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Regulation of male fertility by the endocannabinoid system

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Abstract

Mammalian conception is a complex process regulated by both sexual behavior and reproductive performance. Alcohol, marijuana and tobacco are among the main factors which affect negatively fertility in women and men. Several studies have demonstrated that marijuana impairs the male copulatory activity, and that smokers of this illegal drug show reduced fertility due, for instance, to decrease in sperm concentration, defective sperm function or alteration of sperm morphology.

The discovery of endocannabinoids and all components responsible for their metabolism has allowed to collect valuable information on the effects of these endogenous lipids, able to mimic the actions of delta-9-tetrahydrocannabinol (THC), in reproductive functions.

The purpose of this review is to describe the actions of cannabinoids and endocannabinoids on the control of procreation and hormonal release during the fertilization process in males.

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Keywords: Endocannabinoids, Fertility, Hormones, Sperm cells, Testis.

The endocannabinoid system

The best characterized endocannabinoids are *N*-arachidonylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). Both are endogenous ligands of cannabinoid (CB) receptors, thus mimicking several actions of the natural *Cannabis sativa* component delta-9-tetrahydrocannabinol (THC), which accounts for the majority of the reproductive hazards in marijuana users (Piomelli, 2004). Two are the cannabinoid receptors that respond to THC and that have been identified and cloned. These receptors, indicated as CB1 and CB2, belong to the superfamily of G-protein coupled receptors (Matsuda et al., 1990; Munro et al., 1993). CB1 has been found mainly in the central nervous system, but it is also present in ovary, uterine endometrium, testis, vas deferens, urinary bladder, and other peripheral endocrine and neurological tissues (McPartland, 1999; McPartland and Pruitt, 1999). CB2 receptors have been identified mainly in immune cells (Munro et al., 1993) but are expressed also in brainstem (Van Sickle et al., 2005).

Biochemical studies have revealed that AEA is produced by a transacylase-phosphodiesterase-mediated synthesis, starting from the precursor *N*-arachidonoylphosphatidylethanolamine (NArPE) through the action of *N*-archidonoyl-phosphatidylethanolamine (NAPE) hydrolysis catalysed by a specific phospholipase D (NAPE-PLD) (Okamoto et al., 2004).

2-AG acts as a potent and full agonist for both CB1 and CB2 and, like AEA, it is not stored in intracellular compartments, but is produced on demand and is released from neuronal membranes through several pathways, including phospholipase C-dependent and independent routes (Di Marzo et al., 1999).

The biosynthetic pathway of 2-AG provides for rapid hydrolysis of inositol phospholipids by a specific phospholipase C (PLC); this enzyme generates diacylglycerol (DAG), which is subsequently converted to 2-AG by a *sn*-1-DAG lipase (Stella et al., 1997; Bisogno et al., 2003).

The pharmacological effects of AEA and 2-AG depend on their life span in the extracellular space, which is limited by a rapid transport through the membrane. Both compounds have been proposed to be taken up by cells through a specific carrier, the EMT (Endocannabinoid Membrane Transporter) (Beltramo et al., 1997, 2000), but there is as yet controversy regarding the mechanism by which AEA is transported (Mechoulam and Deutsch, 2005). Once inside the cells, endocannabinoids can be metabolized by multiple pathways; AEA is a substrate for FAAH, that breaks the amide bond and releases arachidonic acid (AA) and ethanolamine (Mc Kinney and Cravatt, 2005), whereas 2-AG is degraded to AA and glycerol mainly by a specific monoacylglycerol lipase (MAGL).

MAGL has been isolated from the porcine brain (Goparaju et al., 1999) and cloned and characterized in rat (Dinh et al., 2002) and human brain (Ho et al., 2002). Rat brain MAGL is a 33-kDa protein and, unlike FAAH, is localized in the cytosol (Dinh et al., 2002).

AEA and 2-AG differentially activate cannabinoid receptors. Both CB1 and CB2 belong to the rhodopsin family of G protein-coupled seven trans-membrane spanning receptors.

Signal transduction pathways regulated by CB-coupled G_i/o proteins include inhibition of adenylyl cyclase (Paria et al., 1995; Matsuda et al., 1990), regulation of ionic currents (Wang et al., 2003), activation of focal adhesion kinase (Derkinderen et al., 2001) and of MAPK (Wang et al., 2003).

