpr54, both mRNA (Fig. 3G) and protein (Fig. 3H), was lower in AEA injected animals ($P < 0.01$ *vs.* control group). As in diencephalon, the signal completely disappeared by omitting Gpr54 primary antibody (-Gpr54 AbI, Fig. 3H). Interestingly, also in testis, AEA inhibited Kiss1 expression ($P < 0.01$ *vs.* control group) (Fig. 3I).

All the reported AEA dependent effects were completely counteracted by the pre-treatment with SR141716A (Fig. 3A–I).

3.4. AEA effects on gonadotropin receptors

To test the responsiveness of Sertoli and Leydig cells to endocannabinoids, we analyzed the testicular expression of *fshr* and *lhr* after AEA injection. Such a treatment induced the expression of both receptors (Fig. 4A, B) ($P < 0.01$ *vs.* control group). This effect was Cb1 mediated as it was completely counteracted by SR141716A.

3.5. Intra-testicular levels of testosterone and 17β-estradiol in AEA treated frogs

Since the *in vivo* AEA treatment decreased the expression of *cyp17* and *3ß-hsd*, encoding key enzymes of steroidogenesis (Chianese et al., 2014), we measured the intra-testicular levels of both testosterone and 17β-estradiol after AEA injection by EIA method. *Via* Cb1, AEA significantly inhibited testosterone production and increased 17β-estradiol levels (Table 1).

3.6. AEA effects on Cyp19 expression

Due to the above results, we evaluated the possible AEA dependent regulation of Cyp19, also known as aromatase, the main enzyme that catalyzes the transformation of androgens into estrogens.

Quantitative PCR and Western blot analyses carried out on testis of AEA injected frogs revealed the increase of Cyp19 mRNA (Fig. 5A) and protein (Fig. 5B) ($P < 0.01$ *vs.* control group); such an effect was completely counteracted by the treatment with SR141716A. The specificity of Western blot signal was determined by preabsorption of the antibody with a large excess (five-fold excess) of the blocking peptide. Disappearance of the 58 kDa band was used as criterion of signal specificity to identify Cyp19 protein (Fig. 5B).

In order to assess a direct effect on testis, the expression of Cyp19 mRNA and protein was also analyzed in *in vitro* AEA treated testis. Interestingly, such a treatment also caused a significant increase of both Cyp19 mRNA (Fig. 5C) and protein (Fig. 5D) ($P < 0.01$ *vs.* control group). The combined treatment with SR141716A completely returned Cyp19 levels to the control ones, whereas treatment with SR141716A alone had no effect on Cyp19 mRNA vs. control group (Fig. 5C, Inset).

3.7. AEA and 17β-estradiol effects on Faah expression

With the intent that AEA or estradiol may regulate endocannabinoid tone *via* Faah enzyme, we verified the effects of *in vivo* and *in vitro* AEA treatments on Faah protein levels. The *in vivo* AEA injection failed to have any effect on Faah (Fig. 6A); by contrast, *in vitro* AEA treatment increased Faah expression (Fig. 6C) ($P < 0.01$ *vs.* control group). The specificity of Western blot signal was determined by preabsorption of the antibody with a large excess (five-fold excess) of the blocking peptide. Disappearance of the approximately 63 kDa band was used as criterion of signal specificity to identify Faah protein (Fig. 6A).

Interestingly, such an increase was also observed after *in vitro* E_2 treatment (Fig. 6B) ($P < 0.01$ *vs.* control group). Faah protein levels returned to the control ones in the combined treatment AEA and SR141716A (AS) (Fig. 6C), in SR141716A treatment (SR) alone (Fig. 6D), as well as in E_2 and ICI treated group (EI) (Fig. 6B). Then, we checked if AEA used estrogens to activate a self-control by modulating Faah expression through a combined treatment with AEA and ICI (AI). Interestingly, such a treatment reduced Faah levels to control values (Fig. 6C).

