The Endocannabinoid System: An Ancient Signaling Involved in the Control of Male Fertility

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The effects of cannabinoids on human health have been known since the antiquities when the extract of the plant *Cannabis sativa* was used because of its psychoactivity. The scientific story of the cannabinoids started in the 1960s with the isolation and characterization of the active component of the plant. After the synthesis of cannabinoid analogues, the analysis of structure–effect relationships was implemented, and this had a similar effect to a positive "Pandora's box" opening. To date, numerous roles have been ascribed to the "endocannabinoid system." Here we describe its involvement in the control of male reproduction, taking into consideration possible evolutionary speculations. Indeed, the endocannabinoid system is a very ancient signaling system, being clearly present from the divergence of the protostomian/deuterostomian.

Key words: **endocannabinoid system; reproduction; sperm motility; amphibian**

Introduction

The use of the plant *Cannabis sativa* is very ancient, dating back thousands of years, as a result of its psychoactivity. In the beginning of the 19th century, many researchers tried to identify the active principle of the plant but they were unsuccessful because extraction was difficult. In the 1940s two different groups, working independently, isolated cannabinol and cannabidiol, two molecules with moderate and null psychoactivity, respectively.[1] Two decades later, Mechoulam and Gaoni[2] isolated the main active component of *C. sativa*, Δ^9-tetrahydrocannabinol (THC), identifying its structure. This early work revitalized research in *C. sativa*, and other cannabinoids were isolated from the plant, giving a strong impetus to an important field of investigation on the effects and mechanisms of action in several experimental animal models.

With the synthesis of cannabinoid analogues, the analysis of structure–effect relationships was implemented and it became possible to correlate the psychoactivity of cannabinoids in humans.[3] The effects from THC suggested the existence of specific receptors, but, because of the lipid nature of the molecule, the demonstration of such receptors needed to be clarified. Only with the synthesis of a more hydrophilic analogue, known as CP-55,940, it was possible to develop a membrane-binding assay.[4] Binding sites were then characterized in rat brain and named cannabinoid (CB) receptors. Five years later, a new receptor type,

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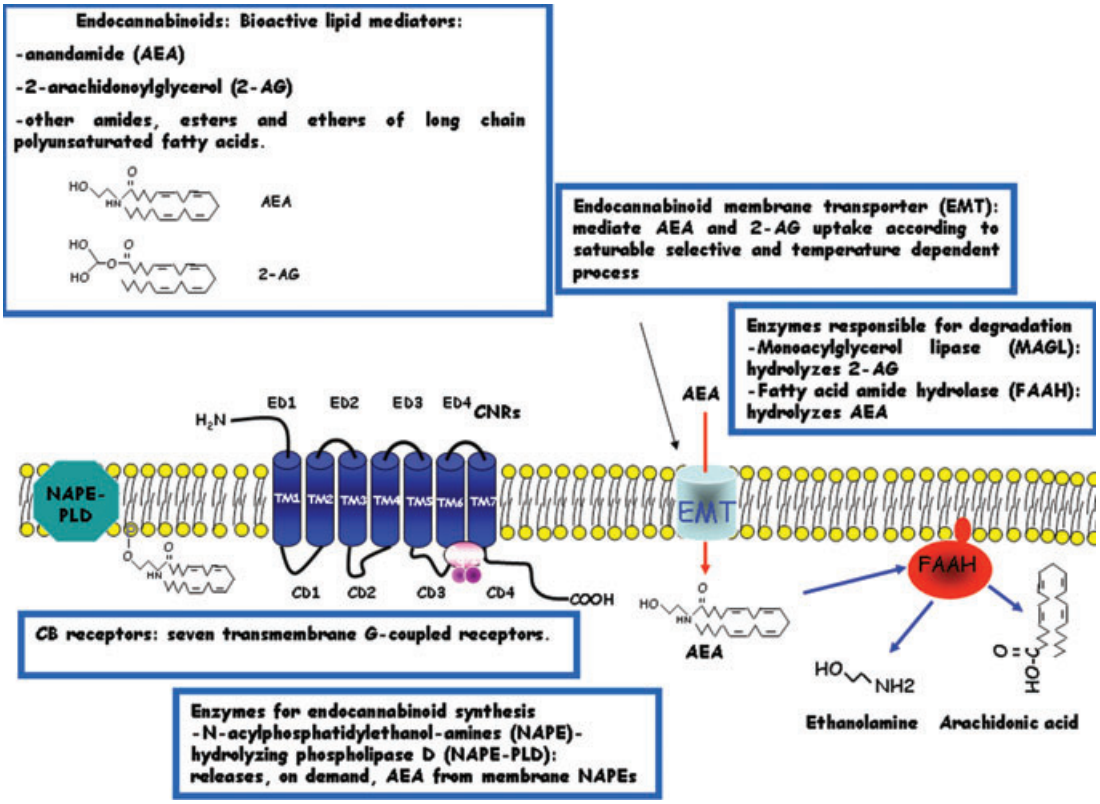