AEA is also considered a true “endovanilloid” (De Petrocellis et al., 2001), in fact several studies have suggested a physiological role for AEA as a transient receptor potential vanilloid 1 (TRPV1) agonist; in contrast, 2-AG is unable to bind to and activate TRPV1 (Szallasi and Blumberg, 1999). Taken together, AEA and 2-AG, their congeners and metabolic enzymes, their purported transporters and molecular targets form the “endocannabinoid system (ECS)”.

The ECS in male tissues: sperm cells

The failure to achieve a pregnancy is a common problem, affecting more than 15% couples attempting their first pregnancy; among the couples experiencing difficulty with conception, male infertility is the primary cause in ~ 40% of cases. Anatomical and genetic defects, testicular injury and disease, sperm disorders, hormonal dysfunctions are the main causes of fertility problems.

AEA has been identified in human reproductive fluids, but also in invertebrates and mammalian spermatozoa. First findings on the effect of AEA on fertilizing capacity were reported in 1992, using sea urchin sperm as a useful model system to study the mating mechanisms (Schuel et al., 1994). The steps to insure a species-specific fertilization in sea urchin include chemotaxis, sperm activation and sperm/egg adhesion. Previously, Schuel’s group had reported the ability of THC to block the acrosome reaction, normally stimulated by a ligand present in the jelly coat of the egg, and they had shown that THC was unable to affect the physiological functions of sperm (Schuel et al., 1987). Then, they demonstrated that AEA treatment mimicked in sea urchin sperm the results obtained with

THC, and that the effects on fertilization were fully reversible, much alike those of THC. Interestingly, AEA and AA exhibited an opposite effect on egg and sperm; in fact, while AEA did not affect the receptivity of pretreated eggs towards sperm, exogenous AA reduced the fertility of eggs and it did not influence the acrosome reaction. Most likely, AEA and THC block the egg-jelly-induced acrosome reaction by affecting an event in the stimulus-secretion-mating mechanism of the sperm prior to the opening of ion channels. In fact, treatment with ionomycin and nigerin, which opens Ca^{2+} and K^{+} respectively, induced artificially the acrosome reaction in sperm pretreated with AEA, leading to the general idea that the cAMP signaling cascade could be affected by endocannabinoids in order to promote the acrosome reaction.

Recently, it has been reported that CP55.940, a CB receptor ligand, is able to bind with high affinity to membrane fractions of human sperm and the analysis of the binding constants (B_{max} and K_d) suggested that the receptor expressed in spermatozoa is indeed a true CB1; the same receptor type is also present in invertebrate and mammalian sperm cells (Schuel et al., 2002a). The identification of cannabinoid receptors prompted the researchers to investigate also the presence of the endogenous ligand in fluids of the human reproductive tracts and, at the same time, to examine the role of CB1 in regulating capacitation and fertilizing potential of human sperm (Schuel et al., 2002b). AEA was detected in seminal plasma, but also in oviductal and follicular fluid, suggesting a major role of this lipid during the journey of sperm to the site of fertilization. A recent paper by Cobellis and colleagues has shown the ECS in the testis of the frog (*Rana esculenta*), demonstrating its expression in the tubular compartment (Cobellis et al., 2006). Interestingly, they examined the existence of both FAAH and CB1 proteins during the

