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Review

The role of endocannabinoids in gonadal function and fertility along the evolutionary axis

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ABSTRACT

Endocannabinoids are natural lipids able to bind to cannabinoid and vanilloid receptors. Their biological actions at the central and peripheral level are under the tight control of the proteins responsible for their synthesis, transport and degradation. In the last few years, several reports have pointed out these lipid mediators as critical signals, together with sex hormones and cytokines, in various aspects of animal and human reproduction. The identification of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) in reproductive cells and tissues of invertebrates, vertebrates and mammals highlights the key role played by these endogenous compounds along the evolutionary axis. Here, we review the main actions of endocannabinoids on female and male reproductive events, and discuss the interplay between them, steroid hormones and cytokines in regulating fertility. In addition, we discuss the involvement of endocannabinoid signalling in ensuring a correct chromatin remodeling, and hence a good DNA quality, in sperm cells.

Contents

Abbreviations: 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; ACTH, corticotrophin; AEA, *N*-arachidonoylethanolamine; ArKO, *aromatase*-nullmice; cAMP, cyclic adenosine monophosphate; CB1, type-1 cannabinoid receptor; CB2, type-2 cannabinoid receptor; CB3, type-3 cannabinoid receptor; CNS, central nervous system; COX, cyclooxygenase; CRE, cAMP responce element; CREM, cAMP responce element modulator; CREτ, cAMP responce element *tau*; DAG, diacylglycerol; DAGL, diacylglycerol lipase; DNMT, DNA methyl-transferase; ECS, endocannabinoid system; eCBs, endocannabinoids; ELISA, enzyme-linked immunosorbent assay; EMT, endocannabinoid membrane transporter; ERE, estrogen responsive element; EtNH2, ethanolamine; FAAH, fatty acid amide hydrolase; FSH, follicle stimulating hormone; GABA, γ-aminobutyric acid; GH, growth hormone; GnIH, gonadotropin-releasing hormone inhibiting hormone; GnRH, gonadotropin-releasing hormone; GPR55, G protein receptor 55; HDAC, hystone deacetylase; ICM, inner cell mass; IL, interleukin; INF-γ, interferon-γ; IVF, *in vitro* fertilization; LH, luteinizining hormone; LIF, leukemia inhibitory factor; MAGL, monoacylglycerol lipase; MAPK, mitogen-activated protein kinase; MAR, matrix attachment region; NAAA, *N*-acylethanolamine-hydrolyzing acid amidase; NAPE-PLD, *N*-arachidonoyl-phosphatidylethanolamine phospholipase D; NArPE, *N*-arachidonoyl-phosphatidylethanolamine; NAT, *N*-acyltransferase; NK, natural killer; OEA, *N*-oleoylethanolamine; OVX, ovariectomized; PEA, *N*-palmitoylethanolamine; PKA, protein kinase A; PLC, phospholipase C; PLD, phospholipase D; PRL, prolactin; PRM, protamine; SNBP, sperm nuclear basic protein; SR141716A, *N*-(piperidino-1-yl)-5-(4-chlorphenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide hydrochloride; Th1/2, type-1/2 helper; TBP, tata-box protein; TGF-β, transforming growth factor-β; THC, delta-9-tetrahydrocannabinol; TNF-α, tumor necrosis factor-α; TNP, transition protein; TRPV1, transient receptor potential vanilloid-1.

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1. The endocannabinoid system (ECS) and reproduction: an overview

Growing evidence has been accumulating to show the central role of the endocannabinoid system (ECS) in controlling reproductive functions in mammals and humans, demonstrating that the precise and orchestrated steps, which follow one another to guarantee a successful pregnancy, are under the supervision of this endogenous system (Bari et al., 2010; Karasu et al., 2010; Maccarrone, 2009; Wang et al., 2006). The ECS, already present in primordial organisms, includes anandamide (AEA) and 2-arachidonoylglycerol (2-AG), their main molecular targets (cannabinoid receptors type-1 (CB_1) and type-2 (CB_2), vanilloid receptor (TRPV1), and the synthesizing- and hydrolyzing-enzymes, as well as a putative transporter, involved in their metabolism. Endocannabinoids (eCBs) are released from membrane phospholipid precursors through the activation of specific phospholipases (Ahn et al., 2008), that are in turn activated "on demand". Although AEA synthesis may occur via multiple biosynthetic pathways (Leung et al., 2006; Liu et al., 2006; Simon and Cravatt, 2006, 2008, 2010; Ueda et al., 2010b), the most prominent route starts from a transacylation by a Ca^{2+}-dependent *N*-acyltransferase (NAT) to produce *N*-arachidonoyl-phosphatidylethanolamine (NArPE) (Jin et al., 2007), which is subsequently converted into AEA through the activity of a specific phospholipase D (NAPE-PLD) (Okamoto et al., 2004). Similarly, the formation of 2-AG involves a rapid hydrolysis of inositol phospholipids by a specific phospholipase C (PLC) to generate diacylglycerol (DAG), which is then converted into 2-AG by an *sn*-1-DAG lipase (DAGL) (Bisogno et al., 2003). After re-uptake through a purported specific transporter, called endocannabinoid membrane transporter (EMT) (Kaczocha et al., 2009), eCBs signalling is terminated by hydrolysis due to two fatty acid amide hydrolases (FAAH and FAAH-2) (Cravatt et al., 1996; Wei et al., 2006) and to *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) (Tsuboi et al., 2005; Ueda et al., 2010a). These hydrolases break the amide bond of AEA to release arachidonic acid (AA) and ethanolamine ($EtNH_2$). 2-AG, on the other hand, is cleaved into AA and glycerol by a specific monoacylglycerol lipase (MAGL) and by FAAH (Dinh et al., 2002; Ho et al., 2002). All ECS elements are schematically depicted in Fig. 1. Nowadays, these elements have been identified in the central and peripheral nervous system and with a resultant anatomical map of their distribution, which has enabled a better understanding of the physiological functions of the ECS in mammalian reproduction. In this context, AEA has been found in female reproductive fluids (Schuel et al., 2002) and in plasma, where high levels of AEA are required at ovulation (El-Talatini et al., 2009b), whereas low levels are necessary to achieve successful pregnancy (Fonseca et al., 2010b; Habayeb et al., 2004; Taylor et al., 2010). In addition, AEA, its metabolic enzymes and receptors have been demonstrated in human endometrium (Horne et al., 2008), ovary (El-Talatini et al., 2009a), and during the stages of embryo development (Paria and Dey, 2000), implicating AEA as a fertility signal in folliculogenesis, preovulatory follicle maturation, oocyte maturity and ovulation (El-Talatini et al., 2009a). Analogously, ECS has also been identified in seminal plasma (Schuel et al., 2002), male reproductive tissues (Gye et al., 2005), Leydig and Sertoli cells (Cacciola et al., 2008a; Maccarrone et al., 2003c; Rossi et al., 2007) and in male germ cells (Cacciola et al., 2008b; Maccarrone et al., 2003c, 2005; Ricci et al., 2007; Rossato, 2008; Rossi et al., 2007; Schuel et al., 1991) from spermatogonia to mature spermatozoa (Grimaldi et al., 2009), both in mammalian and non-mammalian organisms (Cobellis et al., 2006; Cottone et al., 2008; Schuel et al., 1991). The role of 2-AG is still under investigation, even though it has been recently measured in mesometrial decidua, suggesting the existence of a regulatory tone for 2-AG in uterine remodelling (Fonseca et al., 2010a). With regards to male fertility it has been reported that mouse epididymal spermatozoa, in their transit from *caput to cauda*, are in close contact with decreasing concentrations of 2-AG (Cobellis et al., 2010). Some women smoking marijuana have fertility problems, higher risks of embryotoxicity or reduced birth rates; the consumption of *Cannabis* in men decreases the quality of sperm and negatively affects their reproductive health (Bari et al., 2011).

