General and Comparative Endocrinology 205 (2014) 88–93

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

# Hypothalamus–pituitary axis: An obligatory target for endocannabinoids to inhibit steroidogenesis in frog testis

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ARTICLE INFO

*Article history:*
Available online 22 February 2014

*Keywords:*
Endocannabinoid system
Spermatogenesis
Amphibian
Steroidogenesis

ABSTRACT

Endocannabinoids – primarily anandamide (AEA) and 2-arachidonoylglycerol (2-AG) – are lipophilic molecules that bind to cannabinoid receptors (CB1 and CB2). They affect neuroendocrine activity inhibiting gonadotropin releasing hormone (GnRH) secretion and testosterone production in rodents, through a molecular mechanism supposed to be hypothalamus dependent. In order to investigate such a role, we choose the seasonal breeder, the anuran amphibian *Rana esculenta*, an experimental model in which components of the endocannabinoid system have been characterized.

In February, at the onset of a new spermatogenetic wave, we carried out *in vitro* incubations of frog testis with AEA, at $10^{-9}$ M dose. Such a treatment had no effect on the expression of *cytochrome P450 17alpha hydroxylase/17,20 lyase* (*cyp17*) nor *3-β-hydroxysteroid dehydrogenase/Δ-5–4 isomerase* (*3β-HSD*), key enzymes of steroidogenesis.

To understand whether or not the functionality of the hypothalamus–pituitary axis could be essential to support the role of endocannabinoids in steroidogenesis, frogs were injected with AEA, at $10^{-8}$ M dose. Differently from *in vitro* experiment, the *in vivo* administration of AEA reduced the expression of *cyp17* and *3β-HSD*. Whereas the effect on *3β-HSD* was counteracted by SR141716A (Rimonabant) – a selective antagonist of CB1, thus indicating a CB1 dependent modulation – the effect on *cyp17* was not, suggesting a possible involvement of receptors other than CB1, probably the type-1 vanilloid receptor (TRPV1), since AEA works as an endocannabinoid and an endovanilloid as well.

In conclusion our results indicate that endocannabinoids, *via* CB1, inhibit the expression of *3β-HSD* in frog testis travelling along the hypothalamus–pituitary axis.



## 1. Introduction

Several findings underscore the critical role played by the endocannabinoids (eCBs) in the control of reproductive functions (Battista et al., 2012; Chianese et al., 2011a; Fasano et al., 2009; Meccariello et al., 2014; Pierantoni et al., 2009a).

These lipid molecules – such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) – mimic some deleterious effects of $\Delta^9$-tetrahydrocannabinol (THC), the major active ingredient of marijuana. They exert biological activities by activating cannabinoid receptors (CB1 and CB2) (Matsuda et al., 1990; Munro et al., 1993), both localized in the central nervous system and in peripheral tissues. Unlike 2-AG, AEA also works as an endovanilloid since it is able to bind to type 1 vanilloid receptor (TRPV1), a cation channel receptor also activated by capsaicin (CAP, 8-methyl-*N*-vanillyl-6-nonenamide), the pungent compound of hot chili pepper (van der Stelt and di Marzo, 2005).

In the scenario of reproduction, eCBs play the part of foe, acting at both hypothalamus–pituitary and gonad levels. Most information about eCBs involvement in male reproduction came from CB1 knockout ($CB1^{-/-}$) mice (for a recent review see Cacciola et al., 2013c). This animal model efficiently synthesizes luteinizing hormone (LH) at pituitary level, but shows low levels of LH and testosterone in the bloodstream, thus suggesting a hypothalamus dependent molecular mechanism at the basis of such a suppressive modulation (Wenger et al., 2001). Since spermatogenesis is dependent on the presence of an adequate intratesticular level of testosterone (Cobellis et al., 2003), several abnormalities have been demonstrated in $CB1^{-/-}$ animals. In particular, they display down-regulation of the neuroendocrine axis (Cacciola et al., 2013a), developmental decrease of Leydig cell number (Cacciola et al., 2008),