annual reproductive cycle, showing that they are expressed significantly in September, when the spermatids appear and are weakly expressed during the maturation process from spermatogonia to the last stage (Cobellis et al., 2006). Indirect evidence showed that endocannabinoids might affect also the motility of frog spermatozoa; in fact, it has been underlined that AEA treatment reduces, in a CB1-mediated dose-dependent manner, the number of motile spermatozoa during the journey from the cloacal fluid to the aquatic environment (Cobellis et al., 2006), without affecting the death of cell, but inhibiting exclusively their motility. More recently, using CB knock-out mice, it has been demonstrated that CB1 receptors in epididymus play a central role during the acquisition of motility, preventing that sperm cells might get this ability too early (Ricci et al., 2007). The physiological state of spermatozoa, assessed by motility, acrosomal integrity and binding to zona pellucida, is important to predict if these cells can undergo fertilization. Studies *in vitro* showed that hyperactivated motility is modulated by methanandamide (Met-AEA), the stable analogue of AEA, and that, depending on the concentration, it exhibits a stimulatory or inhibitory effect on motile behavior of human sperm. Yet, acrosomal cap modification is important for enabling the sperm to fertilization and it must be accomplished to allow the spermatozoa to bind to the appropriate egg. This physiological change might be completely blocked by both Met-AEA and THC, and the effects depend on the capacitation time and on the subpopulation of sperm analyzed (Schuel et al., 2002a). Finally, the tight binding of capacitated spermatozoa to hemizona is a predicting factor about the sperm ability to fertilize the egg, and it has been reported that treatment with Met-AEA reduces this sperm ability down to 50% of control, confirming the significant role of AEA-signaling in regulating human sperm functions.

Lately, the signaling pathway activated by AEA in order to inhibit the capacitation-induced acrosome reaction has been clarified by using specific antagonists of CB1, it has been demonstrated that this receptor has a direct involvement in this process (Rossato et al., 2005). Immunoblotting analysis and RT-PCR technique confirmed the expression of CB1 receptor protein in human sperm, and immunofluorescent data showed its localization mainly in the sperm head and middle piece, whereas no fluorescent signals were detectable in the tail. In addition, it has been shown that AEA reduces, in a dose-dependent manner, human sperm motility by reducing mitochondrial activity and, at higher concentrations, by decreasing sperm viability (Rossato et al., 2005).

Recently, we characterized the endocannabinoid system in sperm cells of boar (*Sus scropha*), an animal model system used in reproductive physiology, because of its similarity to humans (Maccarrone et al., 2005). Notably, we found that these cells possess all biochemical tools to synthesize, degrade and bind AEA and that the whole system is down-regulated in capacitated sperm. Figure 1 summarizes the elements of ECS expressed by mammalian sperm cells. In particular, FAAH activity was reduced to ~ 10% of control, whereas NAPE-PLD activity was reduced to ~ 60%. In agreement with these results, endogenous levels of AEA in capacitated spermatozoa were increased up to ~ 200% of resting sperm cells. Moreover, we confirmed that a CB1-dependent signaling pathway was triggered during capacitation also in a time-dependent manner. In fact, the inhibitory effect of Met-AEA on this event, needed to render spermatozoa competent to fertilize the oocyte, was completely removed by SR141716, as assessed by chlortetracycline staining assays (Maccarrone et al., 2005). It should be recalled that cAMP is an important regulator of sperm capacitation, and it is known that, among the

several biological activities of endocannabinoids, the binding of AEA to CB1 receptor triggers also cAMP signaling pathway (Howlett et al., 2002). Noteworthy, we found that treatment of spermatozoa with 1 μ M Met-AEA reduced significantly this intracellular second messenger and that the inhibitory effect was blocked by addition of SR141617. CB receptors are involved in the control of the acrosomal reaction which occurs when spermatozoon encounters the zona pellucida proteins of the egg, allowing the fusion between sperm and egg. Instead, TRPV1 seems to regulate the “spontaneous AR”, that might cause “out-of-place” activations of sperm cells. Overall, there must be a tight control that prevents “multiple attacks” of the egg or unproductive acrosome reaction. Endocannabinoids, by acting either at CB1R or at TRPV1, play a key-role in spatiotemporal control of these events. Figure 2 schematically represents the role of ECS in regulating sperm physiology.

The ECS in male tissues: testis

Spermatogenesis is the process by which male spermatogonia develop into mature spermatozoa, that are the male gametes in many sexually reproducing organisms. Spermatogenesis is highly dependent on several conditions, that need to reach an optimum for the process to occur correctly. The starting point of this process is in the seminiferous tubules of testes, where the spermatogonial stem cells, through several meiotic and mitotic divisions, develop into spermatids, during the phase of spermiogenesis. When mammalian sperm are released from seminiferous epithelium, they are still immature and incapable of fertilizing an egg. Sperm maturation occurs in

the epididymis, where spermatozoa acquire motility and undergo substantial changes in function, composition and organization. At all stages of differentiation, the spermatogenic cells are in close contact with Sertoli cells, which are thought to provide structural and metabolic support to the developing sperm cells.