1.1. ECS and mammalian fertility

Consistently, available data shows that the aberrant ECS impairs pregnancy, embryo development, sperm motility and that, under physiological conditions, eCBs signalling is involved in various female and male reproductive events (from oogenesis to oviductal transport, implantation, pregnancy and labour, sperm/oocyte interaction and the capacitation process) suggesting a crucial role for eCBs in the regulation of early and late phases of fertility (Bari et al., 2011). Data reported in animal and human studies show that tightly regulated endogenous AEA content is associated with a successful implantation, which is the result of an intimate "cross-talk" between the active blastocyst and the receptive uterus (Maccarrone, 2009). In fact, low AEA levels have been detected during the implantation window in women with successful pregnancy after *in vitro* fertilization (IVF) treatment (El-Talatini et al., 2009b) and in women with normal menstrual cycles (Habayeb et al., 2004), as well as in the mouse receptive uterus on day 4 of pseudopregnancy as compared to its levels in the non-receptive uterus on days 5 and 6 of pseudopregnancy (Schmid et al., 1997). Data obtained from women, who underwent spontaneous miscarriage in the first trimester of gestation, have shown a decreased AEA hydrolase activity and expression in peripheral lymphocytes (Maccarrone et al., 2000b), and low expression levels of NAPE-PLD and FAAH in human placenta (Trabucco et al., 2009). This accumulated evidence therefore supports the concept of detrimental effects caused by high AEA levels for a normal pregnancy outcome. In addition, low AEA content is required during trophoblast growth, differentiation and invasion (Fonseca et al., 2009; Sun et al., 2010), and during the spontaneous onset of labour in opposition to elevated plasma AEA levels in women with incipient preterm labour (Nallendran et al., 2008, 2010). Although the functional role of AEA during the latest stages of parturition is still undefined (Nallendran et al., 2010), it has been speculated that maternal AEA up-regulates CB_1 expression in placenta of

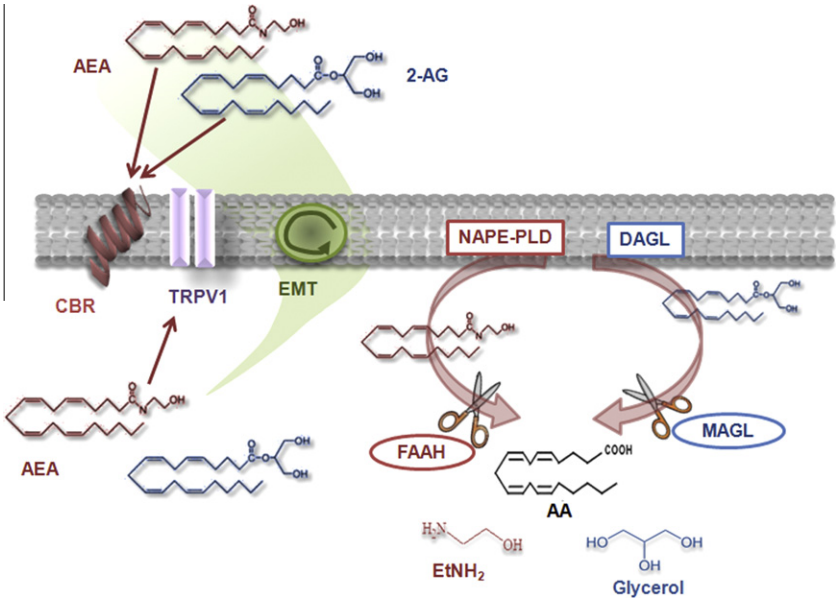

Fig. 1. Schematic representation of the best characterized elements of the endocannabinoid system. *N*-arachidonoyl-ethanolamine (AEA) is produced by a two-step mechanism through the activity of *N*-acyltransferase (NAT, not shown) and NAPE-specific phospholipase D (NAPE-PLD), which releases AEA and phosphatidic acid. The biological actions of AEA are terminated by its cellular uptake through a purported endocannabinoid membrane transporter (EMT), followed by intracellular degradation by fatty acid amide hydrolase (FAAH) to ethanolamine ($EtNH_2$) and arachidonic acid (AA). 2-Arachidonoylglycerol (2-AG) is also released from membrane lipids through the activity of diacylglycerol lipase (DAGL), and can be hydrolyzed by a cytosolic monoacylglycerol lipase (MAGL) that releases glycerol and AA. The transport of 2-AG across the cell membrane may be mediated by the same EMT that takes up AEA, or by other membrane carriers. Both AEA and 2-AG trigger several signal transduction pathways by acting at their targets, CB_1, CB_2 and purported CB_3 receptors. AEA, but not 2-AG, also binds intracellularly to TRPV1, which is the natural target of capsaicin, the pungent ingredient of hot chili peppers.

non-labouring patients, in order to guarantee gonadotropin release and to keep uterine quiescence (Acone et al., 2009). On the other hand, CB_1 downregulation is required in the placenta when patients get close to delivery. Concerning male fertility, high AEA levels are responsible for decreasing sperm motility and viability, and the induction of apoptosis in Sertoli cells, the latter are in charge of providing nutrients during sperm development. Therefore, AEA metabolizing enzymes, especially FAAH, are fundamental to ensure proper AEA levels to avoid severe impairment of fertility signals networks. It has been demonstrated that *cb1* and *cb2* knock-out mice suffer pregnancy loss (Sun and Dey, 2008; Wang et al., 2004) and CB_1 deficiency results in embryo retention in the oviduct for an extended period, and hence in ectopic pregnancy and reduced fertility of $cb1^{-/-}$ mice (Wang et al., 2004). Instead, the correct expression of CB_1 ensures also a normal oviduct-to-uterus transport of embryos. Interestingly, while embryonic CB_1 contributes to normal embryo development and oviductal CB_1 directs the timely transport of embryos, CB_2 seems to be involved only in embryo development (Wang and Dey, 2005). Female mice lacking CB_2 do not present embryos correctly hatched to the implantation sites (Wang et al., 2004). Since CB_2 is expressed in the embryonic stem cells, but not in trophectoderm-derived trophoblast stem cells, it is conceivable that CB_2 plays a role in specifying pluripotent inner cell mass (ICM) cell lineage during blastocyst formation (Sharov et al., 2003). The fertilizing ability of sperm and the sperm-oocyte interaction depend on AEA binding to either CB or TRPV1 receptors. In fact, experimental data on boar sperm demonstrated that AEA, by a CB_1-mediated mechanism, inhibits the capacitation-induced acrosome reaction, whereas the activation of TRPV1 reduces spontaneous acrosome reaction, thus preventing "out of place" acrosomal enzymes (Maccarrone et al., 2005). In addition, TRPV1 drives sperm-oocyte fusion, as evaluated by the progesterone-enhanced hamster egg penetration test (Francavilla et al., 2009). All these observations, reported in Table 1, suggests that aberrant eCBs signalling, either silenced or enhanced, impairs embryo transport and sperm motility, and highlight the major role of eCBs on male and female reproduction. It is worth mentioning that the ability of the ECS elements to critically regulate embryo implantation and development or to coordinate the capacitation process, is part of a more complex network which includes sex hormones and cytokines.

1.2. ECS and non mammalian fertility

Although mammalian models and human specimens are mainly used in the investigation of the ECS in reproduction, the ECS has also been identified in cells and reproductive organs of non-mammalian vertebrates, like teleosts and amphibians (Cacciola et al., 2008b; Cottone et al., 2008). Sea urchin sperm are the most studied to explore the stimulation–secretion coupling mechanism, and the first evidence on the inhibitory effect of AEA in reducing sperm fertilizing ability was obtained using *Strongylocentrotus purpuratus* sperm (Schuel et al., 1991). These data were supported by the discovery of AEA-metabolizing enzymes in the ovaries of *Paracentrotus lividus* (Bisogno et al., 1997). In line with this, a putative role of eCBs in regulating sea urchin embryogenesis and echinoderm development has been recently proposed (Buznikov et al., 2010). In fact, an AEA endogenous tone, measured in 8–16 cell embryos and in mid-blastula two stage, increased in several species of sea urchins, showing a trend similar to that in early mouse embryos, zebrafish and frogs. Moreover, its teratogenic action in sea urchins was found to depend on the embryo stage. Since the presence CB receptors in orthologs is still debatable (Elphick and Egertová, 2005; Elphick et al., 2003; Matias et al., 2005), it remains to be clarified whether any of the candidate receptors, identified *in silico*, might act as eCBs-binding receptor in developing sea urchins and sperm (Cacciola et al., 2008b). Strong evidence on the primary function of CB_1 in controlling sperm motility came from the frog, *Rana esculenta*, where CB_1 protein has been detected in the testis during the annual reproductive cycle and its role has been demonstrated during the

Table 1
Main biological actions mediated by cannabinoid and vanilloid receptors in female and male reproductive events.

