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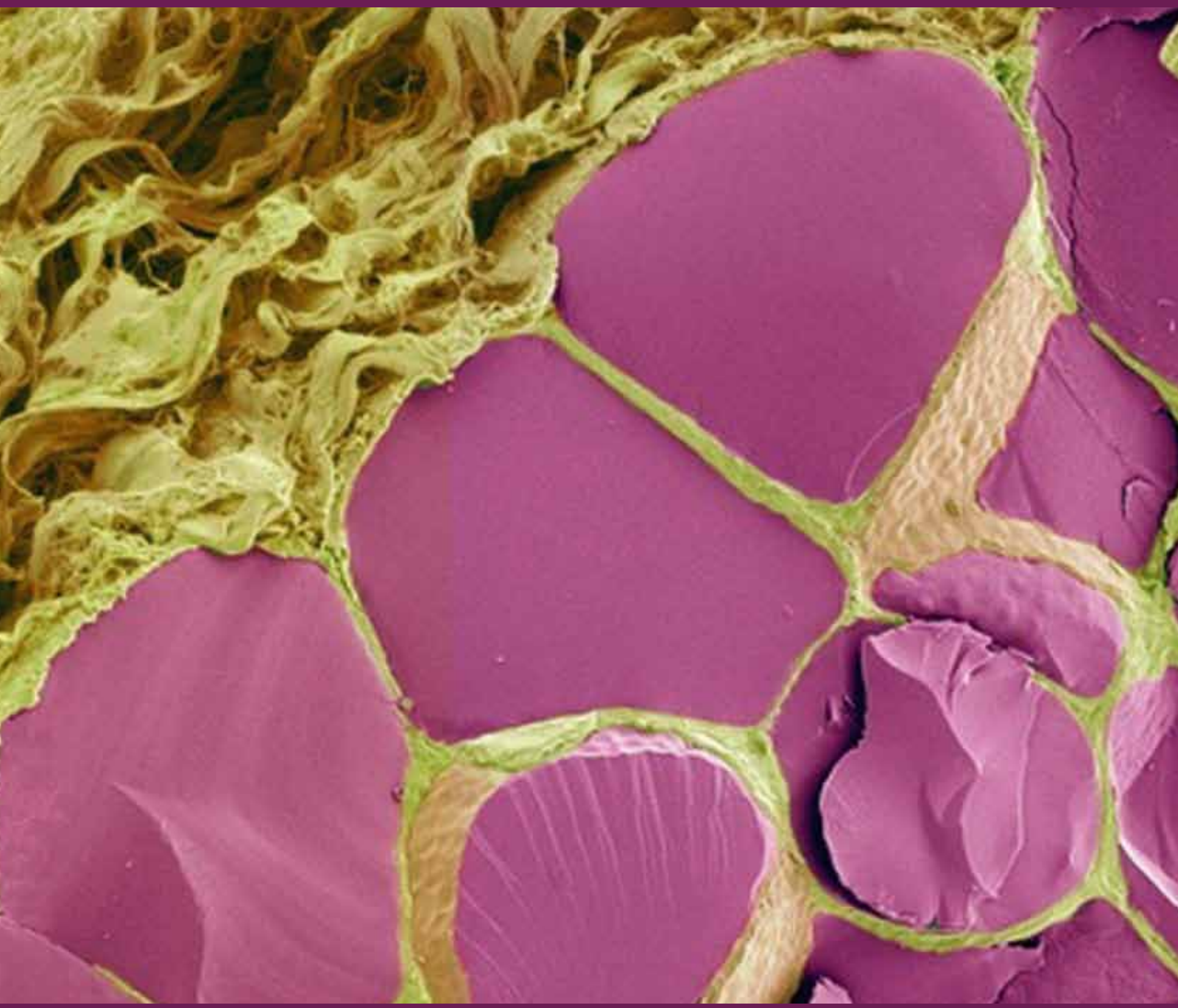
Endocannabinoids and Reproduction

Guest Editors: Rosaria Meccariello, Natalia Battista, Heather B. Bradshaw, and Haibin Wang



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Editorial Board

Contents

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Editorial
Endocannabinoids and Reproduction

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Infertility is a worldwide reproductive health problem whose consequences have deep psychological and social impact in health, demographic change, and wellbeing. Thus, the knowledge of basic, conserved modulators of reproduction might contribute to the discovery of new potential target for the exploitation of drugs to treat infertility in humans. Besides the well known effects of endocannabinoids in the control of pain and visceral functions, in the last decade the deep involvement of endocannabinoid system in the control of reproductive functions in both males and females emerged. In fact, endocannabinoids, endogenous lipids that bind to cannabinoid receptors, modulate reproductive axis at both central and local level. Endocannabinoid signalling is critical for gonadotropin release and sex steroid biosynthesis, for the formation of functional male and female gametes, for fertilization, preimplantation embryo development, implantation, and postimplantation embryonic growth, and for labouring delivery as well. Endocannabinoids are also involved in the neuroendocrine control of reproduction functions through the modulation of stress, food intake, appetite, and sexual behaviour. Recently, new roles in sperm "startup" and gamete quality emerged and impairment of the physiological endocannabinoid tone and signalling has been reported in clinical cases of human infertility. Our hope is that this special issue may be important and timely since a deep knowledge of endocannabinoid system in reproduction might open new perspectives in clinical applications, pointing to endocannabinoid signalling as a novel target for correcting infertility, and for improving reproductive health in humans.

The papers submitted to this special issue in the International Journal of Endocrinology take into account the multifaceted aspects of reproduction. Basic and evolutionarily conserved mechanisms of endocannabinoid activity in reproduction have also been included. E. Cottone et al. submitted an extensive review on the role of the endocannabinoids in the central regulation of reproduction in nonmammalian vertebrates, especially fish and amphibian; they correlate the morphofunctional distribution of cannabinoid receptors to key molecules involved in the control of reproductive functions, such as Gonadotropin Releasing Hormone (GnRH), dopamine, aromatase, and pituitary gonadotropins.

The role of the endocannabinoid system as an ancient signalling system, that has been evolved over 500 million years, is highlighted in the paper of R. Chianese et al. that reports the presence of endocannabinoids in a nonmammalian model, the anuran amphibian *Rana esculenta*, and the functional crosstalk between these bioactive lipids and the GnRH system, shedding light on their different regulation in the brain and in the testis. In particular, a new role for vanilloid receptor emerged in the modulation of testicular GnRH system (both ligands and receptors) providing evidence that an opposite regulation occurs *via* type-1 cannabinoid receptor and vanilloid receptor signalling.

The paper of G. Cacciola et al. is a very interesting and comprehensive review on the pivotal role played by type-1 cannabinoid receptor in spermiogenesis and on its involvement in the chromatin remodelling process that might affect negatively the sperm quality. The emerging evidences

on estrogen activity in sperm quality are deeply detailed in a knock-out animal model, opening new intriguing perspectives in the clinical practice for the treatment of male infertility.

The deep involvement of endocannabinoid signalling in driving the neurophysiological outcomes of mating behaviours has been reported in the research article submitted by J. M. Stuart et al. By means of lipidomic techniques, this group demonstrates that the levels of endocannabinoids, prostaglandins, and *N*-acylethanolamines rapidly change in specific brain areas in relationship to different mating strategies providing evidence that the endogenous cannabinoid systems is rapidly modified in response to changes in environment.

In the context of the signalling events of pregnancy, B. M. Fonseca et al. presented the most recent progress on the endocannabinoid regulatory functions during decidualization and placentation. They summarized that while the endocannabinoid machinery was found to be expressed in decidual and placental tissues, aberrant endocannabinoid signalling was associated with pregnancy disorders, highlighting the content that the endocannabinoid signalling is a potential player coordinating successful decidualization and placentation.

All of these papers have illustrated the potential regulatory interactions of sex steroid hormones with the endogenous cannabinoid system and how they allow reproduction to optimally function. The outstanding review by T. Ayakannu et al. that details the latest understandings of how sex steroids and the endogenous cannabinoid system work synergistically in a variety of cancers truly illustrates how the malfunctions of these signalling mechanisms can have dire effects. Using data from prostate, breast, and endometrial cancers, the review from Konje's group provides compelling evidence that to understand how to fix pathophysiology we must work towards understanding basic functioning physiology in the first place.

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Review Article

Updates in Reproduction Coming from the Endocannabinoid System

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The endocannabinoid system (ECS) is an evolutionarily conserved master system deeply involved in the central and local control of reproductive functions in both sexes. The tone of these lipid mediators—deeply modulated by the activity of biosynthetic and hydrolyzing machineries—regulates reproductive functions from gonadotropin discharge and steroid biosynthesis to the formation of high quality gametes and successful pregnancy. This review provides an overview on ECS and reproduction and focuses on the insights in the regulation of endocannabinoid production by steroids, in the regulation of male reproductive activity, and in placentation and parturition. Taken all together, evidences emerge that the activity of the ECS is crucial for procreation and may represent a target for the therapeutic exploitation of infertility.

1. Introduction

Reproductive functions are under a fine regulation exerted at multiple levels along the hypothalamic-pituitary-gonadal axis. The formation of high quality gametes, followed by a successful pregnancy event, is the result of deep cell to cell communications. In this respect, the list of potential modulators of reproductive activity is still growing. In the last two decades the upcoming role of lipid mediators that share some of the effects with delta-9-tetrahydrocannabinol (Δ^9-THC), the active principle of marijuana plant, *Cannabis sativa,* emerged. These bioregulators, collectively named endocannabinoids (eCBs), are amides, esters, and ethers of long-chain polyunsaturated fatty acid and have been detected in most reproductive tissues and fluids [1–3].Besides ligands, a wide range of receptors, biosynthetic and hydrolyzing enzymes, and putative membrane transporters (EMT) all together form the endocannabinoid system (ECS) (Figure 1), a master system that is deeply involved in the central and local control of male and female reproduction. Since their discovery, research made giant strides in the comprehension of physiological, cellular, and molecular events in reproduction driven by eCBs. Many inputs in the field came from studies conducted in invertebrates and nonmammalian vertebrates, indicating that ECS is an evolutionarily conserved master system deeply involved in the control of reproductive functions. Thus, the aim of this review is just to provide new insights into the complex field of eCBs and reproduction.

2. ECS and Reproduction: An Overview

Smoking marijuana has always represented a warning for the long lasting effects not only on physical and mental performances but also on the reproductive events. The effects of Δ^9-THC on pregnancy was highlighted for the first time at the beginning of 1970 [4] and, since then, numerous papers have been focused on the potential aversive effects caused by

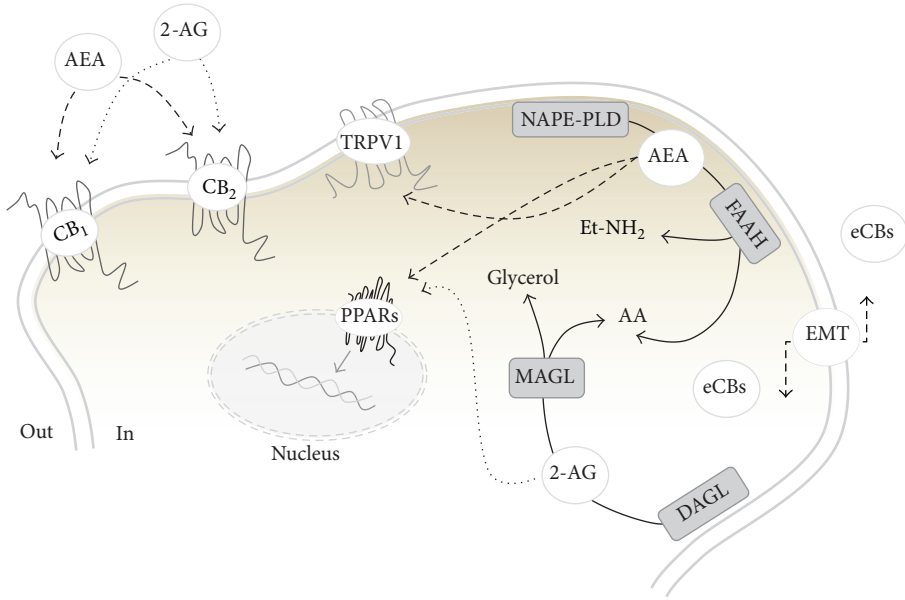

Figure 1: Schematic representation of the ECS. *N*-arachidonoyl-ethanolamine (AEA) is mainly produced by the activity of an *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD), whereas its degradation is due to a fatty acid amide hydrolase (FAAH), which releases ethanolamine (Et-NH_2) 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 cellular uptake from the extracellular to the intracellular space is ascribed to a purported "endocannabinoid membrane transporter (EMT)" that is likely to take up both AEA and 2-AG. Both eCBs trigger several signal transduction pathways by acting at their targets, CB_1, CB_2, GPR55, and nuclear peroxisome proliferator-activated receptors (PPARs). AEA, but not 2-AG, binds intracellularly also Transient Receptor Potential Cation Channel type 1 (TRPV1).

the use of recreational drugs during gestation and in offspring born from cannabis users.

In 1992, *N*-arachidonoylethanolamine (also known as anandamide, AEA), a cannabinoid-like compound, was identified to compete with exocannabinoid ligands for type 1 and type 2 cannabinoid receptors (CB_1 and CB_2, resp.) [5, 6] and, few years later, the group of Dr. Schuel and Dr. Das reported the ability of AEA to affect negatively both male and female fertility [7, 8]. In the next years, the endocannabinoid signaling was demonstrated to play a key role in the preimplantation embryo development [9–12] and it was supposed that AEA content could be critical for a timely embryo implantation [13]. Nowadays, it is clear that, in order to guarantee a receptive uterine environment, AEA levels must be kept low [14], and this is accomplished through a tight regulation mediated by an *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD), the enzyme responsible for its synthesis, and fatty acid amide hydrolase (FAAH), in charge of its degradation to arachidonic acid and ethanolamine [15–18]. Further confirmations on the harmful effects caused by high AEA levels for a normal pregnancy outcome were obtained from measurements of AEA levels in plasma samples from women with normal menstrual cycle and laboring patients [19] and NAPE-PLD and FAAH analysis performed on human placenta [20].

In the same timeframe, several experimental studies highlighted the ability of AEA in regulating sperm functions required for fertilization [7, 21], by reducing sperm motility, inhibiting capacitation-induced acrosome reaction and mitochondrial activity [21, 22]. Cannabinoid and vanilloid (in particular transient receptor potential cation channel type 1, TRPV1) receptors mediate the physiological action of AEA with double effects. On the one hand, AEA binding to CB_1 affects the capacitation process in mammals [23–25]; on the other hand activation of the intracellular site of TRPV1 inhibits spontaneous acrosome reaction in porcine [23] and human sperm cells [26]. Lately, TRPV1-mediated activities of AEA were also reported in capacitated mouse spermatozoa (SPZ), where elevated intracellular levels of AEA are due to a reduced FAAH activity [27]. In the last decade, many studies have been focused on the involvement of the CB_1/CB_2-signaling in follicle maturation, oviductal-uterine embryo migration, implantation and (neuro)development, placentation, and parturition onset, showing that any aberration of endocannabinoid signaling can severely affect these processes (for a review see [28]). Specific and selective antagonists of CB_1/CB_2 and/or CB_1/CB_2 knockout mice ($CB_1^{-/-}$ and $CB_2^{-/-}$, resp.) have always been useful tools that allowed researchers to better understand which target is critical to achieving correctly all reproductive events from sperm-oocyte fusion to the birth of offspring. In this context, we should recall that short and long term exposure to HU210, a selective agonist for CB_1 and CB_2, showed how the deregulation of the ECS markedly reduces total sperm count, depletes spermatogenic efficiency, and impairs sperm motility [29]. A recent paper by Fonseca and workers proposed a functional role of GPR55

receptor in the uterine remodeling and in immune processes activated during fetoplacental development [30]. The differential spatiotemporal expression pattern of GPR55 found in decidual and natural killer (NK) cells might implicate possible interactions of this target with other endocannabinoid-like compounds (i.e., *N*-palmitoylethanolamide), since the main eCBs lack affinity for this receptor [31].

Besides AEA, 2-arachidonoylglycerol (2-AG) is the other main representative of this family of bioactive lipids and its role in fertility seemed unknown up to few years ago, when its impact on mouse spermatogenesis [32], fetoplacental development [33], epididymal start-up [34], and mouse sperm capacitation [27] has been remarked. In fact, it has been reported that transcriptional and translational levels of 2-AG synthesizing (diacylglycerol lipase, DAGL) and hydrolyzing enzymes (monoacylglycerol lipase, MAGL) are finely tuned in various processes of male and female reproduction. This metabolic equilibrium is required in order to guarantee an appropriate 2-AG tone in reproductive stages; in this context, low 2-AG levels were detected in seminal plasma of infertile men, suggesting a reduced sperm fertilizing capacity through a mechanism yet to be explored [35].

To date, we have good knowledge about the existence of a definite network, including eCBs, hormones, prostaglandins, and cytokines that warrant a successful pregnancy in animals and humans. In particular, the involvement of the eCBs in lymphocyte-mediated control of the hormone-cytokine crosstalk at the fetal-maternal interface was reported for the first time by the group of Dr. Maccarrone, showing that FAAH activity and protein were lower in women who miscarried and who underwent IVF treatment [36–38], whereas cannabinoid receptor binding and AEA-carrier were not altered during gestation [36, 39, 40]. Moreover, it seems that steroid hormones primarily regulate AEA levels, with estradiol (E_2) increasing the levels and progesterone suppressing them, and that an *equilibrium* between profertility Th2 cytokines and antifertility Th1 cytokines is requested to establish blastocyst implantation, trophoblast growth, and pregnancy maintenance. On the male side, follicle stimulating hormone (FSH) regulates the expression of FAAH in Sertoli cells through an estrogen-mediated pathway [41], and, in turn, E_2 levels induce, directly or indirectly, epigenetic modifications at the *FAAH* promoter site [42] and influence, via CB_1 [43], chromatin remodeling of spermatids with a clear impact on spermatogenesis [44, 45]. A schematic chronological overview of local activity of eCBs in male and female reproduction is depicted in Figure 2.

3. Evolutionary Aspects of ECS

The study of physiological mechanisms by comparative approach is a fundamental tool to build general models. Key events in reproduction such as the activity of estrogen—classical female hormone—in spermatogonial proliferation [46] or nongenomic action of steroids themselves have been firstly discovered in nonmammalian species and then confirmed in mammals [47, 48].

In this respect, enzymes involved in endocannabinoid biosynthesis and/or degradation occur throughout the animal kingdom including deuterostomian (i.e., sea urchin), protostomian (i.e., crustaceans and nematodes), and basal (i.e., cnidarians and placozoans) invertebrates [49]. Conversely, molecular cloning of CB_1/CB_2 receptor orthologs has produced positive results only in urochordates (the sea squirt, *Ciona intestinalis*), in cephalochordata (the amphioxus, *Branchiostoma floridae)*, in nonmammalian vertebrates (fish, amphibians, reptiles, and birds), and in mammals, with duplication of CB_1 or CB_2 genes found in fish [49–51]. Thus, given that CB_1/CB_2 are unique to chordates, the molecular nature of endocannabinoid signaling in noncordate invertebrate is currently under investigation and may be related to primitive neuronal functions; conversely, the appearance of multiple receptors and receptor splicing forms coming from invertebrates to humans may indicate the subsequent occurrence of functional partitioning. However, the recent identification of candidate *TRPV1* orthologs in the genome of the sea urchin *Strongylocentrotus purpuratus* [52] and of the annelid, the leech *Hirudo medicinalis* [53], confirms the existence of an ancient non-CB_1/CB_2-mediated endocannabinoid signaling.

The functional conservation of ECS is not limited to the central nervous system (CNS) but also extends to the modulation of gonadal functions. The first direct evidence of endocannabinoid activity on male reproduction came from studies conducted in sea urchin to assess the mechanisms of acrosome reaction and polyspermy ([54] for review). In this respect, the endocannabinoid-signaling similarity in neurotransmitter release and acrosomic granule exocytosis let Meizel in 2004 speculate that the sperm may be a "neuron with a tail" adapted to fertilize egg cell [55]. However, in the last 10 years, evidences of endocannabinoid activity have been provided in testis and/or sperm of both invertebrates and vertebrates, including sea urchin, fish, frogs, mice, rats, boars, bulls, and humans [7, 21, 23, 26, 56–63]. AEA inhibitory effects on sperm motility and acrosome reaction have been conserved from sea urchin to mammals and elsewhere properly reviewed [54, 56]. A retrograde AEA signaling is involved in sperm-egg interaction in sea urchin [54], whereas CB_1 and/or CB_2 are differentially expressed in fish [64, 65] and frog ovary [60]. Interestingly, CB_1 signaling is likely involved in the process of testicular regression in the gilthead seabream, *Sparus aurata*, a hermaphrodite species in which the gonadal tissues first develop as testes and then as functional ovary [66]. As described in paragraph 6, in mammals—human included—most female reproductive events, from ovogenesis and fertilization to successful pregnancy and parturition, require a functional endocannabinoid signaling, once again confirming the conservation of functions related to reproduction.

4. eCBs, Hypothalamic GnRH, and Steroids

Three main lines of evidence suggest that the eCBs and gonadal hormone signaling systems interact. (1) eCBs and their receptors are present throughout the hypothalamic-pituitary-gonadal (HPG) axis, (2) changes to the ECS cause

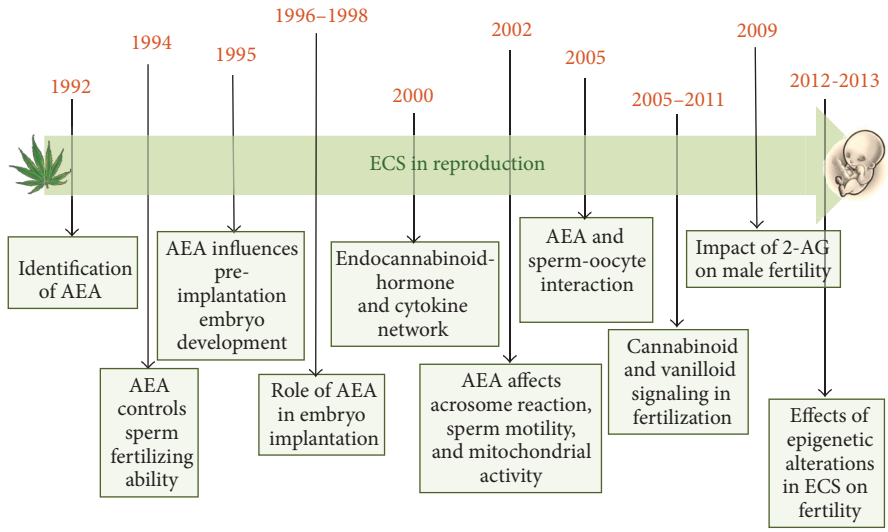

Figure 2: Major breakthroughs in male and fertility reproduction.

changes in the HPG and changes in the HPG axis can affect the expression of eCBs, and (3) the ECS mediates behaviors, which are also mediated by gonadal hormones, such as motivation or reproduction (reviewed in [67]).

The CB_1 partial agonist, Δ^9-THC, has been implicated in negative reproductive outcomes, including the inhibition of ovulation in women [68] and lower serum luteinizing hormone (LH) and testosterone (T) in men [69]. The inhibitory effects of eCBs on gonadal hormone production suggest that eCBs help regulate this circuitry. Leydig cells in the testes contain CB_1. $CB_1^{-/-}$ mice show reduced serum T levels and abnormal Leydig cell development. These results suggest that endocannabinoid signaling is essential for the organization of the reproductive system [70]. Upcoming observations in the hypothalamic control of reproductive functions and gonadal sex steroid production are described below.

4.1. Insights into the Hypothalamic Control of GnRH Activity. In the CNS eCBs are well known retrograde signals that modulate neuronal communications inhibiting presynaptic release of neurotransmitters including γ-aminobutyric acid (GABA) and glutamate. Postsynaptic synthesis of 2-AG or AEA is a phylogenetically widespread phenomenon described from mammals to annelids [49] which modulates neural activity through cannabinoid or vanilloid receptors. Brain maps of CB_1, CB_2, and TRPV1 have been provided in fish, amphibians, and mammals [57, 58, 71–73], with CB_1/TRPV1 colocalization in specific hypothalamic nuclei in mammalian brain [72]. A master system in the central control of reproductive activity is the gonadotropin releasing hormone (GnRH), a hypothalamic decapeptide responsible for gonadotropin discharge and steroid biosynthesis [47, 48, 74]. Inhibitory effects of phytocannabinoids, cannabinoids and eCBs upon the endocrine control of reproduction have been largely described in the literature [51, 75]. Immortalized neuronal cell lines (GT1) possess a complete ECS and are themselves targets of endocannabinoid signaling, since the *in vitro* activation of cannabinoid receptors suppresses the pulsatile release of GnRH [76]. Furthermore, in the mediobasal hypothalamus of male rats, AEA intracerebroventricular injection suppresses GnRH release [77]. The importance of CB_1 in negative modulation of reproductive axis has been demonstrated by altered GnRH signalling in $CB_1^{-/-}$ mice [44]. However, 2-AG is able to suppress LH secretion in wild-type but not in $CB_1^{-/-}$ mice [78], whereas AEA decreases LH levels also in $CB_1^{-/-}$ [78]. Thus, receptors other than CB_1—that is, TRPV1—might be involved in such a modulation.

Despite these observations, only recently has the mechanism involving direct/indirect endocannabinoid activity on the hypothalamic GnRH secreting neurons been provided. From fish to mammals GABA is a modulator of GnRH secreting neurons in the adult ([79, 80] and references inside). Metabolic, sex steroid, and circadian cues are usually conveyed to the GnRH system; involvement of metabotropic glutamate receptor located on astrocytes [81], eCBs [80] or GnRH itself [82] has been described in these routes. A 2-AG dependent inhibitory activity on the release of GnRH has been recently proposed in male mice [80]. At the molecular level, GnRH secreting neurons release 2-AG that directly acts as a retrograde signal on CB_1 receptor located on GABAergic presynaptic neurons and inhibits the release of GABA (Figure 3(a)). As a consequence, GABA receptors located on GnRH secreting neurons are not activated and GnRH is not released [80]. Since astrocytes express CB_1 [83] and eCBs can alter astrocyte transmitter uptake [84], a simplified alternative mechanism involving endocannabinoid-dependent modulation of glial cell functions (i.e., prostaglandin production) has been postulated [85]. In such a model, glutamate release by GnRH neurons may stimulate astrocyte to produce prostaglandins; these, in turn, may induce the synthesis of eCBs and/or the exposure of presynaptic CB_1, thus modulating GABA release (Figure 3(b)).

Functional crosstalk among eCBs and GnRH neuronal systems has been described also in fish and amphibians [51,

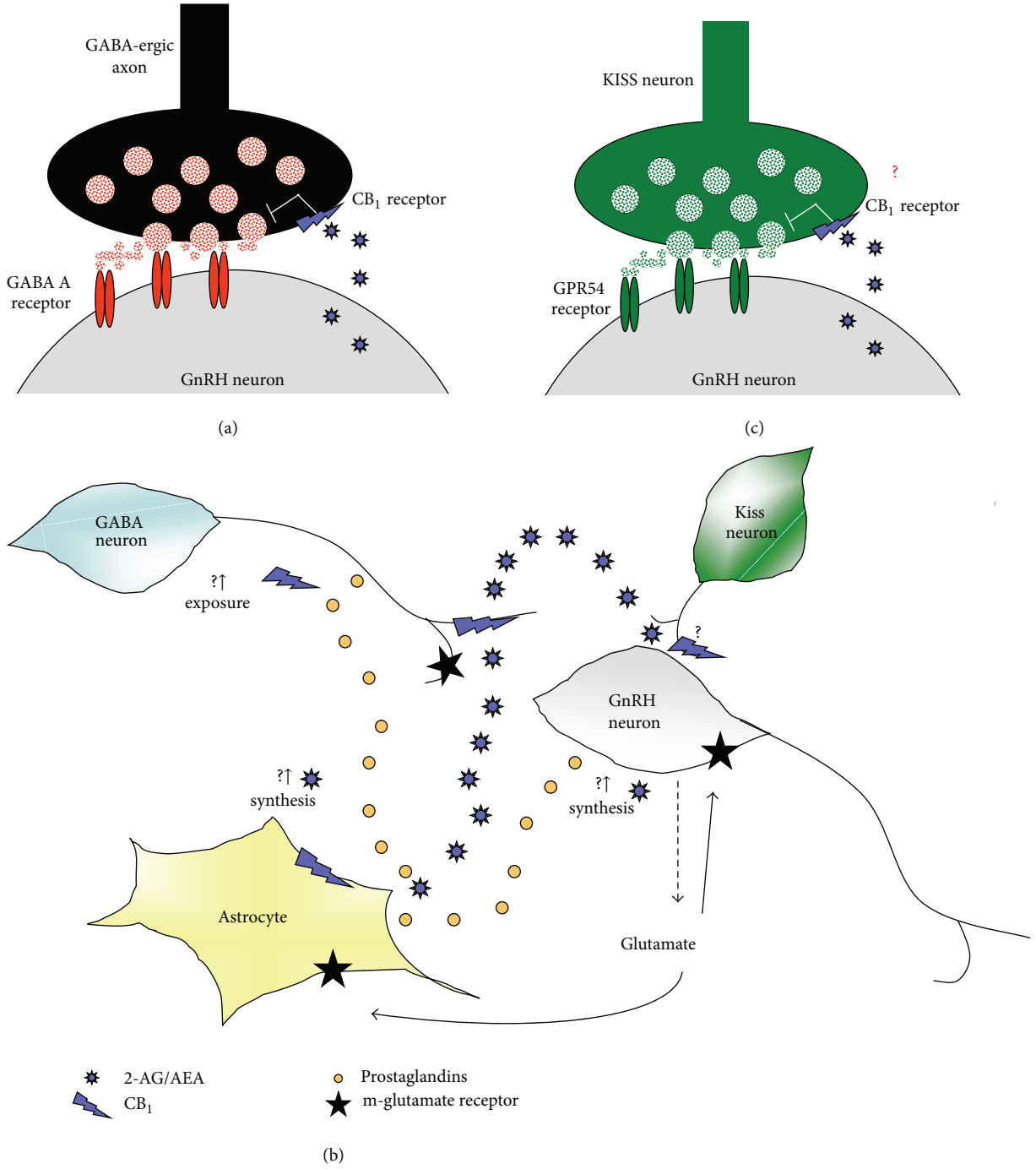

Figure 3: Possible mechanisms for the modulation of GnRH secreting neurons by eCBs. (a) GABAergic modulation of GnRH secreting neurons. GnRH secreting neurons release eCBs that, as retrograde signals, directly act on CB_1 located on GABAergic presynaptic neurons and inhibit the release of GABA; as a consequence, GnRH secreting neurons do not receive GABAergic input and do not release the GnRH. (b) Possible involvement of glial cells in eCBs/GABA/GnRH circuitry. Glutamate release by GnRH neurons may stimulate astrocytes to produce prostaglandins which in turn induce the synthesis of eCBs and/or the exposure of presynaptic CB_1, thus modulating GABA release. (c) Hypothesis: are there neuronal systems other than GABAergic able to modulate endocannabinoid/GnRH crosstalk? Kisspeptins stimulate gonadotropin discharge in several species modulating the activity of GnRH secreting neurons via the activation of GPR54 receptor located on GnRH neurons. Might AEA also act as retrograde signal upon kisspeptin neurons in order to suppress GnRH secretion?

57, 86–88], indicating that this is an evolutionarily conserved master system. In nonmammalian vertebrates, GnRH saga is more intricate since, as in humans, at least two distinct GnRH molecular forms (i.e., GnRH-I and GnRH-II) and one GnRH receptor (GnRH-R) have been described [47]. New insights in the central control of male reproduction emerged from nonmammalian vertebrates. CB_1 has been localized in fish forebrain—the encephalic area mainly involved in the control of GnRH secretion and gonadotropin discharge— but in teleosts colocalization was observed in GnRH-III secreting neurons [57]. In the diencephalon of the anuran amphibian the frog *Rana esculenta*, CB_1 dependent modulation of GnRH system expression rate (both ligands and receptors) has been reported [87, 88]. In particular, in the basal hypothalamus, *via* CB_1, AEA significantly decreases *GnRH-I* and *GnRH-II* expression and upregulates *GnRH-RI* and *GnRH-RII* mRNA without any effect upon *GnRH-RIII* [87]. Twenty percent of hypothalamic GnRH-I secreting neurons possess CB_1, and buserelin, a long acting GnRH analog, increases CB_1 expression and inhibits those of *GnRH-I* [88]. The opposite profiles of CB_1 and GnRH-I proteins [86] seem to confirm such AEA-dependent self-modulation route in which GnRH secreting neurons might produce eCBs to suppress the production of GnRH.

Conversely, as in the mouse, most frog GnRH-I secreting neurons are surrounded by CB1 immunopositive fibers, [76, 88] confirming the conservation of endocannabinoid-dependent retrograde signalling. GABAergic transmission, however, is not the only neuronal system that might be involved in the modulation of endocannabinoid/GnRH crosstalk. In this respect, one of the possible candidates is the kisspeptin signaling system. Kisspeptins, RFamide peptides encoded by the *kiss1* gene, stimulate LH and, to a lesser extent, FSH secretion in several species modulating the activity of GnRH secreting neurons *via* the activation of GPR54 receptor located on GnRH neurons [89]. Preliminary observations in male frogs indicate that *in vivo* administration of AEA suppresses the expression of diencephalic GPR54, turning off the GnRH system and steroidogenesis (Meccariello R., personal communication). Thus, it is not excluded that AEA might also act as retrograde signal upon kisspeptin neurons (Figure 3(c)). Interestingly, in both male and female, kisspeptin neuronal activities are strongly involved in steroid-dependent feedback mechanisms [90, 91].

As described in the next paragraph, AEA-dependent suppression of GnRH release is reversed by E2 administration in female rats [77] whereas, steroids represent the major factor in negative feedback mechanisms in males. Thus, in addition to E2-dependent modulation of endocannabinoid tone via FAAH modulation [42], the investigations concerning the possible crosstalk between kiss/GnRH/cannabinergic neurons [92] might open new insights in the molecular mechanisms of gonadal steroid feedback.

4.2. Interplay between Sex Steroids and eCBs. In the HPG axis, CB_1 regulates sex hormone production. Intracerebroventricular injection of AEA reduced GnRH release in male and ovariectomized (OVX) female rats. However, in the same experiment, estrogen treated females experienced increased plasma LH after AEA injection. Therefore, estrogen possibly reverses the inhibitory effects of AEA [77]. Steroid hormones regulate CB_1 expression in the pituitary [93]. In the rat pituitary there are sex differences; however the same may not be the case for humans. Therefore, in humans, nonsteroid signaling molecules may influence CB_1 expression in the pituitary [94]. The possible direct activity of eCBs upon pituitary gland—evaluated in terms of ECS characterization as well as of eCBs dependent secretion of anterior pituitary hormones—has been suggested in vertebrates, but this issue is still controversial since species specific activities have been observed (for a recent review see [51]). In addition to the pituitary, cannabinoids and gonadal steroid function are linked in the hypothalamus. GnRH neurons in the medial preoptic area can synthesize endogenous cannabinoids, which exhibit negative feedback on GnRH release. However, it has also been noted that relatively few GnRH neurons contain CB_1 mRNA, so eCBs must be exerting influence over neighboring cells [76]. Providing further evidence for a link between eCBs and sex hormones, endocannabinoid levels in the rat hypothalamus have been shown to fluctuate over the hormonal cycle. AEA levels reached a maximum during diestrous in the hypothalamus. Also, males showed significantly lower levels of 2-AG than females [95].

In a relationship critical to female reproductive success, an estrogen response element exists in the *FAAH* gene sequence. When estrogen binds to this response element, *FAAH* gene transcription is downregulated and AEA levels should remain elevated [96]. High doses of estrogen can have an anxiolytic effect. Hill et al. proposed that the anxiolytic effect is mediated by alterations in FAAH [97]. It may be that eCBs regulate the onset of puberty. eCBs may contribute to the peripubertal inhibition of GnRH neurons. Lopez hypothesizes that estrogen release from the ovaries at the time of puberty helps remove the endocannabinoid "brake" on reproductive functioning [69]. The overall relationship between estrogen and eCBs can be described as "bidirectional." In one direction, endocannabinoid activity downregulates HPG axis activity, leading to reduced estrogen levels. In contrast, decreasing FAAH activity and modulating CB_1 expression, estrogen up- regulates AEA production [67].