Our group reported that Sertoli cells, obtained from 4 to 24 day-old mice, possess the biochemical machinery to synthesize, transport, degrade and bind both AEA and 2-AG. In particular, Sertoli cells express on their surface functional CB2 receptors, whose levels remain constant for at least 16 days (Maccarrone et al., 2003). On the other hand, FAAH activity and the uptake of AEA through EMT were found to decrease in an age-dependent manner, due to down-regulation of gene expression. Moreover, as previously reported for other human peripheral cells (Maccarrone et al., 2000), the transport of AEA across the plasma membrane was increased by nitric oxide (NO) donors. Since NO has a potential role in both normal spermatogenesis and inflammation-mediated male infertility, its effect on the up-regulation of EMT in Sertoli cells deserves to be further investigated *in vivo*.

Accumulating evidence points out the high preservation of some elements of ECS, such as FAAH and CB receptors, in phylogenetically distant species. Orthologues of CB receptors and synthesizing and degrading enzymes of endocannabinoids were identified in sea squirt *Ciona intestinalis* (Matias et al., 2005). Afterwards, the ability of rat testis to synthesize endocannabinoids (Sugiura et al., 1996) and the expression of CB1 in mouse testis (Gye et al., 2005) have been also demonstrated.

Collected together, these data draw attention to the ECS as a new target in regulating male fertility and present new perspectives to the understanding and treatment of male fertility problems.

ECS, sex hormones and male fertility

Follicle-stimulating hormone (FSH) and luteinising hormone (LH) are glycoproteins secreted by the anterior pituitary, that act directly on the testis to stimulate somatic cell function in support of spermatogenesis. In males, FSH receptor expression is limited to the testicular Sertoli cells (Rannikki et al., 1995), while LH receptors are found primarily in the Leydig cells, although receptor staining is also observed in spermatogenic cells (Eblen et al., 2001; Lei et al., 2001). In particular, the primary role of FSH in spermatogenesis is stimulation of Sertoli cell proliferation during prepubertal development (Heckert and Griswold, 2002), whereas LH acts on regulation of testosterone (T) synthesis within the adult testis. Humans who lack functional FSH receptors develop smaller testis than do normal males, in agreement with the reduction in Sertoli cell number observed in the testis of mice lacking this receptor (Dierich et al., 1998). These men also exhibit disturbed spermatogenesis (oligozoospermia and teratozoospermia), although they remain fertile (Tapanainen et al., 1997). T and its metabolites, dihydrotestosterone and estradiol, are representative of sex hormones. T plays a significant role in the maintenance of male fertility acting on morphological development and reproductive function. Spermatogenesis is dependent on the presence of an adequate intratesticular level of T (Russell and Clermont, 1977), so that it needs

androgen receptors, expressed in Sertoli cells, for a proper sexual differentiation (Lindzey et al., 1994). More recently, Hall and colleagues reported the adverse action of THC on the release of T and on the production, motility and viability of sperm (Hall, 1998). A significant decrease in serum T concentration has been found in *Rhesus* monkeys, after THC administration. Acute and chronic doses of THC cause significant depression of T formation by rat testis microsomes (List et al., 1977), and decrease testicular weight (Harclerode et al., 1978). In rodents, high THC doses cause a modest increase in abnormally formed sperm. Moreover, long-term cannabinoid exposure in male mice disrupted spermatogenesis and induced aberrations in sperm morphology (Zimmerman et al., 1978).

Previous studies have demonstrated the acute effects of THC on the secretion of LH and FSH at the hypothalamic-pituitary levels, with an inhibitory effect on gonadal function (Ayalon et al., 1977; Wenger et al., 1987). In fact, in males who smoke cannabis, serum LH levels are lower than in non-smoking controls (Kolodny et al., 1974) or pre-smoking baseline values (Cone et al., 1986). Indeed, direct intracerebroventricular administration of THC produces decreased plasma LH levels and increases hypothalamic levels of gonadotropin releasing hormone (GnRH) (Wenger et al., 1987), suggesting that decreased release of GnRH into the pituitary portal vasculature is responsible for the suppressed levels of LH after THC exposure. These data were further confirmed by evaluating the inhibitory effect of AEA on the release of LH in both hypothalamus and pituitary gland (Wenger et al., 1999a; Wenger et al., 1999b).