Receptor	Effects on male fertility	Effects on female reproduction
CB_1	Control of sperm viability and acquisition of motility Modulation of energy metabolism Inhibition of ZP–induced AR Control of adult Leydig cell number	Transport of embryos from uterus to oviduct Embryo survival or programmed death Embryo implantation and development Immunoregulation of gestation Placentation Labour
CB_2	Induction of spermatogenesis Regulation of sperm motility speed Anti-apoptotic effect in Sertoli cells	Embryo development Blockade of trophoblast cell proliferation

AR = Acrosome reaction; ZP = Zona pellucida.

dilution mechanism that allows the acquisition of spermatozoa motility. Recently, it has been reported that a functional 2-AG gradient along the epididymis promotes sperm start-up by releasing spermatozoa from the inhibition exerted by CB_1 activation, and controls sperm potential to become motile during its voyage in the epididymis (Cobellis et al., 2010). In the following sections we will discuss the existing relationships between ECS and the other components involved in the control of reproductive events, and will focus on the evolutionary aspects of regulatory mechanisms.

2. ECS regulation in reproductive events: evolutionary aspects

The ECS is an ancient system, well-characterized in mammalian and non-mammalian vertebrates. Nevertheless there is evidence of ECS activity also occurs in invertebrates (Buznikov et al., 2010; Fasano et al., 2009; McPartland et al., 2006). Since the discovery of Δ^9-tetrahydrocannabinol (Δ^9-THC), many groups have reported on the effects of this compound in several invertebrate animal models. For instance, in the protozoan *Tetrahymena pyriformis*, Δ^9-THC affects cell division; in the lobster it affects neurotransmitter release and in the sea urchin, an *Echinodermata*, it inhibits fertilizing ability of sperm cells (Fasano et al., 2009 and references inside; Schuel et al., 1991). Recently, evidence has emerged for eCBs production in molluscs, sea urchins and starfish (Buznikov et al., 2010; Fasano et al., 2009 and references inside). By contrast, the search for CB receptor orthologs in invertebrate genomes has been carried out without any success in both insects and nematodes; similarly, data obtained in molluscs are still questionable. Although the search of CB_1/CB_2 in sea urchins has been negative, candidate TRPV1 orthologs have recently been identified in *S. purpuratus* genome (Buznikov et al., 2010). Molecular cloning of CB receptors orthologs has produced positive results in the sea squirt, *Ciona intestinalis*, an urochordates, and in the amphioxus, *Branchiostoma floridae*, a cephalochordate; in vertebrates, a partial or complete molecular characterization of the ECS has been provided from fishes to birds and mammals, with duplication of CB_1 or CB_2 receptor genes found in fishes (Fasano et al., 2009 and references inside). To date, an elegant approach to assess the phylogenetic history of the ECS, has been carried out by McPartland and coworkers (2006). This group suggests an ECS evolution from the searching of functional orthologs in the genomes of twelve organisms that spanned the phylogenetic "Tree of Life" (McPartland et al., 2006). The heterogeneous distribution of the ECS in invertebrates, vertebrates and mammals is reported in Table 2, where a differential origin of ligands, receptors and enzymes is evidenced. In fact, such authentic analysis has documented that functional TRPV1 and GPR55 receptors are limited to mammals; CB_2 and DAGLβ are limited to vertebrates; MAGL and COX2-like enzymes are limited to chordates; CB_1-like receptors and DAGLα are limited to bilaterian animals; NAPE-PLD is limited to the opisthokonta (animalia and fungi), and FAAH is limited to eukaryotes. Besides evolutional conflicting data on the ECS, this comparative approach has been recognized to yield a deep insight on adaptative phenomena that lead to evolutionary track, besides providing information on physiological mechanisms that build general models. In this respect, the use of mammalian and non-mammalian experimental models provided useful indications on the functional aspects of the ECS in reproduction.

3. ECS and reproductive hormones

Reproductive functions, from gamete formation to lactation, are under neuroendocrine control and require a tight crosstalk between the hypothalamus, pituitary and gonads. It is well-known that both cannabinoids and eCBs interfere with neuroendocrine control of reproductive functions in animal models and marijuana smokers, in that they affect gonadotropin [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] production, gonadic steroid production (testosterone in males, estradiol and progesterone in females), spermatogenesis, ovulation, the menstrual cycle, embryo development and implantation, as well as sexual behaviour (Murphy et al., 1998; Pagotto et al., 2006; Wang et al., 2006).

3.1. The cross-talks between ECS and pituitary hormones

At the central level, a consensus opinion is that both cannabinoids and eCBs negatively modulate male and female gonadotropin discharge by inhibiting the release of hypothalamic gonadotropin-releasing hormone (GnRH). Thus, they may perturb reproductive functions through a direct or indirect action upon GnRH secreting neurons. For instance, Δ^9-THC inhibits the activity of the neuronal system facilitating GnRH release, such as norepinephrine and glutamate, and stimulates the activity of the neuronal systems able to inhibit GnRH, through dopamine, γ-aminobutyric acid (GABA), endogenous opioid peptides and corticotrophin-releasing hormone, well-known signals involved in stress response (Murphy et al., 1998; Pagotto et al., 2006; Wang et al., 2006). Thus, the current view considers Δ^9-THC, AEA and 2-AG as retrograde signals that, by acting at the pre-synaptic level via CB_1, inhibit the release of specific neurotransmitters. The hypothalamus contains fewer eCBs-binding sites than other areas of the central nervous system (CNS), and receptor concentration differs in the hypothalamic nuclei (Tsou et al., 1998). In the brain, eCBs activity has mostly been due to CB_1 activation, since only recently CB_2 and TRPV1 have been localized in neurons (Cristino et al., 2006; Gong et al., 2006). Nevertheless, profiling neurotransmitter receptor expression in mouse GnRH secreting neurons reveals CB_2 expression in diestrous adult females (Todman et al., 2005), and CB_1/TRPV1 co-localization has been reported in mouse hypothalamic paraventricular nucleus (Cristino et al., 2006). Recently, an interesting study from Farkas and coworkers explained eCBs-mediated GnRH inhibition via GABAergic fibers in male mice (Farkas et al., 2010). GnRH-secreting neurons tonically release 2-AG in presynaptic fissure, which in turn activates CB_1 receptors located on GABAergic afferents, in tight relationship with GnRH neurons. The activation of CB_1 inhibits spontaneous GABA release, while postsynaptic GABA receptors (GABAA and GABAB), located on GnRH-secreting neurons, are not activated; as a consequence, GnRH is not released (Farkas et al., 2010). Since the inhibitory effect of AEA on GnRH-secreting neurons is reversed by estrogens

Table 2
ECS elements in invertebrates, vertebrates and mammals.

ECS elements	 Invertebrates	 Vertebrates	 Mammals
NAPE-PLD	✓	✓	✓
FAAH	✓	✓	✓
DAGL	×	✓	✓
MAGL	×	✓	✓
CB_1	× ✓ *Branchiostoma floridae*	✓	✓
CB_2	×	✓	✓
TRPV1	× ✓ *Srongylocentrotus purpuratus*	✓	✓

✓ = present; × = absent.