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http://dx.doi.org/10.1016/j.ygcen.2014.02.010
0016-6480/

low sperm chromatin quality (Cacciola et al., 2013b; Chioccarelli et al., 2010) and abnormal epididymal sperm motility acquisition (Ricci et al., 2007; Cobellis et al., 2010). Besides the observations in genetically modified experimental models, in mammals eCBs negatively affect gonadotropin releasing hormone (GnRH) release (Scorticati et al., 2004), downstream regulating LH production (Wenger et al., 2001), whereas in the anuran amphibian *Rana esculenta* AEA-dependent transcriptional modulation of GnRH system (both ligands and receptors) has been reported in the diencephalon and in the testis (Chianese et al., 2011b, 2012; Meccariello et al., 2008). Interestingly, in frog gonad, an opposite regulation of each component of the GnRH system – two ligands (GnRH1 and GnRH2) and three receptors (GnRH-R1, -R2, -R3) (Chianese et al., 2011b, 2012) – occurs activating CB1 or TRPV1, thus providing evidence that two different AEA-dependent signalling pathways might modulate the activity of testicular GnRH (Chianese et al., 2013). Thus, a central query to be resolved is whether AEA might directly affect the steroidogenesis *via* testicular GnRH or this action exclusively requires the downregulation of hypothalamic GnRH and pituitary gonadotropins.

In order to investigate the specific molecular mechanism by which eCBs modulate testosterone production in vertebrates, we choose as experimental model just *R. esculenta*, a seasonal breeder in which components of the endocannabinoid system have been characterized. In particular, *cb1* has been cloned (Meccariello et al., 2007) and its expression has been analyzed in both brain and testis during the annual sexual cycle (Meccariello et al., 2006, 2008) with testicular CB1 mRNA/protein (Chianese et al., 2012; Cobellis et al., 2006; Meccariello et al., 2006) detected in parallel to FAAH – the enzyme involved in AEA degradation – in germ cells, especially in elongated spermatids and spermatozoa. Together with the ability to degrade AEA, frog testis has been shown to be able to produce eCBs during the annual reproductive cycle as suggested by the expression and localization of *Nape-pld*, the enzyme capable to synthesize AEA (Chianese et al., 2012, 2013). Furthermore, frog spermatogenesis shows peculiar features (Pierantoni et al., 2002b); in fact, it proceeds through cysts formations – consisting of Sertoli cells enveloping cluster of germ cells at a synchronous stage – and orchestrated during the year by endocrine, environmental and gonadal factors (Pierantoni et al., 2002a; Rastogi, 1976).

Thus, we carried out *in vitro* incubations of *R. esculenta* testis and *in vivo* treatment with AEA and then analyzed the expression of *cytochrome P450 17alpha hydroxylase/17,20 lyase* (*cyp17*) and *3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase* (*3β-HSD*), key enzymes of steroidogenesis. Since the *in vivo* treatment only had effect, we conclude that the functionality of the hypothalamus–pituitary axis is essential to support the role of eCBs in the regulation of steroidogenesis in frog testis.

## 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 the Italian Ministry of Education, University and Research.

### 2.1. Animal and tissue collection

*R. esculenta* male frogs were collected in February in the neighbourhood of Naples (Italy). The animals were anaesthetized with ethyl-3-aminobenzoate methanesulfonate salt (MS222, Sigma–Aldrich, Milan, Italy) and euthanized by decapitation immediately after capture to minimize stress. Testes were removed, flash frozen in liquid nitrogen and stored at −80 °C until used for RNA extraction.

### 2.2. Total RNA extraction and cDNA preparation

Total RNA was extracted from frog testes using Trizol Reagent (1 ml/50–100 mg tissue) (Life Technologies, Paisley, UK) according to the manufacturer's instructions. RNA samples were treated with DNaseI (10U/sample) (Amersham Pharmacia Biotech) at 37 °C for 30 min to eliminate any contamination of genomic DNA. Total RNA purity and integrity were determined by spectrophotometer analyses at 260/280 nm and by electrophoresis.

Complementary DNA (cDNA) was obtained by reverse transcription using 5 μg of total RNA, 0.5 μg of oligo $dT_{(18)}$, 0.5 mM dNTP mix, 5 mM DTT, 1X first-strand buffer (Life Technologies), 40U RNAse Out and 200U SuperScript-III RNAseH$^-$ Reverse Transcriptase (Life Technologies) in a final volume of 20 μl, following the manufacturer's instructions. As negative control, total RNA not treated with reverse transcriptase was used.