In addition to estrogen interactions, endocannabinoid activity attenuates progesterone release from the corpus luteum. Administration of AEA to pregnant rats caused a decrease in serum progesterone, as well as serum LH. Therefore, it appears that eCBs regulate the release of progesterone in two ways: (1) by directly binding onto receptor sites in the corpus luteum and (2) by directly controlling LH release in the CNS [98]. Like estrogen, progesterone can interact with a promoter region in the *FAAH* gene in that progesterone has been shown to increase *FAAH* expression in T-cells and human lymphoma U937 cells. In contrast, progesterone had no effect on *FAAH* expression in human neuroblastoma CPH100 cells [99]. Blocking progesterone receptors with antisense oligonucleotides eliminated the facilitating effect of Δ^9-THC on female rodent mating behavior. In addition,

blocking CB_1, using SR141716, and blocking dopamine (DA) D1 receptors, using antisense nucleotides, also eliminated the effects of Δ^9-THC on mating behavior [100]. Therefore, an interaction between progesterone, DA signaling, and cannabinoid signaling is necessary for female reproductive behavior. It is not known whether this could apply to human females.

As an example of how the ECS is involved in sexual motivation that is driven by sex steroids, studies with phytocannabinoids have been shown to affect sexual motivation. For example, exogenous CB_1 agonist treatment in male rodents attenuates both appetitive and consummatory aspects of sexual behavior. However, studies in humans have been less conclusive. Men who use marijuana show great variation in sexual response [67]. For females, the effects of cannabinoids on sexual motivation and performance are much less clear. It appears that acute blocking Δ^9-THC administration in female rats increased sexual receptivity at lower doses but decreased sexual receptivity at higher doses [101]. These findings are similar to Δ^9-THC's effects on anxiety. Estrogen and DA have a complex relationship. Estrogen enhances dopaminergic activity in the nucleus accumbens via enhanced DA release and downregulates autoreceptor inhibition. Thus, eCBs could elicit a strong DA response in the nucleus accumbens and striatum. The effect could possibly overpower the motivational value of sex steroids and increase the likelihood of mate-seeking behavior [67].

On average, Δ^9-THC affects males and females differently. This is not to say that there is not a large variation in response within the sexes, but there have been enough differences shown to suggest gonadal steroid modulation of exogenous cannabinoid reward. After showing that CB_1 agonists induce stronger analgesic and motor suppressing effects in female rats than in male rats, Craft and colleagues investigated whether activational effects of gonadal hormones were responsible for these differences. In males, T attenuated the motor effects of Δ^9-THC. In females, estrogen was linked to increased antinociception. OVX females showed less analgesia in response to Δ^9-THC than OVX females given estrogen. In addition, intact estrous females showed more antinociception than diestrous females [102]. Likewise, Fattore and colleagues determined that female rats found the CB_1 agonist WIN55,212-2 (WIN) more rewarding than male rats. Compared to male rats and OVX females, female rats showed faster acquisition of WIN self-administration and higher overall drug intake. However, gonad-intact female rats showed faster extinction for WIN self-administration. One explanation for Fattore's work is that there is a higher hedonistic value on cannabinoids for females [103]. On the other hand, estrogen may attenuate the disruptive effects of Δ^9-THC on learning, leaving female rats less affected by a negative side effect [104]. It is possible that the greater response to Δ^9-THC seen in female rats is due to estrogen modulation of DA signaling in the ventral tegmental area and nucleus accumbens. However, precise interactions between cannabinoids and estrogen are not well understood [67].

5. Insights in the Progression of the Spermatogenesis and the Acquisition of Sperm Functions

The suppression of LH levels in marijuana smokers as well as in animal models is related to the impairment of hypothalamic GnRH secretion ([51, 105, 106] for reviews). However, the presence of eCBs in reproductive fluids [1, 107, 108] and the ubiquity of testicular endocannabinoid activity are critical for the activity of Sertoli and Leydig cells, for germ cells progression and sperm quality [51, 105, 106].

Interstitial Leydig cells were the first target of CB_1 activity to be identified [70, 109]. Such CB_1 dependent modulation has been confirmed in $CB_1^{-/-}$ mice, where a decreased number of Leydig cells [110] and low estrogen levels [44] have been observed. Consistingly, also in nonmammalian vertebrates *CB_1* mRNA [111], but not CB_1 protein [59], has been localized in interstitial compartment. In the germinal compartment AEA reduces the spermatogenetic output by inducing apoptosis of Sertoli cells [41], a process reversed by FSH-dependent activation of aromatase and by E_2-dependent activation of FAAH [41, 42]. The involvement of endocannabinoid signaling in the progression of spermatogenetic stages has been only recently elucidated. In *Rana esculenta* increasing levels of CB_1 and FAAH have been detected in postmeiotic stages [59, 60], whereas *NAPE- PLD* has been detected by *in-situ* hybridization in Leydig cells and mitotic and early-meiotic stages [111]. In mice, CB_1, CB_2, and TRPV1 fluctuate in a stage specific manner [32, 109]. During the first spermatogenetic wave transcriptional downregulation of *CB_1* has been observed as soon as meiotic events occur [109] whereas the expression peak has been observed in postmeiotic stages [32, 109]. Besides the control of sperm function required for the fertilization (i.e., sperm motility, capacitation, and acrosome reaction) CB_1 activity in chromatin remodeling during the spermiogenesis has been recently reported [43–45]. Interestingly, CB_2, the receptor with higher affinity to 2-AG than CB_1, is highly expressed in mitotic/meiotic stages and the protein is retained in residual body at the end of the spermiogenesis [32], indicating CB_2 participation in meiotic progression. Consistently to the above observations, the levels of eCBs, especially 2-AG, decrease throughout the progression of spermatogenesis, being higher in the spermatogonia and reaching minimal level in spermatids [32]. Lastly, an intriguing matter of debate is the high expression of *TRPV1* observed in meiotic stages [32] and the massive germ cell depletion observed in mice lacking the receptor [112]. Thus, a possible role in the protection of meiotic stages has been postulated for TRPV1.

In such a context, the gonadal activity of neurohormones such as GnRH might be critical. In human testes two GnRH molecular forms and two GnRH-Rs have been detected [47, 113, 114], with *GnRH-RII* gene postmeiotically expressed in round and elongating spermatids. Beside a central query to be resolved is whether these transcripts are functional in sperm the mRNA levels of *GnRH-I, GnRH-II, GnRH-R, cytochrome P450 side-chain cleavage* (*CYP11A1*), and *3beta-hydroxy-steroid dehydrogenase type 2* enzyme (*HSD3B2*) as

well as the intratesticular T levels are significantly increased in patients with spermatogenic failure [115] indicating that testicular GnRH may locally act to regulate spermatogenesis and steroidogenesis in humans. Once again, data obtained in nonmammalian vertebrates as well as in mollusks confirmed the involvement of local GnRH in processes such as Sertoli-Leydig cells communication, estradiol dependent spermatogonia proliferation, and sperm release [47, 48, 74, 116, 117] whereas evidences in frogs and rats suggest the participation in sperm functions related to fertilization [111, 118]. Only recently has AEA-dependent modulation of local GnRH system been provided in amphibian testes. In fact, during the annual sexual cycle eCBs, via CB_1 activation, modulate GnRH activity in frog testes in a stage dependent manner [111]. When the upsurge of a new spermatogenetic wave occurs (February), *in vitro* AEA treatment specifically upregulates *GnRH-II* and *GnRH-RIII* mRNA and downregulates *GnRH-RII*. Conversely, in postreproductive period (June), *in vitro* AEA treatment significantly decreases *GnRH-I* and *GnRH-RII* mRNA, whereas it stimulates the transcription of *GnRH-II* and *GnRH-RI*. *GnRH/GnRH-R* localization in frog testes clearly indicates a functional distribution with a GnRH-I/GnRH-RII system mainly involved in the control of germ cell progression and Leydig/Sertoli cell communication and a GnRH-II/GnRH-RII system mainly involved in the control of sperm functions [111]. Thus, the differential AEA-dependent modulation of hypothalamic and testicular GnRH systems may reflect the functional divergence of GnRH molecular forms in testes. In such a picture, TRPV1 signaling should be also considered since, in postreproductive period, the activation of TRPV1 modulates the transcription of testicular *GnRHs* and of *GnRH-RI* and *GnRH-RII*, but in an opposite way compared to that of AEA ([119] in this issue).

Focusing on sperm functions, recently, a fertilization strategy adapted mechanism (external or internal fertilization) has been characterized for the control of sperm motility. In amphibians, exhibiting fertilization in aquatic environment, endocannabinoid activity in cloacal fluid may keep SPZ in a quiescent stage; the addition of CB_1 antagonist SR141716A [120] and/or the dilution of cloacal fluid soon increase SPZ motility, in a fashion that mimics the quick activation of SPZ in the aquatic environment during the mating [59]. Such a "dilution-activating mechanism," in mammals adapted into a 2-AG functional gradient inside the epididymus, the anatomical structure in which SPZ acquire the motility. High 2-AG level has been measured in the caput where SPZ are immotile whereas low level has been detected in the cauda, where SPZ acquire the ability to become motile [34, 121]. Accordingly, (1) the SPZ of $CB_1^{-/-}$ mice early acquire the motility in the caput epididymus [62], (2) the pharmacological inactivation of CB_1 drives the same effects observed in knockout animals, and (3) the administration of EMT inhibitors results in the falling down of cauda motile SPZ in normal mice [34].

A tight control of eCBs levels in SPZ and seminal plasma is required to assure the correct progression of multiple steps involved in the fertilization process. In fact, it has been reported that in *FAAH* null mice ($FAAH^{-/-}$) elevated AEA levels [122] impair the sperm fertilizing ability and motility, and the administration of HU-210, a synthetic analogue of Δ^9-THC, to rats has adverse effects on both spermatogenesis and sperm motility, suggesting that heightened AEA signaling in the male reproductive tract compromises some sperm cells features [29]. Recently, low 2-AG or AEA levels were measured in seminal plasma of infertile men [35, 123], thus suggesting a key role of eCBs in the acquisition of sperm functions and opening new perspectives in the treatment of male infertility.

The importance to keep AEA content at physiological concentrations in cells, tissues, and fluids involved in male and female reproductive events might be related to the existence of an eCBs gradient. In this context, several papers highlighted the involvement of eCBs signaling in the spatiotemporal control of sperm-egg fusion [26, 63, 108, 124]. Analogously to human menstrual cycle phases [125, 126], fluctuations of AEA levels, in combination with sex hormones oscillations, were detected in the various stages of bovine oestrus cycle [108], strengthening the idea that oviductal AEA content is crucial to avoid impairments in the normal sperm-oocyte interaction.

6. eCBs and Pregnancy: A Focus on Placentation and Parturition

In the past few decades, a large amount of evidence has demonstrated that endocannabinoid signaling via cannabinoid receptors is an important player in various female reproductive events, including sperm-egg fusion as fertilization, preimplantation development of embryos and their timely transport from the oviduct into the uterus, attainment of uterine receptivity, embryo-uterine crosstalk during implantation and decidualization, trophoblast differentiation and placental development, and initiation of parturition. In this section, we will briefly introduce the involvement of endocannabinoid signaling in early pregnancy events, with a focus on its pathophysiological significance during trophoblast development and placental formation as well as the labor onset.

6.1. Endocannabinoid Signaling in Early Pregnancy Events. In mammals, the beginning of a new life is seeded at fertilization. The fertilized egg undergoes serial cell divisions to form the 2-cell embryo, 4-cell embryo, 8-cell embryo, morula, and eventually the blastocyst with the first two differential cell lineages, the inner cell mass (ICM), and the trophectoderm [127–129]. During the past two decades, molecular and genetic studies have demonstrated that the ECS is tightly associated with early pregnancy events [130]. For example, cannabinoid receptors are expressed in the preimplantation mouse embryo, as well as in the oviduct and uterus. In mice, CB_1 mRNA is primarily detected from the four-cell embryo through the blastocyst stages, while CB_2 mRNA is present from the zygote through the blastocyst stages [8, 9, 131]. These results indicate that preimplantation embryo is a potential target for endocannabinoid signaling. Activation of CB_1 by cannabinoid ligands interferes with preimplantation embryo

development in culture [9]. On the other hand, asynchronous preimplantation embryo development is also observed in mice lacking CB_1 [131]. This pharmacological and genetic evidence pointed toward a tightly regulated endocannabinoid signaling during preimplantation embryo development [9, 11, 131].

During early pregnancy, another critical event occurring in parallel with preimplantation embryo development is the timely transport of preimplantation embryos from the oviduct into the uterus. In mice, embryos at the late morula or early blastocyst stage enter the uterus, where they develop and differentiate to gain implantation competency, escape from the zona pellucida, and implant into the receptive uterus. Therefore, normal oviductal embryo transport is one of the prerequisites for on-time implantation. In $CB_1^{-/-}$, a large portion of embryos are retained in the oviduct on day 4 of pregnancy and thus fail to initiate on-time implantation [132]. Moreover, wild-type mice treated with methanandamide, a CB_1 agonist, also exhibit a similar phenomenon, collectively suggesting that a tonic endocannabinoid signaling is essential for normal embryo transport from the oviduct into the uterus prior to blastocyst implantation. The endogenous levels of AEA, one of the primary endocannabinoid, are maintained by its synthesis and degradation activity. In this respect, $FAAH^{-/-}$ mice exhibit an elevated level of AEA in the oviduct during early pregnancy, accompanied with a derailed oviductal embryo transport [133]. Thus, an aberrant cannabinoid signaling impairs the oviductal transport of embryos, preventing on-time implantation [132, 133]. This finding is clinically relevant to human ectopic pregnancy, since high AEA levels and aberrant expression of FAAH and CB_1 in fallopian tubes have been observed in women with ectopic pregnancy [130, 134, 135]. Synchronized embryo development to blastocyst and uterine differentiation to receptive state are important for successful implantation. In the mouse, at pregnant day 1 to day 4 (day 1 = vaginal plug), the ovarian hormones estrogen and progesterone control the uterine undergoing from prereceptive to receptive stage. In this respect, lower levels of AEA in the receptive uterus and at the implantation site have been observed in contrast to its high levels in the nonreceptive uterus [13, 131]. Moreover, the CB_1 expression in activated blastocyst is significantly lower than that in dormant blastocysts [12, 131]. These observations suggest a biphasic role of endocannabinoid signaling in synchronizing trophoblast differentiation and uterine preparation to the receptive state for implantation. Also in female rats, ovarian hormones operate in conjunction with the blastocyst intrinsic programme, in order to regulate the synthesis of AEA in a specific manner during the crucial reproductive events that may compromise pregnancy outcome [136]. However, the interaction between lysophosphatidic acid, prostaglandins, and ECS during the window of implantation in the rat uterus has also been reported [137]. Indeed, employing delayed implantation model, previous studies have further demonstrated that AEA at low level renders the blastocyst competent for implantation viaactivating mitogen-activated protein kinase (MAPK) signaling, whereas at a higher concentration it inhibits calcium channel activity and blastocyst reactivation for implantation [12]. This finding has high clinical relevance, since the circulating level of AEA is well associated with pregnancy outcome in women with threatened miscarriage [36, 138]. Taken together, endocannabinoid signaling is an important player directing the normal preimplantation embryo development, activation, and uterine differentiation during the peri-implantation embryo-uterine dialogue.

6.2. Endocannabinoid Signaling Regulates Trophoblast Development and Placentation. With the initiation and progression of implantation and decidualization, trophectodermal epithelium, the wall of spherical blastocyst, will further develop into the extraembryonic tissues and eventually form the placenta. In mice, while the mural trophectoderm penetrates the uterine stromal, forming primary trophoblast giant cells, the polar trophectoderm, adjacent to the ICM, continues to proliferate and forms the ectoplacental cone (EPC) of the early conceptus and the extraembryonic ectoderm [129, 139]. Thereafter, the extraembryonic ectoderm develops to form the chorionic epithelium, which will be further fused with the allantois. Soon after, the chorionic trophoblast and its associated fetal blood vessels undergo extensive villous branching to create a functional mature placenta [140, 141]. Placenta serves as an interface for the exchange of nutrients, gases, and wastes between the maternal and fetal compartments. Moreover, placenta can secrete many hormones and growth factors conducive to the success of pregnancy establishment and maintenance [140, 141].

Increasing evidence suggests that the placenta is also a target of endocannabinoid signaling. In mice, CB_1 and FAAH are expressed in the EPC, and later in the spongiotrophoblast cells [142]. $CB_1^{-/-}$ placentas exhibit compromised spongiotrophoblast development with reduced expression of Mash2 and trophoblast-specific protein α (Tpbpa). This reduced population of Tpbpa positive trophoblast cells is due to an attenuated proliferation of spongiotrophoblast cells in the absence of CB_1 receptors [142]. This is consistent with the observations that CB_1/CB_2 null mutant trophoblast stem (TS) cells show remarkably slower cell proliferation compared with that in wild-type TS cells [142, 143]. It has been further demonstrated that endocannabinoid signaling regulates trophoblast cell proliferation via PI3 K/AKT signaling pathway [142]. Endocannabinoid signaling is also operative during human placental development, since CB_1, FAAH, and NAPE-PLD have been demonstrated to be expressed in human placentas [144–147]. For example, CB_1 receptors are present in all layers of the membrane, with particularly strong expression in the amniotic epithelium and reticular cells. Moderate expression is observed in the chorionic cytotrophoblasts. Moreover, FAAH is highly expressed in the amniotic epithelial cells, chorionic cytotrophoblast, and maternal decidua layer [145]. Besides, emerging evidence suggests that the levels of CB_1, FAAH, and NAPE-PLD in first trimester placentas are highly associated with the term pregnancy outcomes. The expression levels of CB_1 and FAAH are significantly lower or even absent, whereas the *NAPE-PLD* mRNA expression is aberrantly higher in spontaneous

miscarriage women [20]. Higher level of AEA is also detected in plasma of nonviable pregnancies than in viable pregnancies [147]. Most recent study further demonstrates that aberrant endocannabinoid signaling plays an important role in the pathophysiology of preeclampsia. The placental expression of NAPE-PLD is significantly higher in preeclamptic pregnancies, while FAAH exhibits an opposite result [148]. Moreover, AEA and Δ^9-THC have been shown to be able to inhibit human trophoblast BeWo cell proliferation and the transcription of genes involved in growth and apoptosis [138, 149]. These findings reinforce the notion that a tightly regulated endocannabinoid signaling is conducive to normal trophoblast development and placentation in humans.

6.3. Endocannabinoid Signaling Is Operative during Labor Onset. Preterm birth is defined as the birth of a baby which is less than 37 weeks of gestational age in humans [150]. In the world, 15 million babies are born prematurely [151]. Preterm birth is among the top causes of death in infants worldwide, which is the greatest health burden associated with pregnancy and childbirth [152]. Preterm labor may be caused by many factors, for example, genetics, infection, chemical substances, environmental contaminant or other factors [153–157], but the cause of preterm birth in many situations is elusive and unknown.

Progesterone and corticotropin-releasing hormone (CRH) are the most important mediators of labor. Progesterone has an essential and multifaceted role in the maintenance of myometrial quiescence during pregnancy and its withdrawal induces labor. The functions of progesterone are mediated by the nuclear progesterone receptors (PR-A and PR-B) in myometrial cells [158]. Progesterone has been advocated for the prevention of preterm labor [159]. Treatment with progesterone reduces the rate of spontaneous early preterm delivery in the midgestation period in women [159, 160]. CRH also has a critical role in pregnancy and labor, which is produced by the placenta during pregnancy [161–163]. CRH acts on the fetal pituitary-adrenal axis and directly on myometrial cells to facilitate labor, which determines the length of gestation and the timing of parturition and delivery. In this respect, previous studies have demonstrated that endocannabinoid signaling can modulate the activities of the hypothalamic-pituitary axis [77, 164–166] and thus is associated with normal onset and duration of labor in both mice and women [19, 167].

In mice, as described above, loss of CB_1 impairs the normal oviductal embryo transport, leading to deferral of on-time embryo implantation [132]. Therefore, it was generally thought that the labor onset would accordingly be delayed. However, surprisingly, the day of birth of CB_1 null mutant females is almost one day earlier than that in wild-type mice [167]. Similar premature birth can be induced in wild-type mice receiving CB_1-selective antagonist SR141716, but not a CB_2-selective antagonist SR144528 [120, 168]. The levels of progesterone and estrogen are largely alerted in the CB_1 deficient mice. An early drop of serum progesterone levels is observed on day 19 in the CB_1 null mutant mice, while the estrogen level increases on days 16–18. Subsequent analysis further reveals that cytochrome P450 aromatase and 17β-HSD7, which primarily contribute to ovarian estrogen biosynthesis during gestation in mice, are upregulated in CB_1 null ovaries, whereas levels of 20α-HSD, which metabolize progesterone into biologically inactive 20α-dihydroprogesterone, are substantially increased in CB_1 null mutant ovaries on day 19 of pregnancy. The premature birth in mice lacking *CB1* can be restored by subcutaneous injection of progesterone on day 18. This finding suggests that endocannabinoid signaling is essential for the maintenance of normal progesterone/estrogen ratio prior to the onset of parturition. Another interesting finding is that loss of CB_1 overrides cyclooxygenase- (COX-) 1 deficiency-induced delayed parturition and remarkably improves the survival rate of newborn pups. These results suggest that CB_1 signaling has a unique role in regulating normal parturition that is independent of COX-1-derived prostaglandin F2α, but CB_1 deficiency can correct the effects produced by COX-1 deficiency [167]. There is evidence that eCBs via CB_1 can upregulate *COX-2* expression and thus prostaglandin E_2 production in human gestational membranes during late pregnancy [169]. Prostaglandin E and F have an important function to regulate uterine contractions in labor, and the function of prostaglandin was through prostaglandin receptor expressed in myometrial tissue [170]. It remains to be determined whether COX-1 deficiency-induced delayed parturition is associated with aberrant cannabinoid-CB_1 signaling in mice. In addition, loss of CB_1 induces aberrant CRH-driven endocrine activities leading to preterm labor in mice, Antalarmin hydrochloride, a selective CRH antagonist, is able to restore the normal parturition timing in CB_1 deficient mice, and enhanced corticosterone activity on days 14–18 induces preterm birth with impaired fetal growth in wild-type mice. These observations show the concept that CB_1 signaling is crucial for maintaining normal CRH-corticosterone activities and onset of labor in mice [167].

In women, the chronic use of marijuana is often associated with fetal abnormalities and early pregnancy termination [36, 37, 133]. Plasma AEA levels have been shown to be associated with onset of labor. Plasma AEA levels are significantly increased in laboring term than those in nonlaboring term [19, 171, 172]. Meanwhile, a significantly higher expression of CB_1 has been observed in placental villous from nonlaboring compared to laboring women 173]. This finding indicates that the higher AEA level and lower placental CB_1 expression are essential for the timely onset of labor.

Collectively, endocannabinoid signaling is crucial for the normal initiation of parturition. Epidemiological studies should pay a close attention to *CB1* or *FAAH* gene polymorphism or mutation in women with preterm labor in clinical practice.

7. Closing Remarks

In the past few years ECS has emerged as an essential player in male and female reproduction. Nowadays, eCBs together with their synthesizing and degrading enzymes, EMT, and

molecular targets have been identified in reproductive cells, organs, and fluids of invertebrates, vertebrates, and mammals, highlighting the key role played by these endogenous compounds in reproduction processes along the evolutionary axis. Therefore, it comes out that the disruption of the normal physiological action of the ECS impairs the function of the male and female reproductive system and that altered AEA and/or 2-AG content is crucial during the various stages of procreation with relevant and interesting implications in the therapeutic exploitation.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Endocannabinoid System and Sex Steroid Hormone-Dependent Cancers

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The "endocannabinoid system (ECS)" comprises the endocannabinoids, the enzymes that regulate their synthesis and degradation, the prototypical cannabinoid receptors (CB1 and CB2), some noncannabinoid receptors, and an, as yet, uncharacterised transport system. Recent evidence suggests that both cannabinoid receptors are present in sex steroid hormone-dependent cancer tissues and potentially play an important role in those malignancies. Sex steroid hormones regulate the endocannabinoid system and the endocannabinoids prevent tumour development through putative protective mechanisms that prevent cell growth and migration, suggesting an important role for endocannabinoids in the regulation of sex hormone-dependent tumours and metastasis. Here, the role of the endocannabinoid system in sex steroid hormone-dependent cancers is described and the potential for novel therapies assessed.

1. Introduction

Cancer is characterised by an imbalance in cell cycle regulation leading to uncontrolled cell division and reduced cell death. Previous findings, suggesting that endocannabinoids play a vital role in cell proliferation, differentiation, and/or cell survival [1, 2], indicate that modulation of endocannabinoid action may provide an effective novel therapy for the amelioration of cancer symptoms or provide a method for continuous chemoprevention against cancer. This review will focus on describing connections between the endocannabinoid system and sex steroid hormone-dependent cancers.

1.1. The Endocannabinoid System. Endocannabinoids and their receptors are found throughout the body: in the brain, lungs, digestive system, connective tissues, hormone releasing glands, skin/hair, bone, the immune system, and the reproductive organs. The endocannabinoid system is a multifaceted endogenous signalling arrangement that influences multiple metabolic pathways [3]. It is composed of transmembrane endocannabinoid receptors (G-protein-coupled [CB1 and CB2] receptors), their endogenous ligands (the endocannabinoids), and the proteins involved in their biosynthesis and degradation [4]. The main active ingredient of cannabis, Δ^9-tetrahydrocannabinol (Δ^9-THC), mediates its effects through binding and activation of CB1 [5–7] and/or CB2 receptors [8, 9]. Because THC and its analogues have been used in palliative treatments where they inhibit tumour cell growth [10], research dedicated to the potential role of THC and the modulation of the endocannabinoid system in cancer treatment has increased [10–12].

1.2. Endocannabinoid Synthesis and Degradation. Endocannabinoids are unsaturated fatty acid derivatives, which are mainly considered to be synthesised "on demand" from phospholipid precursors residing in the plasma membrane [13] but may also be synthesised and stored in intracellular lipid droplets and released from those stores under appropriate conditions [14]. The most well-characterised endocannabinoids are anandamide (*N*-arachidonoylethanolamide, AEA) [15] and 2-arachidonoylglycerol (2-AG) [16], whose synthesis occurs through the action of a series of intracellular

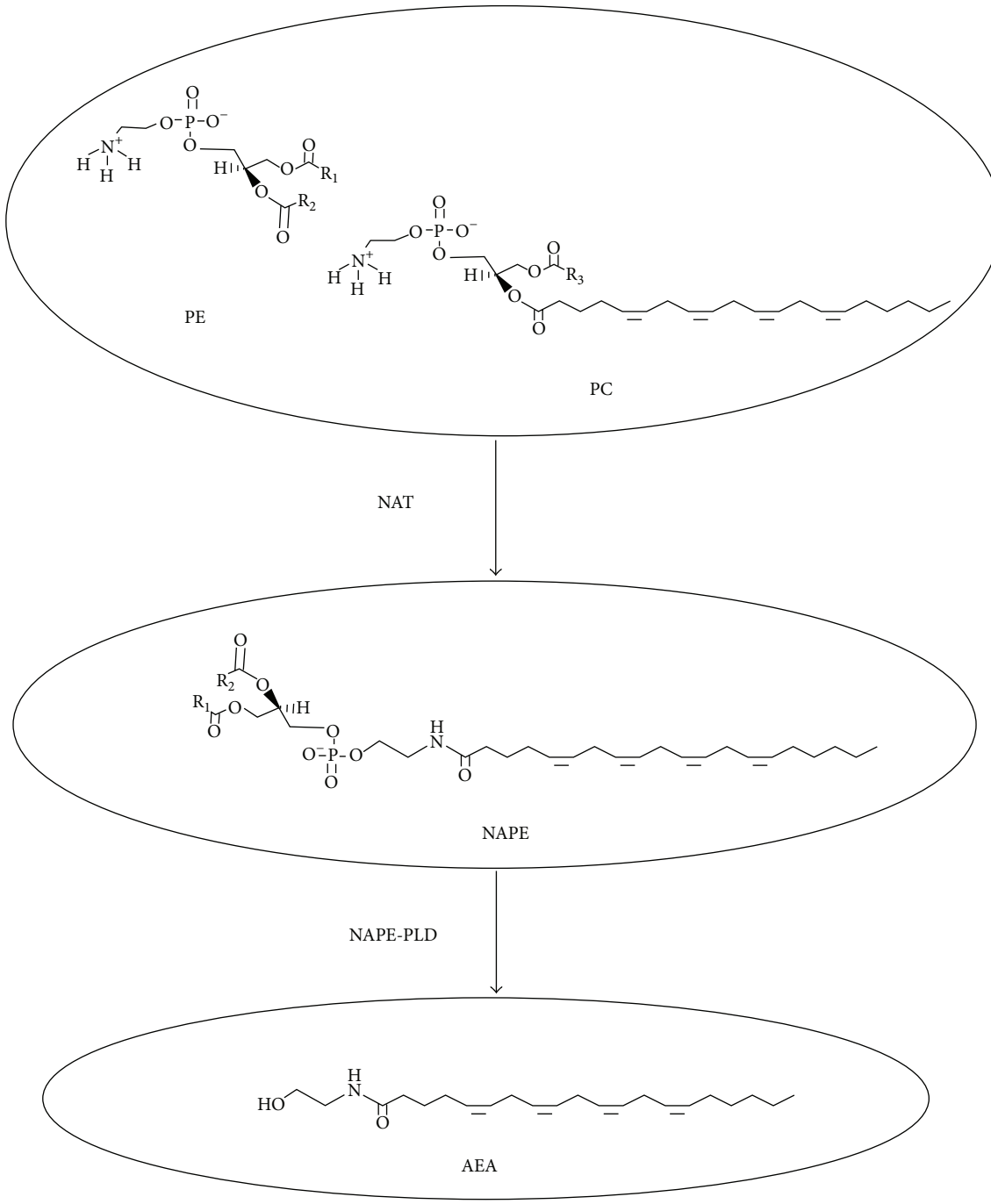

Figure 1: Synthesis of *N*-arachidonoylethanolamine (AEA). *N*-acyltransferase (NAT) catalyzes the transfer of arachidonic acid (AA) from phosphatidylcholine (PC) to phosphatidylethanolamine (PE) to form *N*-arachidonoyl-phosphatidylethanolamine (NAPE). NAPE is then converted into AEA in a one-step hydrolysis reaction catalyzed by the NAPE-specific phospholipase D (NAPE-PLD).

enzymes activated in response to a rise in intracellular calcium levels [17–19]. AEA was the first endogenous ligand identified for cannabinoid receptors and remains the most frequently investigated endocannabinoid [15]. AEA is produced via at least four separate pathways but the pathway that is most active in nonneuronal cells is the one where *N*-arachidonoyl phosphatidylethanolamine is directly converted to anandamide by the actions of *N*-arachidonoyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD [20] (Figure 1)) that has little in common with other phospholipases [21].

The second most often studied endocannabinoid is 2-AG, which is synthesised from diacylglycerol by the sequential actions of phospholipase C and two calcium sensitive *sn*-2-selective diacylglycerol lipases (α and β DAGL) (Figure 2) [22]. When released from cells, AEA and 2-AG act in

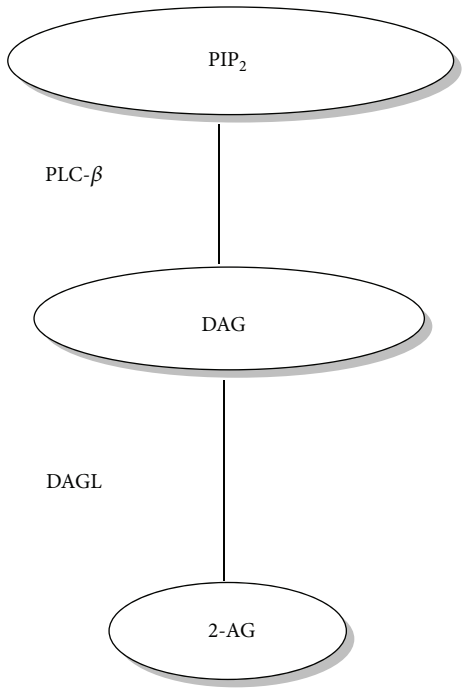

Figure 2: Synthesis of 2-AG. Phosphatidylinositol-4,5-bisphosphate (PIP2) is hydrolysed by phospholipase C-β (PLC-β) to form diacylglycerol (DAG). The DAG is then hydrolysed to 2-AG by diacylglycerol lipase (DAGL).

an autocrine or paracrine manner to stimulate signalling through interaction with various extracellular and intracellular receptor targets (Figure 2). To facilitate endocannabinoid reuptake and attenuate signalling, a diverse number of transport systems have been postulated, such as cellular endocytosis, simple diffusion, and specific carrier proteins [23], but none are yet proven. Both AEA and 2-AG are degraded through the action of specific enzymes; AEA is predominantly metabolised to arachidonic acid and ethanolamine by the enzyme fatty acid amide hydrolyse (FAAH-1) (Figure 3) [24] and to a lesser extent by FAAH-2 (not present in rodents). Although 2-AG is also metabolised by FAAH-1 and to a lesser extent by α,β-hydrolase-6 (ABHD6) and α,β-hydrolase-12 (ABDH12), it is not metabolised by FAAH-2; the predominant enzyme involved in its degradation is monoacylglycerol lipase (MAGL) (Figure 4) [25, 26]. Once inside the cell, AEA is considered to be moved around the cell by an intracellular FAAH-like protein (FLAT-1), that is, catalytically silent, does not bind 2-AG, and delivers it to FAAH-2 on microsomal membranes [27].

Several other endocannabinoids have been identified, including *N*-arachidonoyl-dopamine (NADA) [28], noladin ether, and virodhamine [29]. In addition, structural analogues of endocannabinoids with low affinities for cannabinoid receptors such as *N*-oleoylethanolamine (OEA), *N*-palmitoylethanolamine (PEA), *N*-stearoylethanolamine (SEA), and linoleoylglycerol have also been identified in human, rat, and mouse tissues [30, 31]. These compounds produce an "entourage effect" through being alternative substrates for FAAH and MAGL and thereby increasing the potency of

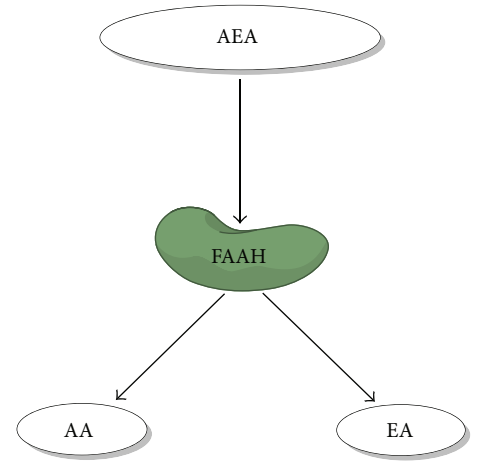

Figure 3: AEA is hydrolysed into arachidonic acid (AA) and ethanolamine by fatty acid amide hydrolase (FAAH).

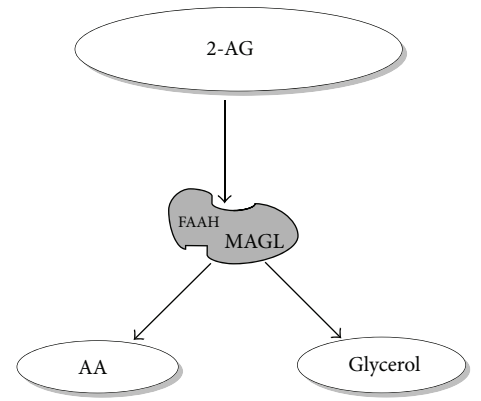

Figure 4: 2-AG is hydrolysed by FAAH and MAGL. As indicated by the size of the letters, MAGL is the major enzyme degrading 2-AG.

endocannabinoids, such as AEA and 2-AG whose degradation by these enzymes is inhibited [32, 33]. Endocannabinoids may also undergo oxidative metabolism by a number of fatty acid oxygenases, such as cytochrome P450 en-zymes (CYP450) [34, 35], lipoxygenases (LOX) [36, 37], and cyclooxygenase-2 (COX-2) [38, 39]. Stimulation of CYP450s, LOXs, and COX-2 in tumour cells and inflammation sites could thus reduce the levels of naturally occurring antiproliferative and anti-inflammatory mediators [40, 41].