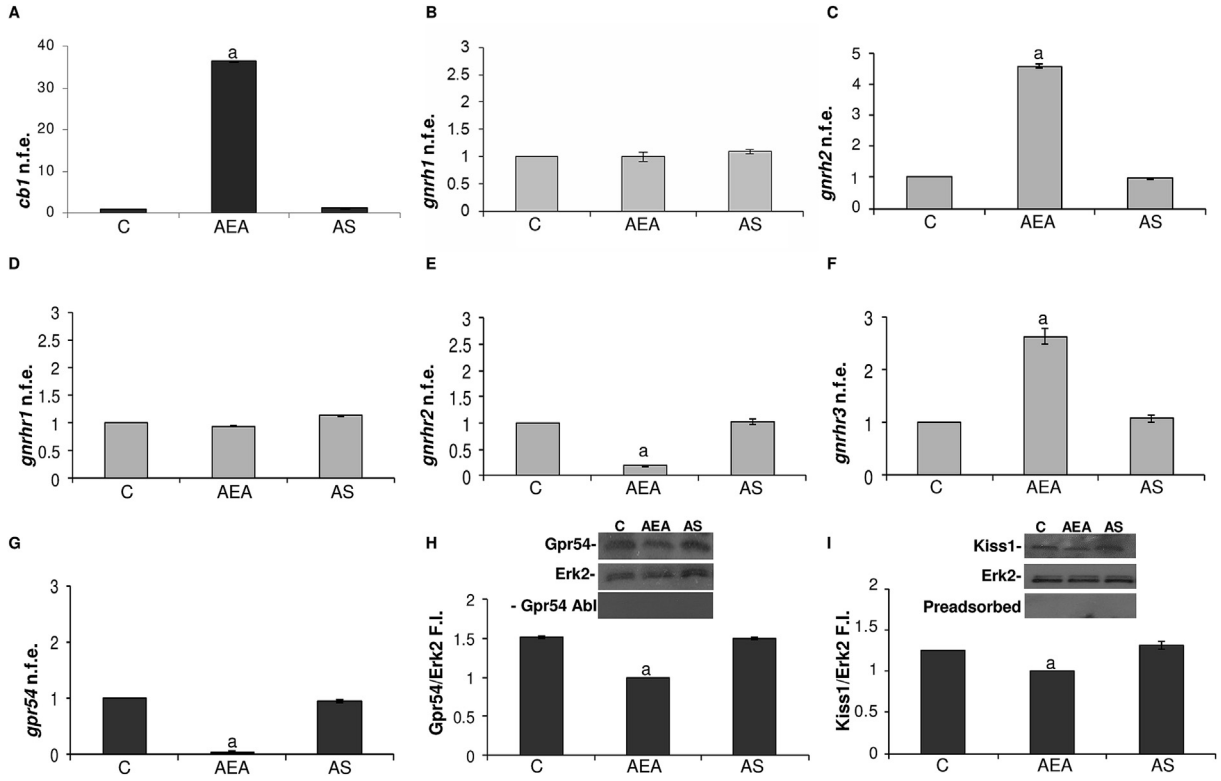

Fig. 3. Effects of AEA treatment on the expression of *cb1*, *gnrhs*, *gnrhrs*, Gpr54 and Kiss1 in frog testis. Quantitative PCR for *cb1* (A), *gnrh1* (B), *gnrh2* (C), *gnrhr1* (D), *gnrhr2* (E), *gnrhr3* (F) and *gpr54* (G); Western blot for Gpr54 with the corresponding analysis of signal specificity by omitting primary antibody (H); Western blot for Kiss1 in AEA treated frogs with the corresponding analysis of signal specificity by reprobing the same filter with the Kiss1 antiserum preabsorbed with a five-fold excess of the blocking peptide (I). Western blot signals were normalized against Erk2. C, control group, testis collected from frogs injected with KRB alone; AEA, testis collected from frogs injected with 10^{-8} M AEA; AS, testis collected from frogs injected with both 10^{-7} M SR141716A and 10^{-8} M AEA. Quantitative PCR data are reported as normalized mean fold expression (n.f.e.) ± s.e.m.; Western blot data are reported as mean fold increase ± s.e.m. Data are representative of three separate experiments at least. "a" indicates statistically significant differences among samples ($P < 0.01$).

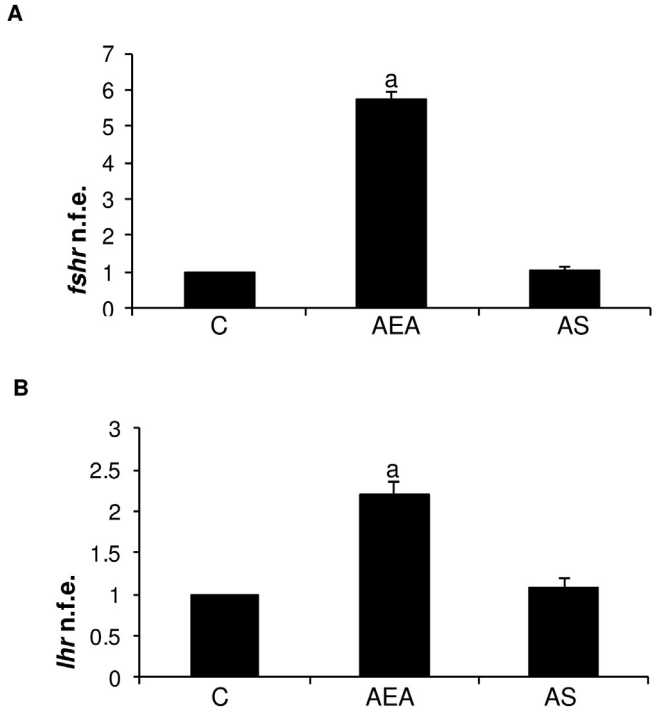

Fig. 4. Effects of AEA treatment on the expression of gonadotrophin receptors in testis. *Fshr* (A) and *lhr* (B) in frog testis evaluated by qPCR. C, control group, testis collected from frogs injected with KRB alone; AEA, testis collected from frogs injected with 10^{-8} M AEA; AS, testis collected from frogs injected with both 10^{-7} M SR141716A and 10^{-8} M AEA. Data are reported as normalized mean fold expression ± s.e.m. (n.f.e.) and are representative of three separate experiments at least. "a" indicates statistically significant differences among samples ($P < 0.01$).

4. Discussion

In vertebrates, endocannabinoids and kisspeptins exert their activity at multiple levels along the HPG axis. In fact, the lack of kisspeptin signaling causes idiopathic hypogonadotropic hypogonadism in humans and experimental animal models (d'Angelmont de Tassigny et al., 2007; de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003). In addition, the phenotype of CB1 knockout ($CB1^{-/-}$) animals is characterized by the down-regulation of the neuro-endocrine axis activity (Cacciola et al., 2013a), the decrease of Leydig cell number (Cacciola et al., 2008), the impairment of the acquisition of sperm motility (Cobellis et al., 2010; Ricci et al., 2007) and the poor packaging of chromatin in spermatozoa (Cacciola et al., 2013b; Chioccarelli et al., 2010). In the hypothalamus, GnRH secreting neurones represent the direct target of kisspeptin activity (Oakley et al., 2009). Nevertheless, direct and indirect mechanisms have also been proposed to explain the endocannabinoid dependent modulation of the release, biosynthesis and signaling of GnRH (Battista et al., 2012; Bovolin et al., 2014; Meccariello et al., 2014a; Pagotto et al., 2006). Consistently, in amphibians, the transcriptional down-regulation of *gnrhs* and the up-regulation of *gnrhrs* has been reported in the

Table 1
Intra-testicular testosterone (T) and estradiol (E) levels in AEA treated frogs.