Recently, it has been demonstrated that serum LH and T levels are significantly decreased in CB1R knockout (*cb1^{-/-}*) mice, indicating that CB1 activation is needed for

the effects of endocannabinoids on the regulation of reproductive functions. In fact, they found that AEA suppressed LH and T secretion in wildtype (*cb1^{+/+}*) mice, but it had no effect in receptor-inactivated animals (Wenger et al., 2001). The expression of CB1 in the anterior pituitary gland is subjected to sex steroid-dependent influence. CB1 receptor mRNA levels exhibited differences between males and females, and it was demonstrated that males are more sensitive than females to cannabinoid-induced changes in pituitary hormone secretion (Bonnin et al., 1993; Bonnin et al., 1994). Gonzalez reported higher amounts of CB1 receptor mRNA transcripts in the anterior pituitary gland of males respect to those observed in females, because of fluctuations in the ovarian cycle (Gonzalez et al., 1999). In males, castration reduced CB1 receptor mRNA levels in the anterior lobe of the pituitary gland, suggesting a possible stimulatory effect of androgens in the expression of this gene. Replacement with dihydrotestosterone did not recover the levels found in intact males, thus suggesting that CB1 receptor gene might be under the stimulatory effect of androgens only after its aromatization to estradiol.

On the other hand, in males chronic THC treatment caused a marked reduction of CB1 receptor-mRNA transcripts in intact and orchidectomized animals (Gonzalez et al., 1999). The effect was reversed by replacement of dihydrotestosterone in orchidectomized animals, suggesting the involvement of androgens. AEA binding to postsynaptic CB receptors is known to modulate the release of mediators; for instance AEA may increase the release of GABA, a neurotransmitter involved in the inhibition of luteinising hormone releasing hormone (LHRH) (Fernandez-Solari et al., 2004). Therefore, a stimulation of GABA release could be involved in the inhibition of basal LHRH release by AEA in males (Scorticati et al., 2004). These data are in keeping with previous reports on the

switch from inhibitory to stimulatory of the effect of AEA on LH release following the acute administration of estrogen to ovariectomized female rats (Scorticati et al., 2004).

The prenatal exposure of males to estrogens affects their later fertility (Stillman, 1982), but the recent discovery that testicular cells express estrogen receptors indicates a physiological relevance of the estrogenic action on the testis (Carreau, 2002; Aschim et al., 2004). Immature Sertoli cells may themselves produce estrogens via their aromatase (ARO) activity (Rommerts et al., 1982), whereas in adulthood Leydig and germ cells become sources of testicular estrogen (Carreau et al., 1999). Also FSH can contribute to estrogen production, by enhancing the *aro* promoter (McDonald et al., 2006). FSH binding to its receptor is known to activate different signalling pathways (Walker and Cheng, 2005). The activation of cAMP and, subsequently, the activation of protein kinase A (PKA) are responsible for the stimulation of Sertoli cell proliferation. FSH also causes Ca^{2+} influx into Sertoli cells that is mediated by cAMP and perhaps PKA modification of surface Ca^{2+} channels. In addition, the initiation of phosphoinositol-3-kinase signalling by FSH stimulates the ARO expression, acting at the transcriptional level. T and other androgens can undergo peripheral conversion to estrogen and its derivatives through the action of ARO; for instance, the oral treatment with testolactone and anastrozole, inhibitors of ARO, prevents the conversion of T to estradiol and, thus, blocks the inhibitory effects of estrogen on spermatogenesis (Kumar et al., 2006).

Recently, both PKA- and ARO-mediated signalling pathways have been shown to be involved in the modulation of FAAH by FSH. In fact, FSH inhibits dose-dependently apoptosis induced by AEA, by enhancing only FAAH activity and expression (Rossi et al., 2007). In particular, using specific enzyme inhibitors, it was demonstrated that PKA-

dependent pathway acts directly on FAAH activity, by phosphorylating transcription factors and accessory proteins which, in turn, activate the enzyme. On the other hand, the ARO-dependent pathway increases FAAH expression at the transcription and translation levels (Rossi et al., 2007). Figure 3 depicts these effects of FSH on Sertoli cells.