1.3. Receptors. Two subtypes of cannabinoid receptors belonging to the Gi/o family of seven trans-membrane G-protein-coupled receptors (GPCR) have been described. The first, CB1, or the central receptor was first described as being predominantly expressed in the central nervous system but is also present in a variety of peripheral tissues at much lower levels [42–44]. The second, CB2, or the spleen-type receptor was originally isolated from splenic cells and is primarily expressed in immune and blood cells, although it has also been found in various brain areas [45] and other tissues [46, 47]. Both receptors are distributed in human tissues including the brain, testis, sperm, leucocytes, placenta, fetal membranes, endothelial cells, anterior eye, pituitary gland, breast,

and reproductive tissues [17, 48–54]. Surprisingly, they share little sequence homology, only 44% at the protein level or 68% in the trans-membrane domains, which are thought to contain the binding sites for cannabinoids [55]. Pharmacological studies have strongly suggested the existence of novel cannabinoid receptor subtypes [56, 57], and recently two orphan G-protein-coupled receptors (GPR55 and GPR119) have been proposed as cannabinoid receptors. GPR55 has been identified in the brain and peripheral tissues such as the gut, spleen, and adrenals, and, of the endocannabinoids, 2-arachidonoylglycerol (2-AG) and PEA have the greatest affinity for this receptor. GPR119 on the other hand has a narrow distribution having been described predominantly in the pancreas and intestinal tissues. The endocannabinoid with greatest affinity for GPR119 is OEA [58]. Cannabinoids can also inhibit various types of calcium channels [59, 60] and activate certain potassium channels [61]. In addition, the transient receptor potential vanilloid subtype 1 (TRPV1), a ligand-gated Ca^{2+} permeable ion channel, usually activated by stimuli such as acidity and heat and involved in the transmission and modulation of pain [62], is also activated by both AEA and acyl dopamine referred to as endovanilloids in this context [62]. Lately, the peroxisome proliferator-activated receptors (PPARs) have been included in the lists of the endocannabinoid targets, as they are stimulated by endocannabinoids under both physiological and pathological conditions [63].

2. Endocannabinoids and Cancer

Adjuvant cannabinoid use in the treatment of adverse side effects from chemotherapy, such as neuropathic pain, loss of appetite, nausea, and vomiting, is the most studied potential therapeutic application for these compounds [64]. Beyond the palliative effects induced by cannabinoids, these molecules and endocannabinoids are increasingly recognised for their role in the regulation of the key processes involved in the development of cancer. For example, the endocannabinoid system is reported to induce apoptosis [11, 65, 66], cell cycle arrest [67–69], and the inhibition of angiogenesis and metastasis [70–72] in animal models and cell lines. Further research is however needed to substantiate these antineoplastic effects in humans. Furthermore, there is a suggestion that endocannabinoid signalling in the tumorigenic cell differs from that of its "normal" counterpart.

2.1. The Endocannabinoid System: Cancer versus Normal Cell. When considering the development of a novel anticancer treatment that "selectively targets tumour cells," thereby improving the therapeutic index of anticancer strategies, a comparison of the action of the drug in cancer cells with respect to that in normal cells represents a crucial step that must be carefully explored. Where this shows significant effects on the cancer, but not on the normal cells, such a drug will have potential benefits.

Evidence suggests that the actions of the endocannabinoid system indeed are selective in cancerous rather than in noncancerous cells [73–75]. These are affected by different components of the endocannabinoid system and result in a wide range of actions. In cancer cells such as those of the breast, melanoma, lymphoma, pancreas, and thyroid, there is increased sensitivity to endocannabinoids due to an increased level of endocannabinoid receptors in these cells compared to normal cells found in adjacent tissue obtained from the same specimen [73, 75–81]. For example, met-fluoro-anandamide (Met-F-AEA) increases the levels of CB1 receptors in both K-ras-transformed FRTL-5 (KiMo1) cells and in KiMo1-derived tumours in nude mice, whereas in FRTL-5 cells (a thyroid-differentiated epithelial cell line), Met-F-AEA produced downregulation of CB1 receptors [75]. Treatment of human prostate cancer (LNCaP) cells with the CB agonist WIN-55,212-2 showed significantly higher expression of both CB1 and CB2 receptors in these cells when compared to normal cells and interestingly a significant decrease in cell viability when treated with 1–10 μM of WIN-55,212-2 for 24–48 hours, whilst similar doses had no effect on prostate epithelial (PrEC) cells [78].

Furthermore, the RAS-MAPK/ERK pathway in brain cells is one signalling pathway, which has been reported to be differentially regulated by cannabinoids in the cancerous cell when compared to the normal cell [82], where THC induces ceramide synthesis and glioma cell death via a CB1-mediated effect, whilst astrocytes are protected from ceramide-induced sensitisation to oxidative stress-related damage [83]. Similarly, proapoptotic and antiproliferative effects of cannabinoids on cancer cells and not on healthy tissue have been recorded in animal studies [65, 76], whilst cultured oligodendroglial cells are protected from various proapoptotic stimuli [84]. Furthermore, THC induces apoptosis in several human cancer cell lines [11, 74, 85], whilst endocannabimimetic substances inhibit the proliferation of KiMol cells more robustly than FRTL-5 cells and *in vivo*, Met-F-AEA inhibits the growth of KiMol-induced tumours in athymic mice, an effect that was accompanied by a reduction in p21ras action [75]. In addition, ligand-induced activation of CB2 receptors reduces human breast cancer cell proliferation, whereas in normal breast tissue the expression of CB2 receptor was significantly less and the proliferation was thus much less affected [78].

Furthermore, elevated levels of AEA and 2-AG have been documented in several other cancerous tissues when compared to normal healthy counterparts, such as prostate and colon cancer, endometrial sarcoma, pituitary adenoma, and highly aggressive human cancer tissues [52, 53, 86, 87]. Recently, an increase in FAAH expression in prostate cancer cell has been reported when compared to that in the noncancerous prostate cell [88]. The expression level of MAGL is higher in androgen-independent versus androgen-dependent human prostate cancer cell lines and RNA-interference disruption of MAGL impairs prostate cancer aggressiveness [89], suggesting that 2-AG has a role to play in the aggressiveness of some types of prostate cancer.

The effect of endocannabinoids on tumorigenesis may depend on the stage of differentiation of the malignant tissues under investigation. In the human colon cancer cell line Caco2, endocannabinoids failed to show any proliferative effect via CB1 receptors in differentiated cells [90]; however, in undifferentiated cells, cannabinoids were strongly antiproliferative via CB1 and this was not because of alterations

in the levels of CB1 receptors. Intriguingly, an alteration in CB1 glycosylation that probably affected cell signalling was suggested [90]. However, similar elevations in CB receptor glycosylation could not be found in human bladder, pancreas, or small intestine cancer cells [86, 91], suggesting this is not the main reason for the antiproliferative effects observed in the undifferentiated Caco2 cells. The small difference in CB receptor expression, although not significant in these studies, could possibly be applicable in other hormone-dependent cancer cells.

2.2. Endocannabinoid Receptor Signalling Actions and Consequences in Cancer. The variability of endocannabinoid effects in different tumour models is highly incongruous and may be a consequence of the differential expression of cannabinoid receptors, where it is envisioned that differential expression of cannabinoid receptors between cancerous and normal tissues may play a determining role in the progression and/or inhibition of malignancy. For example, high levels of CB1 and CB2 mRNA were detected by *in situ* hybridization in well-differentiated human hepatocellular carcinoma and in cirrhotic liver samples, while the expression of these receptors in poorly differentiated hepatocellular carcinoma was low [92]. In addition, increased expression of CB1 and/or CB2 has been noted in human mantle cell lymphoma [80, 81], breast cancer [79], acute myeloid leukaemia [93], hepatocellular carcinoma, and prostate cancer cell lines; however, the levels of both receptors were similar in malignant and nonmalignant human astroglial cancer cells [94] and in malignant and nonmalignant nonmelanoma skin cancer cells [70].

In general, a relationship between CB receptor expression and the outcome of cancer has been documented. In astrocytoma cells, for example, it has been shown that 70% of cells express CB1 and/or CB2 with the extent of CB2 receptor expression correlating directly with the degree of tumour malignancy [66], whilst in gliomas a higher expression of CB2 receptor compared to CB1 receptor was found and related to tumour grade [66]. In addition, tumour-associated endothelial cells demonstrated immunoreactivity for CB1 receptors similar to that of the cancer cells [95]. Similarly, increased expression of both CB1 and CB2 receptors has been documented in non-Hodgkin lymphoma when compared to reactive lymph nodes [80], whilst CB1 expression is increased in mantle cell lymphoma [96]. In contrast, a significantly reduced expression of CB1, but not of CB2, was noted in colon cancer compared with the normal adjacent mucosa [97]. Taken together, these studies imply a role for CB1 and CB2 receptors and their expression in relation to disease prognosis and outcome and that this is greatly dependent on the type/specific cancer being studied.

In breast carcinoma, a relationship between CB2 expression, the histological grade of the cancer, and other markers of prognostic and predictive value, such as ErbB2/HER-2 oncogene, oestrogen, and progesterone receptors, has been reported [98]. CB1 receptor expression in the human prostate cancer cell lines LNCaP (androgen-sensitive), DU145 and PC3 (androgen-insensitive) has been reported to be higher than in the normal human prostate epithelial cells [78]. This was confirmed in prostate cancer tissues where the expressions of CB1 and TRPV1 receptors were upregulated and furthermore correlated with increasing cancer grade [99]. Moreover, the level of CB1 receptor expression in cancer specimens has been shown to correlate with the disease severity at diagnosis and outcome [100]. In human pancreatic cancer, higher levels of CB1 receptor expression are related to a shorter survival time (median 6 months) than lower CB1 receptor (median 16 months) [91]. In contrast, the overexpression of CB1 and CB2 receptors found in human hepatocellular carcinoma was associated with improved prognosis [92].

The mechanism by which endocannabinoid receptor expression is modulated in relation to cancer has not been fully examined; however, several studies have revealed important evidence for further relationships between cannabinoid receptors and cancer, where transcription factor involvement has been postulated. Indeed, it has been shown that THC induces a CB2-receptor-dependent transcription of the CB1 gene in human T cells and T cell lymphoma lines, mediated via IL-4 release through activation of the transcription factor STAT6 [101]. In addition, expression of CB2 is induced following the oral administration of specific *Lactobacillus* strains in colonic epithelial cells, through the NF-κB pathway [102], whilst it has been reported that CB1 receptor expression in human colon cancer was induced by 17β-oestradiol through an oestrogen-receptor-dependent mechanism [103]. In alveolar rhabdomyosarcoma, CB1 receptor expression has greatly increased and this was evident in chromatin immunoprecipitation studies, which have demonstrated that the CB1 gene is a transcriptional target of PAX3/FKHR, a chimeric transcription factor found in this condition [104]. Another hypothesis, that has been examined, is that alternatively spliced isoforms of CB1 (CB1a and CB1b), which could reflect differences in its functionality in normal and cancerous tissues, are responsible for the variability in the response described above [105].

2.3. The Endocannabinoid System and Sex Steroid Hormones. The endocannabinoid system is widespread throughout the central nervous system (CNS) and peripheral regions and regulates a large range of physiological functions and behaviour. The same can also be said of the sex steroid hormones. As stated above, there is evidence suggesting that the two systems interact extensively (Figure 5).

2.3.1. The Role of Progesterone. Progesterone is a C-21 steroid hormone that is produced predominantly by the ovarian corpus luteum after ovulation and is possibly involved in the regulation of endocannabinoid signalling. Progesterone has been shown to upregulate human lymphocyte FAAH activity through the transcription factor Ikaros [106, 107] and thereby decreases plasma AEA levels [108]. Whether this is a general phenomenon or a T cell specific effect needs clarification since the expression of Ikaros transcription factors seems to be confined to the T cell [109]. Furthermore, while progesterone increases FAAH expression and its activity in immortalized human lymphoma U937 cells, but not in immortalized human neuroblastoma CPH100 cells [110], it has been

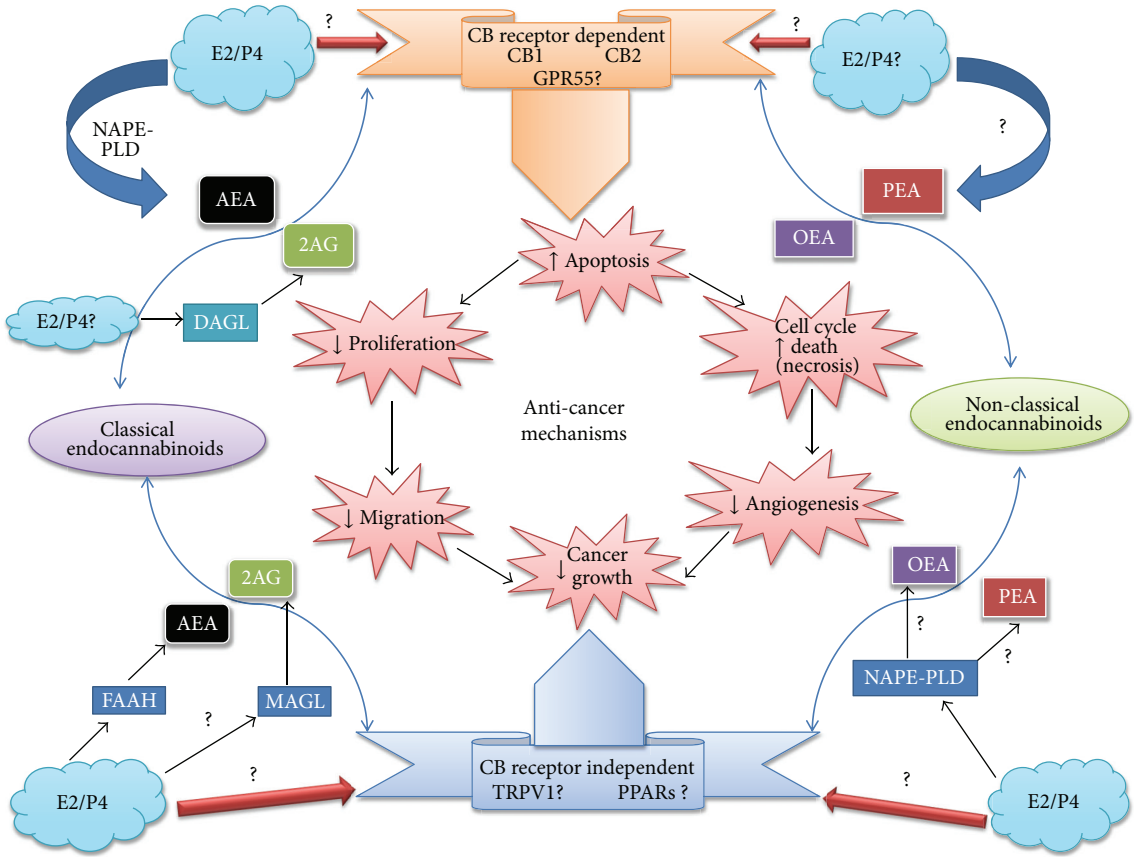

Figure 5: Known and putative interactions between gonadal sex steroids and the endocannabinoid system in sex hormone-dependent cancers. Endocannabinoid synthesis occurs through the actions of specific enzymes (blue boxes and broad arrows) through enzymatic hydrolysis of membrane specific NArPE lipid precursors. The role of the sex steroid hormones oestradiol (E2) and progesterone (P4) (blue clouds) is only speculative. The classical (anandamide (AEA) and 2-arachidonoylglycerol (2-AG)) and nonclassical endocannabinoids (*N*-oleoylethanolamine (OEA) and *N*-palmitoylethanolamine (PEA)) independently or collectively bind at both classical (CB1, CB2, and GPR55) and nonclassical (TRPV1 and PPAR) receptors (ribbons) to affect anticancer mechanisms (pink starbursts in the centre of the figure), which may be linked (arrows) or may not be linked to each other, but all result in reduced cancer cell mass. Known interactions between sex steroid hormones and the endocannabinoid system in the anticancer mechanisms are shown, whereas speculative or unknown interactions are indicated by the presence of a question mark. Up and down arrows indicate either an increase or decrease in the activity of that particular anticancer mechanism.

reported to have a minimal effect on EMT, NAPE-PLD, and CB1 expression in lymphocytes [106, 107]. Progesterone has also been documented to downregulate uterine NAPE-PLD expression in mice, leading to a decrease in tissue AEA levels [111]. In the pregnant mouse uterus, it has also been reported to downregulate FAAH activity [112], and when taken together with reduced NAPE-PLD expression in mice these data suggest that the NAPE-PLD : FAAH activity ratio in the mouse uterus may be key to the regulation of local AEA levels and thus maintenance of pregnancy or endometrial pathologies, such as cancer. At the same time, the actions of progesterone on local AEA levels in the rat uterus are more complex with progesterone stimulating its production in the ovariectomised animal [113]. These authors concluded that "...the effect of ovarian hormones on the synthesis of anandamide depends on different physiological conditions, (including the) oestrous cycle and early pregnancy, and on the presence of the activated blastocyst...," but the precise feedback mechanisms that might be involved are unknown. For example, treatment with either CB1 or CB2 receptor agonists reduces the levels of serum progesterone, corpus luteum weights, corpus luteum LH receptor mRNA content, and corpus luteum LH receptor density in sheep [114]. It has also been reported that the levels of serum progesterone and LH content are decreased following chronic administration of AEA in pregnant rats [115], suggesting that both positive and negative feedback loops are involved in the coregulation of endocannabinoid and progesterone function.

2.3.2. The Role of Oestrogen. Oestrogens are also steroid hormones, produced predominantly by the ovarian follicle during the early stage of the menstrual and oestrous cycles. Once inside the cell, oestrogens bind to and activate specific oestrogen receptors, resulting in the regulation of the expression of multiple gene targets involved in cellular proliferation, apoptosis, and autophagy [116]. Although FSH and LH stimulate the synthesis of oestrogen in the ovaries, there are other nonovarian sources of oestrogens such as the breast,

the adrenal glands, and the liver, but the levels produced are relatively small and probably only have local actions [117]. The most potent growth stimulating oestrogen is 17β-oestradiol (E2), which has been linked directly and indirectly with the endocannabinoid system, where E2 stimulates NAPE-PLD and inhibits FAAH synthesis and directly stimulates the release of AEA from endothelial cells [108, 118, 119]. By contrast, another study revealed that NAPE-PLD is downregulated in the uterus by oestradiol, suggesting that it results in decreased anandamide levels, although this was not directly tested [111]. However, other studies revealed that E2 decreases the activity of FAAH in the mouse uterus [108, 118], whilst also regulating the expression of FAAH [112]. Moreover, in cancers, such as glioma, breast, and colon, oestrogens appear to regulate the ECS [120]. Evidence of cannabinoid and oestrogen receptor coexpression has been documented in colorectal carcinoma and normal colonic epithelium [90] and in the human anterior pituitary gland [121], where E2 regulates CB1 mRNA expression, a feature it also shared within the rat hypothalamus [121]. These differential effects of oestrogens on components of endocannabinoid signalling pathways reveal an intricate interaction which may play an important role in sex steroid hormone-dependent tumours.

3. The ECS and Breast Cancer

Breast cancer is the most common cancer in women [122]. It is dependent on hormones, such as oestrogen and progesterone, for initial growth and survival. Risk factors for the development of breast cancer include lower fertility, nonbreast-feeding, genetic predisposition, higher hormone levels, and iodine deficiency [123]. Bones, lungs, and lymph node are among the sites where breast cancer cell may spread [124, 125], but the cancer itself normally develops from breast tissues surrounding the milk ducts (ductal neoplasm) or breast lobules (lobular neoplasm) [126]. Cannabinoid receptors have been documented to be present in breast tissue; CB1 immunoreactivity was expressed in 28% of human breast cancer samples [127] and immunohistochemistry studies have revealed the presence of CB1 in 14% of human breast cancer tissues expressing ErbB2, which is a member of the epidermal growth factor (EGF), but interestingly with no relationship between CB1 and ErbB2 expression [128]. CB1 receptors have also been documented in human breast tissues using Western blot, immunofluorescence, and/or RT-PCR techniques as well as in various breast cancer cell lines (T-47D, MCF-7, MDA-MB-231, TSA-E1, and MDA-MB-468) [69, 103, 127, 129, 130].

In contrast, CB2 immunoreactivity was documented in 72% of human breast tissues [128], and is present in 91% of ErbB2-positive cancer tissues (in contrast to CB1 receptor), suggesting a relationship between CB1 receptor and the ErbB2-positive cancer cell phenotype [128]. CB2 immunoreactivity was noted in 35% of human breast cancer tissues [127] while CB2 receptors were expressed in a variety of breast cancer cell lines (T-47D, MCF-7, MDA-MB-231, MDA-MD-468, EVSA-T, and SkBr3) and human breast tissues as determined by Western blot, RT-PCR, and/or immunofluorescence techniques [69, 103, 127, 129, 130]. In addition, FAAH transcripts are present in the EFM-19 and MCF-7 cancer cell lines, as determined by northern blotting [131] and RT-PCR [132] techniques, whilst GPR55 is highly expressed in the MDA-MB-231 and MCF-7 breast cancer cell lines [133].

Recent research has identified a role for the endocannabinoid system in the regulation of breast cancer growth, with induction of apoptosis and control of cancer neovascularization in breast cancer being key control points [127, 128, 130, 134] and importantly a critical relationship with oestrogen [116]. These effects are achieved through a variety of mechanisms; for example, CBD seems to involve direct TRPV1 activation and/or CB2 indirect activation (via FAAH), induction of oxidative stress [130], and the ability to decrease ID-1, an inhibitor of basic helix-loop-helix transcription factor. The expression of ID-1 in breast cancer cells was associated with its efficacy in reducing proliferation, migration, and invasion [135]. In these types of cell lines, the endocannabinoid system induces growth arrest by downregulating prolactin receptor expression [67]. Therefore, breast cancer proliferation depends on signalling via the CB1 receptor, which has been revealed to downregulate the prolactin receptor and indirectly inhibit cell growth [67]. Furthermore, these effects were also noted when FAAH activity was blocked [67], suggesting that AEA is somehow involved in this process. In addition, the antiproliferative effects of the endocannabinoid system were shown to be mediated through downregulation of the high affinity NGF receptor [69]. Similarly, the ECS inhibits breast cancer growth *in vivo*, by acting through the CB1 receptor [134]. Finally, a recent study showed that CBD induces a concentration-dependent cell death of both oestrogen receptor-positive and oestrogen receptor-negative breast cancer cells through a mechanism involving a CB1-, CB2-, and TRPV1-independent receptor activation [116]. Furthermore, the gender-specific actions of E2 in the hippocampus, where the ERα-specific inhibition of CB1-dependent signalling in a subset of neurons occurs only in female and not in male mice, whilst ERβ-specific stimulation occurs in both genders, suggest that there may be a complex interaction between oestrogen and cannabinoid signalling, at least in the rat hippocampus [136]. Considering that the E2-activated ERα promotes human breast cancer cell growth [137] and the E2-activated ERβ receptor inhibits human endometrial cancer cell growth [138] and that cannabinoids may affect both breast and endometrial cancer growth and development, then a potential interaction between these two signalling pathways seems plausible and should be investigated further.

4. The ECS and Prostate Cancer

Prostate carcinoma is the second most common cancer diagnosed in men [122]. Factors such as diet, genetic predisposition, medical exposure to hormones, and viral infections are all implicated in the incidence of prostate cancer [139]. The majority of prostate cancers have a slow progression, although cases of aggressive prostate cancer do occur. Common sites of prostate metastasis are particularly in the bones and lymph nodes [139, 140]. CB1 expression is upregulated

in prostate cancer tissues [99] and the levels of the receptor are associated with cancer severity and outcome [100]. In addition, RT-PCR, Western blot, and immunofluorescence studies have shown that prostate cancer cell lines, PC-3, DU-145, LNCaP, CWR22Rv1, and CA-HPV-10, and human prostate tumour tissues express CB1 [69, 78, 87, 99, 100, 141, 142]. CB2 receptors are also present in the same prostate cancer cell lines [69, 78, 87, 142] and these cancer cell lines express higher levels of CB1 and CB2 than benign prostate epithelium [78]. In addition, FAAH expression has been demonstrated in the prostate cancer cell lines (PC-3, DU-145, and LNCaP) and human prostate cancer tissues [88, 97, 125, 142, 143]. GPR55 is also expressed in the PC-3 and DU-145 prostate cancer cell lines [144]. Thus, various cannabinoid receptor subtypes and endocannabinoid hydrolysing enzymes are known to be located in prostate tissue and synthetic cannabinoids, endocannabinoids, and related compounds appear to inhibit prostate tumour cell proliferation and induce apoptosis via CB1 and/or CB2 receptor activation.

Prolactin (PRL) is necessary for the prostate to be completely formed. Prolactin is a hormone produced by the anterior pituitary gland in lactotroph cells and its gene is located on chromosome 6 [145]. Prolactin plays a vital role in prostate cell proliferation, differentiation, and survival, in normal as well as in malignant cells [146]. This has led to the suggestion that prostate cancer may express prolactin receptors and proliferate in response to prolactin levels and this response can be inhibited by cannabinoids. When induced by exogenous PRL, the proliferation of prostate DU-145 cells was potently inhibited (IC50 = 100–300 nM) by anandamide, 2-AG, and HU-210. Anandamide was also noted to downregulate the levels of prolactin in DU-145 cells [69].

Several intraepithelial or invasive prostate cancers have exhibited increased expression of epidermal growth factor receptor (EGF-R), tyrosine kinase, and EGF. The EGFR levels can be downregulated by micromolar concentrations of AEA via the CB1 receptor and this results in the inhibition of proliferation at day 3 and cell death by apoptosis/necrosis on day 5, with this effect being manifest through both CB1 and CB2 receptors [147]. Cannabinoids have also been documented to downregulate androgen receptor expression and prostate specific antigen (PSA) [148]; however, the sensitivity to cannabinoids seems to be inconsistent in different prostate cancer cell lines, even in the presence of CB receptors [128, 147, 148].

5. The ECS and Endometrial Cancer

Worldwide, endometrial cancer is the seventh most commonly diagnosed malignancy [149] and the 4th most common gynaecological cancer diagnosed in 2008 in the UK [150]. Endometrial cancer refers to several types of malignancies that arise from the endometrium, and early menarche, late menopause, obesity, nulliparity, and the use of oestrogen-only hormone replacement therapy have all been identified as risk factors for the development of endometrial carcinoma, which suggest that greater lifetime exposure to oestrogen, unopposed by progesterone, plays a vital role in the aetiology of endometrial cancer [151, 152]. Exposure to endogenous or exogenous oestrogens and the use of unopposed progesterone lead to an increase in the mitotic activity of endometrial epithelial cells and increased DNA replication and repair errors, which in turn leads to various somatic mutations that may ultimately result in endometrial hyperplasia, which may finally result in the development of malignancy [153, 154]. Chronic inflammation has also been implicated as a vital player in the relationship between obesity, menstrual disorders, and endometrial cancer [153]. Moreover, conditions such as uterine fibroids and endometriosis may produce an increased risk of endometrial cancer because these disorders have been linked to both pelvic inflammation [155–157] and an excess of oestrogen [158, 159]. Based on clinicopathological and molecular characteristics, there are two types of endometrial cancers. The first is the type I or oestrogen-dependent endometrioid carcinomas (EECs), which constitute approximately 80% of the cases of endometrial carcinoma. These tumours express oestrogen (ER) and progesterone (PR) receptors and arise in younger pre- and postmenopausal women [160]. Type I is also strongly associated with either endogenous or exogenous unopposed oestrogen exposure and is usually of low grade and characterised by a favourable prognosis. The second group, the type II or nonendometrioid endometrial carcinomas (NEECs), are comprised of the high grade papillary serous and clear cell carcinomas [161]. These arise in relatively older women and are not usually preceded by an unopposed oestrogen exposure history but have an aggressive clinical course and a worse prognosis than type I cancers [161].

The expression of endocannabinoid receptors in different endometrial cancer tissues/cell lines has been described, where CB2 expression was detected by immunohistochemistry only in the endometrial cancer cells and not in the normal endometrial tissue taken from the same biopsy [162]. Immunoblotting analysis showed that CB2 protein expression was significantly elevated in the endometrial cancer tissues when compared to healthy endometrial tissues [162] and no significant differences were noted in CB1 expression [162]. A mass spectrometry study showed selective upregulation of 2-AG in endometrial cancer tissues compared to healthy endometrial tissues, whilst no significant increases in the levels of AEA or PEA were noted [162]. Similarly, immunoblotting revealed a selective downregulation of MAGL expression in endometrial cancer tissues compared with healthy tissue and, interestingly, there were no significant differences in FAAH protein expression [162]. Furthermore, evidence suggests that CB2 receptor regulation is dysregulated in endometrial cancer, because CB2 levels were significantly higher in the AN3CA human endometrial carcinoma cell line compared to control cells when transfected with a plasmid containing the cDNA for the endocannabinoid receptor CB2 [162]. From these data, it has been concluded that CB2 receptors might play a vital role in the growth of endometrial cancer [163].

Recent research has shown that the complete endogenous pathway for CB2 was altered significantly in endometrial adenocarcinoma, which may thus be one of the underlying factors for endocannabinoid system regulation in the aetiology of endometrial cancer. The marked elevation in CB2

receptor expression and 2-AG in endometrial cancer tissues might be due to the underlying imbalance in the oestrogen/progesterone ratio, which is one of the aetiological factors for the development of endometrial cancer [162], but this has not been fully tested. What has been tested was the effect of CB2 elevation in transfected AN3CA cells where CB2 caused a 40% reduction of cell mitochondrial function when compared to the control cells [163]. This effect was not improved by the CB2 receptor agonist, JWH133, but was fully prevented by the CB2 receptor antagonist SR144528.

The specific increased expression of CB2 receptors in only the tumour developing cells might represent a novel indicator for the diagnosis of endometrial cancer. Therefore, selective CB2 agonists might represent the foundation for the development of new antitumour compounds against endometrial carcinoma, because they have the ability to kill the affected cancer cells without damaging their normal counterparts [164].

6. Conclusions and Future Directions

Currently available studies suggest that the endocannabinoid system may be targeted to restrain the development and progression of breast, prostate, and endometrial carcinoma. The endocannabinoid system exerts a variety of interesting effects that are dependent on the cell line and/or tumour type under investigation, where the ECS, for example, inhibits cancer cell proliferation, angiogenesis, cancer growth, metastasis, and apoptosis. The prevailing data suggest that an imbalance in the endocannabinoid system and its interaction with sex steroid hormone homeostasis may promote cancer development, proliferation, and migration. Therefore, for this vital reason, and although it is early days, the endocannabinoid system has become an attractive novel target for pharmacological intervention in the fight against many hormone-related cancers.

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Research Article

Brain Levels of Prostaglandins, Endocannabinoids, and Related Lipids Are Affected by Mating Strategies

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Background. Endogenous cannabinoids (eCBs) are involved in the development and regulation of reproductive behaviors. Likewise, prostaglandins (PGs) drive sexual differentiation and initiation of ovulation. Here, we use lipidomics strategies to test the hypotheses that mating immediately activates the biosynthesis and/or metabolism of eCBs and PGs and that specific mating strategies differentially regulate these lipids in the brain. *Methods*. Lipid extractions and tandem mass spectrometric analysis were performed on brains from proestrous rats that had experienced one of two mating strategies (paced or standard mating) and two nonmated groups (chamber exposed and home cage controls). Levels of PGs (PGE2 and PGF2alpha), eCBs (AEA and 2-AG, *N*-arachidonoyl glycine), and 4 related lipids (4 *N*-acylethanolamides) were measured in olfactory bulb, hypothalamus, hippocampus, thalamus, striatum, midbrain, cerebellum, and brainstem. *Results*. Overall, levels of these lipids were significantly lower among paced compared to standard mated rats with the most dramatic decreases observed in brainstem, hippocampus, midbrain, and striatum. However, chamber exposed rats had significantly higher levels of these lipids compared to home cage controls and paced mated wherein the hippocampus showed the largest increases. *Conclusions*. These data demonstrate that mating strategies and exposure to mating arenas influence lipid signaling in the brain.

1. Introduction

Decades of studies on mating behavior in laboratory rats (typically *Rattus norvegicus*) provide a wealth of knowledge about developmental and motivational roles of various neurotransmitter systems in sexual differentiation and/or manifestation of reproductive behaviors [1]. Standard mating procedures for rats in laboratory environments typically involve placing a sexually experienced male rat in a testing chamber (these vary in size but are usually around 60 × 50 × 40 cm aquaria) with a female in behavioral estrus (the time of ovulation and sexual receptivity in female rats). In this situation, typically rats mate with the timing of sexual contacts being driven by the male until he ejaculates. When mating chambers are large enough, males often participate in a common preintercourse sequence of sniffing, hopping, and then mounting [2]. In this context, these behaviors are both initiated and regulated by the male in that the male is able to "pace" his interactions with the female, which may engender intrinsic reward, associating the females as a conditioned incentive [2]. Although this standard procedure for mating is rewarding for males, female rats that cannot pace their sexual interactions typically do not develop a conditioned place preference as opposed to their female counterparts that "pace" their sexual contacts in a mating strategy called paced mating [2, 3]. In the laboratory, "paced mating boxes" are larger mating arenas, which have a divider with a small hole. This apparatus allows females to engage and withdraw from males and have some control over the receipt of copulatory stimuli. This paradigm has been used to ascertain neurophysiological mechanisms associated with paced mating and/or standard mating (partition is removed).

Endocannabinoids are endogenous lipid neurotransmitters that activate cannabinoid receptors and play a role in

regulating motivated behaviors, such as feeding, anxiety, drug seeking, pain, and reproduction [4, 5]. The most studied of the endogenous ligands are *N*-arachidonoyl ethanolamine (anandamide; AEA), 2-arachidonoyl glycerol (2-AG) [6], and more recently the endogenous metabolite of AEA *N*-arachidonoyl glycine (NAGly) was shown to activate the GPR18, which is a putative cannabinoid receptor [7–9]. Cannabinoid agonist, WIN 55,212-2 (WIN), administered to male rats reduced intromission frequency and increased intervals between ejaculations [4]. Injecting proestrus, but not hormone primed, rats with a CB_1 (cannabinoid receptor 1) antagonist/inverse agonist and GPR18 antagonist, AM251 facilitated sexual motivation [4]. Levels of endocannabinoid ligands (AEA, NAGly, and 2-AG) change significantly in rodent brain with the estrous cycle and show sex differences, suggesting a preparatory role for mating [10]. Indeed, progesterone can also upregulate CB_1 receptor activity in the hypothalamus [11]. Together these findings suggest a mutual regulation between the endocrine system and endocannabinoid system which may play a role in the neuronal control of mating and its rewarding properties.

A structurally similar lipid signaling system to the endocannabinoids and the prostaglandins, specifically PGE2, may act in the hypothalamus by inhibiting release of a prolactin-secretion-inhibiting factor during mating, which could contribute to induction of prolactin surges [12]. PGE2 can facilitate lordosis in response to mounting among estrogen primed, ovariectomized, and adrenalectomized rats [13]. In paced mating paradigms, the interval between sexual contacts is directly related to how much stimulation females receive and increases with each encounter [14]. Among female guinea pigs (also spontaneous ovulators), prostaglandin release in response to mating may disrupt hypothalamus stimulatory norepinephrine signaling, which leads to the postmating inhibition of sexual behavior [12]. Prostaglandins are key components in the mechanism leading up to the follicular rupture involved in ovulation at the site of the ovary [15]. Their influence is demonstrated at the level of the hypothalamus and pituitary by releasing luteinizing hormone (LH), a gonadotropin essential for the onset of ovulation [15, 16].

