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

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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 Δ^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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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.

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