Figure 1. The endocannabinoid system is composed of ligands, receptors, transporters, and metabolic enzymes. (In color in *Annals* online.)

showing 44% amino acid sequence identity with the human brain CB (herein referred to as CB1), was identified in immune cells and called CB2.[5] CBs are typical 7-transmembrane domain G protein-coupled receptors. The discovery of CB2 provided an explanation for the cannabinoid effects on the immune system. The next step in cannabinoid research was to develop selective agonists and/or antagonists specific for the two receptor types[6,7] and, later on, the production of knockout (KO) animals. CB1 KO mice, generated by two different groups, show absence of response to cannabinoids, as expected,[8,9] and CB2 KO mice are characterized by the lack of cannabinoid immunomodulatory effects while behavioral effects from CB1 involvement are normal.[10] More recently, the existence of additional cannabinoid-binding sites has been found and two receptors, GPR55 and GPR119, have been postulated as novel CB receptors.[11]

Apart from the intrinsic importance of the discovery of CB receptors, their activity strongly suggested the existence of endogenous ligands (endocannabinoids). The first two endocannabinoids isolated were arachidonoylethanolamide [anandamide (AEA); ananda means "beatitude" in Sanskrit] and 2-arachidonoylglycerol (2-AG).[12–14]

The Endocannabinoid System

The endocannabinoid system (ECS) is composed of ligands, AEA, 2-AG, their congeners, target receptors, metabolic enzymes, and transporters; a short description is needed to discuss the ECS role in several physiological activities (Figs. 1 and 2). To date there are four endogenous substances characterized as endocannabinoids: AEA, 2-AG, 2-arachidonoylglycerol ether, and virodhamine

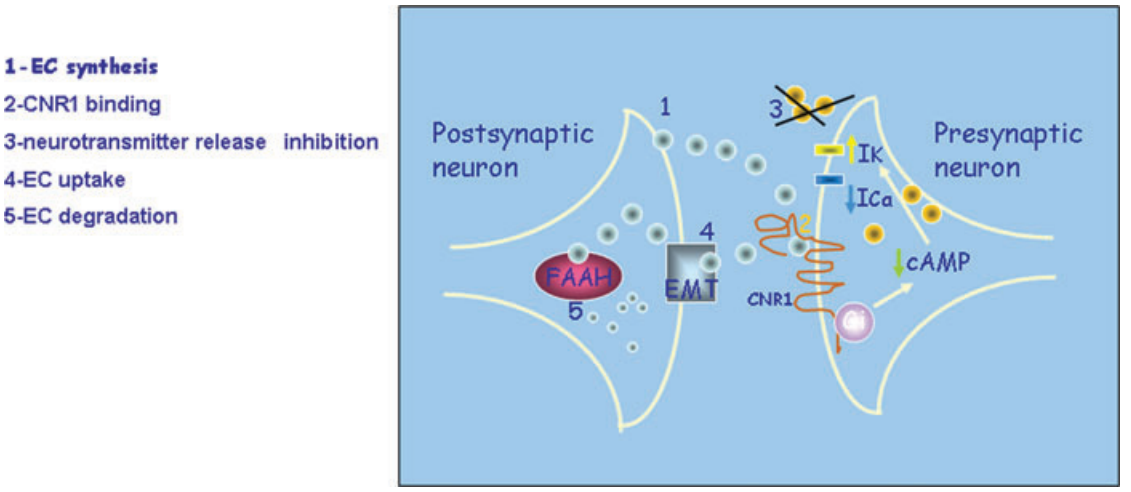

Figure 2. Endocannabinoids (EC) acts as retrograde synaptic messengers, decreasing neurotransmitter release. 1, EC synthesis; 2, CB1 receptor binding; 3, neurotransmitter release inhibition; 4, EC uptake; 5, EC degradation. (In color in *Annals* online.)