(Scorticati et al., 2004), it is not considered that neuronal systems other than GABAergic transmission might modulate GnRH-secreting activity via eCBs. Differential effects of AEA and 2-AG on LH and prolactin (PRL) secretion have recently been reported in mice. 2-AG is able to suppress LH in wild-type, but not in *cb1*$^{-/-}$ mice while AEA decreases both LH and PRL in the same animals, indicating that receptors other than CB_1 are involved in this signalling pathway (Olàh et al., 2008). Therefore, a TRPV1-mediated AEA effect on LH and PRL secretion has been suggested in *cb1*$^{-/-}$ mice (Olàh et al., 2008); this effect should be exerted at the hypothalamic level, since the pituitary does not express TRPV1. Such an intricate neuronal network can be even more complex, as also stress and food intake, well-known processes under eCBs control (Pagotto et al., 2006), interfere with GnRH secretion. In addition, a GnRH-inhibiting hormone (GnIH), able to inhibit gonadotropin synthesis and secretion through actions on GnRH-secreting neurons and gonadotrophs, and able to modulate sexual behaviour, has recently been identified in amphibians, fishes, birds, sheep, rodents, primates (Kriegsfeld et al., 2006; Tsutsui et al., 2000, 2010). Interestingly, stress suppresses GnRH secretion through the activation of GnIH in male rats (Kirby et al., 2009). GnRH activity has also been shown to be regulated by the kisspeptin system, that is conserved within the CNS. Kisspeptin (the product of *kiss1* gene) has a direct stimulatory effect on GnRH neurons via its GPR54 receptor (Oakley et al., 2009). The hypothalamus fails to drive adequate secretion of gonadotropins, when mutations in *kiss1* or *GPR54* genes occur, a pathological condition known as hypogonadism. Kisspeptin-secreting neurons are located within the hypothalamus of several mammalian and non-mammalian species, and provide direct excitatory input to GnRH neurons possessing GPR54 (Tsutsui et al., 2010). Lastly, the kisspeptin system modulates GnRH release in a steroid-dependent fashion (Oakley et al., 2009 for review). An intriguing question is what is the possible involvement of ECS in GnIH/kisspeptin/GnRH crosstalk? In lower vertebrates both hypothalamic GnRH and CB_1 signalling have been described (Cottone et al., 2008; Meccariello et al., 2008), but here the scenario is more intricate than in rodents, because in lower vertebrates (and also in birds and in humans), multiple forms of GnRH have been detected, and each species possesses at least two GnRH isoforms. To date, mammalian GnRH (also named GnRH-I) is clearly involved in gonadotropin discharge and is the only form detected in rodents; chicken GnRH (also named GnRH-II) mostly expressed in the hindbrain, is widely distributed among vertebrates and is often associated to the modulation of sexual behaviour; salmon GnRH (also named GnRH-III) is mostly expressed in the telencephalon of fishes (Kah et al., 2007 for review). CB_1 has been mapped in the forebrain of adult teleosts (*Carassius aurata* and *Pelvicachromis pulcher*) and anuran amphibians (*Xenopus laevis* and *Rana esculenta*) (Cottone et al., 2003, 2005; Meccariello et al., 2008; Valenti et al., 2005), as well as in zebrafish and *Xenopus laevis* embryos (Lam et al., 2006; Migliarini et al., 2006), and similarities with CB_1 distribution in mammals have emerged (Tsou et al., 1998). Besides the involvement in sensory integrative activities like the olfactory processes in amphibians, and food response in bony fishes, eCBs interfere with neuroendocrine machinery. In fact, in *Carassius auratus* and *Pelvicachromis pulcher* CB_1 and GnRH-III share a topographical co-distribution in basal telencephalon and anterior preoptic area (Cottone et al., 2008); the latter is the encephalic area mainly involved in the control of reproductive functions. In amphibians, the same areas and the septum of the telencephalon share colocalization of GnRH-I and CB_1 proteins (Cottone et al., 2008; Meccariello et al., 2008). A morpho-functional relationship between GnRH-I and CB_1 has been described in the male frog, *Rana esculenta*, brain. As reported in male mice (Farkas et al., 2010) and also in *Rana esculenta* male forebrain a number of CB_1-containing cell bodies and terminals, together with GnRH-I immunostained nerve cells and fibres, are codistributed. Colocalization is observed in 20% of the GnRH-I immunopositive neurons, and GnRH-positive nerve fibres close to CB_1 neurons have also been observed (Meccariello et al., 2008). At the molecular level, mRNA and protein profiles of CB_1 and GnRH-I are opposite in frog diencephalon during the annual sexual cycle (Chianese et al., 2008; Meccariello et al., 2008). GnRH-I/CB1 coexpression in a subset of hypothalamic neurons raises the possibility that eCBs might influence GnRH release directly from GnRH neurosecretory terminals. As reported in male and female rats (Scorticati et al., 2004), incubation of male frog diencephalons with AEA decreases the expression of GnRH-I mRNA; such an effect is mediated by CB_1, since pretreatment with SR141716A, the specific CB_1 antagonist also known as rimonabant (Rinaldi-Carmona et al., 1994), prevents AEA-induced GnRH-I mRNA down-regulation. In turn, treatment with buserelin, a GnRH long-lasting analogue inhibits the synthesis of GnRH-I mRNA and induces an increase of CB_1 transcription (Meccariello et al., 2008). Therefore, enhanced eCBs biosynthesis in frog forebrain might be suggested, and a self-negative modulation of GnRH neuronal activity might be exerted via CB_1. Immortalized GT1 neurons are both a source and target of eCBs; *in vitro* they produce and secrete 2-AG and AEA, are able to take up and degrade eCBs, and possess CB_1 and CB_2, whose activation leads to the inhibition of pulsatile GnRH release (Gammon et al., 2005). Nevertheless, such observations have not been confirmed *in vivo*, although GnRH-secreting neurons are close to cannabinergic fibers and scantly express CB_1 (Gammon et al., 2005). Stimulation of milk production and maintenance of lactation after pregnancy are under PRL control. At the central level, Δ^9-THC/eCBs interfere with pituitary PRL production, acting on tubero-infundibular dopaminergic neurons that are known to exert inhibitory effects on pituitary PRL release (Fernández-Ruiz et al., 1997; Murphy et al., 1998). In normal cycling female rats, the plasma PRL response to eCBs depends on the stage of the estrous cycle (Bonnin et al., 1993). Intracerebroventricular injections of AEA suppress PRL release in male rats and have little effect in ovariectomized (OVX) female rats but by contrast, treatment of OVX rats with estrogens increases PRL levels and reverses the inhibitory effect of AEA on PRL release (Scorticati et al., 2003). Since AEA injection differentially affects dopamine turnover in males and females, a sex-dependent mechanism has been suggested. In male rats such an effect is mediated through CB_1 activation in dopaminergic neurons of mediobasal hypothalamus; in female rats AEA likely acts as a modulator of the neuronal system involved in the decrease of the inhibitory control of PRL release (Scorticati et al., 2003). Still controversial is the direct activity of eCBs on the pituitary, based on the demonstration of gene expression, specific binding and immunoreactivity for CB_1, as well as of putative eCBs

biosynthetic enzymes in the pituitary pars distalis (Gonzales et al., 1999, 2000; Lynn and Herkenham, 1994; Murphy et al., 1998; Wenger et al., 1999). For example, Δ^9-THC induces PRL release and increases cyclic adenosine monophosphate (cAMP) levels in primary rat pituitary cell (Rodriguez de Fonseca et al., 1999); similarly, in rats AEA exhibits differential effects on the *in vitro* secretion of LH, PRL, corticotrophin (ACTH) and growth hormone (GH) from the anterior pituitary (Wenger et al., 2000). CB_1 has been detected in the pituitary pars distalis of several species, from amphibians to humans (Cesa et al., 2002; Fernández-Ruiz et al., 1997; Gonzales et al., 2000; Pagotto et al., 2001; Wenger et al., 1999; Yasuo et al., 2010a,b) but its cellular localization is quite different among species. For instance, in amphibians CB_1 immunolabelling is detected in lactotroph, gonadotroph and thyrotroph, but not in corticotroph, cells (Cesa et al., 2002); in rats CB_1 maps to lactotroph and LH-secreting cells (Wenger et al., 1999), and only in corticotroph and folliculo-stellate cells in humans (Yasuo et al., 2010b). CB_1 receptor expression in rat pituitary is higher in males than in females. In females, it is dependent on the phase of the ovarian cycle, being highest on the second day of diestrus and lowest on estrus. In addition, AEA content fluctuates in female pituitary, with the highest levels detected in the estrus and lowest on the first day of diestrous and proestrous. AEA profile in the female hypothalamus during the ovarian cycle is opposite to that observed in pituitary. CB_1 expression depends on steroids, since it is reduced in both orchidectomized male and estradiol-replaced OVX female rats (Gonzáles et al., 2000). Recently, in mammals an important role for the pars tuberalis has been shown within the neuroendocrine networks of the hypothalamus–hypophysis system; this brain area is located between the median eminence, the pituitary portal vessels and the pituitary pars distalis. A retrograde pathway, from the pars tuberalis to the hypothalamus, is crucial for photoperiodic response of gonads in birds and mammals (Nakao et al., 2008; Unfried et al., 2009; Yasuo et al., 2010a). Beside melatonin in mammals such a pathway involves the thyrotropin β-subunit, which activates enzymes controlling the local concentration of thyroid hormones in the third ventricle, and hence GnRH release (Unfried et al., 2009; Yasuo et al., 2010c). The pars tuberalis is also a key station for the anterograde signalling toward the pituitary, and eCBs are included among tuberalins, the messengers supposed to be secreted from the pars tuberalis to target the pituitary pars distalis (Yasuo et al., 2010c). In fact, in both hamsters and humans, pars tuberalis produces high levels of 2-AG and low levels of AEA, *N*-palmitoylethanolamine (PEA) and *N*-oleoylethanolamine (OEA), and expresses NAPE-PLD, DAGL, FAAH and MAGL, while the pituitary pars distalis possesses CB_1 (Yasuo et al., 2010b, 2010c). Additionally, DAGL increases 2-AG production in the pars tuberalis of hamsters kept under long-day conditions (Yasuo et al., 2010c).