### 2.3. Cloning of R. esculenta cyp17 and 3β-HSD

To clone *cyp17* and *3β-HSD*, 1 μl of diluted (1:5) cDNA was used for standard PCR analysis in combination with 10 pmol of oligonucleotide primers designed on *Rana rugosa* nucleotide sequence (*cyp17* S: 5′-cgctgtgtatgttcggtgaagg-3′ and AS: 5′-ggtctcgagctgccactgact-3′, accession number in GenBank AB284119.2; *3β-HSD* S: 5′-gactcaatgctccaaccttcacag-3′ and AS: 5′-ggacctctggcaggtcctca-3′, accession number in GenBank AB284117.1). The predicted amplificate size was 331 and 237 bp, for *cyp17* and *3β-HSD*, respectively. PCR conditions were: 94 °C, 5 min, 1 cycle; 94 °C, 30 s, 58 °C, 30 s, 72 °C, 30 s, 30 cycles; 72 °C, 7 min. PCR products were sub-cloned in pGEM-T Easy Vector (Promega Corp., Madison, WI). DH5α high-efficiency competent cells were transformed and recombinant colonies were identified by blue/white colour screening. Recombinant plasmid DNA was extracted by using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA), the insert size was controlled by restriction analysis with *EcoRI* (Fermentas, St. Leon-Rot, Germany) and then the inserts were sequenced on both strands by Primm Sequence Service (Primm srl, Naples Italy).

### 2.4. Treatment of frog testes with AEA

#### 2.4.1. Experiment 1. In vitro incubations with AEA

Testes of male frogs ($n$ = 5 animals/treatment) collected in February were quickly removed, immediately frozen in liquid nitrogen, stored at −80 °C and used as fresh control (C0) or treated for 1 h as follow: Krebs–Ringer bicarbonate buffer for amphibians (KRB) alone, control group, C; $10^{-9}$ M KRB/AEA, treatment group, AEA; $10^{-8}$ M KRB/SR141716A (SR/Rimonabant) – a selective antagonist of CB1 (Rinaldi-Carmona et al., 1994), treatment group, SR; $10^{-8}$ M KRB/SR for 30 min and then $10^{-9}$ M KRB/AEA and $10^{-8}$ M SR for 1 h, treatment group, AS.

AEA doses and incubation times were chosen on the basis of previous experiments carried out in both rats (Scorticati et al., 2004) and frogs (Chianese et al., 2011b, 2012; Meccariello et al., 2008). After the incubation, testes were pooled and processed for RNA extraction and quantitative PCR (qRT-PCR).

#### 2.4.2. Experiment 2. In vivo injections of AEA

Male frogs ($n$ = 5 animals/treatment) collected in February were divided in three groups and injected in the dorsal sac with KRB alone (control group, C), $10^{-8}$ M AEA (treatment group, AEA) and $10^{-8}$ M AEA/$10^{-7}$ M SR (combined treatment, AS). In the last treatment, frogs – preliminarily injected with $10^{-7}$ M SR – were injected with $10^{-8}$ M AEA/$10^{-7}$ M SR after 30 min. AEA dose chosen for *in vivo* treatment was ten times more than *in vitro* one. 2 h after injections testes were removed and processed for RNA extraction and qPCR analysis.

### 2.5. qRT-PCR

qRT-PCR analysis was conducted on AEA-treated testes of February to evaluate eCBs effects on *cyp17* and *3β-HSD* expression. All qRT-PCRs were prepared in a final volume of 20 μl containing 1 μl of 1:5 diluted cDNA, 0.5 μM each primer 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 cDNAs were replaced by water was also included. All assays included a melting curve analysis for which all samples displayed single peaks for each primer pair. Quantitative PCR efficiencies values were 99.7% ($R^2$ = 0.992, slope = −3.330) for *3β-HSD* and 99.5% for *cyp17* ($R^2$ = 0.990, slope = −3.335). Genes of interest were normalized to the reference gene *fp1* [S: 5′-tacgagcgtccatcacacac-3′ and AS: 5′-agaccaaagcccatgtcatc-3′; T (°C) annealing 56 °C; amplificate size 356 bp], whose boundary as housekeeping gene has been previously demonstrated (Chianese et al., 2011b, 2012); the relative quantification of the mRNA levels was performed using the comparative $C_q$ method with the formula $2^{-\Delta\Delta Cq}$. Data were then reported as mean fold change ± SD over the value one assigned to control group, C ($n$ = 6). ANOVA followed by Duncan's test for multi-group comparison was carried out to assess the significance of differences.