Taken together, these data emphasize a main role for CB receptor and FAAH in the interaction between ECS and sex hormones during male reproduction, and underline their value as promising target to treat infertility problems (Table 1).

Closing remarks

In conclusion, this review has presented the ECS in males, and its link with sex hormones in controlling fertility in invertebrates, mammals and humans. The detection of endocannabinoids, the presence of specific enzymes for their synthesis and degradation, and the identification of CB receptors in sperm cells and male tissues suggest a critical role for endocannabinoids in modulating sperm functions during fertilisation, and open novel perspectives in their therapeutic exploitation for the treatment of male infertility.

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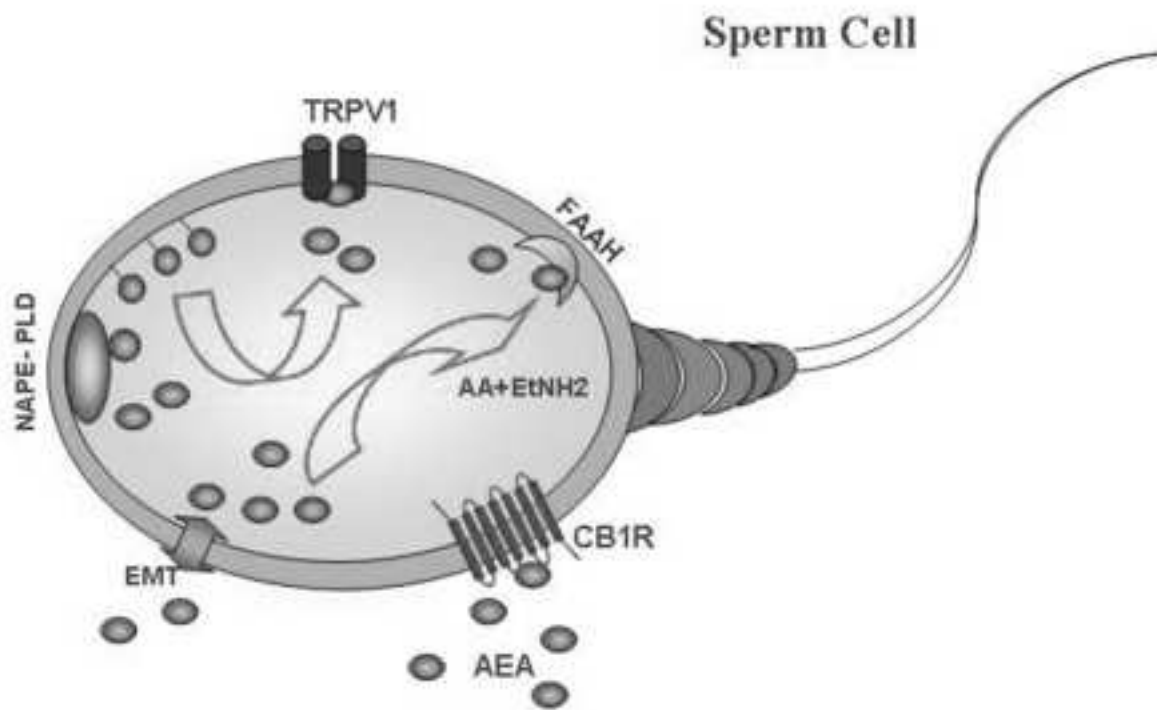
Figure legends

Figure 1. The endocannabinoid system in sperm cell. The synthesis of AEA from membrane is catalyzed by the activity of specific phospholipase D (NAPE-PLD), which releases AEA and phosphatidic acid. AEA is transported in both directions through the cell membrane by a purported carrier (EMT) and, once taken up, is hydrolyzed by fatty acid amide hydrolase (FAAH) to ethanolamine (EtNH₂) and arachidonic acid (AA). The main molecular targets of AEA are cannabinoid receptors (CB), showing an extracellular binding site, and type-1 vanilloid receptors (TRPV1), showing an intracellular binding site.