	Control (pg/mg tissue)	AEA (pg/mg tissue)	SR + AEA (pg/mg tissue)
T	100.14 ± 16.5	49.18 ± 8.7**	88.56 ± 7.1
E	0.560 ± 0.069	1.944 ± 0.312**	0.709 ± 0.228

**$P < 0.01$ *vs.* control group and animals pretreated with SR141716A.

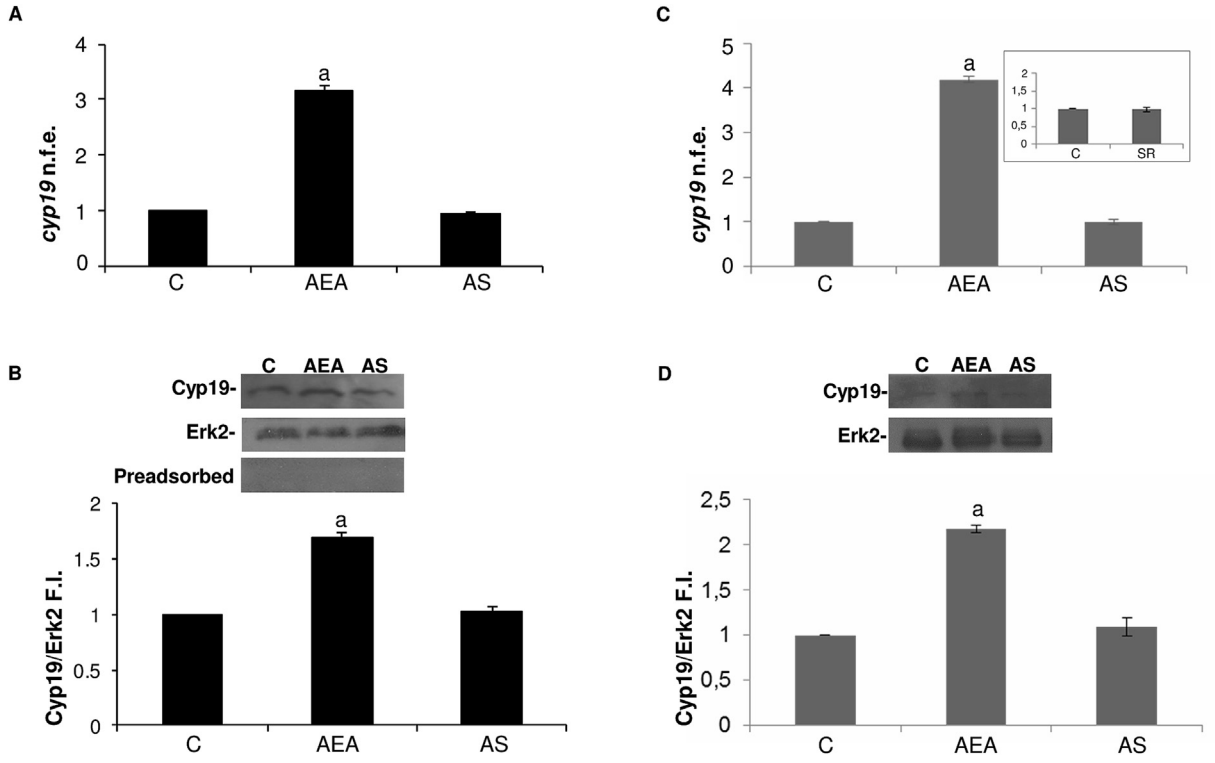

Fig. 5. Effects of AEA treatment on the expression of Cyp19. Quantitative PCR (A, C) and Western blot (B, D) analyses. The specificity of Western blot signals was evaluated by reprobing the same filter with the Cyp19 antiserum preabsorbed with a five-fold excess of the blocking peptide. Western blot signals were normalized against Erk2 (B, D). C, control group, testis collected from frogs injected with KRB alone; AEA, testis collected from frogs injected with 10^{-8} M AEA (A, B) or *in vitro* treated testis with 10^{-9} M AEA (C, D); SR, testis *in vitro* treated with 10^{-8} M SR141716A alone (C, inset); AS, testis collected from frogs injected with both 10^{-7} M SR141716A and 10^{-8} M AEA (A, B) or *in vitro* treated testis with both 10^{-8} M SR141716A and 10^{-9} M AEA (C, D). Quantitative PCR data are reported as normalized mean fold expression ± s.e.m.; Western blot data are reported as mean fold increase ± s.e.m. Data are representative of three separate experiments at least. "a" indicates statistically significant differences among samples ($P < 0.01$).

diencephalon of male frogs incubated with AEA during the post-reproductive period (Chianese et al., 2011b). Here, for the first time in a vertebrate species, we suggest the involvement of kisspeptin activity in the AEA dependent modulation of GnRH circuitry during the pre-reproductive period, when GnRH1 and GnRH2 are released to sustain the onset of a new spermatogenetic wave (Di Matteo et al., 1996; Fasano et al., 1993). In fact, in the diencephalon the *in vivo* administration of AEA decreases the transcription level of GnRH ligands and receptors, but also negatively affects Gpr54 (both mRNA and protein) and Kiss1 protein levels. Gpr54 is expressed in the forebrain and diencephalon of non-mammalian and mammalian vertebrates, with morpho-functional relationship and colocalization in GnRH secreting neurones in fish and mammals (Lee et al., 2009; Oakley et al., 2009). In *Xenopus laevis*, kisspeptin system is abundantly expressed in the brain, notably in the hypothalamus (Lee et al., 2009). Nevertheless, the activity of Gpr54 has been detected in bullfrog hypothalamus and pituitary (Moon et al., 2009) and in *P. esculentus* testis (Chianese et al., 2013a, 2015), as well.