## 3. Results

### 3.1. Cloning of cyp17 and 3β-HSD cDNA fragments from R. esculenta testis

We partially cloned from frog testis two cDNA fragments of 331 and 237 bp long, encoding *cyp17* and *3β-HSD*, respectively. Alignments – conducted by LALIGN and CLUSTALW multiple alignments – revealed a nucleotide identity of 95% against *Rana dybowskii* and *R. rugosa* and 52.4% against *Xenopus laevis* sequence, for *cyp17*, whereas the nucleotide identity was of 90% against *R. rugosa* sequence, for *3β-HSD*.

Alignments – conducted by CLUSTALW multiple alignments tool – revealed the following amino acid identity: 94% against *R. dybowskii*, 85% against *X. laevis*, 74% against *Danio rerio*, 31% against *Mus musculus* and 41% against *Homo sapiens* for CYP17, whereas 79% against *R. rugosa*, 48% against *D. rerio*, 56% against *M. musculus* and 53% against *H. sapiens* for 3β-HSD.

### 3.2. Testicular cyp17 and 3β-HSD expression after AEA in vitro incubation

Frog spermatogenesis starts in the late winter/early spring when seminiferous tubules gradually populate of proliferating spermatogonia; then, in late spring/summer, meiotic and postmeiotic stages are visible. In autumn, massive spermiogenesis events occur, thus during the winter stasis only non proliferating spermatogonia and spermatozoa can be detected (Rastogi et al., 1976).

To assess a possible effect of eCBs on steroidogenesis in frog, we carried out *in vitro* incubations of testis collected in February – at the onset of a new spermatogenetic wave – with AEA, at $10^{-9}$ M dose, for 1 h and evaluated the expression levels of *cyp17* and *3β-HSD*, key enzymes in steroidogenesis pathway. The first one converts pregnenolone and progesterone to the corresponding 17α-hydroxy forms, and subsequently converts 17α-hydroxypregnenolone and 17α-hydroxyprogesterone to dehydroepiandrosterone (DHEA) and androstenedione, respectively; the second one converts pregnenolone to progesterone, 17α-hydroxypregnenolone to 17α-hydroxyprogesterone, DHEA to androstenedione and androst-5-ene 3β, 17β-diol to testosterone (Fig. 1).

AEA treatment failed to have any effect on *cyp17* and *3β-HSD* expression. Similar result was detected after the treatment with SR at $10^{-8}$ M dose alone or in combination with AEA (Fig. 2A and B).

### 3.3. In vivo treatment of frog with AEA

Since the *in vitro* incubation of frog testis had any effect upon *cyp17* nor *3β-HSD* expression, we envisaged a possible involvement of the hypothalamus–pituitary axis in support to eCBs role in steroidogenesis. Thus, we injected AEA, at $10^{-8}$ M dose, in dorsal sac of frogs collected in February. After 2 h from the injection, testes were removed and processed for qPCR analysis.

Differently from the *in vitro* experiment, AEA *in vivo* treatment inhibited both *cyp17* and *3β-HSD* expression ($P < 0.01$) (Fig. 3A and B). Furthermore a different modulation was observed: whereas the effect on *3β-HSD* was completely counteracted by SR (Fig. 3B) – thus indicating a CB1 dependent modulation – the effect on *cyp17* was not ($P < 0.01$) (Fig. 3A), suggesting a possible involvement of receptors other than CB1.