3.2. ECS-hormone interactions in the sexual behavior

Many experiments indicate that eCBs act as modulators of sexual behaviour in vertebrates, including humans. The influence of cannabis intake on sexual behaviour and arousability appears to be dose-dependent in both men and women, although women are far more consistent in reporting facilitatory effects. Furthermore, evidence from non-human primates indicates somewhat more beneficial than debilitating effects of cannabinoids on female sexual perceptivity and receptivity, while suggesting predominantly detrimental effects on male sexual motivation and erectile function (Gorzalka et al., 2010). To illustrate this, it is known that the facilitatory effects of Δ^9-THC on sexual receptivity in female rats require the cross-talk between CB_1, membrane progesterone receptor and dopamine D1B receptor (Mani et al., 2001). In rough-skinned newts, *Taricha granulosa*, the cannabinoid agonist levonantradol inhibits both newt spontaneous locomotor activity and courtship clasping behavior, in a dose-dependent fashion (Soderstrom et al., 2000). In addition, the rapid suppression of male sex behaviour in response to acute stress or elevated corticosterone concentration, involves activation of eCB signalling in the hindbrain and alters sexual behaviour by modulating the excitability of medullary circuits (Coddington et al., 2007). GnRH/GnRHII act as neuromodulators in the hindbrain, affects sexual behaviour via GnIH but the possibility of additional networks involving eCBs cannot be excluded.

3.3. The involvement of ECS at the level of gonadal physiology

At the gonadal level, an intricate crosstalk between eCBs and hormones (gonadotropins and steroids) is involved in the control of several activities. In men and rodents, chronic use of cannabinoids decreases testosterone production and secretion, depresses spermatogenesis, and reduces the weight of testes and accessory reproductive organs (Wang et al., 2006). Serum LH and testosterone levels, as well as *in vitro* basal secretion of testosterone, are significantly decreased in *cb1*$^{-/-}$ mice. AEA, in turn, suppresses LH and testosterone secretion in wild-type mice, but it is ineffective in knockout animals (Wenger et al., 2001). The localization of eCB receptors and metabolic enzymes in germinal cells (Grimaldi et al., 2009; Gye et al., 2005; Maccarrone et al., 2003a; Rossi et al., 2007), and the presence of CB_1 in Leydig cells and its association with testosterone secretion (Wenger et al., 2001) confirm the importance of eCBs in testis endocrinology (Maccarrone and Wenger, 2005). Nevertheless, the presence of few adult Leydig cells in *cb1*$^{-/-}$ mice may explain *in vitro* the lower basal testosterone secretion in *cb1*$^{-/-}$ testes compared with wild-type counterparts, and points to eCBs as potential modulators of Leydig cell proliferation (Cacciola et al., 2008a). In fact, during the first wave of spermatogenesis, spatiotemporal localization of CB_1 protein in rat testis interstitium negatively correlates with cell division of adult Leydig cells, measured as bromodeoxyuridine uptake. Experiments on serial sections clearly demonstrate that mitotic immature Leydig cells do not possess CB_1; by contrast, immature non-mitotic Leydig cells are CB_1 immunopositive. A link between testicular steroidogenesis and CB_1 has also been postulated in vertebrates other than mammals. In *Rana esculenta*, a seasonal breeder, CB_1 is expressed all over the annual sexual cycle with the highest expression in November and March, thus correlating with plasma and intratesticular testosterone levels (Meccariello et al., 2006). Furthermore, CB_1 mRNA is detected by *in situ* hybridization in the interstitium (Meccariello, unpublished observations). An intriguing question is whether the dose-dependent antiapoptotic effect of FSH upon Sertoli cells is highly related to the endogenous AEA content? Immature Sertoli cells are the major source of testicular estrogens; FSH induces estradiol production by Sertoli cells through enhancement of P450-aromatase transcription, the enzyme involved in the switch from androgens to estrogens (McDonald et al., 2006). In mice, Sertoli cells have the biochemical machinery to produce, bind, transport and degrade eCBs at various developmental stages (Maccarrone et al., 2003c; Rossi et al., 2007). Until the establishment of the blood testis barrier, FSH exerts proliferative effects on Sertoli cells, whereas, AEA induces their apoptosis via activation of TRPV1 (Maccarrone et al., 2003c). Interestingly, treatment of Sertoli cells with FSH enhances the activity and the expression of FAAH, whereas it does not affect the enzymes that synthesize AEA and 2-AG, nor the levels of CB_2 and TRPV1 receptors (Rossi et al., 2007). An increase in FAAH activity reduces endogenous levels of AEA, thus protecting Sertoli cells against its pro-apoptotic potential. An interesting mechanism involving protein kinase A (PKA) and aromatase activity has been suggested for FAAH stimulation by FSH (Maccarrone et al., 2003c; Rossi et al., 2007). Indeed, FSH-dependent PKA activation might lead to the activation of accessory

proteins able to induce FAAH activity; on the other hand, P450-aromatase, converting testosterone to estradiol, could directly activate FAAH transcription, because an estrogen responsive element (ERE) has been found in the promoter of murine FAAH gene (Waleh et al., 2002).

Deleterious effects of eCBs on female reproduction, from ovulation to arrest of implantation and growth, are well-known and have been reviewed elsewhere (Battista et al., 2008; Sun and Dey, 2008; Tsou et al., 1998; Wang et al., 2006). For instance, in female rats intraperitoneal administration of Δ^9-THC in the afternoon of proestrus prevents the pre-ovulatory surge of both LH and FSH, thus blocking ovulation which in rabbits treated with Δ^9-THC prior to mating, the coitus-induced LH surge and subsequent ovulation are blocked. In rhesus monkeys and humans, Δ^9-THC has inhibitor effects on LH surge and ovulation in the follicular and pre-ovulatory phases. Nevertheless, drug tolerance with the restoration of normal cycles has been reported in both monkeys and women who were long-term marijuana users (Murphy et al., 1998; Wang et al., 2006). In women AEA tone changes during the menstrual cycle; a peak of plasma AEA occurs at ovulation and positively correlates with estradiol and gonadotropin levels, suggesting that FSH and LH may be involved in the regulation of AEA content (El-Talatini et al., 2010). Consistently, during the luteal phase FAAH activity increases in lymphocytes (Lazzarin et al., 2004). AEA is produced in the human ovary under hormonal control and plays a role in folliculogenesis, preovulatory follicle maturation, oocyte maturation and ovulation (Fonseca et al., 2010b), processes which are all driven by well-known reproductive hormone cycling. Immunohistochemical analysis of normal human ovaries indicates that ECS components are widely expressed in the ovarian medulla and cortex, with more intense CB_2 than CB_1 immunoreactivity in the granulosa cells of primordial, primary, secondary and tertiary follicles, as well as in corpus luteum and corpus albicans. Instead, FAAH and NAPE-PLD are only found in growing secondary and tertiary follicles and corpora lutea and albicantes (El-Talatini et al., 2009a). High levels of AEA adversely affect embryo development and implantation thought to be via CB_1 activation, whereas low levels of AEA promote embryonic growth and differentiation (Sun and Dey, 2008; Wang et al., 2006). Moreover FAAH activity, by modulating AEA levels, is pivotal to make the uterus receptive for pregnancy. Consistently, FAAH activity changes in mouse uterus during the estrous cycle (Klinger et al., 2006), and ovarian steroids like estradiol and progesterone are negative modulators of it (Battista et al., 2008; Maccarrone et al., 2000a), once again confirming a tight crosstalk between eCBs and sex hormones. All data reported above are summarized in Table 3.