Beside the receptor, present results confirm the presence of Kiss1 protein in the brain of *P. esculentus* and add new insights in the modulation of HPG axis. In fact, data suggest the existence of a double route in the modulation of GnRH secreting neuron activity by endocannabinoids: i) indirectly *via* the down-regulation of kisspeptin neuron activity; ii) directly *via* the Cb1 dependent down-regulation of *gpr54* expression. Consistently, in *P. esculentus* Cb1 immunoreactive fibers not only surround GnRH secreting neurones, but 20% of GnRH secreting neurones coexpresses Cb1 (Meccariello et al., 2008). Evidences that in amphibians kisspeptins may regulate GnRH release pre-synaptically at the level of the median eminence have recently emerged (Oakley et al., 2009; Tena-Sempere et al., 2012), but Gpr54 localization in GnRH secreting neurons has never been investigated so far. Further experiments will surely clarify this issue in the next future, but, at present, as far as we are aware, this is the first report concerning the possible interplay between endocannabinoid and kisspeptin systems in the modulation of hypothalamic GnRH activity under physiological conditions. In fact, the only data available in literature concern the endocannabinoid dependent decrease of serum LH secretion in male rats under stress conditions *via* the transcriptional down-regulation of *kiss1* (Karamikheirabad et al., 2013).

Following AEA *in vivo* administration, the suppression of hypothalamic Gpr54/*gnrh* system activity causes in the testis the decrease of intratesticular testosterone levels, the transcriptional increase of *lhr* and *fshr* mRNA, the modulation of the GnRH local system and the decrease of Gpr54 (both mRNA and protein) as well as Kiss1 protein levels. Leydig cells represent the first target of endocannabinoids to have been discovered in testis (Wenger et al., 2001) and in frog the transcription of either *cb1* and *N-acyl phosphatidylethanolamine-specific phospholipase D*, the enzyme responsible for AEA biosynthesis, occurs in interstitial compartment (Chianese et al., 2012). The decrease of testosterone levels here reported well parallels with the decrease of *cyp17* and *3β-hsd* expression previously observed in *in vivo* but not in *ex vivo* AEA treated testes (Chianese et al., 2014). Accordingly, the increased transcription of gonadotropin receptors might represent a compensatory mechanism due to gonadotropin depletion in order to improve testis receptivity. Hence, the differential modulation of *gnrhs* and *gnrhrs* here observed in testis fully parallels those previously reported in *ex vivo* testis in pre-reproductive period

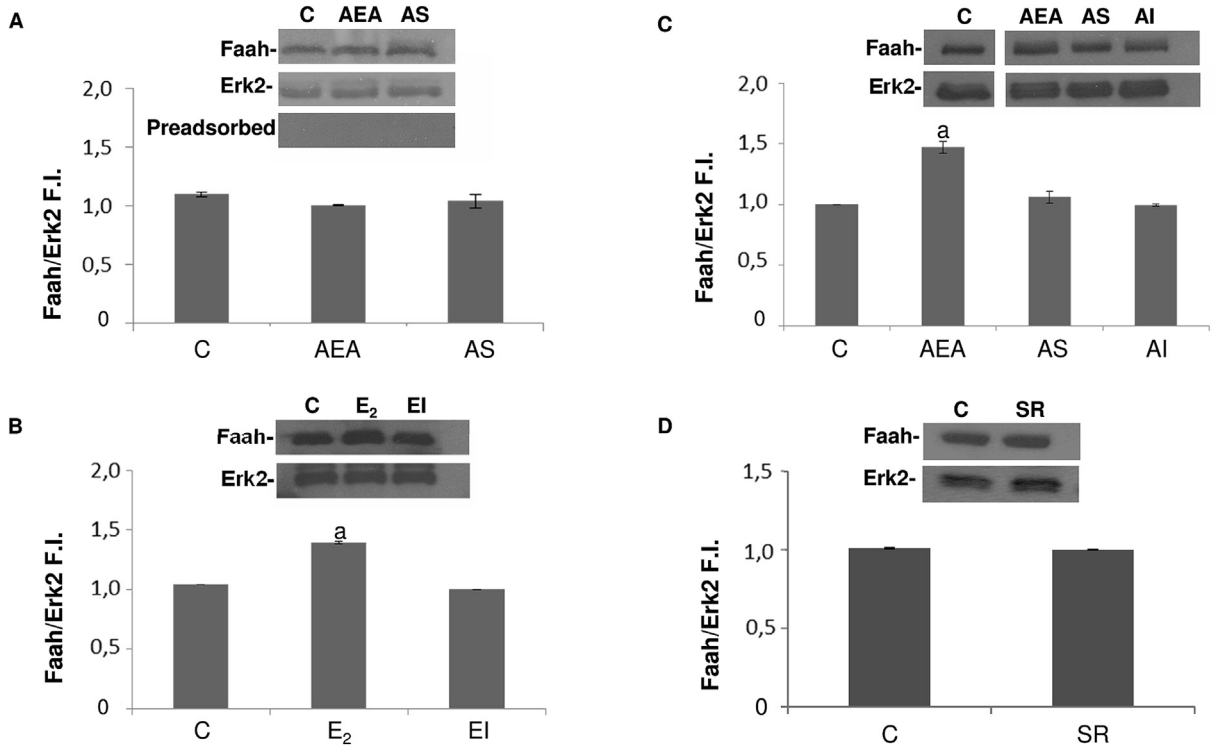

Fig. 6. Effects of AEA and E_2 treatment on the expression of Faah. Western blot analysis for Faah. *In vivo* AEA injection of frogs with the corresponding analysis of Faah specificity by reprobing the same filter with the antiserum preabsorbed with a five-fold excess of the blocking peptide (A). C, control group, testis collected from frogs injected with KRB alone; AEA, testis collected from frogs injected with 10^{-8} M AEA; AS, testis collected from frogs injected with both 10^{-7} M SR141716A and 10^{-8} M AEA. *In vitro* E_2 treatment of frog testis (B). C, control group, testis treated with KRB alone; E_2, testis treated with 10^{-6} M E_2; EI, testis treated with both 10^{-5} M ICI and 10^{-6} M E_2. *In vitro* AEA treatment of frog testis (C, D). C, control group, testis treated with KRB alone; AEA, testis treated with 10^{-9} M AEA; SR, testis treated with 10^{-8} M SR141716A alone; AS, testis treated with both 10^{-8} M SR141716A and 10^{-9} M AEA; AI, testis treated with both 10^{-5} M ICI and 10^{-9} M AEA. Western blot data are reported as mean fold increase ± s.e.m. Data are representative of three separate experiments at least. "a" indicates statistically significant differences among samples ($P < 0.01$).