## 4. Discussion

Steroid signalling has a fundamental role to sustain the spermatogenesis in males. In this regard, the deleterious effects of both THC and eCBs on the neuroendocrine system were extensively studied in the past decades in both humans and animal models. In marijuana smokers, serum LH and testosterone levels are lower than in non-smoking controls; indeed, in animal models, direct intracerebroventricular administration of THC/AEA produces decreased plasma LH levels thought the downregulation of GnRH secreting neurons (Scorticati et al., 2004; Wenger et al., 1987). By reflex, a significant decrease in serum testosterone concentration has been observed in THC/AEA treated animals, with reduction of copulatory behaviour and of weight of testes and accessory reproductive organs (Battista et al., 2012; Murphy et al., 1998; Wang et al., 2006).

Functional crosstalk among eCBs and GnRH neuronal systems has been described in vertebrates and, at hypothalamic level, several molecular mechanisms have been formulated. Postsynaptic release of 2-AG or AEA is a phylogenetically conserved phenomenon, leading to the inhibition of presynaptic neurotransmitter release; in this respect eCBs directly – as retrograde signal – or indirectly – *via* the modulation of glial cells – might impair the release of well known modulators of GnRH secreting neurons, i.e. γ-aminobutyric acid (GABA) (Farkas et al., 2010; Glanowska and Moenter, 2011; Meccariello et al., 2014). An additional AEA-dependent self modulation of GnRH secreting neurons activity has been suggested in amphibians, since (1) 20% of GnRH secreting neurons express CB1, (2) *in vitro* AEA inhibits the transcription of GnRHs and (3) buserelin – a long acting GnRH analogue – upregulates the transcription of *CB1* (Chianese et al., 2011b; Meccariello et al., 2008). Besides the molecular mechanisms, the key role of CB1 in the negative modulation of neuroendocrine axis has been demonstrated by the modifications in GnRH signalling in $CB1^{-/-}$ mice, explaining low serum LH concentration, low testicular testosterone secretion, low circulating testosterone and E2 levels, high pituitary GnRH-R and low Follicular stimulating hormone beta subunit (FSHβ) mRNA levels, low testicular FSH receptor and P450 aromatase mRNA levels (Cacciola et al., 2013c).

An intriguing matter of debate is the role exerted by well known neurohormones in extrabrain areas. Data obtained from mollusks to mammals (Chianese et al., 2011a; Pierantoni et al., 2002a, 2009b; Sharpe, 1986; Treen et al., 2012) provide evidences that testicular GnRH is involved in processes such as the commu-