(see Ref. 15). Several additional lipid mediators, such as N-oleoylethanolamine (OEA) and N-palmitoylethanolamine, are now included in the list of endocannabinoid-like molecules or "entourage compounds" that are able to improve AEA and 2-AG activity by inhibiting their degradation (see Ref. 15). Both AEA and 2-AG are natural ligands for CBs and they show the same effects as THC, the main psychoactive component of *C. sativa*. To date, three receptor types have been found by molecular cloning: the transient receptor potential vanilloid type 1 receptor (TRPV1), CB1, and CB2.[5,16] The ligand fishing method, used to identify additional receptor forms (perhaps mediating non-CB1/CB2 effects), evidenced two novel CB receptors, GPR55 and GPR119. They are involved in physiological processes, the former in the reduced mechanical nociception following inflammation and the latter in the regulation of energy balance and body weight.[11]

Localization studies have shown that CB1 is mainly expressed in the central nervous system, although it is also present in extra brain tissues, gonads included.[17,18] CB2 receptors have been identified in immune cells.[5] Later, its expression was also detected in brain stem[19] and other tissues. TRPV1, a ligand-gated, nonselective, cationic channel, shows an intracellular binding site for AEA.[20–22] Of the two orphan receptors recently identified, GPR55 is suggested to be involved in pain signaling; however, whether its site of action is in the brain or in immune cells is still an open question. GPR119 is mainly expressed in the pancreas and gastrointestinal tract; this localization prompted the search for a synthetic agonist able to affect the metabolic status.[11]

The enzyme responsible for AEA production is a specific phospholipase D (NAPE-PLD),[23] which catalyzes the hydrolysis of *N*-arachidonoylphosphatidylethanolamine. 2-AG is derived from the hydrolysis of inositol phospholipids as a result of a specific phospholipase C (PLC) and the subsequent conversion of diacylglycerol (DAG) as a result of a *sn*-1-DAG lipase.[24,25] Furthermore, for its synthesis, other pathways have been hypothesized, including PLC-dependent and independent routes.[26]

AEA and 2-AG are produced on demand and released in the extracellular space through a specific carrier, the endocannabinoid membrane transporter (Refs. 27, 28), the existence of which appears to be controversial.[29] In fatty acid amide hydrolase (FAAH) KO cells, AEA uptake is not reduced by putative

transport inhibition, thus suggesting a simple diffusion mechanism for AEA to cross the plasma membrane.[30]

The re-uptake inside the cell induces the degradation of AEA by FAAH, producing arachidonic acid (AA) and ethanolamine.[31] 2-AG is degraded to AA and glycerol mainly by a specific mono-acylglycerol lipase (MAGL). MAGL has been cloned and characterized in rat and human brain.[32,33]

The signaling pathway is started by the binding of AEA or 2-AG to the receptors (CB1 or CB2). This induces, through the Gi/o proteins, the inhibition of adenylyl cyclase,[16,34] regulation of ionic currents,[35] activation of focal adhesion kinase,[36] and mitogen-activated protein kinase.[35]

Unlike 2-AG, AEA is also able to bind intracellular sites (a ligand-gated and nonselective cationic channel) as TRPV1 receptors or the T-type Ca^{2+} channel.[37] The current hypothesis is that AEA shares molecular similarities with capsaicin, the exogenous ligand of TRPV1. This receptor is expressed at both brain and extra-brain areas.[20–22]

The orphan receptors, recently found and named GPR55 and GPR119, are activated by several cannabinoid ligands and OEA, respectively.[11,38]

Is the ECS Conserved during Evolution?