(Chianese et al., 2012), suggesting the existence of local and not centrally mediated mechanisms. Steroidogenesis requires local activity of GnRH whereas data concerning the involvement of kisspeptin are quite controversial (Meccariello et al., 2014b), even if in frog kisspeptin signaling is involved in the transcriptional up-regulation of estrogen receptors *erα/β* (Chianese et al., 2013a, 2015) and *gpr54* is expressed in Leydig cells (Chianese et al., 2013a). Furthermore, the intra-testicular production of kisspeptins in amphibians has not yet assessed so far, but here we suggest that frog testis produces Kiss1 and we demonstrate that, *via* Cb1, AEA decreases Kiss1 and Gpr54 levels suggesting a new interesting aspect in the control of testis physiology.

Interestingly, in AEA injected animals, the decrease of intra-testicular testosterone parallels the increase of both intra-testicular estradiol and aromatase Cyp19 levels. Accordingly, $CB1^{-/-}$ mice have low rate of testosterone secretion *in vitro* (Wenger et al., 2001), low circulating testosterone and estradiol levels (Cacciola et al., 2013a; Wenger et al., 2001) and low testicular *cyp19* expression (Cacciola et al., 2013a), confirming the need of endocannabinoid signaling in testosterone into estradiol conversion. All the effects observed at central and testicular level occur *via* Cb1. In fact, not only a specific Cb1 antagonist restores the functionality of HPG axis, but AEA induces the transcription of its own receptors in both hypothalamus and testis (Chianese et al., 2011b, 2012).

Taking in account previous and present results, the activity of AEA along the HPG axis may be exerted at multiple levels. Acting at central level, AEA suppresses the hypothalamic activity of kisspeptin and GnRH system leading to the decrease of gonadal testosterone production. Concomitantly, in testis the treatment further suppresses testosterone levels by means of Cyp19 switch on which in turn promotes the conversion of testosterone into estradiol. In amphibians, estrogens negatively feedback at hypothalamus and pituitary in order to suppress gonadotropin discharge (Pavgi and Licht, 1989, 1993); consistently, in mammals kisspeptin neurons located in the arcuate nucleus are the main target of sex steroid mediated negative feedback in males (Clarkson and Herbison, 2009; Pinilla et al., 2012). Interestingly, in frogs, intragonadic estradiol levels fully parallel the fluctuation of plasmatic estradiol (Varriale et al., 1986). Thus, present data address for the first time kisspeptin neurons as new upstream target of AEA activity in the modulation of GnRH circuitry and in sex steroid dependent feedbacks.

The increase of estradiol biosynthesis following AEA *in vivo* administration well correlates to the direct AEA and estradiol activity in testis here checked by *in vitro* treatments. Interestingly, the modulation of AEA tone by FAAH is the major checkpoint of endocannabinoid signaling in vertebrates and FAAH is the direct target of estrogens in mammalian testis (Rossi et al., 2007). Present data demonstrate that *in vitro* treatment with 17β-estradiol induces the production of Faah protein and extend to a non-mammalian vertebrate the estradiol dependent up-regulation of FAAH, thus demonstrating the existence of evolutionarily conserved mechanisms and confirming the deep contribute of non-mammalian vertebrates in the discovery of highly conserved master systems (Chianese et al., 2011a). *In vitro* AEA has also the ability to induce the production of Faah protein and such an effect is fully counteracted by pre-treatment with ICI182780, further confirming estrogen mediated mechanisms in Faah biosynthesis (Grimaldi et al., 2012). A part from estradiol effect on Faah, AEA has also direct

effect on Cyp19 protein production (up-regulation observed in both *in vivo* and *in vitro* treatments). In this respect we suggest a local fine regulatory loop in the modulation of AEA tone in testis (see graphical abstract). In fact, high AEA tone, *via* Cyp19, induces estradiol biosynthesis with subsequent Faah production and AEA decrease; low AEA tone inhibits testosterone aromatization into estradiol with subsequent low production of Faah. Such a modulation occurs in a time window critical for the upsurge of a new spermatogenetic wave, a process that in amphibians just requires estradiol signaling (Cobellis et al., 1999, 2002; Minucci et al., 1997; Pierantoni et al., 2002), aromatase activity (Caneguim et al., 2013) and local GnRH and kisspeptin system (Fasano et al, 1990; Chianese et al., 2015; Cobellis et al., 2003).

In conclusion, we provide the first evidence that AEA regulates the reproductive activity centrally acting through kisspeptin system and in testis modulating its tone *via* the biosynthesis of estradiol which in turn targets Faah protein.

Conflicts of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data deposition

Nucleotide sequences of *fshr, lhr* and *cyp19* were deposited in GenBank under the accession numbers KM983558, KM983559, KM983560, respectively.

References

Battista, N., Meccariello, R., Cobellis, G., Fasano, S., Di Tommaso, M., Pirazzi, V., Konje, J.C., Pierantoni, R., Maccarrone, M., 2012. The role of endocannabinoids in gonadal function and fertility along the evolutionary axis. Mol. Cell Endocrinol. 355, 1–14.

Bovolin, P., Cottone, E., Pomatto, V., Fasano, S., Pierantoni, R., Cobellis, G., Meccariello, R., 2014. Endocannabinoids are involved in male vertebrate reproduction: regulatory mechanisms at central and gonadal level. Front. Endocrinol. (Lausanne) 5, 54.