Lipidomics techniques, in which lipid extracts from tissues are analyzed using tandem mass spectrometry, allow us to measure multiple different lipids from the same tissue and determine relative amounts of lipid between brain areas and treatment groups. Here, we test the hypothesis that production of the prostaglandins PGE2 and $PGF_{2\alpha}$, as well as the endocannabinoid ligands AEA, 2-AG, and NAGly, and structurally related lipids and signaling molecules *N*-palmitoyl ethanolamine (PEA), *N*-oleoyl ethanolamine (OEA), N-docosahexaenoyl ethanolamine (DHEA), and *N*-stearoyl ethanolamine (SEA) are differentially regulated acutely by mating strategies in the female rodent brain. Brains from female rats that were either paced or standard mated and two control groups (chamber exposed and home cage control) were analyzed using high-performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) for production levels of the lipids listed above in eight different brain regions (olfactory bulb, hypothalamus, hippocampus, thalamus, striatum, midbrain, cerebellum, and brainstem). Overall, levels of these lipids were significantly lower among paced compared to standard mated rats in the majority of brain areas with the most dramatic decreases observed in brainstem, hippocampus, midbrain, and striatum. However, chamber exposed rats had significantly higher levels of these lipids than did home cage controls wherein the hippocampus showed the largest increases. These data demonstrate that mating strategies and exposure to mating arenas influence lipid signaling in the brain and imply that eCBs, *N*-acylethanolamines, and PGs are involved in driving the neurophysiological outcomes of mating behaviors.

2. Methods

2.1. Materials. Arachidonoyl ethanolamide-d4 (d4-AEA) was purchased from Tocris Bioscience (St. Louis, MO). AEA, PEA, SEA, OEA, DHEA, and 2-AG were purchased from Cayman Chemical (Ann Arbor, MI). NAGly was purchased from Biomol (Plymouth Meeting, PA). HPLC-grade water and methanol were purchased from VWR International (Plainview, NY). HPLC-grade acetic acid and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO).

2.1.1. Animals—Animal Subjects Used in This Experiment Were Housed at the State University of New York (SUNY) Albany. Age-matched and littermate female Long-Evans rats (4–6 months old) in behavioral estrus ($n = 6$ per group) and sexually experienced male rats were maintained on a 12 : 12 h reversed dark-light cycle (08:00 dark and 18:00 light). Food and water were available *ad libitum*. Vaginal cytology of rats was obtained daily to assess phase of the estrous cycle. On the day of testing, all females (even control groups) with a proestrous smear were vaginally masked and were behaviorally assessed with a male to make sure they were sexually receptive, which was determined by responding to one male mount with lordosis. Only sexually receptive females were used as test subjects. They were then returned to their home cages for a minimum of 2.5 hours before testing.

Experimental Conditions. This protocol was according to Erskine, 1985 [14]. It was performed at the SUNY Albany in the Laboratory of Cheryl Frye.

Animals were tested during the dark phase of the cycle between the hours of 08:00 and 16:00. The animals were transported from the animal housing room to the testing area in their home cages, where they were placed outside the testing room on a rack until testing began. Experimental subjects had vaginal masks affixed to the perineum to minimize mating-induced changes. Behavioral analyses and manipulations were taken by an observer who was unaware of the hypotheses and experimental conditions.

2.2. Paced Mating. Testing was conducted in a white melamine chamber (37.5 × 75 × 30 cm) that was divided into two compartments via a Plexiglas divider that had been cleaned with quatricide and allowed to dry. This apparatus was also constructed and tested to confirm it was functioning

properly before testing began. The Plexiglas divider had a small (5 cm) hole in the bottom center that was large enough for a female to pass through but not large enough for a male. The males had also been previously conditioned to stay away from the hole. This allowed the females to self-administer or "pace" their mating by controlling the frequency of mating contacts and the amount of time between mounts, intromissions, and ejaculation. The males were habituated to the testing chamber first, followed by the female in the opposite side of the chamber. Their sexual interaction was observed for 15 minutes or until the first ejaculatory emission was reached. Lordosis quotients, aggression quotients, proceptivity, and percent exits were measured. Once the fifteen minutes or the first ejaculatory emission was reached, the female was immediately removed from the chamber and decapitated. The brain was then removed and immediately frozen on dry ice. The chamber was then cleaned with quatricide and allowed to dry before the next trial was done.

2.3. Standard Mating. The same 37.5 × 75 × 30 cm melamine chamber that was used for paced mating was also used for this test group. The Plexiglas divider was removed for this experiment. This allowed the male, instead of the female, to control how often mating was administered. The females were vaginally masked to prevent pregnancy and other mating-induced changes.

2.4. Chamber Exposed. A female was placed in the 37.5 × 75 × 30 cm melamine chamber with the Plexiglas divider (same as paced mating chamber design) inserted, for 15 minutes. She was then immediately removed from the chamber and decapitated. The brain was removed and immediately flash-frozen on dry ice. The chamber was then cleaned with quatricide and allowed to dry before the next trial was done.

2.5. Home Cage Control. Females were taken from their housing chambers and decapitated. The brains were removed and immediately flash-frozen on dry ice. They had no social exposure (to males) and no chamber exposure.

2.6. Tissue Dissection. After all the tissues were collected, the tissue was sent overnight on dry ice from Albany, NY, to Bloomington, IN, where it was stored in a −80°C freezer until brains were dissected and processed. Tissue dissection and storage were performed as previously described by Bradshaw et al., 2006 [10]. In brief, the frozen brains were thawed for approximately 5 minutes on an ice cold tin foil covered dissection plate. Once thawed, brains were dissected into the following regions: olfactory bulb, hypothalamus, striatum, thalamus, hippocampus, midbrain, brainstem, and cerebellum. Each region was then placed in a 1.5 mL microfuge tube and flash-frozen with liquid nitrogen. They were stored in the −80°C freezer until used for lipid extractions.

2.7. Lipid Extraction. Each brain area was processed separately and all tissues from a specific brain area were processed together, although the order of processing was randomized, as previously described [10]. The samples were removed from the −80°C freezer. After being shocked with liquid nitrogen, they were weighed and placed in centrifuge tubes on ice. Furthermore, 40 : 1 volumes of methanol were added to each tube followed by 10 μL of 1 uM d4-AEA. d4-AEA was added to act as an internal standard to determine the recovery of the compounds of interest. The tubes were then covered with parafilm and left on ice and in darkness for approximately 2 hours. Remaining on ice, the samples were then homogenized using a polytron for approximately 1 minute on each sample. The samples were then centrifuged at 19,000 ×g at 24°C for 20 minutes. The supernatants were then collected and placed in polypropylene tubes (15 or 50 mL), and HPLC-grade water was added making the final supernatant/water solution 25% organic. To isolate the compounds of interest, partial purification of the 25% solution was performed on a Preppy apparatus (Sigma-Aldrich) assembled with 500 mg C18 solid-phase extraction columns (Agilent Technologies, Santa Clara, CA). The columns were conditioned with 5 mL of HPLC-grade methanol immediately followed by 2.5 mL of HPLC-grade water. The supernatant/water solution was then loaded onto the C18 column and then washed with 2.5 mL of HPLC-grade water followed by 1.5 mL of 40% methanol. The prostaglandins were then collected with a 1.5 mL elution of 70% methanol, NAGly with a 1.5 mL elution of 85% methanol, and the ethanolamides with a 1.5 mL elution of 100% methanol. All were collected in individual autosampler vials and then stored in a −20°C freezer until mass spectrometer analysis.

2.8. LC/MS/MS Analysis and Quantification. Samples were removed from the −20°C freezer and allowed to warm to room temperature and then vortexed for approximately 1 minute before being placed into the autosampler and held at 24°C (Agilent 1100 series autosampler, Palo Alto, CA) for LC/MS/MS analysis. Also 10–20 μL of eluants was injected separately for each sample to be rapidly separated using a C18 Zorbax reversed-phase analytical column (Agilent Technologies, Santa Clara, CA) to scan for individual compounds (mobile phase A: 20% HPLC methanol, 80% HPLC water, and 1 mM ammonium acetate; mobile phase B: 100% HPLC methanol and 1 mM ammonium acetate). Gradient elution (200 μL/min) then occurred under the pressure created by two Shimadzu 10AdVP pumps (Columbia, MD). Next, electrospray ionization was accomplished using an Applied Biosystems/MDS Sciex (Foster City, CA) API3000 triple quadrupole mass spectrometer. A multiple reaction monitoring (MRM) setting on the LC/MS/MS was then used to analyze levels of each compound present in the sample injection. Synthetic standards were used to generate optimized MRM methods and standard curves for analysis. Figure 1 shows a flowchart of the extraction process starting after the animals had been mated.

2.9. Data Analyses. The amount of analyte in each sample was calculated by using a combination of calibration curves of the synthetic standards and deuterium-labeled internal standards obtained from the Analyst software. The standards provided a reference for the retention times by which the

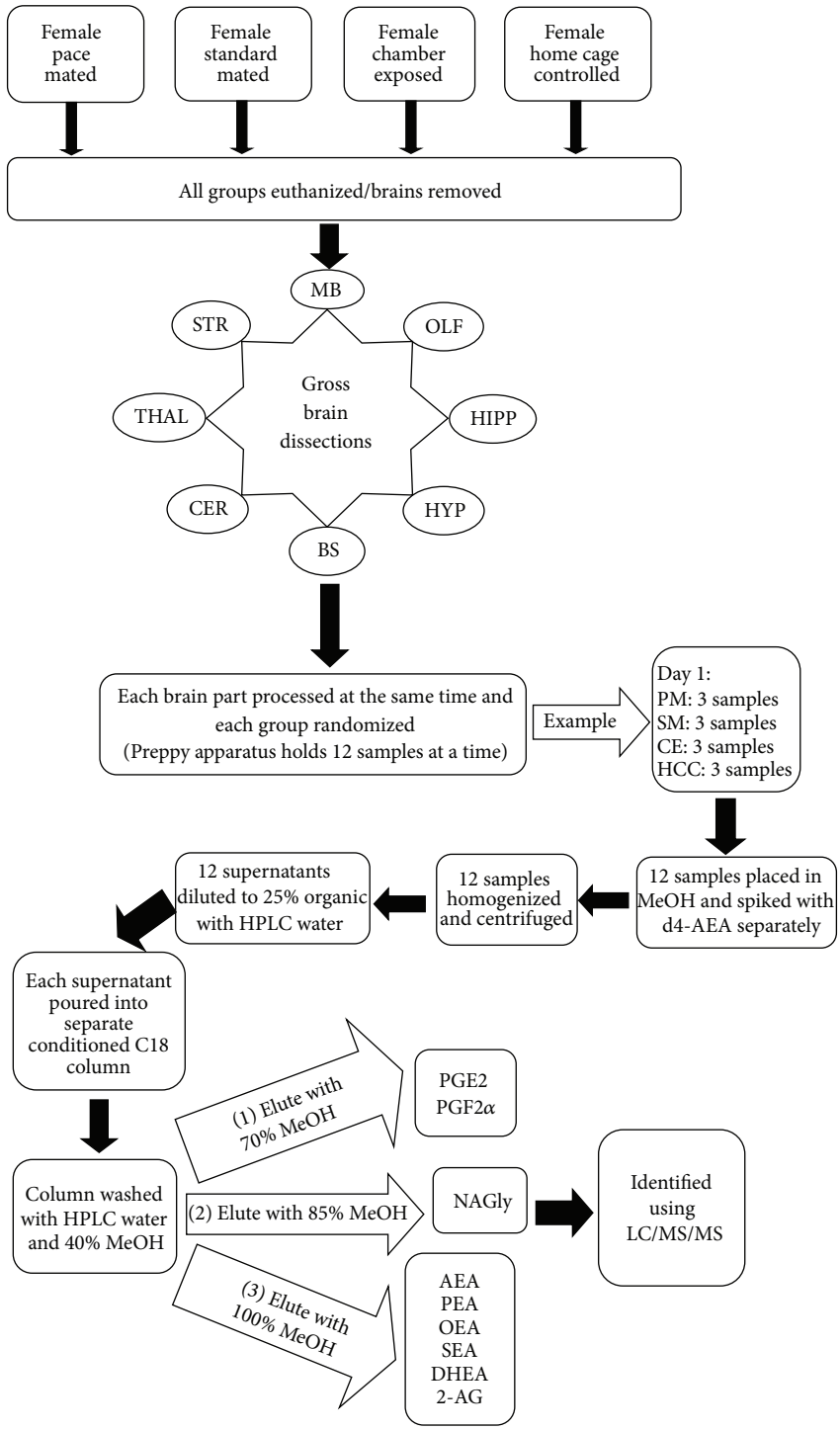

Figure 1: Flowchart of experimental design and lipidomic methodology—starting with the 4 different mating strategies treatment groups, euthanization and collection of brains, gross brain dissection, and then the randomization of the lipid extraction process to the standardized elution and HPLC/MS/MS analysis. See Section 2 for abbreviation definitions.

analytes could be compared. They also helped to identify the specific precursor ion and fragment ion for each analyte which enabled their isolation. These processes provide confidence in the claim that the compounds measured were, in fact, the compounds of interest. The amount of each compound in each tissue was then converted to moles per gram tissue, which is how it was statistically analyzed.

The current study had 4 treatment groups (n = 6/grp) and profiled 9 lipids in 8 different brain regions generating over 1700 data points. In an effort to consider the relatedness

Table 1: General linear model analyses of each lipid profiled by brain area and treatment group.

	GLM analysis							
	BS	CER	HIPP	HYP	MB	OB	STR	THAL
AEA	**0.008**	0.077	0.093	0.319	**0.025**	0.819	**0.017**	0.070
NAGly	**0.037**	0.069	**0.039**	0.224	0.076	0.802	0.241	0.128
PEA	**0.009**	0.119	**0.001**	0.331	**0.039**	0.528	**0.017**	0.143
SEA	**0.016**	0.103	**0.000**	0.665	0.065	0.785	**0.039**	0.589
OEA	**0.017**	0.103	**0.000**	0.588	**0.028**	0.171	**0.012**	0.114
DHEA	**0.042**	0.056	**0.001**	0.284	**0.035**	0.567	**0.009**	0.181
2-AG	**0.000**	0.332	0.572	0.975	**0.020**	0.465	**0.007**	0.224
PGE2	**0.047**	0.283	**0.046**	0.187	0.348	0.597	**0.000**	0.979
PGF2α	0.419	0.113	0.079	0.168	0.306	0.731	**0.000**	0.656

$P \leq 0.05$. Significant differences are shown in bold black.

between analytes in each brain region, general linear models (GLM) were used to consider the experimental conditions between subjects variables and analytes as nested variables across brain regions. Table 1 summarizes the P values from the GLM analysis for each brain region. Using this analysis, it was shown that there were significant interactions between analyte and treatment group in the brainstem (BS), hippocampus (HIPP), midbrain (MB), and striatum (STR). Therefore, post hoc analyses of each individual group to each other were performed using ANOVA, described below. Using this criterion, additional post hoc analyses were not performed on the remaining four brain regions analyzed. Those values are presented in Table 2.

Data from the BS, HIPP, MB, and STR were subsequently analyzed for each brain using the nontested group as the control value compared to the chamber exposed, standard mated, and paced mated group. Follow-up analyses considered the chamber exposed group as the experimental control compared to standard and paced mated groups. Finally, the standard mated group data was compared to the paced mated group. Each comparison was a one-way ANOVA with post hoc Fisher's LSD with a 95% confidence interval for the mean using SPSS software. Data in Tables 3–6 are presented as means ± SE of the means, where $P \leq 0.05$ was considered statistically significant.

3. Results

All analyses indicate that the majority of the changes measured in the 9 lipids profiled here occurred in the brainstem, hippocampus, midbrain, and striatum. To further illustrate this finding, Table 7 combines these analyses and shows the percent increase and decrease when comparing the chamber exposed to home cage control (Table 7(a)), chamber exposed to paced mated (Table 7(b)), and standard mated to paced mated (Table 7(c)). These data potentially represent a shift from appetitive neurochemistry (chamber exposed) to consummatory (paced mated) and indicate the brainstem, hippocampus, midbrain, and striatum as primary brain regions involved in this shift. Described below are the specific findings for each of these brain regions.

3.1. Brainstem Lipids across Treatment Groups. Brainstem levels of lipids showed the most dramatic differences from the home cage controls compared to the paced mated treatment group. Furthermore, 2-AG and PGE_2 significantly increased in the chamber exposed group, whereas there was a significant decrease in SEA and 2-AG in the standard mated group (Table 3(a)). Levels of AEA, NAGly, PEA, SEA, OEA, DHEA, and 2-AG significantly decreased, whereas there was a significant increase in PGE_2 in the paced mated group compared to home cage group (Table 3(a)). Likewise, there was a significant decrease in AEA, NAGly, PEA, SEA, OEA, and DHEA in the paced mated group compared to the chamber exposed group (Table 5(b)), as well as a significant decrease in AEA and 2-AG in the paced mated group compared to the standard mated group (Table 3(c)).

3.2. Hippocampus Lipid across Treatment Groups. Levels of NAGly, PEA, SEA, OEA, DHEA, and PGE2 showed significant increases in the chamber exposed group compared to the home cage group (Table 4(a)), whereas there were significant decreases in NAGly, PEA, SEA, OEA, and DHEA in the standard mated group, as well as significant decreases in AEA, NAGly, PEA, SEA, OEA, DHEA, PGE2, and PGF2α in the paced mated group compared to the chamber exposed group (Table 4(b)). In addition, there was a significant decrease in PEA, OEA, and DHEA in the paced mated group compared to the standard mated group (Table 4(c)).

3.3. Midbrain Lipids across Treatment Groups. Midbrain levels of the eCBs showed a unique profile in that there was a significant increase in AEA and a significant decrease in 2-AG in the chamber exposed group compared to the home cage group. Similarly, there was a significant decrease in 2-AG in the standard mated group compared to the home cage control (Table 5(a)). Comparisons of the chamber exposed group to the mating groups showed a significant decrease in AEA, NAGly, PEA, SEA, OEA, and DHEA in the paced mated group compared to the chamber exposed group (Table 5(b)). Likewise, there was a significant decrease in AEA, NAGly, PEA, SEA, OEA, and DHEA in the paced mated group compared to the standard mated group (Table 5(c)).

Table 2: Levels of lipid production in olfactory bulbs, hypothalamus, thalamus, and cerebellum across the four treatment groups: home cage control, chamber exposed, standard mated, and paced mated.

	Home cage	Chamber exposed	Standard mated	Paced mated
		Olfactory bulbs		
AEA	$8.2E-11 \pm 2.4E-11$	$6.8E-11 \pm 1.0E-11$	$7.1E-11 \pm 1.6E-11$	$9.8E-11 \pm 3.8E-11$
NAGly	$1.8E-11 \pm 5.4E-12$	$2.3E-11 \pm 4.4E-12$	$1.7E-11 \pm 4.9E-12$	$3.1E-11 \pm 2.0E-11$
PEA	$4.1E-12 \pm 1.2E-12$	$3.9E-12 \pm 9.6E-13$	$3.7E-12 \pm 1.3E-12$	$2.2E-12 \pm 3.0E-13$
SEA	$6.2E-11 \pm 1.5E-11$	$6.4E-11 \pm 1.5E-11$	$6.0E-11 \pm 1.8E-11$	$4.5E-11 \pm 6.9E-12$
OEA	$8.4E-11 \pm 1.6E-11$	$9.3E-11 \pm 1.3E-11$	$8.2E-11 \pm 2.1E-11$	$4.7E-11 \pm 4.3E-12$
DHEA	$4.3E-11 \pm 1.5E-11$	$2.7E-11 \pm 3.1E-12$	$5.6E-11 \pm 2.1E-11$	$3.5E-11 \pm 1.4E-11$
2-AG	$2.6E-9 \pm 1.3E-10$	$2.1E-9 \pm 2.9E-10$	$2.6E-9 \pm 3.2E-10$	$2.6E-9 \pm 2.7E-10$
PGE2	$9.1E-10 \pm 1.6E-10$	$8.1E-10 \pm 1.9E-10$	$6.5E-10 \pm 1.8E-10$	$6.5E-10 \pm 9.4E-11$
PGF2α	$3.0E-10 \pm 5.3E-11$	$2.5E-10 \pm 5.5E-11$	$2.1E-10 \pm 5.2E-11$	$3.2E-10 \pm 1.1E-10$
		Hypothalamus		
AEA	$1.2E-10 \pm 8.0E-11$	$1.4E-11 \pm 1.9E-11$	$9.8E-11 \pm 6.6E-12$	$1.1E-10 \pm 2.3E-11$
NAGly	$7.1E-12 \pm 1.6E-12$	$1.1E-11 \pm 2.2E-12$	$6.1E-12 \pm 1.5E-12$	$1.0E-11 \pm 2.0E-12$
PEA	$1.4E-10 \pm 1.9E-11$	$1.0E-11 \pm 1.3E-12$	$7.1E-12 \pm 5.2E-13$	$9.0E-12 \pm 3.1E-12$
SEA	$1.8E-10 \pm 2.5E-11$	$1.7E-10 \pm 2.3E-11$	$1.5E-10 \pm 1.6E-11$	$2.1E-10 \pm 7.8E-11$
OEA	$1.6E-11 \pm 2.2E-12$	$2.6E-10 \pm 2.7E-11$	$1.9E-10 \pm 2.2E-11$	$2.5E-10 \pm 8.9E-11$
DHEA	$7.6E-9 \pm 1.0E-9$	$3.0E-11 \pm 8.9E-12$	$2.4E-11 \pm 3.0E-12$	$3.0E-11 \pm 10.0E-12$
2-AG	$7.6E-9 \pm 1.0E-9$	$8.5E-9 \pm 3.0E-9$	$7.4E-9 \pm 2.3E-9$	$7.2E-9 \pm 1.6E-9$
PGE2	$2.4E-10 \pm 7.1E-11$	$4.0E-10 \pm 7.5E-11$	$2.9E-10 \pm 5.9E-11$	$5.0E-10 \pm 1.3E-10$
PGF2α	$1.6E-10 \pm 3.0E-11$	$2.1E-10 \pm 2.4E-11$	$1.7E-10 \pm 3.0E-11$	$2.7E-10 \pm 5.5E-11$
		Thalamus		
AEA	$3.3E-11 \pm 1.5E-12$	$3.7E-11 \pm 6.8E-12$	$3.7E-11 \pm 4.9E-12$	$2.2E-11 \pm 1.7E-12$
NAGly	$1.6E-11 \pm 2.1E-12$	$2.0E-11 \pm 3.6E-12$	$1.9E-11 \pm 2.2E-12$	$1.2E-11 \pm 5.9E-13$
PEA	$2.4E-11 \pm 1.8E-12$	$3.1E-11 \pm 6.2E-12$	$2.6E-11 \pm 3.8E-12$	$1.8E-11 \pm 1.1E-12$
SEA	$3.5E-10 \pm 2.8E-11$	$4.2E-10 \pm 7.0E-11$	$3.9E-10 \pm 3.5E-11$	$3.5E-10 \pm 2.7E-11$
OEA	$4.9E-10 \pm 3.4E-11$	$6.2E-10 \pm 1.1E-10$	$5.6E-10 \pm 6.7E-11$	$3.8E-10 \pm 2.9E-11$
DHEA	$1.1E-10 \pm 5.7E-12$	$1.4E-10 \pm 1.8E-11$	$1.3E-10 \pm 1.3E-11$	$1.0E-10 \pm 6.5E-12$
2-AG	$1.8E-8 \pm 1.4E-9$	$1.5E-8 \pm 10.0E-10$	$1.8E-8 \pm 1.0E-9$	$1.8E-8 \pm 1.2E-9$
PGE2	$1.8E-10 \pm 2.3E-11$	$1.9E-10 \pm 4.6E-11$	$2.0E-10 \pm 5.1E-11$	$1.9E-10 \pm 2.0E-11$
PGF2α	$2.2E-10 \pm 1.9E-11$	$1.8E-10 \pm 2.7E-11$	$2.0E-10 \pm 3.0E-11$	$1.8E-10 \pm 1.5E-11$
		Cerebellum		
AEA	$3.4E-12 \pm 4.4E-13$	$5.4E-12 \pm 1.1E-12$	$3.2E-12 \pm 2.4E-13$	$4.0E-12 \pm 2.6E-13$
NAGly	$7.3E-12 \pm 3.3E-13$	$9.3E-12 \pm 1.5E-12$	$7.7E-12 \pm 4.7E-13$	$5.9E-12 \pm 4.2E-13$
PEA	$4.4E-12 \pm 2.5E-13$	$6.6E-12 \pm 1.2E-12$	$4.8E-12 \pm 1.7E-13$	$4.5E-12 \pm 5.6E-13$
SEA	$9.5E-11 \pm 5.4E-12$	$1.4E-10 \pm 2.1E-11$	$1.2E-10 \pm 3.5E-12$	$1.1E-10 \pm 1.1E-11$
OEA	$1.1E-10 \pm 3.9E-12$	$1.6E-10 \pm 3.0E-11$	$1.2E-10 \pm 4.9E-12$	$1.1E-10 \pm 9.6E-12$
DHEA	$2.1E-11 \pm 7.0E-13$	$3.1E-11 \pm 4.9E-12$	$2.3E-11 \pm 2.7E-13$	$2.6E-11 \pm 1.9E-12$
2-AG	$3.2E-9 \pm 1.6E-10$	$3.4E-9 \pm 1.7E-10$	$3.3E-9 \pm 1.3E-10$	$3.9E-9 \pm 4.5E-10$
PGE2	$9.5E-11 \pm 1.1E-11$	$8.4E-11 \pm 8.3E-12$	$1.1E-10 \pm 9.2E-12$	$8.0E-11 \pm 1.7E-11$
PGF2α	$5.3E-11 \pm 4.8E-12$	$5.1E-11 \pm 8.3E-12$	$5.6E-11 \pm 3.6E-12$	$3.7E-11 \pm 4.4E-12$

3.4. Striatum Lipids across Treatment Groups. Significant increases in PEA, OEA, DHEA, and 2-AG were demonstrated in the chamber exposed group compared to the home cage group. Additionally, there was a significant increase in SEA, PGE2, and PGF2α in the standard mated group compared to the home cage control (Table 6(a)). In comparison to chamber exposed controls, there were significant increases in PGE2 and PGF2α in the standard mated group and significant decreases in AEA, PEA, OEA, and DHEA in the paced mated group (Table 6(b)). Uniquely, every lipid measured was significantly lower in the paced mated group compared to the standard mated with the exception of the AEA metabolite, NAGly, which was significantly higher (Table 6(c)).

Table 3: Brainstem analysis.

(a) Comparisons of home cage to each group

Brainstem: significance versus home cage control				
	Home cage	Chamber exposed	Standard mated	Paced mated
AEA	$1.7E-11 \pm 1.7E-12$	$2.0E-11 \pm 2.5E-12$	$1.6E-11 \pm 2.1E-12$	$\mathbf{9.6E-12 \pm 9.8E-13}$
NAGly	$2.1E-11 \pm 8.4E-13$	$2.3E-11 \pm 3.0E-12$	$2.0E-11 \pm 1.1E-12$	$\mathbf{1.5E-11 \pm 1.8E-12}$
PEA	$3.4E-11 \pm 1.2E-12$	$3.9E-11 \pm 4.1E-12$	$3.0E-12 \pm 3.7E-12$	$\mathbf{2.3E-11 \pm 2.2E-12}$
SEA	$6.5E-10 \pm 4.5E-11$	$5.8E-10 \pm 5.4E-11$	$\mathbf{5.2E-10 \pm 3.0E-11}$	$\mathbf{4.5E-10 \pm 3.6E-11}$
OEA	$8.3E-10 \pm 3.2E-11$	$8.9E-10 \pm 1.1E-10$	$7.3E-10 \pm 6.3E-11$	$\mathbf{5.4E-10 \pm 7.1E-11}$
DHEA	$1.3E-10 \pm 4.6E-12$	$1.3E-10 \pm 1.5E-11$	$1.2E-10 \pm 7.6E-12$	$\mathbf{9.3E-11 \pm 1.2E-11}$
2-AG	$2.1E-8 \pm 6.9E-10$	$1.4E-8 \pm 1.1E-9^{\dagger}$	$\mathbf{1.6E-8 \pm 7.4E-10}$	$\mathbf{1.2E-8 \pm 8.1E-10}$
PGE2	$2.1E-10 \pm 2.6E-11$	$3.4E-10 \pm 2.8E-11^{\dagger}$	$2.8E-10 \pm 3.5E-11$	$3.3E-10 \pm 4.0E-11^{\dagger}$
PGF2α	$1.7E-10 \pm 1.0E-11$	$2.0E-10 \pm 1.5E-11$	$1.8E-10 \pm 6.8E-12$	$1.6E-10 \pm 2.2E-11$

(b) Comparisons of chamber exposed to standard or paced mating

Brainstem: standard and paced mating versus chamber exposed			
	Chamber exposed	Standard mated	Paced mated
AEA	$2.0E-11 \pm 2.5E-12$	$1.6E-11 \pm 2.1E-12$	$\mathbf{9.6E-12 \pm 9.8E-13}$
NAGly	$2.3E-11 \pm 3.0E-12$	$2.0E-11 \pm 1.1E-12$	$\mathbf{1.5E-11 \pm 1.8E-12}$
PEA	$3.9E-11 \pm 4.1E-12$	$3.0E-12 \pm 3.7E-12$	$\mathbf{2.3E-11 \pm 2.2E-12}$
SEA	$5.8E-10 \pm 5.4E-11$	$5.2E-10 \pm 3.0E-11$	$\mathbf{4.5E-10 \pm 3.6E-11}$
OEA	$8.9E-10 \pm 1.1E-10$	$7.3E-10 \pm 6.3E-11$	$\mathbf{5.4E-10 \pm 7.1E-11}$
DHEA	$1.3E-10 \pm 1.5E-11$	$1.2E-10 \pm 7.6E-12$	$\mathbf{9.3E-11 \pm 1.2E-11}$
2-AG	$1.4E-8 \pm 1.1E-9$	$1.6E-8 \pm 7.4E-10$	$1.2E-8 \pm 8.1E-10$
PGE2	$3.4E-10 \pm 2.8E-11$	$2.8E-10 \pm 3.5E-11$	$3.3E-10 \pm 4.0E-11$
PGF2α	$2.0E-10 \pm 1.5E-11$	$1.8E-10 \pm 6.8E-12$	$1.6E-10 \pm 2.2E-11$

(c) Comparisons of standard mating to paced mating

Brainstem: standard versus paced mating		
	Standard mated	Paced mated
AEA	$1.6E-11 \pm 2.1E-12$	$\mathbf{9.6E-12 \pm 9.8E-13}$
NAGly	$2.0E-11 \pm 1.1E-12$	$1.5E-11 \pm 1.8E-12$
PEA	$3.0E-12 \pm 3.7E-12$	$2.3E-11 \pm 2.2E-12$
SEA	$5.2E-10 \pm 3.0E-11$	$4.5E-10 \pm 3.6E-11$
OEA	$7.3E-10 \pm 6.3E-11$	$5.4E-10 \pm 7.1E-11$
DHEA	$1.2E-10 \pm 7.6E-12$	$9.3E-11 \pm 1.2E-11$
2-AG	$1.6E-8 \pm 7.4E-10$	$\mathbf{1.2E-8 \pm 8.1E-10}$
PGE2	$2.8E-10 \pm 3.5E-11$	$3.3E-10 \pm 4.0E-11$
PGF2α	$1.8E-10 \pm 6.8E-12$	$1.6E-10 \pm 2.2E-11$

Data are moles per gram tissue and are shown as means ± SE. Values in light face have no significant difference among the groups. †Values denote a significant increase, whereas those in bold denote a significant decrease from the treatment group in the far left. $P \leq 0.05$.

4. Discussion

4.1. Our Hypothesis That Lipid Analytes Would Be Altered by Mating Was Supported. Lipidomics, as a field, aims to identify and characterize biologically active lipids and their functional relevance. Here we have used lipidomics techniques to profile 9 signaling lipids throughout the female rat brain as a function of nonmating (chamber exposed and home cage controls) and mating strategies (standard verses paced). Each of the 9 lipids profiled here underwent differential regulation in at least one brain region in relation to the other treatment groups. The most consistent patterns of change were in those analytes measured in the brainstem, hippocampus, midbrain, and striatum.

4.2. Increases in Signaling Lipid Production in the Chambered Exposed Condition. There are at least two ways to interpret the overall increases in lipid signaling molecules during the chamber exposed condition: (1) as the neurochemical response to novelty stress (simply being moved to a new environment) or (2) as a neurochemical correlate to appetitive behavior (each of these rats had been tested for lordosis behavior with a male in this type of chamber 4 hours prior

Table 4: Hippocampus analysis.

(a) Comparisons of home cage to each group

Hippocampus: significance versus home cage control				
	Home cage	Chamber exposed	Standard mated	Paced mated
AEA	$6.3E-11 \pm 7.1E-12$	$7.0E-11 \pm 1.5E-11$	$5.2E-11 \pm 8.2E-12$	$3.6E-11 \pm 3.7E-12$
NAGly	$2.3E-11 \pm 1.6E-12$	$4.2E-11 \pm 1.1E-11^{\dagger}$	$2.5E-11 \pm 2.1E-12$	$1.8E-11 \pm 1.4E-12$
PEA	$6.7E-12 \pm 6.2E-13$	$1.4E-11 \pm 2.2E-12^{\dagger}$	$9.0E-12 \pm 1.1E-12$	$5.0E-12 \pm 3.9E-13$
SEA	$1.2E-10 \pm 1.3E-11$	$2.4E-10 \pm 2.9E-11^{\dagger}$	$1.5E-10 \pm 1.9E-11$	$1.2E-10 \pm 1.1E-11$
OEA	$2.1E-10 \pm 1.8E-11$	$3.2E-10 \pm 3.3E-11^{\dagger}$	$2.5E-10 \pm 2.7E-11$	$1.5E-10 \pm 6.5E-12$
DHEA	$4.2E-11 \pm 3.9E-12$	$6.8E-11 \pm 5.8E-12^{\dagger}$	$5.0E-11 \pm 6.3E-12$	$3.4E-11 \pm 1.6E-12$
2-AG	$9.2E-9 \pm 4.5E-10$	$8.7E-9 \pm 1.4E-9$	$7.4E-9 \pm 1.0E-9$	$8.0E-9 \pm 5.9E-10$
PGE2	$2.7E-10 \pm 4.7E-11$	$5.9E-10 \pm 1.7E-10^{\dagger}$	$3.4E-10 \pm 4.7E-11$	$2.2E-10 \pm 2.9E-11$
PGF2α	$2.9E-10 \pm 2.4E-11$	$3.9E-10 \pm 8.8E-11$	$3.0E-10 \pm 3.1E-11$	$2.0E-10 \pm 2.1E-11$

(b) Comparisons of chamber exposed to standard or paced mating

Hippocampus: standard and paced mating versus chamber exposed			
	Chamber exposed	Standard mated	Paced mated
AEA	$7.0E-11 \pm 1.5E-11$	$5.2E-11 \pm 8.2E-12$	$\mathbf{3.6E-11 \pm 3.7E-12}$
NAGly	$4.2E-11 \pm 1.1E-11$	$\mathbf{2.5E-11 \pm 2.1E-12}$	$\mathbf{1.8E-11 \pm 1.4E-12}$
PEA	$1.4E-11 \pm 2.2E-12$	$\mathbf{9.0E-12 \pm 1.1E-12}$	$\mathbf{5.0E-12 \pm 3.9E-13}$
SEA	$2.4E-10 \pm 2.9E-11$	$\mathbf{1.5E-10 \pm 1.9E-11}$	$\mathbf{1.2E-10 \pm 1.1E-11}$
OEA	$3.2E-10 \pm 3.3E-11$	$\mathbf{2.5E-10 \pm 2.7E-11}$	$\mathbf{1.5E-10 \pm 6.5E-12}$
DHEA	$6.8E-11 \pm 5.8E-12$	$\mathbf{5.0E-11 \pm 6.3E-12}$	$\mathbf{3.4E-11 \pm 1.6E-12}$
2-AG	$8.7E-9 \pm 1.4E-9$	$7.4E-9 \pm 1.0E-9$	$8.0E-9 \pm 5.9E-10$
PGE2	$5.9E-10 \pm 1.7E-10$	$3.4E-10 \pm 4.7E-11$	$\mathbf{2.2E-10 \pm 2.9E-11}$
PGF2α	$3.9E-10 \pm 8.8E-11$	$3.0E-10 \pm 3.1E-11$	$\mathbf{2.0E-10 \pm 2.1E-11}$

(c) Comparisons of standard mating to paced mating

Hippocampus: standard versus paced mating		
	Standard mated	Paced mated
AEA	$5.2E-11 \pm 8.2E-12$	$3.6E-11 \pm 3.7E-12$
NAGly	$2.5E-11 \pm 2.1E-12$	$1.8E-11 \pm 1.4E-12$
PEA	$9.0E-12 \pm 1.1E-12$	$\mathbf{5.0E-12 \pm 3.9E-13}$
SEA	$1.5E-10 \pm 1.9E-11$	$1.2E-10 \pm 1.1E-11$
OEA	$2.5E-10 \pm 2.7E-11$	$\mathbf{1.5E-10 \pm 6.5E-12}$
DHEA	$5.0E-11 \pm 6.3E-12$	$\mathbf{3.4E-11 \pm 1.6E-12}$
2-AG	$7.4E-9 \pm 1.0E-9$	$8.0E-9 \pm 5.9E-10$
PGE2	$3.4E-10 \pm 4.7E-11$	$2.2E-10 \pm 2.9E-11$
PGF2α	$3.0E-10 \pm 3.1E-11$	$2.0E-10 \pm 2.1E-11$

Data are moles per gram tissue and are shown as means ± SE. Values in light face have no significant difference among the groups. †Values denote a significant increase, whereas those in bold denote a significant decrease from the treatment group in the far left. $P \leq 0.05$.

to the experimental treatment and may already associate the chamber with mating), though a combination of both scenarios may also be the case. Data shown here from the midbrain contributes to the theory that the chamber exposed group may be experiencing stress; previous work has shown that midbrain produces both AEA and 2-AG after the onset of stress [17]. Here, the midbrain showed an increase in AEA and a decrease in 2-AG in the chamber exposed group, which is somewhat at odds with the data by Hohmann and colleagues, in which 2-AG levels increase with stress [17]. Their time course for eCB measurement was much longer than the 15 minutes assayed here; therefore, the 15-minute period may have been too brief to show an increase in 2-AG. Alternatively, it could be an indication that the chamber exposed condition is a different type of stressor, for example, one with an ethological relationship to potential mating.