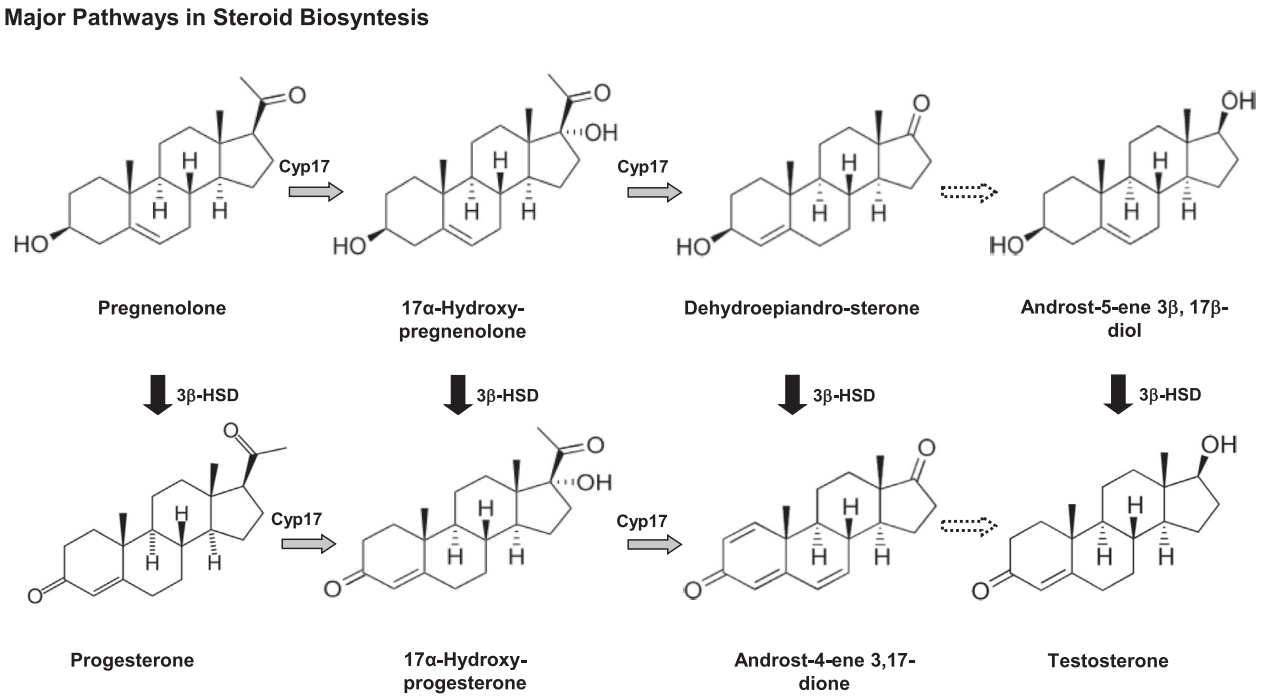


**Fig. 1.** Involvement of cyp17 and 3β-HSD in testosterone biosynthesis. Black and grey arrows indicate the reactions catalysed by 3β-HSD and cyp17, respectively.

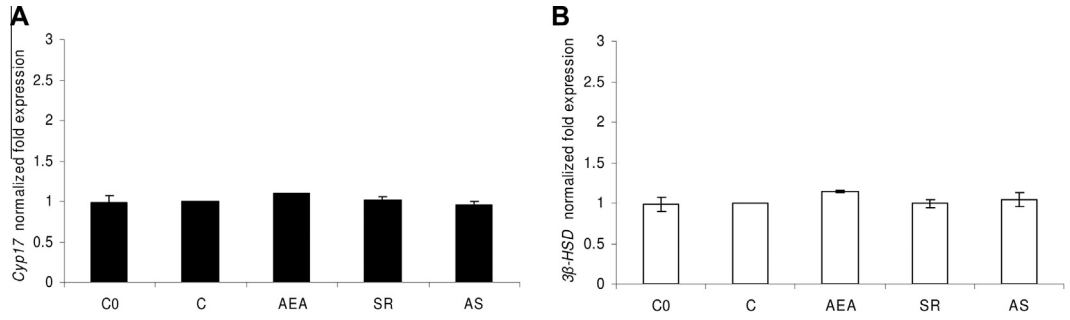


**Fig. 2.** Effects of AEA treatment on *cyp17* (A) and *3β-HSD* (B) expression after 1 h *in vitro* incubations of testes collected from February frogs, evaluated by qPCR C0, fresh control; C, control group, testis incubated with KRB; AEA, testis incubated with $10^{-9}$ M AEA; SR, testis incubated with $10^{-8}$ M SR; AS, testis incubated with both $10^{-8}$ M SR and $10^{-9}$ M AEA. Data are reported as mean fold change ± SD over the value one assigned to the KRB incubated control (C) and are representative of three separate experiments at least ($N = 6$).

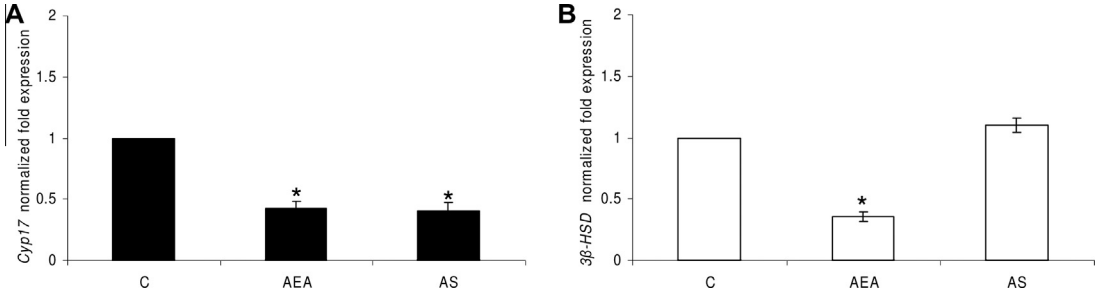


**Fig. 3.** Effects of AEA treatment on *cyp17* (A) and *3β-HSD* (B) expression after 2 h from the injection in the dorsal sac of frogs collected in February, evaluated by qPCR. C, control group, testis collected from frogs injected with KRB alone; AEA, testis collected from frogs injected with $10^{-8}$ M AEA; AS, testis collected from frogs injected with both $10^{-7}$ M SR and $10^{-8}$ M AEA. Data are reported as mean fold change ± SD over the value one assigned to the KRB incubated control (C) and are representative of three separate experiments at least ($N = 6$). * indicates statistically significant differences among samples ($P < 0.01$).