4. ECS and reproductive cytokines

Although it has long been known that ovarian steroids regulate implantation, their actions are largely mediated through a number of other regulatory molecules. It has been demonstrated that several cytokines play functional roles during the establishment of pregnancy, including the development of uterine receptivity (i.e., decidualization and changes in luminal and glandular epithelium), and the control of trophoblast adhesion and invasion. Cytokines are produced by various cells of the immune and hematopoietic systems, and exert a widespread regulation of cellular functions like proliferation and differentiation. They act by binding to surface receptors which lead to the initiation of various signal transduction pathways. Endometrial epithelial, stromal or decidual cells, as well as trophoblast cells and subsets of leukocytes (specifically, macrophages and natural killer (NK) cells), represent a source of cytokines in the uterus of various species. The correct establishment of a hormone-cytokine network is a key-element to guarantee a successful pregnancy (Klein et al., 2004). Peripheral T lymphocytes produce type-1 helper (Th1) and type-2 helper (Th2) cytokines, which have opposite effects on trophoblast growth. In fact, the pro-fertility Th2 cytokines (interleukin (IL)-3, IL-4 and IL-10) are important for the timing of blastocyst implantation and successful pregnancy, by promoting trophoblast growth either directly or indirectly through the inhibition of NK cell activity, and the stimulation of natural suppressor cells. On the other hand, the anti-fertility Th1 cytokines (IL-2, IL-12 and interferon-γ, INF-γ) impair gestation, by causing a direct damage to the trophoblast, by stimulating NK cells and by enhancing tumor necrosis factor-α (TNF-α) secretion by macrophages. Peripheral T lymphocytes also produce leukemia inhibitory factor (LIF), which is known among the IL-6 family to be critical for implantation in mammals (Ahima and Flier, 2000; Maccarrone and Wenger, 2005). The precise mechanisms regulating LIF production remain to be elucidated, but several findings strengthen the idea that LIF release during the secretory phase of the menstrual cycle is probably dependent on tightly regulated local micro-environments, and any defects in local networks may result in fetal loss (Paiva et al., 2009). Further work is required to establish which factors are more critical, since they may provide an opportunity for manipulation of the environment during the "window of implantation". The biological actions of eCBs in reproductive events may occur not only by altering the physiological crosstalk with sex hormones, but also through a direct/indirect modulation of the immune system. In fact, accumulated evidence confirms the involvement of the ECS in the modulation of immune response in various cell-types, their effect on cytokine networks, on the induction of apoptosis in immune cells, and on the downregulation of innate and adaptive immune response (Klein et al., 2004; Tanasescu and Costantinescu, 2010). eCBs have also been demonstrated to increase the production of IL-1, -4, -6 and -10 (Derocq et al., 2000; Kishimoto et al., 2004), or to inhibit the release of TNF-α and IFN-γ-mediated Th1 response in human lymphocytes (Cencioni et al., 2010), suggesting that they may have a opposite effects on cytokine secretion depending on the nature of the pro-inflammatory stimulus, or on the type of cells (Tanasescu and Costantinescu, 2010). In this context, it should be recalled that high circulating levels of AEA inhibit the release of LIF from blood cells in a CB_1-dependent manner, resulting in pregnancy failure and embryo death. It has been demonstrated that IL-4 and IL-10 enhance FAAH activity, whereas IL-2 and IL-12 act as FAAH inhibitors, leading to a decrease or an increase of AEA levels, respectively. Therefore, defective FAAH in peripheral blood has been proposed as a diagnostic marker of human infertility (Maccarrone and Finazzi-Agrò, 2004). In line with this, a recent study has demonstrated that a blood level of AEA above a certain threshold is nearly 100% predictive of miscarriage in women at risk (Habayeb et al., 2008), corroborating the concept that FAAH is a critical "sensor" of reproductive abnormalities in human female fertility (Maccarrone and Finazzi-Agrò, 2004). Also mammalian spermatogenesis is strictly controlled by estrogens, which are produced mainly by Sertoli cells and by FSH, that is a member of the glycoprotein hormone family (Rossi et al., 2007). Immune signals, such as the anti-inflammatory transforming growth factor β (TGFβ), or the pro-inflammatory TNF-α, may also take part in the orchestration of gametogenesis and in early germ cell differentiation in invertebrates and mammals (Chaves-Pozo et al., 2008; O'Bryan and Hedger, 2008). Different molecular pathways are triggered during the formation, maintenance and differentiation of germ cells. Among these TGFβ signalling is highly conserved throughout the six hundred million years of evolution, and is required for fertility from insects to mammals (Loveland and Hime, 2005). TGFβ is critical for spermatogenesis and its disruption causes formation of testicular tumors, thus an important goal remains to ascertain what regulates TGFβ expression and what, in turn, is regulated

Table 3
eCBs and reproductive hormones.

Tissue	eCBs and reproductive hormones
Hypothalamus	eCBs downregulate GnRH transcription and release via direct/indirect action on GnRH secreting neurons
	Estradiol reverses AEA inhibitory effect on GnRH release
	GnRH agonists increase CB_1 transcription
	eCBs reduce PRL through dopaminergic activation of tuberoinfundibular neurons; estradiol reverses AEA inhibitory effect on PRL release
	eCBs increase ACTH and corticosterone, by activating the parvocellular part of PVN
Pituitary	No effect of eCBs on FSH, either *in vivo* or *in vitro*
	eCBs reduce LH both *in vivo* and *in vitro*
	eCBs reduce PRL *in vitro*
	eCBs increase ACTH *in vitro* in a CB_1 independent fashion
	Steroid-dependent CB_1 expression
Testis	eCBs reduce testosterone
	FSH enhances FAAH activity in Sertoli cells
Ovary	eCBs reduce estradiol
Uterus	Estradiol and progesterone reduce FAAH

GnRH = gonadotropin-releasing hormone; PRL = prolactin; ACTH = corticotrophin; PVN = paraventricular nucleus; FSH = follicle-stimulating hormone; LH = luteinizing hormone.

by its signalling. Incidentally, cells treated with Δ^9-THC increase TGFβ mRNA (Teixeria et al., 2010), whereas TGFβ protein decreases after treatment with a specific CB_1 receptor antagonist in cirrhotic rats (Teixeria-Clerc et al., 2006), or by a genetic or pharmacological inactivation of CB_1 receptors in liver fibrosis (Yang et al., 2007). Although there is not proof yet of a direct connection between eCBs and cytokines in male fertility, we cannot rule out that AEA and congeners interfere with the control of sperm maturation and production, by modulating the release of other signalling molecule, and/or by affecting transduction pathways that normally regulate fertility processes (Table 4).