4.3. Decreases in Signaling Lipids in the Paced Mated Condition. Pacing behaviors among female rats may have deep evolutionary roots. For example, colonies of female rats live together in burrows that typically have entryways that are

Table 5: Midbrain analysis.

(a) Comparisons of home cage to each group

Midbrain: significance versus home cage control				
	Home cage	Chamber exposed	Standard mated	Paced mated
AEA	$1.2E-11 \pm 1.1E-12$	$1.8E-11 \pm 2.9E-12^{\dagger}$	$1.5E-11 \pm 1.3E-12$	$1.0E-11 \pm 7.3E-13$
NAGly	$9.7E-12 \pm 1.1E-12$	$1.3E-11 \pm 2.2E-12$	$9.4E-12 \pm 7.1E-13$	$7.8E-12 \pm 2.7E-13$
PEA	$8.8E-12 \pm 6.2E-13$	$1.2E-11 \pm 2.1E-12$	$1.1E-11 \pm 9.4E-13$	$7.3E-12 \pm 4.5E-13$
SEA	$1.6E-10 \pm 6.8E-12$	$2.0E-10 \pm 2.6E-11$	$2.1E-10 \pm 1.7E-11$	$1.5E-10 \pm 10.0E-12$
OEA	$2.2E-10 \pm 1.5E-11$	$3.1E-10 \pm 5.4E-11$	$2.9E-10 \pm 2.5E-11$	$1.8E-10 \pm 1.2E-11$
DHEA	$5.3E-11 \pm 3.1E-12$	$6.9E-11 \pm 9.6E-12$	$6.8E-11 \pm 4.2E-12$	$4.7E-11 \pm 3.3E-12$
2-AG	$1.3E-8 \pm 6.8E-10$	$\mathbf{9.7E-9 \pm 7.9E-10}$	$\mathbf{1.1E-8 \pm 6.4E-10}$	$1.1E-8 \pm 4.0E-10$
PGE2	$3.7E-10 \pm 5.6E-11$	$1.1E-10 \pm 2.2E-11$	$2.4E-10 \pm 2.5E-11$	$2.7E-10 \pm 8.0E-11$
PGF2α	$1.2E-10 \pm 1.6E-11$	$1.3E-11 \pm 2.2E-12$	$8.2E-11 \pm 9.0E-12$	$8.7E-11 \pm 1.4E-11$

(b) Comparisons of chamber exposed to standard or paced mating

Midbrain: standard and paced mating versus chamber exposed			
	Chamber exposed	Standard mated	Paced mated
AEA	$1.8E-11 \pm 2.9E-12$	$1.5E-11 \pm 1.3E-12$	$\mathbf{1.0E-11 \pm 7.3E-13}$
NAGly	$1.3E-11 \pm 2.2E-12$	$9.4E-12 \pm 7.1E-13$	$\mathbf{7.8E-12 \pm 2.7E-13}$
PEA	$1.2E-11 \pm 2.1E-12$	$1.1E-11 \pm 9.4E-13$	$\mathbf{7.3E-12 \pm 4.5E-13}$
SEA	$2.0E-10 \pm 2.6E-11$	$2.1E-10 \pm 1.7E-11$	$\mathbf{1.5E-10 \pm 10.0E-12}$
OEA	$3.1E-10 \pm 5.4E-11$	$2.9E-10 \pm 2.5E-11$	$\mathbf{1.8E-10 \pm 1.2E-11}$
DHEA	$6.9E-11 \pm 9.6E-12$	$6.8E-11 \pm 4.2E-12$	$\mathbf{4.7E-11 \pm 3.3E-12}$
2-AG	$9.7E-9 \pm 7.9E-10$	$1.1E-8 \pm 6.4E-10$	$1.1E-8 \pm 4.0E-10$
PGE2	$1.1E-10 \pm 2.2E-11$	$2.4E-10 \pm 2.5E-11$	$2.7E-10 \pm 8.0E-11$
PGF2α	$1.3E-11 \pm 2.2E-12$	$8.2E-11 \pm 9.0E-12$	$8.7E-11 \pm 1.4E-11$

(c) Comparisons of standard mating to paced mating

Midbrain: standard versus paced mating		
	Standard mated	Paced mated
AEA	$1.5E-11 \pm 1.3E-12$	$\mathbf{1.0E-11 \pm 7.3E-13}$
NAGly	$9.4E-12 \pm 7.1E-13$	$\mathbf{7.8E-12 \pm 2.7E-13}$
PEA	$1.1E-11 \pm 9.4E-13$	$\mathbf{7.3E-12 \pm 4.5E-13}$
SEA	$2.1E-10 \pm 1.7E-11$	$\mathbf{1.5E-10 \pm 10.0E-12}$
OEA	$2.9E-10 \pm 2.5E-11$	$\mathbf{1.8E-10 \pm 1.2E-11}$
DHEA	$6.8E-11 \pm 4.2E-12$	$\mathbf{4.7E-11 \pm 3.3E-12}$
2-AG	$1.1E-8 \pm 6.4E-10$	$1.1E-8 \pm 4.0E-10$
PGE2	$2.4E-10 \pm 2.5E-11$	$2.7E-10 \pm 8.0E-11$
PGF2α	$8.2E-11 \pm 9.0E-12$	$8.7E-11 \pm 1.4E-11$

Data are moles per gram tissue and are shown as means ± SE. Values in light face have no significant difference among the groups. †Values denote a significant increase, whereas those in bold denote a significant decrease from the treatment group in the far left. $P \leq 0.05$.

large enough only for an average-size female, but not an average-size male, to traverse. This enables females to leave their burrows and seek mating opportunities and retreat from males during mating, which allows them to control copulatory contacts [18, 19]. Both wild and laboratory bred rats exhibit these initiation and retreat patterns [19]. Therefore, female rats are able to control the type and number of sexual contacts and the intervals between them using a paced mating strategy. Paced mating, which is rewarding for female rats, facilitates hormonal release that is necessary to enter into an optimal progestational state. Estrogen and progesterone play a role in this feedback loop, as does prolactin, which is required for progestational uterine physiology and is particularly sensitive to paced mating versus standard mating.

Progesterone concentrations have a direct effect on how often a female will remove herself from contact with a male and for how long she stays away (higher progesterone typically means more contact with a male) [20]. One hypothesis to explain this phenomenon is that progesterone can act as an analgesic in genital sensitivity, given that female pacing can be influenced by pelvic nerve modulation [21]. Progesterone may also act as an anxiolytic, which may permit the female

Table 6: Striatum analysis.

(a) Comparisons of home cage to each group

Striatum: significance versus home cage control				
	Home cage	Chamber exposed	Standard mated	Paced mated
AEA	$2.3E-12 \pm 2.5E-13$	$2.9E-12 \pm 3.4E-13$	$3.1E-12 \pm 5.0E-13$	$1.6E-12 \pm 7.3E-14$
NAGly	$5.8E-11 \pm 1.4E-11$	$7.0E-11 \pm 1.2E-11$	$7.8E-11 \pm 1.2E-11$	$9.6E-11 \pm 1.3E-11$
PEA	$6.1E-13 \pm 5.3E-14$	$1.0E-12 \pm 1.6E-13^{\dagger}$	$8.8E-13 \pm 1.3E-13$	$5.3E-13 \pm 2.1E-14$
SEA	$8.4E-12 \pm 7.6E-13$	$1.0E-11 \pm 1.2E-12$	$1.2E-11 \pm 1.4E-12^{\dagger}$	$7.2E-12 \pm 6.0E-13$
OEA	$9.6E-12 \pm 8.1E-13$	$1.5E-11 \pm 2.3E-12^{\dagger}$	$1.4E-11 \pm 2.1E-12$	$8.2E-12 \pm 2.6E-13$
DHEA	$3.1E-12 \pm 2.7E-13$	$4.4E-12 \pm 6.1E-13^{\dagger}$	$4.0E-12 \pm 4.6E-13$	$2.4E-12 \pm 2.0E-13$
2-AG	$3.4E-10 \pm 1.3E-11$	$4.1E-10 \pm 2.8E-11^{\dagger}$	$3.8E-10 \pm 1.7E-11$	$3.1E-10 \pm 1.5E-11$
PGE2	$9.6E-11 \pm 1.5E-11$	$1.2E-10 \pm 2.7E-11$	$2.4E-10 \pm 2.5E-11^{\dagger}$	$9.8E-11 \pm 1.5E-11$
PGF2α	$1.0E-10 \pm 8.5E-12$	$1.2E-10 \pm 1.6E-11$	$1.8E-10 \pm 1.3E-11^{\dagger}$	$8.7E-11 \pm 1.2E-11$

(b) Comparisons of chamber exposed to standard or paced mating

Striatum: standard and paced mating versus chamber exposed			
	Chamber exposed	Standard mated	Paced mated
AEA	$2.9E-12 \pm 3.4E-13$	$3.1E-12 \pm 5.0E-13$	$\mathbf{1.6E-12 \pm 7.3E-14}$
NAGly	$7.0E-11 \pm 1.2E-11$	$7.8E-11 \pm 1.2E-11$	$9.6E-11 \pm 1.3E-11$
PEA	$1.0E-12 \pm 1.6E-13$	$8.8E-13 \pm 1.3E-13$	$\mathbf{5.3E-13 \pm 2.1E-14}$
SEA	$1.0E-11 \pm 1.2E-12$	$1.2E-11 \pm 1.4E-12$	$7.2E-12 \pm 6.0E-13$
OEA	$1.5E-11 \pm 2.3E-12$	$1.4E-11 \pm 2.1E-12$	$\mathbf{8.2E-12 \pm 2.6E-13}$
DHEA	$4.4E-12 \pm 6.1E-13$	$4.0E-12 \pm 4.6E-13$	$\mathbf{2.4E-12 \pm 2.0E-13}$
2-AG	$4.1E-10 \pm 2.8E-11$	$3.8E-10 \pm 1.7E-11$	$\mathbf{3.1E-10 \pm 1.5E-11}$
PGE2	$1.2E-10 \pm 2.7E-11$	$2.4E-10 \pm 2.5E-11^{\dagger}$	$9.8E-11 \pm 1.5E-11$
PGF2α	$1.2E-10 \pm 1.6E-11$	$1.8E-10 \pm 1.3E-11^{\dagger}$	$8.7E-11 \pm 1.2E-11$

(c) Comparisons of standard mating to paced mating

Striatum: standard versus paced mating		
	Standard mated	Paced mated
AEA	$3.1E-12 \pm 5.0E-13$	$\mathbf{1.6E-12 \pm 7.3E-14}$
NAGly	$7.8E-11 \pm 1.2E-11$	$9.6E-11 \pm 1.3E-11^{\dagger}$
PEA	$8.8E-13 \pm 1.3E-13$	$\mathbf{5.3E-13 \pm 2.1E-14}$
SEA	$1.2E-11 \pm 1.4E-12$	$\mathbf{7.2E-12 \pm 6.0E-13}$
OEA	$1.4E-11 \pm 2.1E-12$	$\mathbf{8.2E-12 \pm 2.6E-13}$
DHEA	$4.0E-12 \pm 4.6E-13$	$\mathbf{2.4E-12 \pm 2.0E-13}$
2-AG	$3.8E-10 \pm 1.7E-11$	$\mathbf{3.1E-10 \pm 1.5E-11}$
PGE2	$2.4E-10 \pm 2.5E-11$	$\mathbf{9.8E-11 \pm 1.5E-11}$
PGF2α	$1.8E-10 \pm 1.3E-11$	$\mathbf{8.7E-11 \pm 1.2E-11}$

Data are moles per gram tissue and are shown as means ± SE. Values in light face have no significant difference among the groups. †Values denote a significant increase, whereas those in bold denote a significant decrease from the treatment group in the far left. $P \leq 0.05$.

to withstand longer intromission time with males [22]. Also, estrogen in the striatum influences levels of dopamine and dopamine-mediated behaviors [23]. Dopamine, a neurohormone, at elevated concentrations within the nucleus accumbens (NAc) of the striatum, increases the percent of exits exhibited by female rats, suggesting that it may play a role in the level of neuronal feedback necessary to maintain longer reproductive bouts [23]. It has been predicted that once the level of dopamine begins to decline back to a basal state during mating, the females will return to copulate until the dopamine levels have been restored [23]. Of these two steroids, progesterone was found to have a stronger influence on pacing behavior [20]; however, some effects of progesterone to facilitate, and be increased by, sexual responding may occur through its actions as a prohormone. Progesterone is readily metabolized by sequential actions of 5α-reductase and 3α-hydroxysteroid oxidoreductase to form 5α-pregnan-3α-ol-20-one (3α,5α-THP), which is important in the production of paced mating [13]. 3α,5α-THP, unlike progesterone, does not act on progestin receptors (PRs), has

Table 7: Percent difference in the production of nine lipid signaling molecules in midbrain, striatum, and hippocampus. (a–c) Percent change of *N*-arachidonoyl ethanolamine (anandamide; AEA); *N*-arachidonoyl glycine (NAGly); N-palmitoyl ethanolamine (PEA); N-stearoyl ethanolamine (SEA); N-oleoyl ethanolamine (OEA); N-docosahexaenoyl ethanolamine (DHEA); 2-arachidonoyl glycerol (2-AG); prostaglandin E2 (PGE2); and prostaglandin F2 alpha (PGF2α). Comparisons are from (a) home cage, (b) chamber exposed, and (c) standard mated. Only percent differences of those means that were significantly different are shown. Averages and analysis of significant differences are shown in Tables 4, 5, and 6.

(a)

	Chamber exposed compared to home cage control			
	Hippocampus	Midbrain	Striatum	Brainstem
AEA		↑(50%)		
NAGly	↑(83%)			
PEA	↑(109%)		↑(64%)	
SEA	↑(100%)			
OEA	↑(52%)		↑(56%)	
DHEA	↑(62%)		↑(42%)	
2-AG		**↓(25%)**	↑(21%)	**↓(33%)**
PGE2	↑(119%)			↑(62%)
PGF2α				

(b)

	Paced mating compared to chamber exposed			
	Hippocampus	Midbrain	Striatum	Brainstem
AEA	**↓(49%)**	**↓(44%)**	**↓(45%)**	**↓(52%)**
NAGly	**↓(57%)**	**↓(40%)**		**↓(35%)**
PEA	**↓(64%)**	**↓(39%)**	**↓(47%)**	**↓(41%)**
SEA	**↓(50%)**	**↓(25%)**		**↓(22%)**
OEA	**↓(53%)**	**↓(42%)**	**↓(45%)**	**↓(39%)**
DHEA	**↓(50%)**	**↓(32%)**	**↓(45%)**	**↓(28%)**
2-AG			**↓(24%)**	
PGE2	**↓(63%)**			
PGF2α	**↓(49%)**			

(c)

	Paced mating compared to standard mating			
	Hippocampus	Midbrain	Striatum	Brainstem
AEA		**↓(33%)**	**↓(48%)**	**↓(40%)**
NAGly		**↓(17%)**	↑(23%)	
PEA	**↓(44%)**	**↓(34%)**	**↓(40%)**	
SEA		**↓(29%)**	**↓(40%)**	
OEA	**↓(40%)**	**↓(38%)**	**↓(41%)**	
DHEA	**↓(32%)**	**↓(31%)**	**↓(40%)**	
2-AG			**↓(18%)**	**↓(25%)**
PGE2			**↓(59%)**	
PGF2α		**↓(33%)**	**↓(48%)**	**↓(40%)**

actions via $GABA_A$, NMDA, dopamine receptors, and downstream signal transduction pathways, which may contribute to the reward state associated with paced mating [13].

Diurnal prolactin secretion following mating is more readily instantiated among rats which receive ten or more intromissions during a paced copulatory series as compared to five or fewer sexual contacts in the control, standard mating, paradigm [24]. Controlling the number of intromissions and intervals between them, in paced mating, results in more litters coming to terms and larger litter sizes than what occurs with the standard mating procedure [25]. Enhanced fertility and fecundity with paced, compared to standard, mating may be a result of neurophysiological changes in the brain and reproductive tract [24]. Indeed, female rats engaged in paced mating have earlier termination of estrus [22] and begin twice daily prolactin surges, characteristic of pregnancy, and have higher levels of progestogens, sooner, than do their nonpaced counterparts [20, 25]. This optimal hormonal stimulation in

paced mated female rats may facilitate reward processes and increases the probability of subsequent pregnancies [19, 26].

Rewarding properties of many psychoactive drugs are initiated by activation of the mesolimbic dopaminergic system that begins in the ventral tegmental area of the midbrain (VTA) [27]. Our understanding of how the cannabinoid system plays a role in reward circuitry is growing but still poorly characterized [28]. Increases in AEA and 2-AG production were demonstrated in the midbrain when rats experience chronic alcohol exposure [29], whereas 2-AG and AEA decreased in animals exposed to chronic amphetamine [30]. Both of these results have implications for interactions with the midbrain-striatal dopamine system. Paced mating strategies result in higher levels of midbrain dopamine than standard mating, and this appears to be regulated by estrogen acting on striatal neurons [23]. Here, we show that midbrain AEA and striatal AEA and 2-AG levels are significantly lower after 15 minutes of paced mating compared to standard mating. These data suggest that the paced mating paradigm could be a model for studying the interactions between the cannabinoid and dopaminergic systems in the context of acute rewards without using exogenous pharmacological interventions.

4.4. N-Acylethanolamines Show Dramatic Differences in Regulation in Brainstem, Hippocampus, Midbrain, and Striatum. *N*-Acylethanolamines are a large family of lipid signaling molecules that are ubiquitous in nature. They are primary signaling molecules in plants [31], invertebrates [32], and vertebrates [6]. All of the signaling properties of each of these lipids are not fully understood. PEA, OEA, and DHEA have been shown to have "cannabimimetic" properties in which they are associated with anti-inflammatory and analgesic responses [33]. Recently, OEA was proposed to be the endogenous ligand for GPR119 [34], whereas PEA is an activator of P-PAR-alpha [35]. In our hands, DHEA activates TRPV1 receptors with the same efficacy as AEA (*unpublished results*); however, to date there is no specific molecular target for SEA. Data here show that the metabolism and production of this family of signaling lipids are most acutely changed in female rats that are either placed in the mating area (chamber exposed) or paced mated. Fatty acid amide hydrolase (FAAH) is the primary regulatory enzyme for the family of *N*-acylethanolamine molecules [36]. Recent work from our lab also suggests that FAAH is the rate-limiting factor for NAGly biosynthesis [7]. Earlier evidence suggested that FAAH has an estrogen response element in its promoter region [5]. Therefore, estrogen priming occurring concurrent to ovulation may prime the system to have more FAAH available for rapid degradation of *N*-acyl amides. The rapidity in which these *N*-acylethanolamines appear to first increase in production upon introduction to the mating arena and then rapidly degrade in the context of paced mating suggests a more flexible and timely system than one that requires gene transcription. The wide range of molecular targets of the *N*-acylethanolamines measured here also suggests a variety of regulatory mechanisms that are likely activated with these dramatic changes in signaling ligands.

Overall changes in the majority of lipids profiled here were concentrated in the midbrain, striatum, and hippocampus. Our prior work has demonstrated that other brain neurotrophic factors, neurosteroids, are also changed with mating [26, 37–39]. Indeed, we have consistently seen and reported that levels of allopregnanolone in the midbrain VTA (but also the hippocampus > striatum, cortex) increase with mating these effects are greater with paced mating than; with standard mating, and they occur among gonadally intact rats or rats that are ovariectomized and adrenalectomized and estrogen-primed. Our work on this topic has suggested that estrogen enhances biosynthesis of neurosteroids, which may help prime females and enable them to seek out and/or initiate mating. However, with mating there is a further rise in steroid biosynthesis and then a decline which is necessary for termination of mating (and reproductive success). Interestingly, here we see that other lipid signaling molecules are also labile and changing in response to acute reproductive experience.

5. Conclusions

Neurochemical regulation of complex behavioral patterns, such as the appetitive and consummatory aspects of mating behaviors in females, requires hormonal priming but is rapidly modified within the mating context. Here, we add to this intricate neurophysiological signaling event by demonstrating that eCBs, PGs, and the *N*-acylethanolamines PEA, SEA, OEA, and DHEA are, likewise, modified during these situations. Brain areas that predominate in reward system pathways that are engaged during paced mating show the most dramatic changes in these lipid signaling molecules suggesting a role for these lipids in how this reward system is both activated and maintained. We hope to bear out the functional significance of these changes in our future work.

Authors' Contribution

Jordyn M. Stuart performed all dissections, lipid extractions, and purifications as well as HLPC/MS/MS analysis, statistical analysis, and preparation of the paper; Jason J. Paris performed all behavioral experiments, euthanasia and brain harvesting, statistical analysis, and preparation of the paper; Cheryl Frye coordinated all behavioral experiments, ran statistical analysis, and made preparations of the paper; and Heather B. Bradshaw conducted experimental design, developed MS/MS methodologies, and coordinated all lipid extraction/HPLC/MS/MS analyses, statistical analyses, and preparation of the paper.

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Review Article

Estrogens and Spermiogenesis: New Insights from Type 1 Cannabinoid Receptor Knockout Mice

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Spermatogenesis is a complex mechanism which allows the production of male gametes; it consists of mitotic, meiotic, and differentiation phases. Spermiogenesis is the terminal differentiation process during which haploid round spermatids undergo several biochemical and morphological changes, including extensive remodelling of chromatin and nuclear shape. Spermiogenesis is under control of endocrine, paracrine, and autocrine factors, like gonadotropins and testosterone. More recently, emerging pieces of evidence are suggesting that, among these factors, estrogens may have a role. To date, this is a matter of debate and concern because of the agonistic and antagonistic estrogenic effects that environmental chemicals may have on animal and human with damaging outcome on fertility. In this review, we summarize data which fuel this debate, with a particular attention to our recent results, obtained using type 1 cannabinoid receptor knockout male mice as animal model.

1. Introduction

Spermatogenesis occurs in the testis in a stepwise fashion so that committed spermatogonia proliferate and develop into spermatocytes (SPC) to enter meiosis and produce round spermatids (SPT). These undergo a morphological transformation (spermiogenesis) into mature SPT (i.e., spermatozoa), which are differentially released from Sertoli cells (spermiation) depending on the species. In mammals, further transformations occur in the epididymis to form mature spermatozoa (SPZ) suitable for fertilization [1–4]. Spermatogenesis is a process highly conserved throughout vertebrate species and it is mainly under hypothalamic-pituitary control [5–17]. Indeed, it is a hormonally controlled mechanism; apart from gonadotropins and androgens, numerous endocrine, paracrine, or autocrine factors converge in a complex stage-specific multifactorial control of spermatogenesis [10, 18–24].

Spermiogenesis is the terminal differentiation process of male germ cells, during which haploid round SPT undergo extensive biochemical and morphological changes including acrosome formation, flagellar development, chromatin condensation, and severe nuclear and cellular reorganization [25, 26]. In mouse, morphological criteria have been used to classify spermiogenesis in 16 developmental steps [27]; in particular, round (steps 5–8), elongating (steps 9–11), condensing (steps 12–14), and condensed (steps 14–16) SPT are differentially characterized by acrosomal and flagellar development, as well as by cellular and nuclear shape. During the early steps, round SPT are transcriptionally active; in elongating SPT, transcriptional activity decreases and then turns off; later, in condensing SPT, an extensive chromatin reorganization occurs at molecular and morphological levels [28].

The underlying events that lead to this extensive chromatin reorganization and packaging have been reported in several excellent reviews and here summarized, but very little is known about the molecular mechanisms involved [26, 29–32]. Interestingly, in recent overviews about estrogens (E_2) and spermatogenesis in mammals, the presence of E_2 receptor (ER) and aromatase in somatic and germ cells has been underlined to suggest a possible involvement of this traditionally female hormone in spermiogenesis [2, 33–36].

In this review, we focus on the recent advances in our laboratory about the emerging role of E_2 in SPT chromatin reorganization during spermiogenesis. In particular, new

insight has come out from the study of type 1 cannabinoid receptor knockout ($Cnr1^{-/-}$) mice.

2. Chromatin Reorganization in SPT

Spermatozoa are highly differentiated haploid cells with a particular chromatin organization that results from remodeling events occurring during meiotic and postmeiotic phases of spermatogenesis [26].

Indeed, when cells enter the meiotic prophase, all the somatic histones, except H4 (i.e., H4t-gene protein), are replaced by testis-specific (TH2A, TH2B, TH3, and H1t) or testis-enriched (H2AX, H1a) histone variants [37–39]. Testicular variants of H1 linker are H1t, H1t2, and HILS1 [40–42]. Among these, H1t has been reported to exert the lowest condensing effect on rat testis oligonucleosomes [43]. The high levels of H1t (about 55% of the linker histones) during the pachytene phase until the stage of elongating SPT suggest a role in keeping chromatin in a relatively decondensed state which enables nuclear events. Indeed, during the early steps, round SPT are transcriptionally active and contain H1t-enriched nucleosomal chromatin. In elongated SPT, H1t persists until the transcriptional activity of the genome is still detectable [37]. This histone-variant incorporation step, together with histone posttranslational modifications, such as acetylation, methylation, ubiquitination, and phosphorylation, creates specific chromatin domains characterized by quickly disassembling nucleosomes and by a new "histone code," both facilitating histone displacement [39, 44, 45].

During the postmeiotic stage of spermatogesis, when round SPT are extensively remodelled to form mature SPZ, a gradual and radical change in the chromatin cytoarchitecture is observed (the main events are summarized in Figure 1) [46]. This extensive chromatin reorganization requires (i) expression and storage of specific proteins involved in condensation, such as transition proteins (TNP) and protamines (PRM), (ii) transient DNA strand-breaks which require the topoisomerase enzyme, (iii) displacement and degradation of the nucleosomal structure, (iv) sequential histone replacement, firstly by TNP and then by PRM, (v) transcriptional silencing and DNA repair, and (vi) repackaging of protaminated chromatin into toroidal structures [47, 48]. However, many species retain a small fraction (1% in mouse, 15% in human) of their chromatin in the more relaxed nucleosomal configuration [49] so that SPZ contain at least two differentially packaged chromatin domains: (1) the PRM-based chromatin that organizes the bulk of DNA in a highly compact toroidal configuration, suitable to arrest transcription and mask genome from exogenous and endogenous damage until fertilization [29]; (2) the nucleosome-based chromatin that organizes epigenetically marked developmental loci in a potentially dynamic transcriptional configuration, useful after fertilization [30, 50]. Interestingly, early after fertilization, before activation of the embryonic genome, the paternal pronucleus becomes highly transcriptionally active compared with the female pronucleus [51].

At molecular level, histone-to-PRM exchange requires the expression and storage of specific mRNA involved in condensation. Indeed, transcription and translation are temporally uncoupled. Tnp1/2 and Pmr1/2 mRNAs are synthesized and stored for some days in SPT and later translated, implying a timely controlled process of haploid-regulated transcription and translation [28, 52, 53]. In particular, Tnp1 and Tnp2 mRNA are preserved in translationally inert ribonucleoprotein particles; afterward, they are translated in elongating-condensing SPT (steps 10–15 of spermiogenesis) and then degraded after translation [52, 53]. Temporal and stage-specific appearance of TNP and PRM is strictly regulated and is prerequisite for the correct differentiation of round SPT into mature and motile SPZ with fertilizing capability [54].

Transcriptional regulation of haploid genes depends on potentiation of gene via association with nuclear matrix attachment regions (MARs) [55]. It also depends on DNA methylation and recruitment of transacting factors like TATA-box protein (TBP), Y-box proteins, and cAMP-responsive element modulator (CREM). The latter is a transcription factor which binds as homo- and heterodimers to the regulatory sequence CRE (cAMP-responsive element) [29, 56]. The CREM-encoding gene is highly expressed in the adult testis and shows multiple site of alternative splicing [57]. Levels of CREM transcripts are low in prepubertal testis and only the repressor isoforms (α, β) are detected. However, during puberty, transcripts encoding the activator form CREMtau (CREMτ) appear abundantly expressed only in germ cells, from the pachytene SPC stage onward, while CREMτ protein is not detected in SPC but only in haploid SPT at very high levels [57, 58]. The CREM switch (repressor versus activator) is regulated by the gonadotropin FSH which, acting through Sertoli cells, paracrinally directs the use of an alternative polyadenylation site in SPT, resulting in a more stable CREMt transcript [59]. Many haploid genes have been identified as potential CREM targets since they contain CREs or half CRE in their promoters. Indeed, CREMt regulates gene expression of Tnp and Prm [29, 56].

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. In contrast, the Tnp1 gene shows demethylation in the 5′ region associated with gene activity [38]. In humans, the DNA methyltransferase 1 (DNMT1) is restricted to male germ cells (pachytene SPC and round SPT), and infertile patients showing round SPT maturation arrest also show a specific DNMT1 loss in these cells [60]. Similarly, *Crem*-null mice show round SPT maturation arrest [61, 62]. These observations suggest that methylation and CREM are master controllers of SPT differentiation.

In mouse, histone displacement starts at step 9. Main events promoting histone displacement are phosphorylation of histone H1t [63] and hyperacetylation of H4. The latter process has been largely studied in germ cells of several species [64–66] and is largely conserved during evolution. It has been demonstrated that hyperacetylation of histone tails (steps 8–11 SPT) relaxes nucleosomal DNA-histone interaction, and precedes and overlaps either histone displacement or TNP1/TNP2 presence at nuclear level (step 10–early 15 SPT). Accordingly, core-histones are displaced in their acetylated

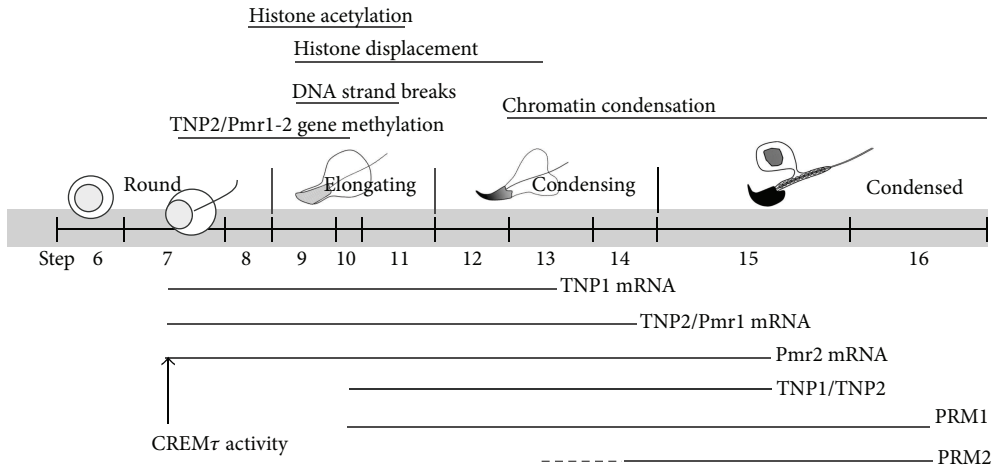

Figure 1: Timing of the main chromatin remodeling events in round, elongating, condensing, and condensed spermatids (the related references are reported in the text).

state [66]. Available reports suggest that the process of histone displacement requires (i) DNA nick/repair induced by topoisomerase which relieves torsional stress associated with histone-to-PRM exchange; (ii) hyperacetylation of histone tails by histone acetyl transferase with a concerted down regulation of histone deacetylase; (iii) histone removal mediated by the recruiting protein BRDT (testis-specific bromodomain protein) able to bind histone acetylated lysines, and (iv) acetylated histone degradation through polyubiquitylation of N-terminal lysines [31]. Histone displacement ends at step 13 SPT [47].

Concurrently to the aforementioned steps, a DNA-binding competition mechanism leads to histone-to-TNP exchange (TNP1-4 in mice, rats, boars, bulls, and men; the best characterized are TNP1 and TNP2) and to final TNP-to-PRM transition (PRM1 in rats; PRM1 and PRM2 in stallions and mice; PRM 4 in humans) [39, 47]. Phosphorylation and dephosphorylation of TNP and PRM trigger their nuclear translocation, their binding to DNA, and eventually chromatin condensation [28, 67]. 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* [68]. In contrast, TNP2 seems to be a DNA-condensing protein [69] and its phosphorylation by protein kinase A greatly reduces its condensation property [70]. Although TNP1 and TNP2 apparently show distinct functions, together with PRM, both are involved in DNA strand-break repair [53, 71–74] and male mice with single *tnp1* or *tnp2* gene deletion demonstrate that TNP1/2 partially complement each other and both affect PRM2 processing before its binding to DNA [75]. Interestingly, in double *Tnp1/Tnp2*-null mice, histone displacement and PRM deposition proceeded relatively normally, chromatin condensation occurs irregularly, and many SPT show DNA breaks, thus demonstrating that although TNP are required for a normal chromatin condensation, they are not essential for the process [76].

Protamines are the most basic DNA-condensing proteins. Most likely, they arise from an ancestral histone H1 gene [77], but, differently from histones, they are characterized by arginine (in eutherians, arginine, and cysteine) rather than lysine residues [78]. This biochemical difference explains PRM greater affinity for DNA, due to a higher hydrogen binding potential of arginine over lysine [79]. These proteins may bind to the major and minor groove of DNA or to the DNA surface by interacting electrostatically with phosphate residues. It has been demonstrated that PRM allow chromatin condensation through arginine residues into toroidal structures at testicular level [29], and further through cysteine residues along the epididymal transit, when inter- and intraprotamines disulphide bonds are formed [80]. In concert with thiol oxidations, PRM also undergo tyrosine phosphorylation during *caput*-to-*cauda* transit [81].