nication between the somatic component of the testis (Sertoli-Leydig cells), the estradiol dependent proliferation of primary spermatogonia, sperm release and, in general, sperm functions related to the fertilization. In humans, in which two GnRH molecular forms and two GnRH-Rs have been detected (van Biljon et al., 2002; White et al., 1998), GnRH1 and 2 are suggested to be local modulators of steroidogenetic activity (Lin et al., 2008a). To support this *in vitro* observation, intratesticular levels of testosterone and the

transcription levels of *GnRH1*, *GnRH2*, *GnRH-R*, *cytochrome P450 side-chain cleavage* (*CYP11A1*), and *3β-HSD type 2* enzyme are changed in patients with spermatogenesis failure (Lin et al., 2008b). In such a context, the recently emerged interplay between eCBs and GnRH in frog testis might be considered as a potential modulator of testicular steroidogenetic ability. Leydig cells were the first target of eCBs activity to be discovered in mammals (Wenger et al., 2001) and CB1$^{-/-}$ mice just display a reduction of Leydig cell numbers (Cacciola et al., 2008). In amphibian testis, whose Leydig cell express *CB1* and have the ability to synthesize eCBs (Chianese et al., 2012, 2013), testosterone is produced all over the annual sexual cycle (Cobellis et al., 1997; D'Istria et al., 1974; Fasano et al., 1989; Polzonetti-Magni et al., 1998) with peak observed in both mating period and in autumn to sustain the release of sperm cells and spermiogenesis events, respectively. In such a context, AEA modulates the expression levels of testicular *GnRH1*, *GnRH2* and of *GnRH-R1*, *-R2*, *-R3* in a stage-dependent manner (Chianese et al., 2012). Thus, using the same samples previously used to assess the AEA-depend modulation of testicular GnRH system, here we evaluated the possible changes in the expression rate of *3β-HSD* and *cyp17*, two key enzymes in testosterone biosynthesis. Such experiments were carried out at end of the winter stasis, when tubules simply contain spermatogonia ready to start a new spermatogenetic cycle and spermatozoa deeply attached to Sertoli cells that will be released during the mating. *In vitro* incubations of testes with AEA during the pre-reproductive period (February) did not gain any changes in *3β-HSD* and *cyp17* expression whereas the upregulation of *GnRH2*/*GnRH-R3* mRNA and downregulation of *GnRH-R2* was previously reported in the same samples (Chianese et al., 2012). Due to *in vitro* results, animals were treated *in vivo* with AEA alone or in combination with Rimonabant, a well known specific antagonist of CB1 (Rinaldi-Carmona et al., 1994). Following *in vivo* treatment, downregulation of both *3β-HSD* and *cyp17* was observed. Interestingly, the preteatment with Rimonabant was able to completely inhibit the downregulation of *3β-HSD*, but not that of *cyp17* indicating the involvement of receptors other than CB1 in the latter phenomena. In this respect, the possible role of TRPV1 signaling should be considered in the next future, since *in vitro* incubations of testes with CAP has recently been reported to modulate GnRH system – both ligands and receptors – but in an opposite way compared to that of AEA (Chianese et al., 2013). Thus, it is not excluded that AEA, acting as an intracellular signal *via* TRPV1, might be centrally or locally involved in the transcriptional modulation of *cyp17*.

In conclusion, AEA *via* CB1 does not directly affect the expression rate of *3β-HSD* and *cyp17*, but the modulation of hypothalamic GnRH activity and gonadotropin discharge is an obligatory step in the regulation of steroidogenesis in frog testis.

## Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

## Data deposition

The sequences of *R. esculenta 3β-HSD* and *cyp17* were deposited in GenBank under the accession numbers KF900149 and KF900150, respectively.

## Acknowledgment

This work was supported by PRIN 2010/2011 to Rosaria Meccariello.

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