Cacciola, G., Chianese, R., Chioccarelli, T., Ciaramella, V., Fasano, S., Pierantoni, R., Meccariello, R., Cobellis, G., 2010. Cannabinoids and reproduction: a lasting and intriguing history. Pharmaceuticals 3, 3275–3323.

Cacciola, G., Chioccarelli, T., Altucci, L., Ledent, C., Mason, J.I., Fasano, S., Pierantoni, R., Cobellis, G., 2013a. Low 17betaestradiol levels in Cnr1 knock-out mice affect spermatid chromatin remodeling by interfering with chromatin reorganization. Biol. Reprod. 88, 1–12.

Cacciola, G., Chioccarelli, T., Altucci, L., Viggiano, A., Fasano, S., Pierantoni, R., Cobellis, G., 2013b. Nuclear size as estrogen-responsive chromatin quality parameter of mouse spermatozoa. Gen. Comp. Endocrinol. 193, 201–209.

Cacciola, G., Chioccarelli, T., Mackie, K., Meccariello, R., Ledent, C., Fasano, S., Pierantoni, R., Cobellis, G., 2008. Expression of type-1 cannabinoid receptor during rat postnatal testicular development: possible involvement in adult Leydig cell differentiation. Biol. Reprod. 79, 758–765.

Caneguim, B.H., Luz, J.S., Valentini, S.R., Cerri, P.S., Sasso-Cerri, E., 2013. Immunoexpression of aromatase and estrogen receptors β in stem spermatogonia of bullfrogs indicates a role of estrogen in the seasonal spermatogonial mitotic activity. Gen. Comp. Endocrinol. 182, 65–72.

Chianese, R., Chioccarelli, T., Cacciola, G., Ciaramella, V., Fasano, S., Pierantoni, R., Meccariello, R., Cobellis, G., 2011a. The contribution of lower vertebrate animal models in human reproduction research. Gen. Comp. Endocrinol. 171, 17–27.

Chianese, R., Ciaramella, V., Fasano, S., Pierantoni, R., Meccariello, R., 2015. Kisspeptin drives germ cell progression in the anuran amphibian Pelophylax esculentus. Gen. Comp. Endocrinol. 211, 81–91.

Chianese, R., Ciaramella, V., Fasano, S., Pierantoni, R., Meccariello, R., 2013a. Kisspeptin receptor, GPR54, as a candidate for the regulation of testicular activity in the frog, *Rana esculenta*. Biol. Reprod. 88, 73.

Chianese, R., Ciaramella, V., Fasano, S., Pierantoni, R., Meccariello, R., 2011b. Anandamide modulates the expression of GnRH-II and GnRHRs in frog, Rana esculenta, diencephalon. Gen. Comp. Endocrinol. 173, 389–395.

Chianese, R., Ciaramella, V., Fasano, S., Pierantoni, R., Meccariello, R., 2014. Hypothalamus-pituitary axis: an obligatory target for endocannabinoids to inhibit steroidogenesis in frog testis. Gen. Comp. Endocrinol. 205, 88–93.

Chianese, R., Ciaramella, V., Scarpa, D., Fasano, S., Pierantoni, R., Meccariello, R., 2013b. Endocannabinoids and endovanilloids: a possible balance in the regulation of the testicular GnRH signalling. Int. J. Endocrinol. 904748.

Chianese, R., Cobellis, G., Pierantoni, R., Fasano, S., Meccariello, R., 2008. Non-mammalian vertebrate models and the endocannabinoid system: relationships with gonadotropin-releasing hormone. Mol. Cell Endocrinol. 286, S46–S51.

Chianese, R., Ciaramella, V., Scarpa, D., Fasano, S., Pierantoni, R., Meccariello, R., 2012. Anandamide regulates the expression of GnRH1, GnRH2, and GnRH-Rs in frog testis. Am. J. Physiol. Endocrinol. Metab. 303, E475–E487.

Chioccarelli, T., Cacciola, G., Altucci, L., Lewis, S.E., Simon, L., Ricci, G., Ledent, C., Meccariello, R., Fasano, S., Pierantoni, R., Cobellis, G., 2010. Cannabinoid receptor 1 influences chromatin remodeling in mouse spermatids by affecting content of transition protein 2 mRNA and histone displacement. Endocrinology 151, 5017–5029.

Clarkson, J., Herbison, A.E., 2009. Oestrogen, kisspeptin, GPR54 and the pre-ovulatory luteinising hormone surge. J. Neuroendocrinol. 21, 305–311.

Cobellis, G., Cacciola, G., Scarpa, D., Meccariello, R., Chianese, R., Franzoni, M.F., Mackie, K., Pierantoni, R., Fasano, S., 2006. Endocannabinoid system in frog and rodent testis: type-1 cannabinoid receptor and fatty acid amide hydrolase activity in male germ cells. Biol. Reprod. 75, 82–89.

Cobellis, G., Meccariello, R., Fienga, G., Pierantoni, R., Fasano, S., 2002. Cytoplasmic and nuclear Fos protein forms regulate resumption of spermatogenesis in the frog, Rana esculenta. Endocrinology 143, 163–170.

Cobellis, G., Meccariello, R., Minucci, S., Palmiero, C., Pierantoni, R., Fasano, S., 2003. Cytoplasmic versus nuclear localization of Fos-related proteins in the frog, Rana esculenta, testis: in vivo and direct in vitro effect of a gonadotropin-releasing hormone agonist. Biol. Reprod. 68, 954–960.

Cobellis, G., Pierantoni, R., Minucci, S., Pernas-Alonso, R., Meccariello, R., Fasano, S., 1999. C-fos activity in *Rana esculenta* testis: seasonal and estradiol-induced changes. Endocrinology 140, 3238–3244.