At morphological level, when histone-to-PRM transition occurs, an extraordinary event is observed in the nucleus of differentiating germ cells: flocculent densities of chromatin coalesce into a coarsely granulofibrillar chromatin, which gradually extends in a centripetal and rostral-to-caudal direction and becomes dense and homogeneous at the end of spermiogenesis [28]. This chromatin condensation in toroidal structures modifies the shape of the whole nuclear compartment and strongly reduces its size promoting development of the peculiar elongated, small, and hydrodynamic sperm head that supports swimming ability. Indeed, by stacking these toroids, the sperm nucleus achieves a higher efficiency in packaging the paternal genome and therefore in reducing its size to an absolute minimum [30]. The mechanism by which PRM induce the conformational change in chromatin packaging is not well understood, but it is probably related to PRM properties and to enzymes involved in chromatin remodeling [29, 56].

Abnormal sperm histone or PRM content can disrupt chromatin organization [82–84]. Indeed, histone retention decreases nucleoprotamine-based chromatin and exposes a more relaxed chromatin to damage [56, 85]. In both humans and animals, abnormal DNA damage is associated with compromised fertility and increased miscarriage rates [56, 76, 86]. Therefore, chromatin quality is an objective marker of

sperm function that provides a significant prognostic factor for male infertility [87–89].

3. Estrogens and Spermiogenesis

The presence of intracellular (ERα and ERβ) and transmembrane (GPR30) E_2 receptors in the testis and in particular the expression of ERβ, GPR30, and aromatase in germ cells have highlighted the physiological role of the E_2 in spermatogenesis [35, 90]. Aromatase knockouts (ArKO) or ERα knockouts (αERKO) have further accentuated the role of E_2 in germ cell progression and maturation. Indeed, the specific phenotypes have demonstrated that in αERKO mice disruption of spermatogenesis appears to be primarily linked to mechanical defect in the epididymis [1], whereas in ArKO mice a specific depletion in developing SPT seems to occur [91]. To date, no helpful information came from ERβ or GPR30 knockout mice [2, 33, 34].

Traditionally, testosterone and E_2 were considered male only and female only hormones, respectively. However, at the beginning of the 1930s, the developmental exposure to high doses of E_2 was reported to induce malformation of the male reproductive tract in mammals [92], thus suggesting that E_2 might regulate male reproduction [2, 93]. It is now accepted that E_2 regulate spermatogenesis (gonocyte and spermatogonia proliferation, meiosis, Sertoli cell function) as well as spermiation, sperm transport, and epididymal sperm maturation. Some of these functions are evolutionarily conserved since they have been observed in mammalian and nonmammalian species [1, 7, 8, 12, 14, 19, 36, 94–99].

The first evidence that E_2 affect spermiogenesis came in 1987, when adult male rats were treated with an ovarian protein able to inhibit aromatase activity. After treatment, animals showed degeneration of round SPT and a massive decrease of elongated SPT [100, 101]. Accordingly, a significant decrease in round and elongated SPT, but not in earlier germ cells, was found in adult male bonnet monkeys treated with an aromatase inhibitor for 150 days [102]. These data are not surprising, given the more intense immunostaining and higher aromatase activity in SPT than in any other testicular cells [103, 104].

To well define the role of E_2 in male germ cell development, mice with a targeted disruption of the *cyp19A1* aromatase gene (ArKO mice) were generated [91]. These animals were initially fertile but developed progressive infertility between 4.5 months and 1 year. Spermatogenesis is primarily arrested at early spermiogenic stages, with the appearance of multinucleated cell into the tubular lumen. Furthermore, an abnormal acrosome development with multiple acrosomal vesicles and uneven spreading over the nuclear surface is also observed [91]. This observation suggests that acrosome biogenesis may be an E_2-dependent process. Accordingly, aromatase is detected at high levels in the Golgi complex of developing SPT [34]. The progressive nature of the phenotype may be intrinsic to the mechanism of E_2 action in adult seminiferous epithelium, as observed also in female ArKO mice, characterized by a progressive phenotype too [91]. Alternatively, the delayed phenotype may be explained by the high content of phytoestrogens in the diet, which may supply sufficient exogenous E_2 to maintain normal spermatogenesis in young animals. Indeed, in young ArKO mice on a phytoestrogens-free diet, the phenotype was more severe than in mice on normal diet [105, 106].

However, ArKO mice are not an ideal model to study E_2-regulated events during spermatogenesis in adulthood because of developmental absence of E_2. Therefore, using rat and mouse, several attempts were carried out to create conditions of high intratesticular E_2 levels and study its effect on spermatogenesis.

Earlier studies on long-term exposure to pharmacological doses of E_2 in adult male rats demonstrated that this treatment suppressed both gonadotropins and testosterone releases and induced complete azoospermia [107]. Then, a second attempt was based on the administration of different doses of exogenous E_2 over a period of 10 days to increase intratesticular E_2 levels with a concomitant deficiency in circulating FSH and both plasma and intratesticular testosterone. By exploiting these experimental conditions, it was suggested that, during spermiogenesis, round SPT differentiation (steps 1 to 6) was largely dependent on E_2, whereas SPT elongation (steps 8 to 19) was androgen dependent [108]. These data were supported by the observation that high intratesticular E_2 levels preserved round SPT steps 1–6 whereas testosterone deficiency, induced by E_2 treatment via a negative central feedback, in turn originated pyknotic bodies in elongated/condensed SPT steps 8–19 [108]. In a further study, a similar treatment in rats significantly decreased testicular levels of CREMτ protein, as well as the CREMτ-inducible TNP1/2 and PRM1 proteins, while the relative mRNA levels were not changed [109]. In the same article, the E_2 treatment was also reported to significantly increase testicular androgen binding protein (ABP) mRNA levels, thus suggesting a specific stimulatory effect of E_2 on ABP gene regulation or RNA stability. Authors concluded that E_2 suppressed the appropriate translation of the spermatidal proteins through an ABP-dependent posttranscriptional mechanism. Surprisingly, no information about testicular morphology was reported [109]. A further confirmation of E_2 activity on SPT came from the observation that GPR30 regulates expression of apoptotic markers in rat pachytene SPC and round SPT [90, 110]. More interestingly, growing pieces of evidence reveal that endocrine disruptors, that is, environmental pollutants able to interfere with endogenous endocrine system, have been demonstrated to negatively affect spermatogenesis; among these, there are disruptors with agonistic and antagonistic estrogenic effects. To date, this is a matter of debate and concern because these compounds may have damaging outcome on animal and human fertility [111].

Recent findings reveal that E_2 restore spermatogenesis in hypogonadal (hpg) mice. Due to a natural *Gnrh* gene deletion, the *hpg* mice are functionally deficient in gonadotropins and sex steroids and show meiosis arrest at pachytene stage. Treatment with E_2 or ERα agonist restores meiosis in these animals which, in absence of testosterone, produce haploid elongated SPT, likely via a mechanism involving a weak neuroendocrine activation of FSH secretion [112–114]. Qualitatively complete spermatogenesis could be also restored in *hpg* mice

by administration of either testosterone or its metabolite, the potent nonaromatizable androgen dihydrotestosterone (DHT), in absence of FSH stimulation [115]. However, in this case, a possible E_2 involvement cannot be excluded. Indeed, it has been reported that DHT can be converted in 3β-diol by 3β-hydroxysteroid dehydrogenase (3β-HSD), which preferentially binds ER rather than androgen receptor [116]. Interestingly, in addition to th results obtained in *hpg* mice [112–114], studies on FSH-receptor knockout mice (FORKO) support an E_2 involvement in spermiogenesis, likely in a synergistic or independent way with FSH and/or androgens [117]. Indeed, FORKO mice show low testosterone and E_2 levels [118], as well as a significant increase of spermatogonia, decrease of elongated SPT, and weight loss of testis, epididymis, and seminal vesicles. Tubular and luminal diameters of *caput* epididymis appear smaller than those of wild-type males with few lumina filled with SPZ [117]. At molecular level, deprivation of FSH signaling greatly decreases TNP/PRM levels as well as chromatin quality of SPZ [119].

We recently characterized the reproductive phenotype of type 1 cannabinoid receptor knockout (*Cnr*1$^{-/-}$) male mice. These mutants exhibit endocrine and phenotypic features which are useful to extend the above studies about the role of E_2 in SPT differentiation and in particular in the maintenance of sperm chromatin quality. Main features are summarized in Table 1 and described below.

Table 1: Reproductive phenotype of *Cnr*1$^{-/-}$ male mice and related references.

Hypothalamus-pituitary-testis axis
(i) Normal pituitary LH content [120]
(ii) Low serum LH concentration [120]
(iii) Low testicular testosterone secretion [120]
(iv) Low circulating testosterone and E_2 levels [120, 121]
(v) High pituitary GnRH-R and low FSHβ subunit mRNA levels [121]
(vi) Low testicular Fsh-R mRNA levels [121]
(vii) Low testicular P450 mRNA levels [121]
(viii) Low P450arom levels in Leydig cells [121]
(ix) Low Tnp2 levels (both mRNA and protein) [85]
Adult Leydig cell
(i) Low number of adult Leydig cells [122]
(ii) Normal 3-βHsd mRNA levels/Leydig cell [121]
Sperm chromatin quality
(i) High number of SPZ with retained histones [85, 121, 123]
(ii) High number of SPZ with uncondensed chromatin [85, 123]
(iii) High number of SPZ with DNA damage [85, 123]
(iv) SPZ with high % of damaged DNA [85]
(v) Increase of DNA damage during epididymal transit from *caput* to *cauda* [85]
(vi) High mean values of sperm nuclear length [123]
Epididymal sperm motility acquisition
(i) High number of potentially motile SPZ in *caput* [4]
(ii) Precocious sperm motility acquisition in *caput* epididymis [4]

4. Reproductive Phenotype of *Cnr*1$^{-/-}$ Male Mice: E_2 Activity on Sperm Quality

Endocannabinoids are lipidic mediators identified in several peripheral tissues (brain, testis, and epididymis) and biological fluids (follicular fluid, maternal milk, and blood) [4, 124–129]. To date, the best characterized are arachidonoylethanolamide (AEA or anandamide) and 2-arachidonoylglycerol (2-AG), but other molecules have been proposed as possible cannabinoid receptor (CNR) agonists [26, 130]. Endocannabinoids regulate reproduction, in both males [16, 17, 20, 26, 122, 131–139] and females [140–145], and specific G-protein-coupled cannabinoid receptors, CNR1 and CNR2 (or CB1 and CB2, resp.), have been localized in male and female reproductive tracts [20, 146–148]. In the testis, CNR1 is present in somatic and germ cells including SPT, from round stage onward [122, 129, 131, 149–154], and recently its involvement in chromatin packaging during SPT differentiation has been reported [26, 85]. However, much remains to be clarified about a direct and/or indirect role. *In vivo* studies, using nonmammalian and mammalian animal models, show that CNR1 acts at both central and local level [155, 156]. In frog, an intriguing CNR1-GnRH (gonadotropin-releasing hormone) interplay occurs at a central level, with CNR1 regulating GnRH synthesis [16]; at testicular level, CNR1 also regulates *GnRH1/2* and *GnRH-R (GnRH-receptor)* expression [157]. In rat, CNR1 regulates the release of hypothalamic GnRH [158], while in mice it increases *Tnp2* expression in the testis [85]. It has been hypothesized [26] that testicular AEA, responsive to E2 and produced by somatic cells [131, 159] and/or by SPT [140], may act as paracrine/autocrine factor on SPT themselves via CNR1, by regulating *Tnp2* mRNA transcription or stability.

Most information about CNR1 involvement in male reproduction came from *Cnr*1$^{-/-}$ mice. An early study reported that *Cnr*1$^{-/-}$ male efficiently synthesizes the gonadotropin LH but shows low levels of LH and testosterone in the bloodstream. Furthermore, *Cnr*1$^{-/-}$ testis produces few testosterone *in vitro* [120]. Recently, we have characterized the reproductive phenotype of *Cnr*1$^{-/-}$ mice and reported that males show normal progression of spermatogenesis [3, 4, 122], produce SPZ [3, 4], and are fertile [85] although they displayed a lot of abnormalities (see Table 1 and references herein) such as (i) downregulation of neuroendocrine axis [121], (ii) developmental decrease of Leydig cell number [122], (iii) low sperm chromatin quality [85, 121], and (iv) abnormal epididymal sperm motility (i.e., potential to move) acquisition [3, 4]. Some of these abnormalities well fit with the early data reported by Wenger et al. [120]. Indeed, at molecular level *Cnr1* gene deletion originates a ligand-dependent downregulation of GnRH-R signaling and this may explain the LH drop originally observed in these animals. In any case, although both LH and testosterone decrease by 50% in serum of *Cnr*1$^{-/-}$ as compared with wild-type mice [120], we found

that LH signaling is sufficient to regulate steroidogenesis supporting testosterone production in Leydig cells. Indeed, the 3β-HSD, a LH-responsive selective marker of Leydig cells, is synthesized at normal levels in individual single cells [121], thus suggesting that in *Cnr*1$^{-/-}$ mice the testosterone decrease, in both *in vivo* and *in vitro* systems [120], is exclusively related to a decrease of Leydig cell number. We also found that GnRH downregulation is accompanied by downregulation of *Fshb*, *Fsh-R, Tnp2,* and *P450arom* mRNA as well as of TNP2 and P450arom protein. The P450arom protein decrease is observed in the interstitial Leydig cells and the low levels are independent by Leydig cells number. Simultaneously, low E_2 levels were detected in the bloodstream suggesting that, in the mutant mice, the downregulation of neuroendocrine axis interferes with gene expression of Tnp2 in SPT as well as of P450arom in Leydig rather than in germ cells with consequent reduction of E_2 levels in the bloodstream [121].

The morphological and biochemical evaluations of epididymal *Cnr*1$^{-/-}$ sperm samples showed a lot of abnormalities. In wild-type animals, a 2-AG gradient (high level in *caput* versus low levels in *cauda*) prevents sperm motility acquisition in *caput* through the inhibitory activation of CNR1. In knockout animals, a high number of SPZ from *caput* epididymis appears motile, thus demonstrating that, in absence of CNR1, SPZ precociously acquire their potential to move [4]. The morphological and biochemical analyses of epididymal *Cnr*1$^{-/-}$ SPZ also showed poor chromatin quality [26, 85]. In particular, we found that the genetic inactivation of *Cnr1* affects chromatin remodeling mechanisms that occur in SPT during spermiogenesis. Indeed, in *caput* epididymis from *Cnr*1$^{-/-}$ animals, the number of SPZ with histone retention as well as the number of SPZ with uncondensed chromatin or with DNA damage is higher than in *Cnr*1$^{+/+}$ and *Cnr*1$^{+/-}$ animals [85, 121, 123] demonstrating that in these animals spermiogenesis is qualitatively inefficient. Despite that, animals retain their fertility [85], likely because of a sufficient number of SPZ with mature chromatin. Correlation analysis and morphological studies also showed that abnormal histone retention is strictly related to uncondensed chromatin or DNA damage as well as it is associated to sperm nuclear size elongation. Intriguingly, histone displacement and chromatin condensation normally occur in a rostral-to-caudal direction [89] and then the failure of these mechanisms might be responsible of the nuclear swelling along its longitudinal axes. Recent experiments suggest that low plasma E_2 levels might be the cause of the sperm chromatin imperfections observed in these animals [123]. Indeed, 24-day postpartum *Cnr*1$^{-/-}$ male mice exposed to low doses of E_2, every other day for a complete cycle of spermatogenesis, showed a weak upregulation of neuroendocrine axis (no effect on *GnRH* mRNA levels; strong increase of *GnRH-R* mRNA; weak increase of *Fshb* subunit, *Fsh-R,* and *P450arom* mRNA; weak increase, about 20%, of P450arom protein in Leydig cells; no effect on *Tnp2* mRNA) and the rescue of sperm chromatin quality indices (histone content, chromatin condensation, DNA damage, and nuclear size), via an ER mediated mechanism [121]. Several studies propose that chromatin condensation and DNA damage are related to each other and are secondary effects associated to disrupted histone displacement [29, 56]. Therefore, it is plausible to conclude that E_2 treatment, through a TNP2-independent effect, primarily affects histone displacement and sequentially induce the rescue of sperm chromatin quality indices to physiological values. In agreement, *caput* SPZ from rat chronically injected with E_2 showed a TNP/PRM1-independent chromatin hypercompaction [109]. In addition, serum concentration of E_2 and free T4 inversely correlates with sperm DNA damage in men from an infertility clinic [160]. Furthermore, it has been reported that E_2 delay testicular cell damage, which leads to functional senescence. Therefore, E_2 are helpful in protecting the reproductive functions from the adverse effects exerted by reactive oxygen species (ROS) produced in large quantities in the aged testis [161].

These results, in combination with aforementioned data, show that E_2, indirectly via stimulatory effects on FSH secretion and/or directly via paracrine actions within the testis, play a key role in spermiogenesis since they preserve chromatin packaging in SPT and then sperm quality. Interestingly, sperm nuclear length, which is related to chromatin quality, appears to be an E_2-responsive morphological parameter [123] and may be used as helpful tool to discriminate "in real time," among morphologically normal SPZ, those with a good chromatin quality. To date, no tool exists to verify "in real time" sperm chromatin quality. Interestingly, in assisted reproduction technique field, abnormal nuclear size of human SPZ is commonly considered to be of poor prognosis [162]. More interestingly, a recent article describes a tight correlation between percentage of SPZ with nuclear form abnormalities, screened by MSOME (motile sperm organelle morphology examination) technique, and DNA fragmentation [163].

5. Last Considerations and Conclusions

Studies on αERKO mice led to conclude that E_2 are involved in epididymal sperm maturation. Our data and those reported in the literature suggest a further and intriguing role for E_2 in spermiogenesis and in maintenance of sperm chromatin quality. The main future endpoint will be the characterization of E_2 mechanisms to better understand whether its action is direct and/or mediated. Indeed, it is still a matter of debate whether E_2 and/or FSH affect chromatin remodeling in SPT in either a synergistic or an independent way with androgens [31]. Gene deletion animal models have revealed that both FSH and testosterone levels are implicated in the regulation of chromatin condensation during spermiogenesis [31]. However, although is emerging idea is that these hormones may act in synergy [31, 164], it has been reported in rat that the inhibition of FSH, resulted from hyperprolactinemia induction, reduces chromatin packaging in an androgen-independent way.

Apart from the divergences, FORKO mice exhibit endocrine and phenotypic features of *Cnr*1$^{-/-}$ male mice, including reduced histone displacement, enlarged sperm head size, decreased chromatin quality of SPZ (low packaging and high DNA damage), and low levels of testosterone and E_2 [117–119].

In *Cnr*1$^{-/-}$, the number of SPZ and epididymal epithelium morphology, both dependent on testosterone [165], are not affected [3] suggesting that, in mutant mice, testosterone ranges within levels sufficient to support spermatogenesis. Therefore, we speculate that, in *Cnr*1$^{-/-}$ mice, E_2 action, (i.e., the rescue of histone displacement in SPT and chromatin quality in SPZ) is independent of testosterone. Our future endpoint will be to confirm if this really occurs and to establish E_2 and FSH roles. The importance of these studies is corroborated by the growing pieces of evidence that E_2 activity can be mimicked or antagonized by estrogenic environmental chemicals with potentially damaging effects on animal and human fertility [111].

Abbreviations

SPC: Spermatocytes
SPT: Spermatids
SPZ: Spermatozoa
E_2: Estrogens
ER: Estrogen receptor
Cnr1$^{-/-}$: Type 1 cannabinoid receptor knockout
TNP: Transition proteins
PRM: Protamines
MARs: Nuclear matrix attachment regions
TBP: TATA-box protein
CREM: cAMP-responsive element modulator
CRE: cAMP-responsive element
CREMτ: CREMtau
DNMT1: DNA methyltransferase 1
BRDT: Testis-specific bromodomain protein
GPR30: Transmembrane E2 receptor
ArKO: Aromatase knockouts
αERKO: ERα knockouts
ABP: Androgen binding protein
Hpg: Hypogonadal
DHT: Dihydrotestosterone
3β-HSD: 3β-Hydroxysteroid dehydrogenase
AEA: Arachidonoylethanolamide
2-AG: 2-Arachidonoyl-glycerol
CNR: Cannabinoid receptor
GNRH: Gonadotropin-releasing hormone
GNRH-R: GnRH-receptor
ROS: Reactive oxygen species
MSOME: Motile sperm organelle morphology examination
FORKO: FSH-receptor knockout mice.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Endocannabinoid System in the Postimplantation Period: A Role during Decidualization and Placentation

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Although the detrimental effects of cannabis consumption during gestation are known for years, the vast majority of studies established a link between cannabis consumption and foetal development. The complex maternal-foetal interrelationships within the placental bed are essential for normal pregnancy, and decidua definitively contributes to the success of this process. Nevertheless, the molecular signalling network that coordinates strategies for successful decidualization and placentation are not well understood. The discovery of the endocannabinoid system highlighted new signalling mediators in various physiological processes, including reproduction. It is known that endocannabinoids present regulatory functions during blastocyst development, oviductal transport, and implantation. In addition, all the endocannabinoid machinery was found to be expressed in decidual and placental tissues. Additionally, endocannabinoid's plasmatic levels were found to fluctuate during normal gestation and to induce decidual cell death and disturb normal placental development. Moreover, aberrant endocannabinoid signalling during the period of placental development has been associated with pregnancy disorders. It indicates the existence of a possible regulatory role for these molecules during decidualization and placentation processes, which are known to be particularly vulnerable. In this review, the influence of the endocannabinoid system in these critical processes is explored and discussed.

1. Cannabinoids: Historical Perspective

Cannabis sativa properties were known for centuries, though only in 1964 its main psychoactive component, Δ^9-tetrahydrocannabinol (THC), was isolated and its chemical structure revealed. Due to its lipophilic nature, it was assumed that the psychotropic effects of THC resulted from interference with membrane fluidity, rather than binding to a specific receptor. However, by the mid 1980s, it was shown that cannabinoid activity was highly stereoselective, which led to the search for a specific receptor and its endogenous ligands [1, 2].

In late 1980s, cannabinoid receptors were discovered. The first cannabinoid receptor (CB1) was isolated from rat brain, [3] and, in 1993, a second receptor (CB2) was cloned from human promyelocytic leukaemia HL-60 cells [4].

Both cannabinoid receptors are G protein-coupled receptors (GPCRs), and their activation reduces adenylyl cyclase activity, leading to diminished cyclic adenosine monophosphate (cAMP) levels [5, 6]. Additionally, both receptors are coupled with intracellular signalling pathways related to activation of mitogen-activated protein kinases (MAPK). The CB1 is also coupled to ionic channels, inhibiting N- and P/Q-type voltage-gated calcium channels, activating A-type voltage-gated calcium channels, and inwardly rectifying potassium channels [5–7]. Furthermore, cannabinoids can modulate sphingolipid-metabolizing pathways by increasing intracellular levels of ceramide, an ubiquitous lipid second messenger [8].

On the other hand, as cannabinoids induced contractility of vascular smooth muscles independently of CB1 or CB2 receptors activation, it was suggested that other cannabi-

noid-like receptors may exist [9, 10]. Later, the orphan receptor GPR55 was suggested to be involved in non-CB1, non-CB2-mediated actions of cannabinoids [11]. Though with limited sequence homology with CB1 (13%) and CB2 (14%), GPR55 was suggested as a new cannabinoid receptor, the CB3 [12].

2. Endocannabinoid System

Besides THC, other molecules have been described to bind and activate cannabinoid receptors [13]. Some of these molecules were found to be produced by the organism and derived from arachidonic acid (AA), thus resulting in a new class of cannabinoids—the endocannabinoids (eCBs).

The first endocannabinoid, *N*-arachidonoylethanolamine, later called anandamide (AEA), was isolated in 1992 from pig brain by Raphael Mechoulam's group [14]. Three years later a second compound, the 2-arachidonoylglycerol (2-AG), was identified [15, 16].

Although cannabinoid receptors constitute the main targets of AEA, this molecule is capable of interacting with other molecular targets, such as the transient receptor potential vanilloid 1 (TRPV1) [17] and the peroxisome proliferator-activated receptors (PPARs) family [18, 19]. In opposition, 2-AG has higher affinity to CB1 and CB2 receptors than AEA, though it does not activate TRPV1.

Although AEA and 2-AG remain the best studied, other endogenous compounds may also bind cannabinoid receptors such as 2-arachidonoylglycerol ether (noladin ether, 2-AGE) [20], O-arachidonoylethanolamine (virodhamine) [21], *N*-arachidonoyl dopamine (NADA) [22], *N*-arachidonoyl glycine (NAGly) [23], and *Cis*-9,10-octadecanamide (oleamide or ODA) [24].

Like these molecules, other lipid mediators share endocannabinoid metabolic pathways. Although they are not able to bind to any of the cannabinoid receptors identified so far, these lipid messengers may influence endocannabinoid metabolism and function. These include the *N*-acylethanolamide family, particularly *N*-palmitoylethanolamide (PEA; C16:0), stearoylethanolamide (SEA, C18:0), and *N*-oleoylethanolamide (OEA; C18:1) [25].

Together with cannabinoid receptors and the endogenous compounds, the endocannabinoid system is also constituted by the putative membrane transporter and the enzymes responsible for the biosynthesis and degradation of endocannabinoids [26].

It is an accepted idea that endocannabinoids are released "*on demand,*" which means they are only produced when they are needed and on locals required. Based on the presence of intracellular AEA binding proteins, recent studies have been trying to prove the existence of AEA storage sites, believed to be adiposomes [27]. This hypothesis refutes the current conviction of an "*on demand*" production, so it must be carefully and extensively analysed.

The major endocannabinoids have different biosynthetic pathways, though both result from membrane precursors through enhanced intracellular Ca^{2+} concentrations. While AEA is synthesized from its precursor, the *N*-arachidonoyl-phosphatidylethanolamine (NAPE), by a specific phospholipase D (NAPE-PLD) [28], 2-AG is produced through a phospholipase C (PLC), producing 1,2-diacylglycerol (DAG), which may be, subsequently, converted to 2-AG by diacylglycerol lipase (DAGL) [29, 30].

Once synthesized, endocannabinoids are released to extracellular environment to target cannabinoid receptors, located in cell membranes, though AEA may also act on intracellular sites, such as TRPV1 receptor and T-type Ca^{2+} channels [31, 32]. Endocannabinoids appear to be inactivated through a two-step process involving the transport across the membrane, followed by two specific hydrolytic systems. Anandamide is primarily degraded by FAAH through hydrolysis into arachidonic acid and ethanolamine [33, 34]. Although FAAH can also degrade 2-AG [35] into glycerol and arachidonic acid, the main enzyme responsible for the inactivation of this compound is monoacyl glycerol lipase (MAGL) [36]. As AEA and 2-AG present structural similarities with polyunsaturated fatty acids, they can also serve as substrate for the inducible cyclooxygenase-2 (COX-2) and various lipoxygenases (LOXs) [37].

The current evidence indicates endocannabinoids as relevant modulators of several physiological functions not only in the central and autonomic nervous system but also in immune system, endocrine network, gastrointestinal tract, and in reproductive system [38].

During the last decade, the role of endocannabinoid system network in female reproduction has attracted major attention. Various evidences indicate a role for endocannabinoid elements during the preimplantation period. Endocannabinoids and both cannabinoid receptors have been described from the earliest stages of embryonic development to be involved in the regulation of blastocyst maturation, oviductal transport, implantation, and pregnancy maintenance. CB1 receptor is expressed in the embryo, in much higher levels than those in the brain [39]. Consistently, AEA was also found in much higher levels in mice nonpregnant uterus than in brain, which together with the changing levels of AEA with pregnancy status was indicative of a possible role for this lipid in early pregnancy events [40].

Endocannabinoid levels contribute to create the appropriate environment conducive to preimplantation embryo transport through the oviduct [41]. In fact, there is a regional regulation with higher expression of FAAH and NAPE-PLD in the ampulla and isthmus, respectively. This differential expression creates the appropriate AEA levels during oviductal transport.

A similar phenomenon is observed in mice uterus during implantation where expression of AEA-metabolizing enzymes in mouse uterus is critical to define their concentration in implantation sites and consequently in the implantation outcome. In fact, just before embryo implantation, AEA declines to barely detectable levels at the site of implantation, and this change is believed to contribute to the receptive uterine state [42]. AEA can also induce differential signals in blastocyst differentiation and outgrowth. At low levels, cultured blastocysts exhibited accelerated trophoblast differentiation

and outgrowth, while higher levels induce opposite effects [43, 44]. Studies regarding the underlying mechanism of these biphasic effects revealed that stimulatory and inhibitory effects on blastocyst function and implantation depend on different signal transduction pathways. While AEA at low doses activates ERK signalling pathway, at high concentrations it inhibits Ca^{2+} influx. Both effects occur through CB1 receptor [44]. The AEA-biphasic effects reveal AEA as a potential "cannabinoid sensor" mechanism, influencing crucial steps during early pregnancy. Nowadays, it is well accepted that the embryo is a target for natural and endogenous cannabinoids, raising the significance of cannabinoid signalling in female fertility.

Whilst endocannabinoid signalling is clearly critical in early pregnancy events, its effects during decidualization and placentation period and implications in pregnancy outcome remain largely undefined.

3. Endocannabinoid System during Decidualization

Essential changes must occur in human endometrium to allow the establishment of pregnancy. These changes occur in the uterine endometrial stromal cells, which undergo a characteristic decidual cell reaction. Decidualization prepares the uterus for the trophoblast invasion that occurs during pregnancy.

In human, decidualization is present in normal menstrual cycle during the late secretory phase [45], whereas in rodents decidualization is only a blastocyst-dependent process in normal pregnancy [46]. At the site of blastocyst attachment, the endometrial stromal cells undergo decidual reaction, in which stromal cells proliferate and differentiate into decidual cells [47]. Morphologically, this process involves the differentiation of elongated fibroblast-like cells into enlarged polygonal epithelial-like decidual cells. Human decidual cells produce specific molecules such as inflammatory mediators like IL-1, IL-6, IL-8, and TNF-α [48], various regulatory factors including relaxin, renin, prolactin (PRL), and insulin-like growth factor binding protein-1 (IGFBP-1), [45, 49] and specific extracellular matrix proteins, such as laminin, type IV collagen, and fibronectin [50].

Anomalies on decidual process predispose to pregnancy complications, including miscarriage, preeclampsia, foetal growth restriction, and preterm labour.

The rat, just like human, exhibits a highly invasive type of placental development with subsequent remodelling of the uterine tissues, being a suitable model for studying the mechanisms of decidualization [46].

Studies in various mammals, including rats and humans, indicate that endocannabinoid system elements are present in decidua, which suggests its involvement in decidua establishment and/or remodelling (Figure 1) [51–55].

Although limited data are available concerning human decidual tissue, *Cb1* mRNA levels were detected in decidua from women with viable pregnancies [56–58], as well as immunoreactivity for CB1, CB2, NAPE-PLD, and FAAH proteins [55]. During the follicular phase of menstrual cycle, AEA plasmatic levels were significantly higher than those in the luteal phase [59], suggesting that steroid hormones may also be involved in the regulation of AEA levels in human pregnancy as previously observed during early pregnancy in mice [60]. Together, these data point to a full functional endocannabinoid system naturally occurring in human decidual tissue during pregnancy. Currently, there are no studies considering the expression of 2-AG metabolic enzymes or 2-AG levels during human pregnancy.

In rodents, the stimulus for decidualization is not spontaneous, being the blastocyst crucial for this process. Detectable levels of proteins and respective mRNAs for metabolic enzymes (*Faah*, *Nape-pld*, Cox-2, *Magl*, and *Daglα*) and cannabinoid receptors (*Cb1, Cb2, Gpr55,* and *Trpv1*) in rat decidua throughout pregnancy [51–54] were found. Among these, CB1 was markedly upregulated during midpregnancy, which corresponds in rodents to the maximum decidua development with subsequent regression to allow placental establishment [51].

Additionally, it was observed that FAAH, but not NAPE-PLD activity, varies significantly throughout pregnancy in rat maternal tissues. In fact, there is an increase in FAAH activity once decidua is fully developed, suggesting that a tight regulation of AEA levels is required during maternal tissues remodelling and supports a successful pregnancy (unpublished data).

The major endocannabinoids, AEA and 2-AG, and the endocannabinoid-like compounds, OEA and PEA, are detected in rat plasma and decidua during the postimplantation period [52, 53]. Contrary to AEA, in which plasmatic levels were increased on day 10, the other analysed compounds (2-AG, OEA, and PEA) remained relatively unchanged during the postimplantation period [52, 53]. However, the tissue levels for all the studied EC fluctuate according to the period of pregnancy. Collectively, the tissue levels indicate that all the studied compounds may be required during normal pregnancy. However, the levels of these molecules in plasma do not reflect the concentrations in uterine tissues, suggesting that they are tissues regulated [52, 53].

Unlike AEA and 2-AG, OEA and PEA are not able to activate CB1 and/or CB2 receptors or modulate cell survival and death [61, 62]. However, they may potentiate endocannabinoid biological actions through interference with their degradation, a so-called "entourage" effect, thereby leading to an enhancement of EC effects [63, 64]. In that way, their levels also need to be tightly regulated otherwise, they could exacerbate AEA actions and consequently impair normal pregnancy.

Besides a full endocannabinoid system present in decidual cells, a functional effect occurring during decidualization as result of CB1 activation was observed. Kesser et al. evidenced that WIN, a synthetic cannabinoid, inhibits the induction of human decidual cell differentiation, by decreasing mRNA levels of various decidualization-specific markers like prolactin, laminin, and IGFBP-1 [56]. Indeed, WIN-exposed cells showed a marked reduction in intracellular cAMP levels causing important changes in the morphology of decidual fibroblasts with DNA fragmentation. All these effects were reversed by the CB1 antagonist indicating that

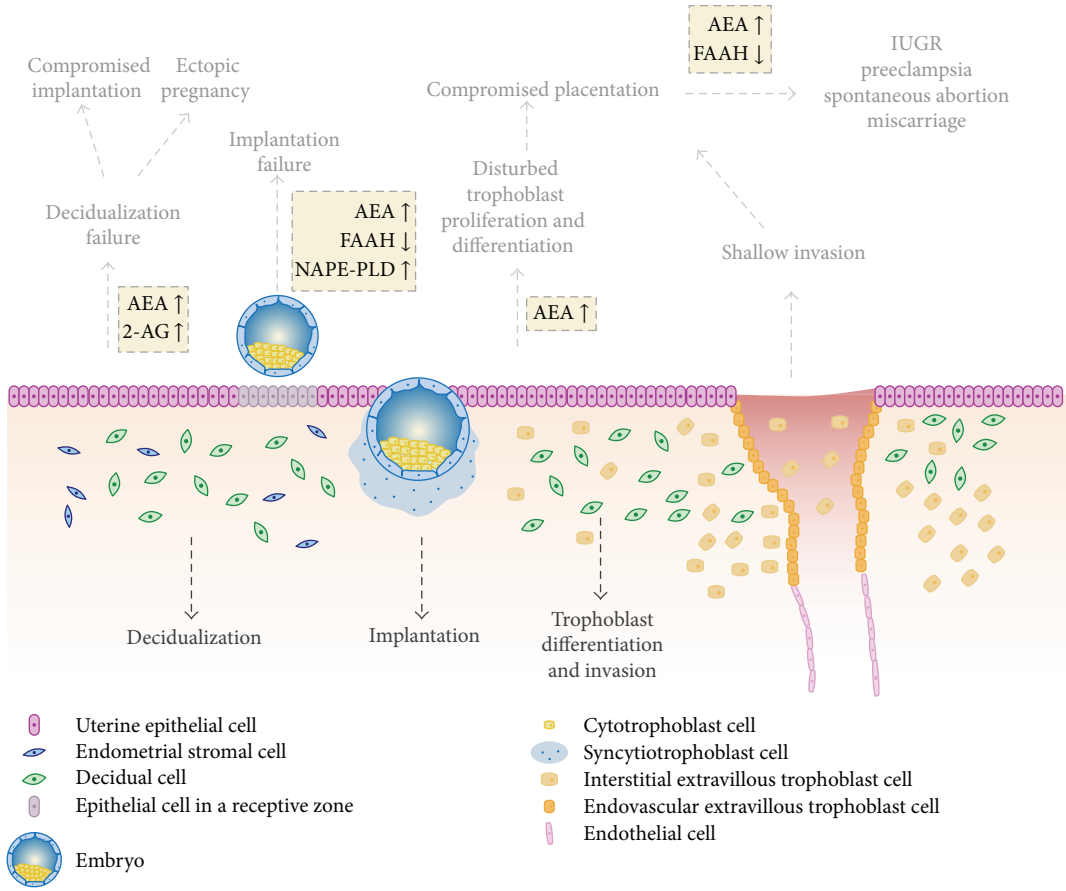

Figure 1: Schematic representation of the fetomaternal interface and potential adverse effects arising from deregulated endocannabinoid signalling based on rodents and human studies. Endometrial stromal cells differentiate into decidual cells, preparing uterine tissues for pregnancy, whereas the invading trophoblast cells critically regulate placental growth and function. All the physiological and molecular processes occurring during those periods are complex but highly organized. Endocannabinoids have reported to be involved in decidualization, implantation, and trophoblast differentiation and invasion. Aberrant endocannabinoid signalling (shown in yellow boxes) is reflected in compromised reprogramming of the endometrial stromal cells, implantation and placentation manifesting in ectopic pregnancy, intrauterine growth restriction, preeclampsia, miscarriage, and spontaneous abortion.

activation of CB1 inhibits human decidualization and stimulates apoptosis by a cAMP-dependent mechanism [56].