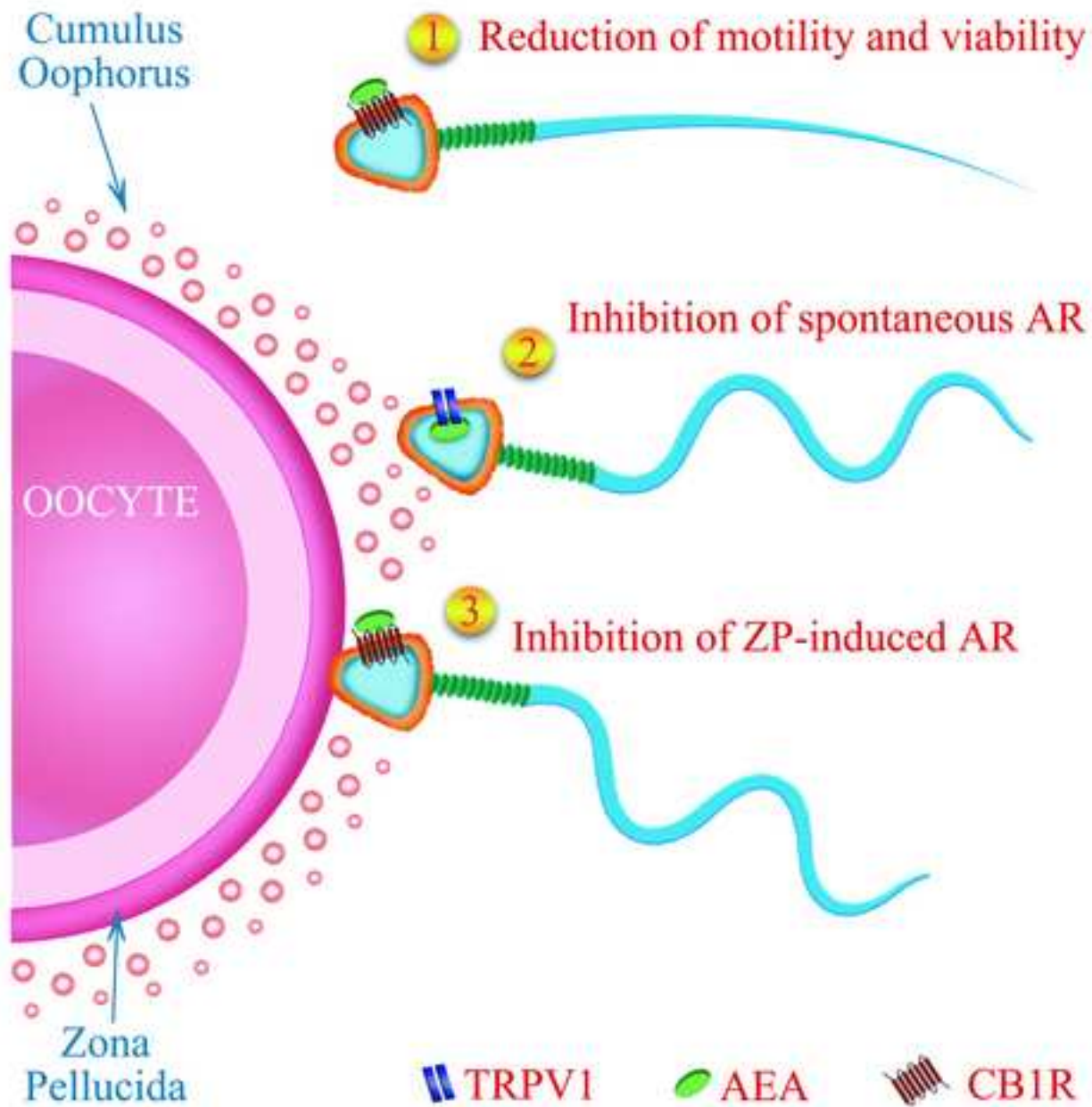
Figure 2. The involvement of cannabinoid and vanilloid receptors in mammalian fertilization. 1) AEA binding to CB1 receptor blocks sperm motility and viability during the capacitation process. 2) Binding of AEA to the intracellular site of TRPV1 inhibits spontaneous acrosome reaction (AR). 3) During AR, the activation of CB1 receptors by AEA reduces binding of sperm cells to zona pellucida (ZP) proteins.