Cobellis, G., Ricci, G., Cacciola, G., Orlando, P., Petrosino, S., Cascio, M.G., Bisogno, T., De Petrocellis, L., Chioccarelli, T., Altucci, L., Fasano, S., Meccariello, R., et al., 2010. A gradient of 2-arachidonoylglycerol regulates mouse epididymal sperm cell start-up. Biol. Reprod. 82, 451–458.

d'Anglemont de Tassigny, X., Fagg, L.A., Dixon, J.P., Day, K., Leitch, H.G.,, Hendrick, A.G., Zahn, D., Franceschini, I., Caraty, A., Carlton, M.B.,, Aparicio, S.A., Colledge, W.H., 2007. Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. Proc. Natl. Acad. Sci. U S A 104, 10714–10719.

de Roux, N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L., Milgrom, E., 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc. Natl. Acad. Sci. U S A 100, 10972–10976.

Di Matteo, L., Vallarino, M., Pierantoni, R., 1996. Localization of GnRH molecular forms in the brain, pituitary, and testis of the frog, Rana esculenta. J. Exp. Zool. 274, 33–40.

Everett, J.W., 2006. Pituitary and hypothalamus: prospectives and overview. In: Knobil, Neill (Eds.), Physiology of Reproduction, third ed. Elsevier.

Fasano, S., Goos, H.J.Th, Janssen, C., Pierantoni, R., 1993. Two GnRH fluctuate in correlation with androgen levels in the male frog, Rana esculenta. J. Exp. Zool. 266, 277–283.

Fasano, S., de Leeuw, R., Pierantoni, R., Chieffi, G., van Oordt, P.G., 1990. Characterization of gonadotropin-releasing hormone (GnRH) binding sites in the pituitary and testis of the frog, Rana esculenta. Biochem. Biophys. Res. Commun. 168, 923–932.

Funes, S., Hedrick, J.A., Vassleva, G., Markowitz, L., Abbondanzo, S., Golovko, A., Yang, S., Monsma, F.J., Gustafson, E.L., 2003. The KISS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem. Biophys. Res. Commun. 312, 1357–1363.

Gammon, C.M., Freeman Jr., G.M., Xie, W., Petersen, S.L., Wetsel, W.C., 2005. Regulation of gonadotropin-releasing hormone secretion by cannabinoids. Endocrinology 146, 4491–4499.

Giang, D.K., Cravatt, B.F., 1997. Molecular characterization of human and mouse fatty acid amide hydrolases. Proc. Natl. Acad. Sci. U S A 94, 2238–2242.

Grimaldi, P., Pucci, M., Di Siena, S., Di Giacomo, D., Pirazzi, V., Geremia, R., Maccarrone, M., 2012. The faah gene is the first direct target of estrogen in the testis: role of histone demethylase LSD1. Cell Mol. Life Sci. 69, 4177–4190.

Hsu, M.C., Wang, J.Y., Lee, Y.J., Jong, D.S., Tsui, K.H., Chiu, C.H., 2014. Kisspeptin modulates fertilization capacity of mouse spermatozoa. Reproduction 147, 835–845.

Irfan, S., Ehmcke, J., Wahab, F., Shahab, M., Schlatt, S., 2014. Intratesticular action of kisspeptin in rhesus monkey (Macaca mulatta). Andrologia 6, 610–617.

Karamikheirabad, M., Behzadi, G., Faghihi, M., Raoofian, R., Ejtemaei Mehr, S., Zuure, W.A., Sadeghipour, H.R., 2013. A role for endocannabinoids in acute stress-induced suppression of the hypothalamic-pituitary-gonadal axis in male rats. Clin. Exp. Reprod. Med. 40, 155–162.

Lee, Y.R., Tsunekawa, K., Moon, M.J., Um, H.N., Hwang, J.I., Osugi, T., Otaki, N., Sunakawa, Y., Kim, K., Vaudry, H., Kwon, H.B., Seong, J.Y., et al., 2009. Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in vertebrates. Endocrinology 150, 2837–2846.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement

with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Maccarrone, M., Wenger, T., 2005. Effects of cannabinoids on hypothalamic and reproductive function. In: Pertwee, R.G. (Ed.), Cannabinoids, Handbook of Exp. Pharm. Springer, Heidelberg, New York, pp. 555–572.
Maruo, K., Suda, M., Yokoyama, S., Oshima, Y., Nakamura, M., 2008. Steroidogenic gene expression during sex determination in the frog *Rana rugosa*. Gen. Comp. Endocrinol. 158, 87–94.
Meccariello, R., Battista, N., Bradshaw, H.B., Wang, H., 2014a. Updates in reproduction coming from the endocannabinoid system. Int. J. Endocrinol. 412354.
Meccariello, R., Chianese, R., Cacciola, G., Cobellis, G., Pierantoni, R., Fasano, S., 2006. Type-1 cannabinoid receptor expression in the frog, *Rana esculenta*, tissues: a possible involvement in the regulation of testicular activity. Mol. Reprod. Dev. 73, 551–558.
Meccariello, R., Chianese, R., Chioccarelli, T., Ciaramella, V., Fasano, S., Pierantoni, R., Cobellis, G., 2014b. Intra-testicular signals regulate germ cell progression and production of qualitatively mature spermatozoa in vertebrates. Front. Endocrinol. (Lausanne) 5, 69.
Meccariello, R., Chianese, R., Cobellis, G., Pierantoni, R., Fasano, S., 2007. Cloning of type 1 cannabinoid receptor in *Rana esculenta* reveals differences between genomic sequence and cDNA. FEBS J. 274, 2909–2920.
Meccariello, R., Franzoni, M.F., Chianese, R., Cottone, E., Scarpa, D., Donna, D., Cobellis, G., Guastalla, A., Pierantoni, R., Fasano, S., 2008. Interplay between the endocannabinoid system and GnRH-I in the forebrain of the anuran amphibian Rana esculenta. Endocrinology 149, 2149–2158.
Minucci, S., Di Matteo, L., Chieffi, P., Pierantoni, R., Fasano, S., 1997. 17 beta-estradiol effects on mast cell number and spermatogonial mitotic index in the testis of the frog, Rana esculenta. J. Exp. Zool. 278, 93–100.
Moon, J.S., Lee, Y.R., Oh, D.Y., Hwang, J.I., Lee, J.Y., Kim, J.I., Vaudry, H., Kwon, H.B., Seong, J.Y., 2009. Molecular cloning of the bullfrog kisspeptin receptor GPR54 with high sensitivity to Xenopus kisspeptin. Peptides 30, 171–179.
Oakley, A.E., Clifton, D.K., Steiner, R.A., 2009. Kisspeptin signaling in the brain. Endocr. Rev. 30, 713–743.
Pagotto, U., Marsicano, G., Cota, D., Lutz, B., Pasquali, R., 2006. The emerging role of the endocannabinoid system in endocrine regulation and energy balance. Endocr. Rev. 27, 73–100.
Pavgi, S., Licht, P., 1989. Effects of gonadectomy and steroids on pituitary gonadotropin secretion in a frog, Rana pipiens. Biol. Reprod. 41, 40–48.
Pavgi, S., Licht, P., 1993. Inhibition of in vitro pituitary gonadotropin secretion by 17 beta-estradiol in the frog Rana pipiens. Gen. Comp. Endocrinol. 89, 132–137.
Pierantoni, R., Cobellis, G., Meccariello, R., Cacciola, G., Chianese, R., Chioccarelli, T., Fasano, S., 2009. CB1 activity in male reproduction: mammalian and non-mammalian animal models. Vitam. Horm. 81, 367–387. G Litwack, Burlington: Academic Press.
Pierantoni, R., Cobellis, G., Meccariello, R., Fasano, S., 2002. Evolutionary aspects of cellular communication in the vertebrate hypothalamo-hypophysio-gonadal axis. Int. Rev. Cytol. 218, 69–141.
Pierantoni, R., Iela, L., d'Istria, M., Fasano, S., Rastogi, R.K., Delrio, G., 1984. Seasonal testosterone profile and testicular responsiveness to pituitary factors and gonadotrophin releasing hormone during two different phases of the sexual cycle of the frog (Rana esculenta). J. Endocrinol. 102, 387–392.
Pinilla, L., Aguilar, E., Dieguez, C., Millar, R.P., Tena-Sempere, M., 2012. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. Physiol. Rev. 92, 1235–1316.
Pinto, F.M., Cejudo-Romàn, A., Ravina, C.G., Fernandez-Sanchez, M., Martin-Lozano, D., Illanes, M., Tena-Sempere, M., Candenas, M.L., 2012. Characterization of the kisspeptin system in human spermatozoa. Int. J. Androl. 35, 63–73.
Ricci, G., Cacciola, G., Altucci, L., Meccariello, R., Fasano, S., Pierantoni, R., Cobellis, G., 2007. Endocannabinoid control of sperm motility: the role of epididymus. Gen. Comp. Endocrinol. 153, 320–322.
Rinaldi-Carmona, M., Barth, F., Hèaulme, M., Shire, D., Calandra, B., Congy, C., Martinez, S., Maruani, J., Nèliat, G., Caput, D., Ferrara, P., Soubrie, P., 1994. SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett. 350, 240–244.
Rossi, G., Gasperi, V., Paro, R., Barsacchi, D., Cecconi, S., Maccarrone, M., 2007. Follicle-stimulating hormone activates fatty acid amide hydrolase by protein kinase A and aromatase-dependent pathways in mouse primary Sertoli cells. Endocrinology 148, 1431–1439.
Salehi, S., Adeshina, I., Chen, H., Zirkin, B.R., Hussain, M.A., Wondisford, F., Wolfe, A., Radovick, S., 2015. Developmental and endocrine regulation of kisspeptin expression in mouse Leydig cells. Endocrinology 156, 1514–1522.
Scorticati, C., Fernández-Solari, J., De Laurentiis, A., Mohn, C., Prestifilippo, J.P., Lasaga, M., Seilicovich, A., Billi, S., Franchi, A., McCann, S.M., Rettori, V., 2004. The inhibitory effect of anandamide on luteinizing hormone-releasing hormone secretion is reversed by estrogen. Proc. Natl. Acad. Sci. U S A 101, 11891–11896.
Seminara, S.B., Messager, S., Chatzidaki, E.E., Thresher, R.R., Acierno, J.S., Shagoury, J.K., Bo-Abbas, Y., Kuohung, W., Schwinof, K.M., Hendrick, A.G., Zahn, D., Dixon, J., et al., 2003. The GPR54 gene as a regulator of puberty. N. Engl. J. Med. 349, 1614–1627.
Suda, M., Kodama, M., Oshima, Y., Yamamoto, K., Nakamura, Y., Tanaka, S., Kikuyama, S., Nakamura, M., 2011. Up-regulation of FSHR expression during gonadal sex determination in the frog Rana Rugosa. Gen. Comp. Endocrinol. 172, 475–486.
Tena-Sempere, M., Felip, A., Gomez, A., Zanuy, S., Carillo, M., 2012. Comparative insights of the kisspeptin/kisspeptin receptor system: lessons from non-mammalian vertebrates. Gen. Comp. Endocrinol. 175, 234–243.
Varriale, B., Pierantoni, R., Di Matteo, L., Minucci, S., Fasano, S., D'Antonio, M., Chieffi, G., 1986. Plasma and testicular estradiol and plasma androgen profile in the male frog *Rana esculenta* during the annual cycle. Gen. Comp. Endocrinol. 64, 401–404.
Wang, H., Dey, S.K., Maccarrone, M., 2006. Jekyll and Hyde: two faces of Cannabinoid signaling in male and female fertility. Endocr. Rev. 27, 427–448.
Wenger, T., Ledent, C., Csernus, V., Gerendai, I., 2001. The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. Biochem. Biophys. Res. Commun. 284, 363–368.