During the past few years, endocannabinoid effects have been extensively studied in several cell types, and, particularly for AEA, a proapoptotic effect has been demonstrated in endothelial cells [65], human neuroblastoma CHP100, and lymphoma U937 cells [66]. However, contrary effects have also been observed, like protecting cells from apoptosis [67] or stimulating proliferation of cancer cells [68].

Concerning decidual cells, AEA and 2-AG were described as proapoptotic compounds in primary rat decidual cells [52, 69]. While lower concentrations induced morphologic and molecular alterations, characteristic of an apoptotic cell death, higher concentrations resulted in a dramatic effect on cell viability and morphology and an increase in LDH release, probably due to a necrotic effect [52, 69]. This suggests a dual effect for endocannabinoids during fetoplacental development, which is dependent on endocannabinoid concentration.

On the other hand, the blockage of CB1 receptor, but not CB2 or TRPV1, was able to reverse the reduction of cell viability and apoptotic features induced by the two main endocannabinoids. Also, the activation of CB1 results in ceramide synthesis *de novo* and p38 phosphorylation, followed by induction of mitochondrial stress and ROS production, leading to apoptosis (Figure 2) [70]. Moreover, methyl-β-cyclodextrin (MCD), a cholesterol membrane depletor, has no effects on AEA/2-AG-programmed cell death [52, 69]. However, it has been referred that MCD blocks AEA-induced apoptosis in glioma cells [71] and hepatocytes [72]. This may result from CB1 redistribution in result of lipid raft disruption, as shown for breast cancer cells [73]. Furthermore, pretreatment with MCD increased decidual cell viability and caused a considerable reduction in LDH release only in the case of high concentrations of AEA and 2-AG [52, 69]. Thus, it is reasonable to suggest that high levels of AEA/2-AG, due to their lipophilic nature, may exert direct effects on rat decidual cells due to greater access through cholesterol-rich

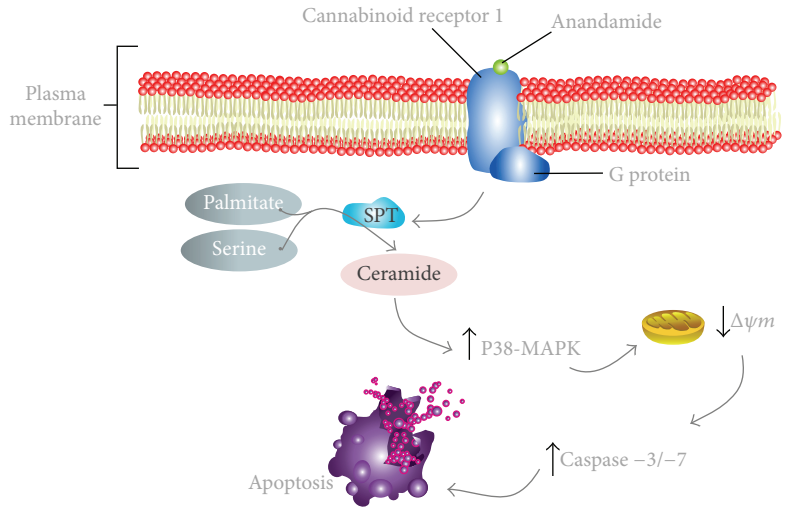

Figure 2: Schematic cartoon portraying the apoptotic signalling pathway triggered by anandamide (AEA) in rat decidual cells. AEA binds and activates the specific G-protein-coupled cannabinoid receptor 1 (CB1). The CB1 activation results in intracellular ceramide accumulation through *de novo* synthesis. This would lead to subsequent increase in phosphorylation of p38 mitogen-activated protein kinase cascade (p38-MAPK), which thus affects the mitochondrial pathway. It is followed by a drop in mitochondrial membrane potential ($\Delta\psi m$), caspase-3/-7 activation, and apoptosis of decidual cells. This CB1 activation mechanism is believed to play a role in decidual cell death, thus affecting uterine remodeling processes occurring during placentation.

lipid rafts or through a membrane transporter present in these cells. Once inside the cell, these molecules induce detrimental effects that result in high cell cytotoxicity. In that way, depletion of membrane cholesterol inhibits this process and consequently inhibits cytotoxic effects without affecting the CB1-mediated apoptosis observed with the lower concentrations.

This evidence clearly indicates that membrane lipid composition and integrity may affect endocannabinoid signalling and uptake as previously observed in hepatic stellate cells. In these cells, alterations of membrane structure and cholesterol content reversed the cytotoxic effect of AEA/2-AG induced via mitochondrial reactive species [74, 75]. Consistently, CB1 activation in trophoblast cells during implantation may trigger different signalling pathways dependent on AEA levels [44].

Consistently with all these observations, an association between endocannabinoid system and decidua-related pregnancy disorders was shown. Lower CB1 expression was observed in decidua and fallopian tubes of women with ectopic pregnancy [76]. Additionally, AEA, OEA, and PEA plasmatic levels were all found to be significantly higher, whereas FAAH activity, but not NAPE-PLD activity, was significantly reduced in ectopic pregnancy [77]. These data suggest that aberrant endocannabinoid signalling in human decidua may result in ectopic pregnancy. Moreover, it points to a potential association between CB1 gene polymorphism and ectopic pregnancy.

Furthermore, AEA induces an increase in nitric oxide (NO) synthesis on decidua, which may implicate endocannabinoids in pathological reproductive events involving infection. These effects were abrogated by either co-incubation with CB1 or CB2 antagonists which suggests that both receptors could be mediating this effect [78].

Interestingly, it was observed that ECS regulates migration of endometrial stromal cell. More precisely, the synthetic cannabinoid methanandamide enhanced endometrial stromal cells migration via CB1, through the activation of PI3K/Akt and ERK1/2 pathways [79]. On the other hand, these observations were accompanied by cytoskeleton reorganization and increased electrical signal generated by K+ channels [79]. This suggests a potential role for endocannabinoids in some pathologic conditions characterized by enhanced endometrial cell invasiveness.

Decidualization process definitively contributes to the complex maternal-fetal relationships within placental bed crucial for normal pregnancy. Taken together, there is now sufficient evidence implicating endocannabinoid elements in decidualization process. On the other hand, a disruption in endocannabinoid levels may interfere with decidual tissue remodelling and consequently with trophoblast differentiation/proliferation or invasion, ultimately impairing placental function.

The significance of COX-2 and prostaglandins for the initiation and maintenance of decidualization is well established. COX-2 is restricted to implantation sites in most species, and targeted disruption of COX-2 in mice results in multiple reproductive impairments including decidualization [80].

FAAH is responsible for the metabolism of AEA to arachidonic acid, which provides a source for prostaglandins production. Anandamide is also a direct substrate to COX-2 oxidative metabolism eventually producing prostaglandin-ethanolamides (PG-EAs).

Some studies have recently shown that AEA is capable of modulating the production of prostaglandins. Consequently, induction of COX-2 expression may represent an underlying mechanism by which PGs may mediate eCB-dependent effects or vice versa [81–83]. In the amnion, AEA caused a

significant increase in PGE2 through CB1 [81, 84]. Similarly, it was described that AEA exerts opposite effects on PGE2 and F2α in mice uterine explants [84]. Moreover, COX-2 derivatives mediate anandamide-inhibitory effect on nitric oxide synthase activity in the receptive uterus [85, 86].

Low FAAH activity and increased AEA levels are apparent in peripheral lymphocytes in women with recurrent miscarriage or poor implantation in women undergoing *in vitro* fertilization [87]. Furthermore, FAAH expression was absent in trophoblasts cells of women who miscarried [88]. Thus, when FAAH activity is absent or low, AEA goes through an oxidative metabolism primarily by COX-2 driving to prostamide production. The longer half-life of prostamides raises the possibility that they might act as mediators, and they are currently the target of studies to explore their potential pathophysiological effects. Endocannabinoid-induced effects were described to be mediated by prostamides in tumorigenic keratinocytes [89] and in other systems [90–92].

A latent biochemical cross-talk between the endocannabinoid and eicosanoid network is manifest. Furthermore, it is possible that aberrant endocannabinoid signalling may overwhelm eicosanoid expression compromising decidualization process and, in that way, fetoplacental development.

4. Endocannabinoid System during Placental Development

The placenta is a specialized pregnancy-specific structure that develops concurrently with the development of the embryo, being comprised of numerous cell types. Among them are specialized cells named trophoblasts, which are the earliest extraembryonic cells to differentiate from the mammalian embryo cells and surround the foetus throughout gestation.

Trophoblast cells are in direct contact with maternal tissues and play key roles in protecting the embryo/foetus from noxious substances, programming maternal support, and preventing maternal immune rejection. At the same time, they ensure appropriate bidirectional nutrient/waste flow required for growth and maturation of the embryo, enabling viviparous development. Thus, placentation is fundamental, creating the milieu, in which the embryo and foetus develop, assures a successful pregnancy, and even influences all the postnatal health and disease.

The balance between molecules synthesized by trophoblasts that promote invasion and inhibitors of this process, produced by decidua, controls the trophoblast invasiveness [93–95]. In turn, imbalances on either side can lead to abnormal invasion, resulting in pregnancy problems. Although the underlying mechanisms of placentation remain largely unknown, endocannabinoid signalling may play an important role in this process (Figure 1).

Supported on experimental models indicating the deleterious action of cannabinoids in early pregnancy, some clinical studies about the effects of endocannabinoids on placentation have been published. Human first trimester placental tissues express FAAH and CB1, indicating human placenta as a target for cannabinoid action and metabolism [96, 97]. The higher levels of FAAH were observed in villous cytotrophoblasts and syncytiotrophoblasts, which correspond to the placental layers closest to the maternal blood [97], indicating that FAAH expression would be essential in the placenta during early pregnancy to protect the foetus from detrimental high levels of maternal AEA.

Some studies have addressed the association between FAAH expression and recurrent miscarriage. One study observed that invasive trophoblasts and decidual cells expressed significantly more FAAH in placenta from women with recurrent miscarriage than in those of normal pregnancies [58]. This indicates an inadequate control of the endocannabinoid system in the uterus of women who experience recurrent miscarriages. However, a contradictory result has been observed with lower FAAH and high CB1 expression in placental samples of spontaneous miscarriage as compared to normal pregnancy [88]. Moreover, this study also revealed *nape-pld* transcripts, providing evidence for a potential endogenous synthesis of AEA by first trimester human placenta [88].

More recently, contrary to FAAH, NAPE-PLD expression was shown to be significantly higher in preeclamptic than in normal placentas, though no differences were observed in CB1 expression [98]. It was also hypothesized that AEA has an important implication in the normal function of placental tissues by modulating nitric oxide synthase (NOS) activity. In fact, it was observed that AEA modulates rat NO placental levels by two independent pathways: by stimulating NO synthesis via TRPV1 or diminishing the NOS activity via cannabinoid receptors, which depends on the production of cyclooxygenase-2 derivatives [85, 99]. Since placental villous from women with preeclampsia presented amplified NOS activity, increased AEA levels may be due to higher NAPE-PLD expression [98].

Also, in rodents a fully endocannabinoid system in placenta was described. The levels of both major endocannabinoids in the placenta gradually increased reaching their maximum level by the end of pregnancy. This increase was accompanied by higher expression of respective synthesizing enzymes, whereas the hydrolysing enzymes remained unchanged in placenta throughout pregnancy [100]. It suggests that, since expression of hydrolysing enzymes was unaffected, the high levels of both endocannabinoids are, therefore, regulated by the synthesizing enzymes. Additionally, FAAH activity was maintained constant during placentation, whereas NAPE-PLD activity increased significantly by the end of pregnancy to support the increased AEA levels observed during labour (unpublished data).

Trophoblast cell differentiation is tightly regulated and endocannabinoid signalling appears to be relevant during such processes. It was found that ablation of CB1 receptor inhibited trophoblast cell proliferation, differentiation, and invasiveness resulting in defective placentation and fetal development. In parallel, an increase in fetal resorption rates in $Cb1^{-/-}$ females was observed, whereas trophoblast cell proliferation and differentiation were modestly affected in $Faah^{-/-}$ females with higher AEA levels [101, 102].

Furthermore, the exogenous cannabinoid THC and AEA have been shown to reduce BeWo trophoblast cell proliferation *in vitro* via CB2 receptor, suggesting that high AEA plasma levels may increase the risk of first trimester miscarriage [103, 104]. This may explain the detrimental effects of *cannabis* consumption, as THC crosses the placenta in a greater extent during early proliferative growth phase, and, unlike endocannabinoids, which are released on demand, THC persists for long periods within the body and thereby may impact normal gestation.

5. Concluding Remarks

Although the adverse effects of cannabinoids in pregnancy have been implicated for years, the exact signalling mechanisms involved remain fairly unclear. In fact, maternal marijuana use has been associated with foetal growth restrictions, spontaneous miscarriage, and cognitive deficits in infancy and adolescence.

With the discovery of cannabinoid receptors, endogenous ligands, and the enzymes involved in their metabolic pathways, a wealth of information is now available regarding the importance of cannabinoid signalling in reproduction. The AEA signalling mediated by CB1 is crucial to various female reproductive events that include embryo development, oviductal transport, and implantation. However, the involvement of endocannabinoids in the molecular dialogue governing both decidualization and placentation only recently started to be depicted.

There is now evidence that endocannabinoid system is fully expressed in maternal tissues and midgestational placentas, and the levels of its constituents fluctuate during normal gestation. Additionally, CB1 receptor stimulation is involved in the inhibition of human decidualization and in the natural remodelling process occurring during this period. Moreover, endocannabinoid signalling was shown to compromise placentation through disturbing trophoblast proliferation and differentiation. CB1 knock-out mice also revealed a deficient trophoblast invasion with consequences to placentation and successful pregnancy.

There is growing evidence supporting the involvement of the endocannabinoid system in decidualization and placentation along with a possible association between polymorphism genotypes of CB1 gene and ectopic pregnancy.

AEA or 2-AG, in higher levels, represents a deleterious factor during this complex process, and a similar mechanism for exocannabinoids may occur during *cannabis* consumption in pregnancy.

This observation raises the question as to whether and how potentially increased levels of these endocannabinoids would affect the process of decidualization. It is possible that sustained higher levels might generate an imbalance in CB1 stimulation that might be responsible for an exacerbated cell death of decidual cells impairing normal placentation. On the other hand and contrary to endocannabinoids, which are synthesized "on demand" and quickly hydrolysed, THC persists for longer periods in the human body and, in that way, can interfere with normal endocannabinoid balance, either through direct stimulation of CB1 receptor and/or indirectly interfering in endocannabinoid metabolism. Thus, exogenous cannabinoid exposure may overwhelm this local protection mechanism and interfere with stromal/decidual cells, trophoblast differentiation/proliferation, and interstitial/endovascular invasion impairing placental function, which may result in intrauterine retardation and low birth weight, some of the adverse effects of *cannabis* consumption during pregnancy.

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Review Article

Role of the Endocannabinoid System in the Central Regulation of Nonmammalian Vertebrate Reproduction

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The endocannabinoid system (ECS) has a well-documented pivotal role in the control of mammalian reproductive functions, by acting at multiple levels, that is, central (CNS) and local (gonads) levels. Since studies performed in animal models other than mammals might provide further insight into the biology of these signalling molecules, in the present paper we review the comparative data pointing toward the endocannabinoid involvement in the reproductive control of non-mammalian vertebrates, focussing in particular on the central regulation of teleost and amphibian reproduction. The morphofunctional distribution of brain cannabinoid receptors will be discussed in relation to other crucial signalling molecules involved in the control of reproductive functions, such as GnRH, dopamine, aromatase, and pituitary gonadotropins.

1. Introduction

The endocannabinoid system (ECS) comprises several components, among which are specific seven transmembrane-domain receptors (i.e., CB1 and CB2 cannabinoid receptors), their exogenous (e.g., Δ^9-THC) and endogenous ligands (e.g., AEA and 2-AG), and a number of biosynthetic and degradative enzymes (for a review, see [1]). CB1/CB2 receptors are present not only in mammals but also in almost all classes of vertebrates and also in urochordates and cephalochordates, but not in the nonchordate invertebrate phyla. However, enzymes involved in the biosynthesis/inactivation of the endocannabinoids occur throughout the animal kingdom (see the reviews [2, 3]). The fact that the ECS has a rather wide phylogenetic distribution points to a fundamental modulatory role of endocannabinoids in the control of central and peripheral activities, for example, neurotransmission, neural development, hormone release and action, appetite regulation, immunomodulation, cardiovascular and respiratory functions, bone formation, and notably reproduction (for a review, see [4]). Indeed, in both humans and other mammals, the potent negative effects of cannabinoids at peripheral and central levels in embryo implantation, maintenance of pregnancy, and hormonal regulation have been widely documented [5, 6]. In addition, a role for the ECS in the modulation of nonmammalian reproduction has come to light in the last years (for a review, see [7]).

In the present work we will first review the data about the morphofunctional distribution of the cannabinoid receptors in the CNS of nonmammalian vertebrates, namely, teleosts and amphibians. In the second part, we will present comparative data about the involvement of the ECS in the central regulation of reproductive activities of bony fish and amphibians.

2. Functional Relevance of Cannabinoid Receptors in Teleost and Amphibian CNS

After the first discovery of CB1 cannabinoid receptors in mammals [8, 9], the cloning of two genes coding for the CB1 receptor subtypes CB1A and CB1B in the bony fish *Fugu rubripes* [10] represented a true milestone in the ECS comparative investigations. Since then, duplicate copies of either the CB1 or CB2 genes (*CNR1, CNR2*) or both have been reported in several teleost species. In the puffer fish *Fugu rubripes*, as already mentioned, two CB1 receptor genes,

but only one CB2 gene, are present [10, 11], consistently with the finding of two CB1 genes and one CB2 gene in another puffer fish species, *Tetraodon nigroviridis* [2, 12]. On the other hand, in the zebrafish *Danio rerio*, only one CB1 gene has been detected [13], while two CB2 genes (CB2A and CB2B) are present [14]. In the goldfish *Carassius auratus*, only one CB1 and one CB2 sequences have been cloned so far [15–17]. In the sole *Solea solea*, two CB1 (CB1A and CB1B) were identified [18], while in *Pelvicachromis pulcher* [19], *Sparus aurata* [20], and *Apteronotus leptorhynchus* [21] only one CB1 gene has been cloned. The presence of duplicate genes is a well known phenomenon in teleosts [22] and can be ascribed to a whole-genome duplication that occurred during evolution of the ray-finned lineage before the teleost radiation. At least three different events have been suggested to occur after gene duplication: (1) one gene of the pair evolves as a pseudogene through degenerative mutations and is eliminated because of its dispensability (most frequent event); (2) duplicate genes are preserved and remain functional; in this case, one copy can acquire a mutation conferring a new and beneficial function (neofunctionalization), whereas the other retains the original function; (3) duplicate genes can be preserved and share the multiple functions of the ancestral gene by complementary loss of subfunctions. Thus far, the functional significance of the differential retention of duplicate CB1 or CB2 genes in different teleost lineages is mostly unknown. In one report [14] the expression of CB2A and CB2B has been compared by RT-PCR in various zebrafish tissues, showing a similar distribution with few exceptions (brain and muscle, where CB2A was very low); however, no functional conclusions can be drawn from this study. Recently Palermo et al. [18] showed that in *Solea solea* in a stress paradigm CB1B mRNA levels were significantly reduced in ovary and brain of stressed fish, while CB1A mRNA remained unchanged, suggesting a possible subfunctionalization of the two duplicate genes.

For what concerns amphibians, CB1 cannabinoid receptors have been cloned and characterized in the rough-skinned newt *Taricha granulosa* [23], in the African clawed frog *Xenopus laevis* [24] and in the green frog *Rana esculenta* [25], while the presence of a CB2 gene has been revealed in *Xenopus tropicalis* [2].

Since the distribution of a receptor could give information on its physiological role, a number of studies have been conducted to characterize the localization of cannabinoid receptors.

2.1. Distribution of CB1/CB2 Cannabinoid Receptors in Teleost CNS. Although the puffer fish was the first nonmammalian species where CB1 receptors were identified, little is known about cannabinoid receptor distribution in this species. The few available data [10] outline, however, that CB1 receptors are highly expressed in the brain and they are present at lower levels in nonneural tissues, thus indicating an evolutionarily conserved feature common to all vertebrates.

The first data on CB1 receptor distribution in the brain of a teleost were obtained in the African cichlid *Pelvicachromis pulcher* [16, 19]. By using immunohistochemical techniques, abundant CB1-immunostainings were observed throughout the telencephalon, in the preoptic area, lateral infundibular lobes of the posterior hypothalamus and pituitary gland, thus suggesting that cannabinoids affect neuroendocrine mechanisms and might indeed be involved in the control of reproduction. An intense CB1-immunoreactivity was also detected in the pretectum and nucleus glomerulosus of the posterior tuberculum, both transitional areas between pro- and mesencephalon, that are involved in the integration of visual-motor activities in order to orient fish toward preys and elicit appetite [26–28]. In the dorsal mesencephalic tegmentum, some large intensely CB1-immunopositive nerve cells, possibly motor neurons of the III cranial nerve, were observed. In the cerebellum, few granule cells and Purkinje cells were stained, as well as a number of α-motoneurons in the spinal cord.

A study in the goldfish [15] showed that CB1-immunoreactivities are distributed through all the forebrain, including the olfactory bulbs. Abundant immunostainings were observed in the inferior lobes of the posterior hypothalamus surrounding the third ventricle lateral recesses; these brain areas are involved in bony fish feeding response, thus providing morphological evidence for the reported involvement of the endocannabinoid system in the goldfish appetite control. The same CB1-immunopositive brain regions are also pivotal for the control of teleost reproduction, the involvement of the ECS being also supported by the finding of CB1-immunopositive fibres in the goldfish infundibulum, but not in the pituitary (Cottone, unpublished data), contrary to what was observed in *Pelvicachromis pulcher*, where pituitary CB1-immunopositive cells were instead observed [29]. In the goldfish, a number of CB1 immunostained cell bodies were also detected in the telencephalon immediately below the ventricular ependyma and were identified as radial glial cells [29, 30]; these cells are very abundant in adult fish, probably due to the absence of astrocytes and ependymal cells and do serve mostly as neural progenitors and newborn neuron migrating scaffolds, as well as neurosteroid-producing cells. The expression of cannabinoid receptors in goldfish radial glial cells does therefore suggest an ECS regulation of neurogenesis in fish, according to what occurred in mammals (for a review [31]).

In zebrafish, CB1 distribution was analysed both in larvae and adult brains, by means of *in situ* hybridization (ISH) [13]. An early CB1 mRNA expression was detected in the preoptic area of the hypothalamus and, later, within the dorsal telencephalon and mesodiencephalon. In postembryonic larvae and adult brain CB1 mRNA is primarily expressed in the dorsal telencephalon, synencephalon, torus longitudinalis, and periventricular hypothalamus, thus suggesting the involvement of the ECS in cognitive processes and neurogenesis.

Very recently, Harvey-Girard et al. [21] presented data on CB1 receptor distribution in the brain of the weakly electric fish *Apteronotus leptorhynchus*. Accordingly with what was observed in the other teleosts, CB1 mRNA is expressed mainly in the telencephalon (especially in subpallial neurons and dorsocentral telencephalon) and in fewer cells in the rest of the brain. CB1 distribution seems to reflect a possible role in the regulation of the electrosensory system and

electrocommunication that are particularly developed and important in gymnotiform fish.

Since CB2 receptor genes have been identified in non-mammalian vertebrates more recently than CB1, data about their distribution are scarce. For instance, Rodriguez-Martin et al. [14] have shown in zebrafish by ISH that CB2 mRNA is expressed in the rostral and proximal pars distalis of the pituitary gland, while expression in the brain was only detected by RT-PCR. In goldfish CB2 mRNA has been detected in the brain, although at levels much lower than CB1 mRNA [17] and the immunostaining of goldfish forebrain sections did actually reveal the presence of CB2-immunopositive cells that were identified as radial glial cells, that is, adult neural progenitors [30].

2.2. Distribution of CB1/CB2 Cannabinoid Receptors in Amphibian CNS. The first report on the occurrence of the ECS in the amphibian CNS concerned the pharmacological and molecular characterization of CB1 receptors in the urodele amphibian *Taricha granulosa* [23]. In this same species, ISH experiments showed CB1 mRNA expression in the telencephalon, in particular in the olfactory bulb, pallium, bed nucleus of the stria terminalis and nucleus amygdalae dorsolateralis; CB1 mRNA is also expressed in regions of the preoptic area, thalamus, midbrain tegmentum and tectum, cerebellum, and the stratum griseum of the hindbrain [32].

The distribution of CB1 receptor has been also investigated in the CNS of adults and embryos/larvae of the anuran amphibian *Xenopus laevis*. In particular, in whole embryos of *Xenopus laevis* CB1 mRNA was first detected at stage 28, and from stage 41 it appears in the rhombencephalon and thereafter also in the olfactory placodes and then in the olfactory bulbs [33]. In adult *Xenopus laevis*, CB1-immunostainings and CB1 mRNA-positive cells were detected in the olfactory bulb, dorsal and medial pallium, striatum and amygdala, thalamus and hypothalamus, mesencephalic tegmentum, cerebellum and spinal cord [24, 34, 35]. Moreover, CB1 receptors were detected in lactotrophs, gonadotrophs, and thyrotrophs of the pituitary gland [36].

In the green frog *Rana esculenta* CB1 immunostained neurons and nerve fibres are abundant in the telencephalic hemispheres, preoptic area, hypothalamus, and a number of hindbrain areas [37, 38]. Interestingly, fluctuations during the annual sexual cycle of CB1 mRNA expression in various regions of the brain, as well as at the testicular level, have been reported and a possible involvement of the ECS in the regulation of gonadal activity has been postulated [38, 39].

On the basis of their neuroanatomical distribution and relationships with a number of other signalling molecules [34, 35], the amphibian CB1 cannabinoid receptors have been mainly considered modulators in sensory and sensorimotor integrations and endocrine and behavioral outputs. Moreover, since the intense CB1 mRNA ISH staining found in *Taricha granulosa* amigdaloid complex [32] well matched the strong CB1-immunoreactivity and mRNA expression in the corresponding nucleus of *Xenopus laevis* [24, 34], the endocannabinoid-mediated modulation of fear, anxiety, and stress responses has also been postulated.

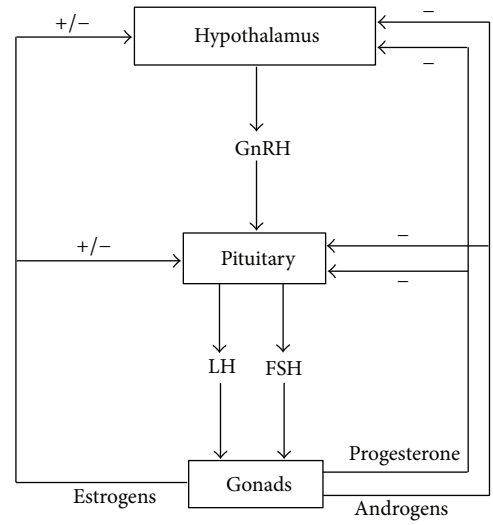

Figure 1: Schematic representation of the hypothalamus-pituitary-gonadal (HPG) axis.

3. The Endocannabinoid System Is Involved in the Central Regulation of Teleost and Amphibian Reproductive Functions

The reproductive functions are regulated by the neuroendocrine system, so that a tight crosstalk between the hypothalamus, pituitary, and gonads takes place (Figure 1). In particular, the decapeptide GnRH is released from the hypothalamus and stimulates pituitary to release gonadotropins. FSH and LH act then at the gonadal levels, regulating spermatogenesis, oocyte growth, and steroidogenesis.

Cannabinoids exert potent negative effects upon both experimental animal and human reproduction, affecting gonadotropin synthesis and release, gonadic steroid production, spermatogenesis, ovulation, embryo development and implantation, sexual behaviour [6, 40, 41]. In vertebrates ECS modulates reproductive functions by acting at multiple levels (for a review, see [42]). Centrally, ECS negatively affects the secretion of pituitary gonadotropin hormones, by acting at the hypothalamic level. Also, a direct action on pituitary is possible, since AEA regulates *in vitro* pituitary hormone secretions in rat [43], and CB1 receptor has been found in mammalian pituitary anterior lobe [44] and in *Xenopus laevis* PRL and FSH cells [36]. The fact that CB1 receptors were detected in the Leydig cells of mouse testis and the endogenous cannabinoid anandamide (AEA) suppressed testosterone secretion by testes in normal but not in CB1 knockout mice [43] was the first evidence that cannabinoids do modulate reproductive functions also at the peripheral level. Recent data also point out a role for CB2 receptor, whose presence was observed in rat and human ovaries [45, 46], in human oocytes [47] and mouse spermatogonia [48]. In addition, endocannabinoids control sperm motility and/or acrosomic reaction [49–53], as well as ovulation, implantation, embryonic development, foetal growth, lactation (see the reviews [6, 54]). It is noteworthy

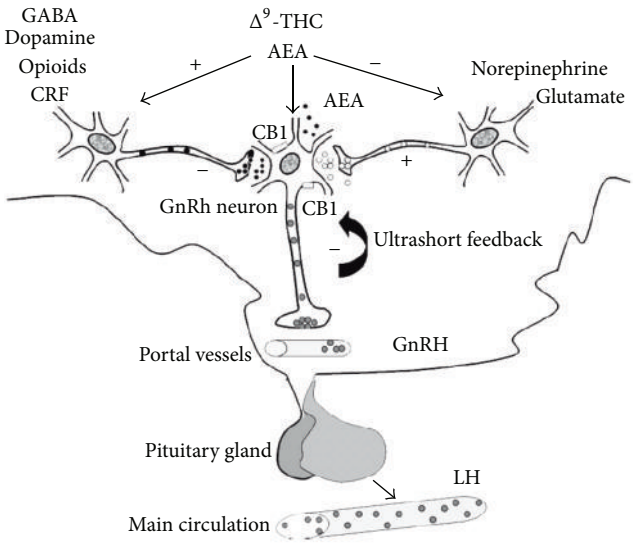

Figure 2: Schematic representation of direct and indirect effects of the ECS in the control of GnRH release (from [57]).

that also in nonmammalian vertebrates basic components of the endocannabinoid system were found and characterized both in the brain and in the gonads. CB1 mRNA expression was in fact detected in the gonads of *Carassius auratus* and *Pelvicachromis pulcher* [55] and *Sparus aurata* [20] and *Danio rerio* [56], as well as *Xenopus laevis* [55]. In *Rana esculenta* the occurrence of both CB1 receptors and FAAH in the gonads was reported and CB1 levels have been shown to fluctuate during the annual sexual cycle [53], consistently with the observations in the brain [38, 39]. Also, the ECS inhibits male courtship clasping behaviour in the newt *Taricha granulosa* [23] and interferes with mammalian copulatory behaviour [6]. At the moment there are scarce or no available data regarding CB2 receptor in nonmammalian vertebrate gonads, with the exception of the teleost goldfish, where CB2 mRNA was found in testis and ovary [17].

At the central level, the action of the ECS on the hypothalamus has been first demonstrated by the observation that both exogenous and endogenous cannabinoids are responsible for the decrease of circulating LH and sexual steroids, by acting through a CB1-mediated inhibition of GnRH-I (formerly called *mammalian*GnRH) discharge (for a review, see [57]). The cannabinoid ligands might inhibit GnRH secreting neurons by activating specific signalling circuitries (e.g., GABA, dopamine, CRF, opioid) and inhibiting others (e.g., glutamate, norepinephrine). Moreover, since immortalized hypothalamic GnRH neurons are capable of releasing endogenous cannabinoids such as AEA and 2-AG and do possess CB1 and CB2 receptors [58], a direct neural control of GnRH-I release has been postulated (see Figure 2). Since GnRH is a key molecule in the gonadotropic regulation of all vertebrates, studies on the morphofunctional relationships between ECS and GnRH were conducted also in nonmammalian vertebrates. In particular, in *Pelvicachromis pulcher*, *Carassius auratus*, *Solea solea*, and *Danio rerio*, a close contiguity between CB1-immunostainings and GnRH-III- (previously called *salmon*GnRH) immununoreactive cell bodies and nerve fibers was observed in brain areas that are pivotal for the control of reproduction, such as the basal telencephalon, the preoptic area, and the hypothalamus ([16, 55, 59] and Cottone, unpublished data). Consistently, in *Rana esculenta* and *Xenopus laevis*, the codistribution of CB1- and GnRH-I-immunoreactivity occurred in brain areas corresponding to those described in the above teleost species [37, 55]. In particular, a subset of frog GnRH-I-immunoreactive neurons in the septum and preoptic area, together with nerve fibres and terminals in the median eminence of the neurohypophysis, were found CB1-immunopositive [38]. Interestingly, during the frog annual sexual cycle, GnRH-I mRNA and CB1 levels have opposite expression profiles in the telencephalon and diencephalon [38, 60]. Also, anandamide is able to inhibit GnRH-I and GnRH-II synthesis and to affect GnRH receptors expression in the diencephalon, as well as in the testis; at the same time, a GnRH agonist inhibits the synthesis of GnRH-I mRNA and induces an increase in CB1 transcription [38, 61, 62], thus suggesting reciprocal relationships between the ECS and the GnRH system.

In line with dopaminergic control of fish reproduction through inhibition of both PRL release and gonadotropin (GTH-I and GTH-II) synthesis [63], CB1-immunoreactivities were found codistributed with TH- (the dopamine biosynthetic rate-limiting enzyme) immunopositive neurons and fibres in the basal telencephalon/preoptic area of *Pelvicachromis pulcher* and *Carassius auratus* [16]. Also, in the diencephalic paraventricular organ (PVO) of the goldfish a number of CB1-immunopositive cerebrospinal fluid (CSF) contacting neurons were found closely adjacent to the TH-positive neurons that innervate the neurointermediate and the distal lobes of the teleost pituitary, thus indicating a CB1-mediated control of PVO dopaminergic neurons and, consequently, a direct or indirect regulation of pituitary activity [29].