Figure 3. Regulation of FAAH by FSH. In Sertoli cells, the scheme shows that follicle-stimulating hormone (FSH) activates the AEA-degrading enzyme FAAH, through protein kinase A (PKA) and aromatase (ARO)-dependent pathways, triggered by FSH receptor (FSH-R). The final outcome is a reduction of endocannabinoid-induced apoptosis. ERE, estrogen response element.

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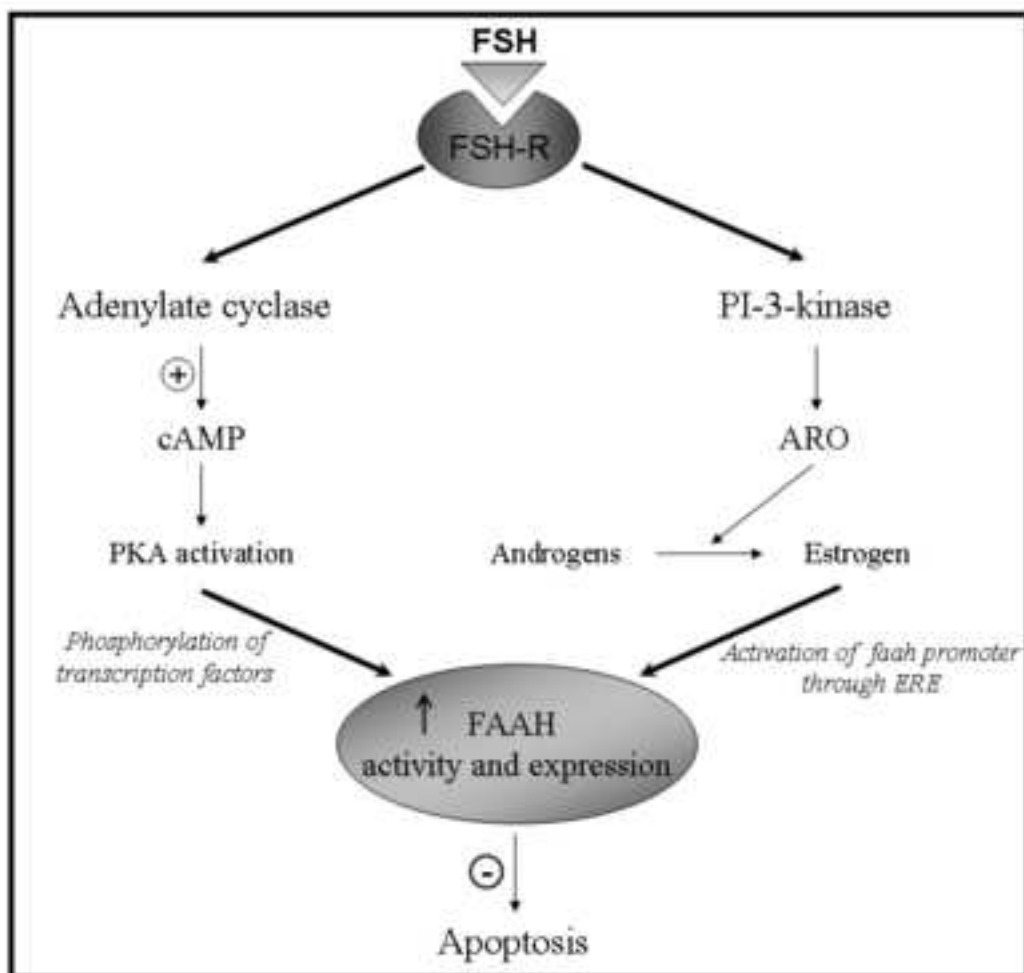


Table 1. Main effects of the interaction between ECS and male hormones.

Male Hormones	Cells	Spermatogenesis function	ECS action	ECS effect
LH	Leydig cells	Regulation of testosterone synthesis	↓ (CB1 mediated)	Inhibition of gonadal functions
FSH	Sertoli cells	Cell proliferation	↓ (FAAH mediated)	Smaller testis Oligozoospermia Teratozoospermia
Testosterone	Sertoli cells	Maintenance of fertility	↓ (CB1 mediated)	Decrease of testicular weight Abnormalities in spermatozoa

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