5. Chromatin remodeling: a new target for ECS

Chromatin remodeling controls genome functioning by altering the local dynamics of chromatin, primarily by regulating DNA accessibility and compactness. This mechanism depends on epigenetic modifications such as DNA methylation, histone acetylation and methylation, incorporation of histone variants, nucleosome remodeling and/or microRNA activity (Kouzarides, 2007). In contrast to oocytes, which maintain a chromatin structure more similar to somatic cells, male germ cells show an impressive chromatin remodeling (Miller et al., 2010), and emerging evidence shows that the sperm nucleus no longer has a limited developmental role (Johnson et al., 2011). Indeed, during spermiogenesis, round spermatids reorganize nuclear chromatin and differentiate into mature elongated cells (mature spermatids or spermatozoa), that carry a tightly packaged chromatin. Depending on the species, histones (somatic or somatic-like) or specific sperm nuclear basic proteins (SNBP) carry out this packaging (Eirìn-Lòpez and Ausiò, 2009). As yet, there is no clear correspondence between the taxonomy of animal species and the type of SNBP found in their sperm. However, chromatin packaging specifically characterizes male gametes and reflects the principal aim of this cell: to preserve DNA and to provide an undamaged genome for fertilization. The specific patterns by which sperm chromatin is packed vary widely among different species. Some species exchange histones with SNBP precursors, later processed into mature proteins; others directly replace histones with mature SNBP (Kurtz et al., 2009). Fishes and birds replace somatic histones with protamines (PRM) (Oliva and Dixon, 1991), that are specific SNBP characterized by high percentage of arginine residues (or arginine and cysteine residues in mammals) (Carrel et al., 2007). A complicated mechanism condenses chromatin in mammals, and requires several steps. Firstly, when cells enter the meiotic prophase, somatic histones are replaced by testis-specific (TH2B, TH2A, TH3, H1t) or testis-enriched (H2AX, H1a) histone variants (Churikov et al., 2004), which organize a less stable nucleosome compared with nucleosomes containing canonical histones (Gaucher et al., 2010). This step creates specific chromatin domains characterized by quickly-disassembling nucleosomes and by a new "histone code", both facilitating histone replacement (Gaucher et al., 2010; Govin et al., 2004). Indeed, histones are displaced through a DNA-binding competition mechanism that involves: (1) transient replacement by transition proteins (TNP1–4 in mice, rats, boars, bulls, men; the best characterized are TNP1 and TNP2), and (2) final TNP-to-PRM exchange (PRM1 in rats; PRM1 and PRM2 in stallions and mice; PRM4, in humans) (Gaucher et al., 2010; Rousseaux et al., 2005). It has been demonstrated that TNP1 has important DNA-nucleosome core destabilizing properties, because it decreases the melting temperature of DNA and relaxes DNA in nucleosomal core particles *in vitro* (Singh and Rao, 1988). In contrast, TNP2 seems to be a DNA-condensing protein (Kundu and Rao, 1996). In mice, hyperacetylation of histone tails (step 8–11 spermatids) relaxes DNA-nucleosome interactions, and precedes and overlaps TNP1/TNP2 presence at the nuclear level (step 10-early 15 spermatids). In combination with transient DNA strand-breaks and histone proteolysis, hyperacetylation facilitates histone displacement, which ends at step 13 spermatids (Rousseaux et al., 2005). Chromatin condensation begins during step 12–13 of spermiogenesis, with a thickening of chromatin fibers and uniform condensation in the anterior-to-posterior direction, that corresponds to the anterocaudal disappearance of the acetylation signal, and to the direction of TNPs deposition. It has been hypothesized that core-histones could be displaced in their acetylated state (Hazzourri et al., 2000). Phosphorylation and dephosphorylation of TNP and PRM trigger their nuclear translocation,

Table 4
ECS and reproductive cytokines.

Cytokine	Cell/tissue	ECS target	ECS effect	Reference
IL-2 and IL-12	T lymphocytes	FAAH	Decreased activity	Maccarrone et al. (2003a,b,c)
IL-4 and IL-10	T lymphocytes	FAAH	Increased activity	Maccarrone et al. (2003a,b,c)
TGFβ	Liver	CB_1	Increased binding	Teixeira et al. (2010), Yang et al. (2007)

their binding to DNA and eventually chromatin condensation (Dadoune, 2003; Sassone-Corsi, 2005). Morphological events of this global chromatin re-organization have been described in mammalian and non-mammalian species (Kurtz et al., 2009), and key-factors are emerging at each step (Carrel et al., 2007).

5.1. Chromatin remodeling in germ cells and spermatids

Recently, eCBs have been pointed out as new actors of this process, thus revealing a new biological activity for these lipid mediators in male germ cells (Chioccarelli et al., 2010), in keeping with previous data in human keratinocytes (Paradisi et al., 2008). Indeed, either Tnp2 (mRNA and protein) levels or histone displacement are affected by *cb1* gene deletion in both heterozygous ($cb1^{+/-}$) and homozygous ($cb1^{-/-}$) mice (Chioccarelli et al., 2010). Complementary experiments carried out *in vivo* on wild-type and $cb1^{+/-}$ mice, and *in vitro* on wild-type mice testis, also show that AEA is able to act locally and to modulate *Tnp2* by increasing mRNA levels through CB_1. It has been hypothesized that testicular AEA, rather than 2-AG (Chioccarelli et al., 2010), produced by Sertoli cells (Maccarrone et al., 2003c) and/or by spermatids (Grimaldi et al., 2009), might act on spermatids themselves. Indeed, it is conceivable that AEA may act as a paracrine/autocrine factor on spermatids via CB_1, by regulating *Tnp2* mRNA transcription (Zhao et al., 2001) or stability (Yu et al., 2005). Both hypotheses are particularly attractive. Tnp1/2 and Pmr1/2 mRNAs are synthesized and stored for some days in spermatids, and are then translated, implying a timely controlled process of haploid-regulated transcription and translation. Transcriptional regulation depends on potentiation of genes via association with nuclear matrix attachment regions (MARs) (Martins et al., 2004), DNA methylation, and/or binding of *trans*-acting factors to promoter regions like TATA-box protein (TBP), cAMP response element modulator (CREM), and Y-box proteins (Carrel et al., 2007). DNA methylation is generally known to decrease gene expression, and histone deacetylase (HDAC) is responsible for part of this repressive effect. It has been demonstrated that treatment of human keratinocytes with agents known to inhibit DNA methylation promotes their differentiation (Maccarrone et al., 2003b and references therein), and that AEA is able to reverse this effect by enhancing DNA methyl-transferase (DNMT) activity (Paradisi et al., 2008). In particular, AEA decreases keratinocyte-differentiating gene expression by increasing DNA methylation through a p38, and to a lesser extend p42/44, mitogen-activated protein kinase (MAPK)-dependent pathway triggered by CB_1 (Paradisi et al., 2008; Pasquariello et al., 2009). In mouse, *Pmr1*, *Pmr2* and *Tnp2* genes are clustered on chromosome 16 and, contrary to the usual paradigm, they are fully methylated when actively transcribed (Choi et al., 1997). Interestingly, in human testis DNMT1 is restricted to male germ cells (pachytene spermatocytes and round spermatids), and infertile patients showing round spermatid maturation arrest also show a specific DNMT1 loss in round spermatids (Omisanjo et al., 2007). Moreover, the promoter region of transition proteins and protamines contains a cAMP response element (CRE), and the CREM-activator isoform *tau* (CREMτ (Foulkes et al., 1992, 1993) regulates the haploid expression of several post-meiotic genes, including the TNP and PRM families (Carrel et al., 2007). Altogether, these data suggest that CB_1 may target *Tnp2* transcription rather than its degradation, by regulating either DNA methylation (Paradisi et al., 2008) or cAMP production (Howlett, 2005). In mice, *in vitro* experiments have demonstrated that AEA upregulates *Tnp2* through a CB_1-PKA/PKC pathway, thus embracing the hypothesis that epigenetic modifications drive the cAMP-dependent CREMτ recruitment to the *Tnp2* promoter region (see the hypothetical model in Fig. 2). Indeed, CREMτ expression (primarily via a cAMP-FSH modulated pathway) and activity (via subcellular localization of the cAMP-dependent coactivator ACT) are both dependent on PKA (Hogeveen and Sassone-Corsi, 2006; Kotaja et al., 2005; Monaco et al., 1995). CREMτ appears to directly influence the fate of spermatids, since *crem-null* mice display a complete block of spermatid differentiation at the first step of spermiogenesis (Blendy et al., 1996; Nantel et al., 1996). To date, it is not clear how the "FSH-signal" internalized in Sertoli cells is transduced into germ cells, in order to induce CREMτ expression and/or activity. Although the existence of FSH receptors on germ cells remains an unsolved question (Baccetti et al., 1998), AEA might be a not-yet characterized "communicating factor" released by Sertoli cell to talk to spermatids. Indeed, Sertoli cells are able to produce and hydrolyze AEA (Maccarrone et al., 2003c), and the expression of their FAAH is regulated by FSH through an estrogen-mediated pathway (Rossi et al., 2007). Therefore, it is conceivable that (1) FSH-induced estrogens might control local AEA levels through FAAH, and that (2) AEA, produced by Sertoli cells, might regulate *Tnp2* expression in spermatids via CB_1. Accordingly, it has been reported that estrogens, directly or indirectly, influence chromatin remodeling which, in turn, primarily promotes the nuclear shape of spermatids. It has been demonstrated that *aromatase*-null mice (ArKO) do not complete elongation of spermatids (Robertson et al., 1999), and gonadotropin-deficient *hpg* mice treated with estrogens, or with a selective ERα agonist, restore meiosis progression and produce haploid spermatid with elongated nuclei (Allan et al., 2010; Ebling et al., 2000). In addition, some data support testosterone/estrogen involvement in TP/PMR expression (Aleem et al., 2004, 2006) and in chromatin condensation. The latter observation is particularly interesting, because $cb1^{-/-}$ male mice, besides showing inefficient histone displacement, also produce spermatozoa with uncondensed chromatin and damaged DNA (Chioccarelli et al., 2010). It is conceivable that chromatin condensation and DNA damage are secondary effects related to disrupted histone displacement, rather than to *Tnp2* decrease. Indeed, $cb1^{-/-}$ male mice show high percentage of spermatozoa retaining histones, a phenotype that seems to be independent of *Tnp2* decrease. In fact, although *Tnp2* transcript decreases by ~50% in both $cb1^{-/-}$ and $cb1^{+/-}$, histone displacement and all chromatin quality indexes examined are highly reduced in $cb1^{-/-}$ as compared with $cb1^{+/-}$ animals (Chioccarelli et al., 2010). Therefore, *cb1* loss causes a double alteration: decrease of *Tnp2* mRNA levels and, through a TNP2-independent mechanism, reduction of histone displacement. Accordingly, Meistrich and coworkers (1992) have demonstrated that *Tnp2*-null mutation does not affect histone removal. In mice, main events promoting histone displacement are phosphorylation of histone H1t (Sarg et al., 2009) and hyperacetylation of hystone H4. The latter process has been studied in germ cells of several species (Kurtz et al., 2009; Meistrich et al., 1992) and is largely conserved during evolution. Because of CB_1 presence in spermatids of several species (Pierantoni et al., 2009), it is conceivable that eCBs may regulate chromatin packaging in vertebrates. Species characterized by chromatin packaging through histones have relatively low levels of acetylation. Indeed, in species where chromatin is packaged through SNBPs or PRMs, a massive acetylation has been observed (Hazzourri et al., 2000; Kurtz et al., 2009). Probably, acetylation of specific lysines, within the N-terminal domain of H4, reduces binding of nucleosomes to DNA, thus leading to chromatin relaxation. This event exposes DNA to binding to more basic proteins (i.e., SNBP/PRM or TP-PRM), or to condensation-promoting factors (Pivot-Pajot et al., 2003), overall resulting in histone displacement/replacement and/or in the next chromatin condensation (Pivot-Pajot et al., 2003). Interestingly, in trout testis it has been demonstrated that hyperacetylation of H4 in condensing spermatids occurs exactly at the same four lysine residues that lead to transcription-associated acetylation (Christensen et al., 1984). Therefore, CB_1 might regulate both *Tnp2* transcription and