Another molecule deeply involved in the reproductive physiology is aromatase (estrogen synthase), the enzyme that catalyzes the transformation of androgens into estrogens (for a review, see [64]). Aromatase is expressed both in gonads and brain; brain aromatase is essential for testosterone-mediated regulation of physiological and behavioural processes, such as sexual differentiation of the brain, activation of male sexual behaviour, and regulation of gonadotropic hormone secretion, as well as neurogenesis. Teleost fish do possess two aromatase genes, codifying for aromatase A, that is specifically expressed in the gonads, and aromatase B, that is strongly expressed in the brain. Indeed, teleosts, compared to other vertebrates, show remarkably high levels of brain aromatase activity and protein and gene expression, due to an autoregulatory loop through which estrogens and aromatizable androgens upregulate aromatase expression [65]. In zebrafish abundant aromatase-positive cells have been observed in the olfactory bulbs, telencephalon, preoptic area, and the hypothalamus, as well as in the thalamus, optic tectum, and around the fourth ventricle [66–68]. Interestingly, brain aromatase expression and activity fluctuate seasonally and with the reproductive state. Peculiarly, in

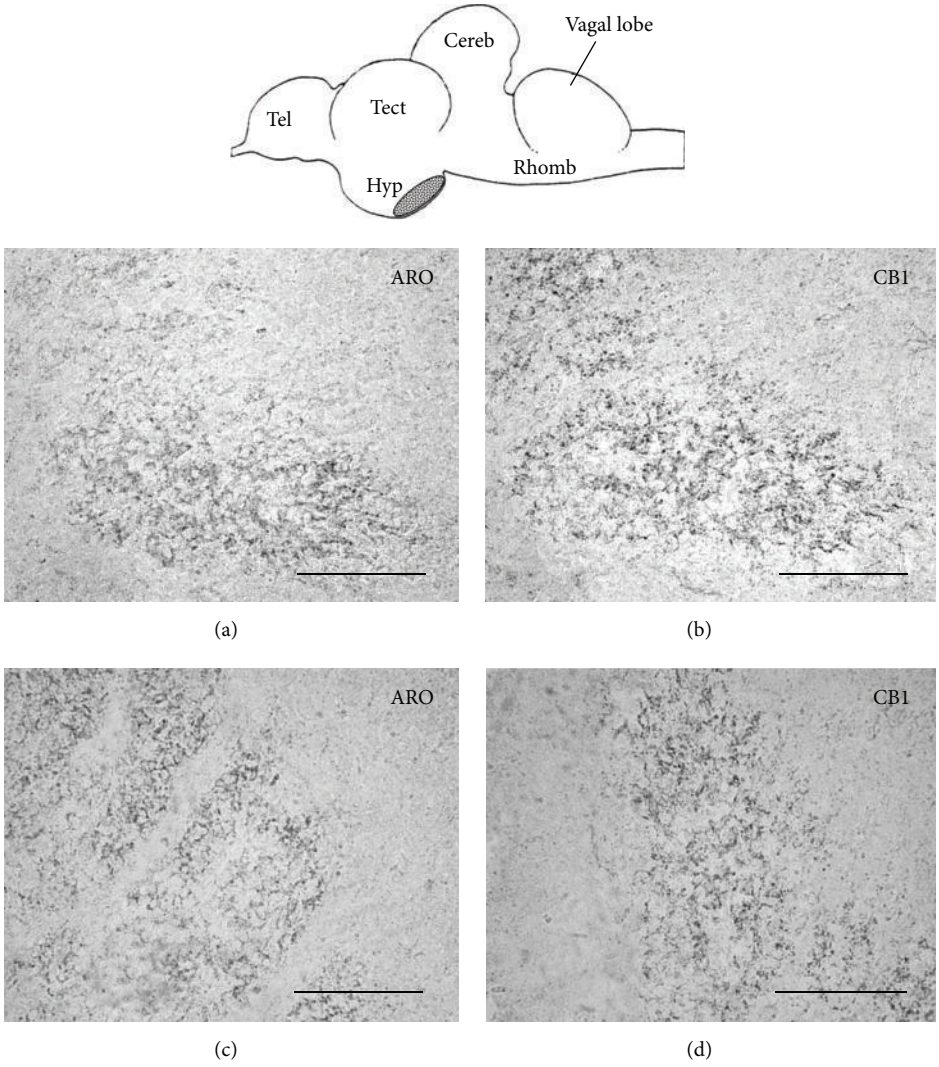

Figure 3: Aromatase/CB1 immunoreactivities in the goldfish lateral recesses of the hypothalamus (depicted in the schematic draw of the goldfish brain, lateral view). (a), (b) and (c), (d): consecutive coronal sections showing in (a) and (c) a large number of aromatase-immunopositive radial glial cell processes codistributed with ((b) and (d), resp.) abundant CB1-immunopositive fibers and cell processes. Calibration bars: 100 μm. Cereb: cerebellum; hyp: hypothalamus; rhomb: rhombencephalon; tect: optic tectum of the mesencephalon; tel: telencephalon; vagal lobe: lobe of the X cranial nerve.

teleosts brain aromatase is not expressed by neurons, as seen in mammals and birds. Instead, it is specifically expressed by radial glial cells [66], the cells that sustain the high neurogenic activity of adult fish; thus, in fish, aromatase is likely to be the key molecule enabling brain growth and brain sexualization throughout life. Given the role of aromatase in reproductive physiology, we evaluated the possible existence of a crosstalk between the ECS and brain aromatase, by analyzing the codistribution of CB1 cannabinoid receptors and aromatase in brain sections of both zebrafish and goldfish. In the preoptic area and periventricular grey of the hypothalamic inferior lobes, a tight contiguity between the two markers was indeed found (Figure 3 and data not shown), thus suggesting a CB1-mediated regulation of aromatase activity at least in bony fish.

4. Concluding Remarks

The data reported in teleosts and amphibians strongly support the modulatory role of brain ECS on several neural circuits, including those involved in the control of reproductive functions.

Although the general physiological role of endocannabinoids is far from being understood in nonmammalian vertebrates, the investigations in organisms different from mammals might provide new insights into the cannabinoid biology. Basic information on the ECS derived from comparative investigations in invertebrates and/or anamniote vertebrates, besides bearing value for evolutionary and wildlife biological studies, could contribute to a better understanding of the mechanisms of action of cannabinoid-related molecules and

stimulate the development of new strategies for their therapeutic use in humans.

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Review Article

Endocannabinoids and Endovanilloids: A Possible Balance in the Regulation of the Testicular GnRH Signalling

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Reproductive functions are regulated both at central (brain) and gonadal levels. In this respect, the endocannabinoid system (eCS) has a very influential role. Interestingly, the characterization of eCS has taken many advantages from the usage of animal models different from mammals. Therefore, this review is oriented to summarize the main pieces of evidence regarding eCS coming from the anuran amphibian *Rana esculenta*, with particular interest to the morphofunctional relationship between eCS and gonadotropin releasing hormone (GnRH). Furthermore, a novel role for endovanilloids in the regulation of a testicular GnRH system will be also discussed.

1. Introduction

Endocannabinoids (eCBs)—such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG)—are lipophilic molecules that work as integral part of the endocannabinoid system (eCS), mimicking several actions of Δ^9-tetrahydrocannabinol (THC), the active principle of *Cannabis sativa*. Although the existence of an intracellular receptor has been suspected, eCBs exert their actions by binding to specific membrane receptors, CB1 and CB2 [1, 2], whose expression is widespread in all species analyzed to date [3]. Unlike 2-AG, AEA also binds to the intracellular site of the type-1 vanilloid receptor (TRPV1), a cation channel receptor also activated by the pungent compound of hot chili pepper, and capsaicin (CAP, 8-methyl-*N*-vanillyl-6-nonenamide) [4]. Other receptors such as GPR55 and GPR119 have been considered putative cannabinoid receptors, however with some hesitation [5].

Although eCBs are lipidic compounds able to traverse plasma membrane by passive diffusion, the existence of a hypothetical eCB transporter has been suggested. In this respect, AEA intracellular carriers belonging to fatty acid binding proteins (FABP) family have been discovered [8]. In addition, eCBs can be also inactivated by a mechanism of cellular reuptake followed by an intracellular degradation mediated by fatty acid amide hydrolase (FAAH) [9] and monoacylglycerol lipase (MAGL) [10]. In neurons, a cytosolic variant of FAAH-1, termed FLAT—which lacks amidase activity but binds AEA with low micromolar affinity—has been considered as an AEA transporter [11]. Endocannabinoid system also includes several enzymes responsible for endocannabinoid biosynthesis such as *N*-acylphosphatidylethanolamine-(NAPE-)specific phospholipase-D (PLD) for AEA [12] and sn-1-diacylglycerol lipase (DAGL) for 2-AG [13].

During the course of the years, the eCS has been characterized and studied from a functional point of view in many species [14–17]. In this regard, the use of nonmammalian animal models has contributed to a better comprehension about the eCS actions, especially in several reproductive events [16, 18–20]. In fact, nonmammalian vertebrates offer a broad spectrum of potentialities, besides, to allow evolutionary speculations. Most of them are seasonal breeders; therefore temperature and photoperiod—easily adjustable in

laboratory—deeply control their gonadal activity. In addition, both brain and gonad architecture show morphological features simpler than mammals thus to easily study relationships between different neuroendocrine/paracrine systems [21].

2. *Rana esculenta*: An Experimental Model to Study the eCS at Both Central and Testicular Levels

The choice of an appropriate animal model is a basic step in the configuration of an experimental approach. Very often the difficulties found in the determination of molecular mechanisms on the basis of important physiological functions—when studied in mammals—incite to select other animal models, especially nonmammalian vertebrates. With this in mind, the anuran amphibian *Rana esculenta* has been a suitable model for the comprehension of endocannabinoid role in reproduction at both central and testicular levels.

During the annual cycle of this seasonal breeder, the gonadotropin-releasing hormone (GnRH)—the main regulator of gonadal activity—accumulates in the brain in the postreproductive period and is slowly released during the winter stasis to sustain the gonadotropin discharge in order to assess the beginning of a new reproductive wave [22–24]. Furthermore, this amphibian shows a laminated type brain—an archetype of those more elaborated of the higher vertebrates—in which GnRH secreting neurons occupy well-known and distinct areas, differently from mammals in which they are quite scattered in the brain [25]. Additionally, frog spermatogenesis proceeds slowly, orchestrated by environmental factors, testicular mediators, and hormonal milieu characterized by cyclic fluctuations. In particular, in specific periods of the annual sexual cycle it is possible to identify in testis a defined and well-known population of germ cells thanks to a very peculiar cystic organization. This consists in Sertoli cells enveloping clusters of germ cells at a synchronous stage [26, 27].

The characterization of eCS in *R. esculenta* begun in 2006 with the molecular cloning and the expression analysis of *cb1* [28, 29]. As indicated above, endocannabinoid activity requires multiple receptors, and this issue is stressed by the discovery of duplicated genes in fish [30, 31], by the detection of several cannabinoid receptor splicing forms [32–34] as well as by the discussed existence of receptors other than CB1/CB2 [5]. In frog, the characterization of *cb1* did not revealed any splicing form but nucleotide differences among brain/testis cDNA and genomic sequences together with the corresponding amino acidic variations [18, 19, 29] as a consequence of a possible editing process. Such a phenomenon seems to occur in other vertebrates and to affect RNA folding, stability and turnover. However, at present, synonymous and nonsynonymous mutations in *cb1*/*cb2* and *Faah* genes have been reported in humans and have been linked to several diseases such as metabolic and reproductive disorders, feeding behaviour, obesity, and schizophrenia [35–40].

In amphibian brain, CB1 is widely distributed in the forebrain [41, 42], the encephalic area mainly involved in the control of reproductive functions, being primarily responsible for the biosynthesis of GnRH [21]. As deeply described in the next paragraph, functional crosstalk between eCBs and GnRH system emerged in frog.

As in other vertebrates and in the central nervous system, *cb1* is widely expressed in frog tissues, gonads included [28]. Fluctuations of *cb1* expression have been reported in both testis and brain during the annual sexual cycle [28] with testicular CB1 mRNA/protein [6, 7, 28] detected in parallel to FAAH in germ cells, especially in elongated spermatids and spermatozoa as observed in other vertebrates (Figures 1(a) and 1(b)) [6, 7, 42–49] and in sea urchin as well [50].

In rodents and in germ cells, CB1 has also been detected in Leydig cells suggesting its possible involvement in Leydig cell ontogenesis and steroidogenetic activity [51–53]; interestingly, in frog *cb1* mRNA was only observed in interstitial compartment (Figures 1(b) and 1(c)), and its expression profile well correlates with seasonal testosterone production [54]. Together with the ability to degrade AEA, frog testis might be able to produce eCBs during the annual reproductive cycle as suggested by *Nape-pld* expression and localization [6]. In the germinal compartment *Nape-pld* mRNA has been observed in secondary spermatogonia and spermatocytes cysts as well as in Sertoli cells surrounding primary spermatogonia; the strongest signal has been found in the interstitium throughout the annual sexual cycle (Figures 1(d)–1(f)).

Taken all together, data in frog clearly confirm a deep evolutionarily conserved involvement of eCBs in germ cell progression and sperm cell functions [43–49, 55–57]. Accordingly, as in human, boar, bull, rodents, and sea urchin, also in frog AEA modulates sperm motility [7, 43, 49, 50, 58, 59], indicating an evolutionarily conserved role in the regulation of such a reproductive function.

3. Relationship between eCS and GnRH System

The presence of *cb1* in frog brain, mainly in the forebrain and midbrain—as also observed from fish to mammals [14, 41, 60, 61]—has suggested that eCS is able to control reproductive functions through a central regulation. This is in line with the discovery that hypothalamic immortalized GnRH secreting neurons possess a complete eCS, CB1 included [62] and that AEA inhibits GnRH release from rat mediobasal hypothalamus [63]. During the annual sexual cycle, *cb1* mRNA fluctuations are opposite as compared to *GnRH-1* [19, 42]; in particular, in frog diencephalons—the encephalic area mainly involved in the release of GnRH—*cb1* expression shows a peak in December, when low levels of GnRH have been detected [22, 24, 64]. The total CB1 protein content has also been assayed in frog forebrain, midbrain, and hindbrain [16, 19] during the year; intriguingly, GnRH release correlates with the minimal levels of CB1 detected in both telencephalon and diencephalon. Accordingly, neuroanatomical and functional relationships between CB1 and GnRH have been discovered in *R. esculenta* brain by immunofluorescence; in particular, CB1 has been found in a subpopulation of the septal and preoptic GnRH-1 neurons [42]. In addition, the *in vitro* treatment of frog diencephalons with AEA has an inhibitory

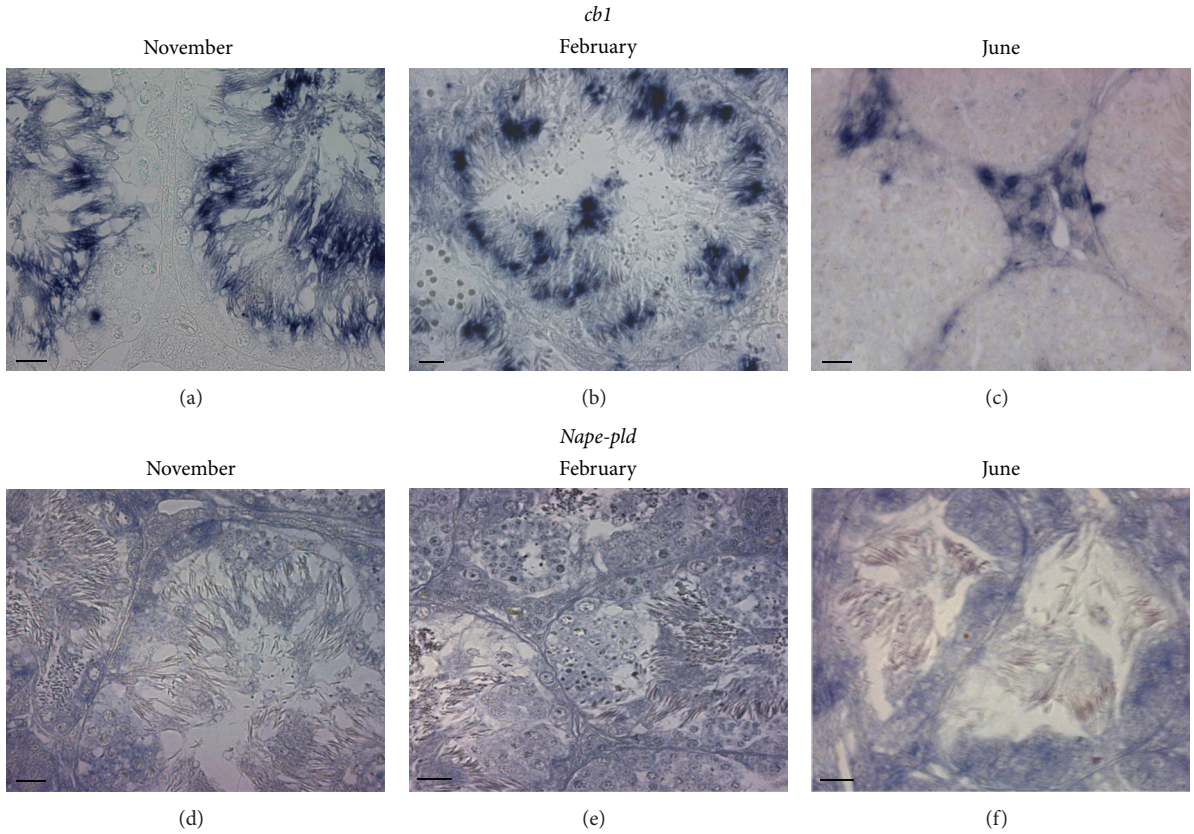

Figure 1: Localization of *cb1* and *Nape-pld* mRNA in the frog testis evaluated by *in situ* hybridization in November (a) and (d), February (b) and (e), and June (c) and (f). Scale bar: 20 μm.

effect upon *GnRH-1* expression, *via cb1* activation [42]. Such a functional crosstalk between the eCS and GnRH is really more complicated, due to the existence of multiple GnRH and gonadotropin-releasing hormone receptor (GnRH-R) molecular forms in *R. esculenta*. In particular, in frog diencephalons, AEA, with a fine CB1-dependent regulation, is able to decrease *GnRH-1* and *GnRH-2* and increase *GnRH-R1* and *GnRH-R2* expression, with no effect upon *GnRH-R3* [65].

In the last years an emerging idea is that the inhibitory action of eCBs on reproductive functions, especially on GnRH neurons activity, might be pondered by new molecules positively affecting reproduction, such as the kisspeptins [66]. Interestingly, the kisspeptin receptor, *GPR54*, has been cloned and characterized in frog [67], and AEA, *in vivo*, inhibits the hypothalamic GnRH system *via* GPR54 [Chianese et al., unpublished results].

In the wake of brain analysis, a deep characterization of GnRH system in relation to eCS has been carried out in frog testis as well [6] (Figure 2). CB1 protein peaks have been observed in periods of the cycle characterized by massive formations of postmeiotic cells (September) and during the breeding season (March) with CB1 mainly localized in postmeiotic stages. Interestingly, the expression profiles of testicular GnRHs clearly indicate their increase in postreproductive period, with *GnRH-1* increased expression occurring from May to July and *GnRH-2* expression presenting a single expression spike in June [6]. Thus, in a period in which both CB1 and FAAH proteins are scantly expresseds GnRH is overexpressed (Figure 2).

GnRH works as a testicular bioregulator affecting spermatogenesis, sperm release, and fertilization [21, 68, 69], processes also driven by eCBs. With this in mind, we carried out *in vitro* incubations of frog testis with AEA choosing two periods of the annual cycle: June (postreproductive period), when testis is reach in meiotic stages; February (end of the winter stasis), when the upsurge of a new spermatogenetic wave occurs. Intriguingly, frog testis shows a quite different modulation of the GnRH system by AEA in comparison to brain. In fact, in frog diencephalon GnRH-1 and GnRH-2—both hypophysiotropic factors [21]—are localized in the anterior preoptic area, and their transcripts are both inhibited by AEA, whereas in testis they are differently expressed, probably working in different reproductive events. In particular, in June, when spermatogenesis slightly proceeds, an opposite regulation by AEA has been observed since AEA decreases *GnRH-1* and increases *GnRH-2* expression (Figures 3(a) and 3(c)), through *cb1* activation (Figure 4(a)) [6]. Furthermore, a specific modulation by AEA has also been observed on *GnRH-Rs* expression, since AEA upregulates *GnRH-R1* and decreases *GnRH-R2* expression, without any effect upon *GnRH-R3* (Figures 4(a), 4(c), and 4(e)). Interestingly, in February, when testis simply contains quiescent spermatogonia and spermatozoa attached to Sertoli cells, AEA affects *GnRH-2* and *GnRH-R2*, a system supposed to

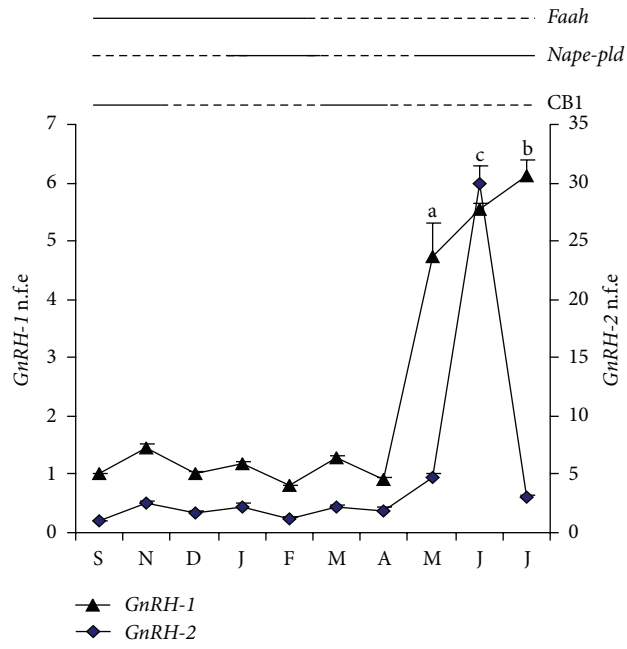

Figure 2: Analysis of *GnRH1*, *GnRH-2*, and some molecular components of the endocannabinoid system in frog testis during the annual sexual cycle. For *GnRH-1*, *GnRH-2*, and *Nape-pld* mRNA data from [6]; for FAAH and CB1 protein data from [7]. Dotted lines: low levels; black lines: high levels. n.f.e.= normalized fold expression. Different letters indicate statistically significant differences.

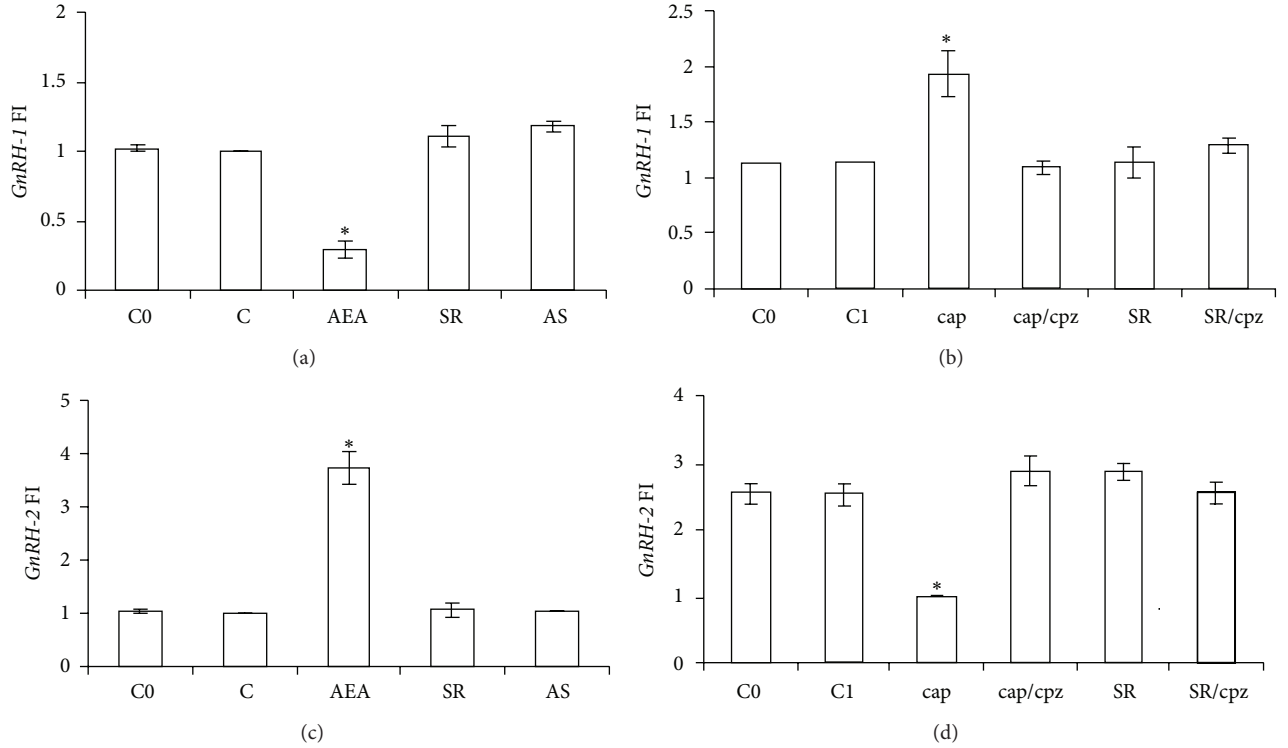

Figure 3: Effects of AEA treatment on *GnRH-1* (a) and *GnRH-2* (c) expression in frog testis collected from June animals ($N = 5$/group) after 1 h of incubation. Incubations have been carried out with AEA 10^{-9} M, SR 10^{-8} M, or both. C0: untreated testis of June; C: control group, testis treated with Krebs-Ringer buffer. Effects of cap treatment on *GnRH-1* (b) and *GnRH-2* (d) expressions in frog testis of June after 1 h of incubation. Incubations have been carried out with cap 10^{-6} M, cpz 10^{-5} M, SR 10^{-8} M, or combinations of cap/cpz and SR/cpz. C0: untreated testis of June; C1: control group, testis treated with Krebs-Ringer buffer. The data in graph are the results of RT-PCR analysis; they are reported as fold increase (FI) calculated comparing the expression of *GnRH-1*/*GnRH-2* to the housekeeping *fp1* and are representative of three separate experiments at least ($N = 6$). Asterisks indicate statistically significant differences.

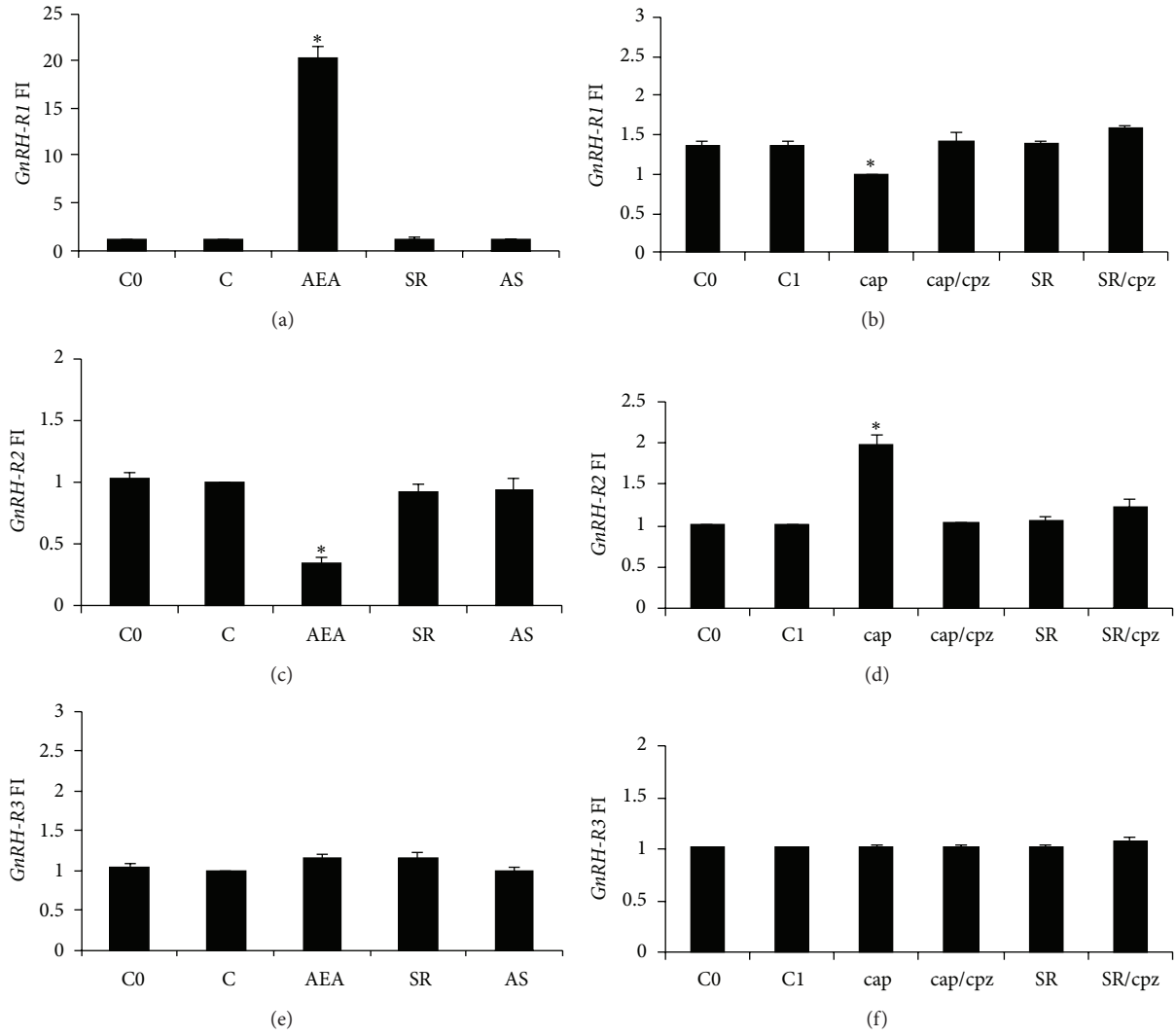

Figure 4: Effects of AEA treatment on *GnRH-R1* (a), *GnRH-R2* (c), and *GnRH-R3* (e) expressions in frog testis collected from June animals ($N = 5$/group) after 1 h of incubation. Incubations have been carried out with AEA 10^{-9} M, SR 10^{-8} M, or both. C0: untreated testis of June; C: control group, testis treated with Krebs-Ringer buffer. Effects of cap treatment on *GnRH-R1* (b), *GnRH-R2* (d), and *GnRH-R3* (f) expressions in frog testis of June after 1 h of incubation. Incubations have been carried out with cap 10^{-6} M, cpz 10^{-5} M, SR 10^{-8} M, or combinations of cap/cpz and SR/cpz. C0: untreated testis of June; C1: control group, testis treated with Krebs-Ringer buffer. The data in graph are the results of RT-PCR analysis; they are reported as fold increase (FI) calculated comparing the expression of *GnRH-Rs* to the housekeeping *fp1* and are representative of three separate experiments at least ($N = 6$). Asterisks indicate statistically significant differences.

be involved in Sertoli-spermatozoa communication, and does not modulate *GnRH-I/GnRH-R1*, a system supposed to be involved in germ cell progression [6]. Therefore, AEA might modulate testicular GnRH signalling at multiple levels and in a stage dependent manner [6].

4. Relationship between Endovanilloids and GnRH System

As mentioned above, AEA has a dual potentiality thanks to the ability to bind to both CB1 and TRPV1 and so working as an endocannabinoid and an endovanilloid as well. In the context of reproduction, this peculiarity makes AEA a dual regulator of acrosome reaction (AR). In boar sperm, AEA—present in both seminal plasma and uterine fluids—prevents, *via* CB1, premature capacitation and inhibits AR [43]. By contrast, a few hours later, when sperm have reached the oviduct, this inhibitory brake becomes less stringent, since AEA concentration progressively reduces. At this time, AEA works as endovanilloid activating TRPV1 [43]. Such an activation prevents spontaneous AR, an uncontrolled phenomenon of exocytosis that leads quickly to cell death [70]. Besides functions related to fertilizing ability due to intracellular AEA signalling, few and contradictory studies have analyzed the effects of CAP, the agonist of TRPV1, in male germ cell progression. In the past, CAP, acting as specific neurotoxin that irreversibly caused degeneration of sensory C fibres of the peripheral nerves, was investigated for its ability to affect testicular descent [71]. However, CAP has been reported to adversely affects the survival of rat spermatogonial cell lines

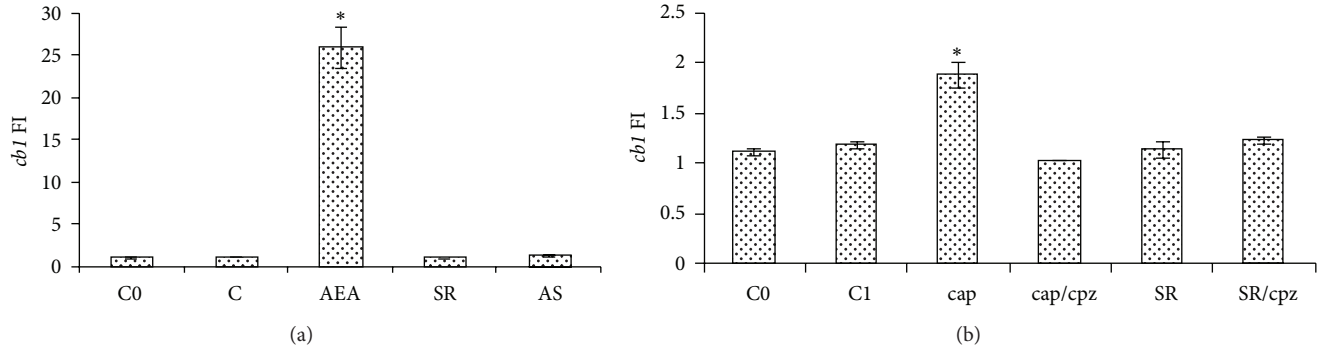

Figure 5: Effects of AEA treatment on *cb1* (a) expression in frog testis collected from June animals ($N = 5$/group) after 1 h of incubation. Incubations have been carried out with AEA 10^{-9} M, SR 10^{-8} M, or both. C0: untreated testis of June; C: control group, testis treated with Krebs-Ringer buffer. Effects of cap treatment on *cb1* (b) expression in frog testis of June after 1 h of incubation. Incubations have been carried out with cap 10^{-6} M, cpz 10^{-5} M, SR 10^{-8} M, or combinations of cap/cpz and SR/cpz. C0: untreated testis of June; C1: control group, testis treated with Krebs-Ringer buffer. The data in graph are the results of RT-PCR analysis; they are reported as fold increase (FI) calculated comparing the expression of *cb1* to the housekeeping *fp1* and are representative of three separate experiments at least ($N = 6$). Asterisks indicate statistically significant differences.

expressing TRPV1 [72], whereas a protective role against heat stress has been suggested for TRPV1 [73]. Conversely in mouse, a diet containing 0.02% CAP enhances testicular cell proliferation and affects the release of both testosterone and ghrelin, the latter being an acylated polypeptide hormone mainly secreted by the endocrine cells of the stomach [74]. Interestingly, in mammals, TRPV1 is expressed in Sertoli cells [75] and germ cells, with high levels of both mRNA and protein detected from spermatocytes to spermatids stages [55]. At present, none has investigated a possible role of endovanilloids in GnRH signalling, either at central level or at testicular level. Once again a simple animal model as *R. esculenta* has shed light on such a mechanism. In parallel to AEA treatment of frog testis, in June, *in vitro* stimulation with CAP has been carried out. Interestingly, the effects observed upon GnRH system have been opposite to those of AEA. In particular, CAP increases *GnRH-1* and decreases *GnRH-2* (Figure 3); then, it decreases *GnRH-R1* and increases *GnRH-R2*, with no effect on *GnRH-R3* (Figure 4). These effects have been completely counteracted by capsazepine (CPZ), a competitive TRPV1 antagonist [76]. No effects have been observed after SR141716A (SR), a CB1 antagonist, alone or in combination with CPZ. Interestingly, CAP affects *cb1* expression as well (Figure 5) suggesting a possible overlapping between the eCB and the endovanilloid system.

5. Closing Remarks

The eCS field is an important example of the kinds of inputs that studies of comparative endocrinology can give to our knowledge. The contribution of lower vertebrate animal models in reproduction research is very strong not only because they make easy the investigation of mechanisms regulating mammalian reproductive physiology but also because they allow to understanding on how these mechanisms have evolved.

The frog *R. esculenta* has been a suitable model for a complete characterization of the eCS. Thanks to its feature as seasonal breeder, *GnRH* and *cb1* expression profiles have been compared indicating the existence of a physiological reverse relationship between the two systems. More interestingly what happens in brain not always can be confirmed in testis; in fact, a different regulation by AEA of the GnRH system has emerged in frog brain and testis. In addition, a novel role can be ascribed to endovanilloids as new regulators of the GnRH system in testis. Furthermore, it is reasonable that eCBs and endovanilloids might work as two different faces of the same medal since an opposite regulation of each component of the GnRH system by these molecules has been described.

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