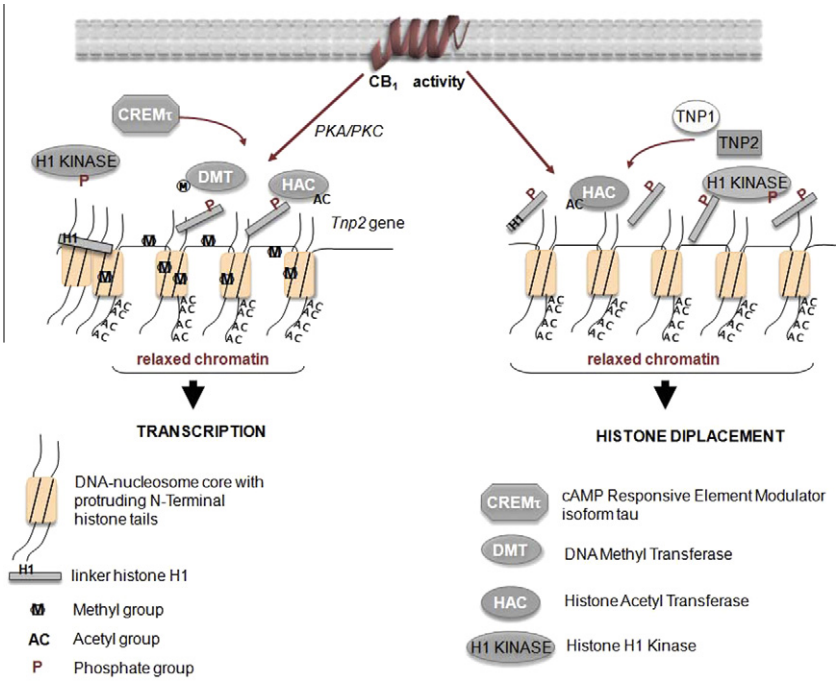

Fig. 2. CB_1 regulates *Tnp2* transcription and histone displacement in mouse round and elongating spermatids, respectively. The scheme describes an hypothetical model in which some of the factors involved in both events are potential targets of CB_1 (in white). Covalent modifications relax chromatin and, in round spermatids, drive the cAMP-dependent CREMt recruitment to the *Tnp2* promoter region (left), while in elongating spermatids they facilitate histone displacement through a DNA-binding competition mechanism involving TNP1 and TNP2 (right). It is possible that factors and enzymes may work in combination in a multiprotein complex, in order to relax chromatin and bring about distinct downstream events (transcription and histone displacement).

histone displacement occurring in spermatids, probably through acetylation. An hypothetical model is depicted in Fig. 2. This hypothesis is in keeping with the emerging idea that a unique machinery, using different combinations of the same multiprotein complexes, may act to remodel chromatin and, therefore, to drive gene expression or packaging (Strahl and Allis, 2000).

6. Conclusions

In this review, we have summarized the current knowledge of the cross-talks between eCBs, steroid hormones and cytokines in female and male fertility, along the evolutionary axis. The available data suggest a tight control of this network of signals for successful implantation and pregnancy maintenance in invertebrates, vertebrates and mammals. They also support the view that the endogenous tone of eCBs, and in particular of AEA, needs to be tightly controlled in a proper spatio-temporal sequence, in order to allow successful implantation and pregnancy maintenance. In this context, any substance able to reduce AEA content, by modulating the activity of its metabolic enzymes (i.e., by down-regulating AEA synthesis through NAPE-PLD, and/or more importantly AEA degradation by FAAH), holds the promise to become a useful fertility enhancer. FAAH is indeed the key-regulator of AEA levels *in vivo*, and has been shown to direct several pre-implantation events, from the development of the fertilized egg to its oviductal transport. In humans AEA levels in blood inversely correlate with FAAH activity in peripheral lymphocytes, and interestingly FAAH activity and expression are under the control of Th1/Th2 cytokines and leptin, as well as of sex hormones like progesterone and estrogen. It remains to be established if and to what extent the epigenetic control of *faah* gene expression, e.g. through DNA methylation or histone acetylation, can contribute to the modulation of enzyme activity by cytokines and/or steroids. Taken together, FAAH and AEA assays in peripheral blood cells (easily accessible for routine immunochemical analyses such as enzyme-linked immunosorbent assay, ELISA) hold the promise to become useful in predicting the outcome of assisted and natural reproduction in pregnant women. Thus, it is highly advisable that public and/or private funding agencies support research efforts aimed at improving our understanding of fertility defects, so that human reproductive potential may be restored, and then preserved against (environmental) insults. Once the value of "natural" reproduction is re-established, our reliance on assisted reproduction technologies could diminish, and appreciation of the underlying mechanisms and safety be improved. On a final note, it seems noteworthy that the interplay between different signals demonstrated in reproductive events, and kept along the evolutionary axis, might also take place in other body organ/system, for instance in the brain during neuroinflammation. In this case, eCBs control neurotransmission through a tight exchange of information with cytokines released from the microglia, and with neurosteroids acting on neurons (Rossi et al., 2010). If crosstalks between cytokines, steroid hormones and eCBs have a more general validity, the lessons learned from investigations into reproductive defects might have a broader interest, opening up avenues to novel therapeutics able to combat several central and peripheral human diseases.

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