The ECS is not restricted to humans or to mammals; in fact, it has been characterized in several phylogenetically distant species, supporting the hypothesis that it can display "master" functions. The ECS appears to be very ancient, occurring in mammalian and non-mammalian vertebrates. Studies have been carried out also in invertebrates, which are considered useful experimental animal models in physiology, genetic, and neurobiological research. For example, the insect *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* are convenient by virtue of their small size and short generation time. Furthermore, other invertebrates, such as *Aplysia californica*, show numerous advantages for neurobiologists by virtue of the relative simplicity of their nervous system.[39] The sea urchin (Echinodermata) has frequently been used to study mechanisms of fertilization[40] and embryonic development.[41]

Starting from the identification of the THC, many groups reported the effects of this compound in several animal models; in the protozoan *Tetrahymena pyriformis*, THC affected cell division[42]; in the lobster, the effect of THC was evidenced on neurotransmitter release. Schuel *et al.*[40] were the first to report an additional effect of inhibited fertilizing ability of sperm cells treated with THC.

These studies concerning the mode of action of cannabinoids did not explain how they exerted their effects. In fact, to answer this question it is necessary to demonstrate the presence of an ECS, in other words to look for ligand biosynthesis and degradation enzymes, receptor presence, and the possible cascade of reactions activated by the interaction of ligand and receptor.

AEA is present in Cnidaria (*Hydra vulgaris*; Refs. 43, 44), in molluscs (*A. californica*; Ref. 43), and in Echinodermata (*Paracentrotus lividus*, Ref. 45). 2-AG shows a similar pattern.[43] However, the presence of these molecules cannot be interpreted as a marker of a cannabinoid signaling system; in fact, AEA has been found, for example, in chocolate (its presence is more likely from the milk used because plants do not contain AA, the normal precursor of AEA), but there is not sufficient evidence to indicate that chocolate has an ECS.[46]

However, as far as the receptors are concerned, *H. vulgaris* contains the elements necessary to identify an ECS. They express selective cannabinoid-binding sites, endogenous CB receptor ligand, AEA, FAAH-like activity, and a putative biosynthetic precursor of AEA.[44] Its proposed role is the modulation of the "feeding response" by which the control of mouth opening/closure is carried out.

In *D. melanogaster*, binding sites for CP-55,940 (a synthetic agonist of CB1) have been evidenced, but the presence of receptors in insects still needs to be demonstrated. Indeed, further research demonstrated that the binding sites were quite different compared to vertebrate CB1/CB2 receptors. In addition, a bio-informatic approach has demonstrated the existence in the genome of some sequences displaying a very high level of similarity with mammalian *cb* but codifying insect catecholamine receptors. As a consequence, we can say that the *D. melanogaster* genome does not contain orthologues of mammalian CB receptor genes. Similar results were obtained from bio-informatic studies carried out with *C. elegans.*[46,47]

Similarly, no definitive data are available on the presence of CB receptors in molluscs. Binding sites have been detected but they may simply reflect a nonselective, but competitive, interaction of cannabinoids with cell membranes and not with specific membrane proteins.[46]

In the urochordates (sea squirt *Ciona intestinalis*[48]) and in the cephalochordates (amphioxus *Branchiostoma floridae*[49]) *cb* orthologues have been cloned. Negative results were obtained from the search of *cb1/cb2* in the sea urchin.[50]

In nonmammalian vertebrates, the presence of binding sites specific for CP-55,940 has been demonstrated in a variety of species, including chicken, turtle, frog, and trout.[51] In 1996, Yamaguchi *et al.*[52] discovered a nonmammalian CB receptor gene in the puffer fish *Fugu rubripes*. Two genes, sharing sequence similarity with mammalian *cb1*, were found and named *fcbA* and *fcb1B*. They are extensively expressed in the brain and moderately expressed in the testis, ovary, and spleen. More recently, a mammalian *cb2* orthologue has been reported in *Fugu.*[53] This is the first *cb2* gene identified in a nonmammalian species, promoting *Fugu* as a suitable model for discovering possible physiological roles for CB2.

The presence of two *cb1* forms in *Fugu* can be explained by whole genome duplication, a phenomenon not unusual in nonmammalian species (*Danio rerio* is another example possessing two paralogue genes of *cb2*, named *cb2a* and *cb2b*; Ref. 54). The presence of mammalian *cb2* orthologues in *Fugu* suggests that the duplication event occurred before the divergence of tetrapods and teleosts.

In the amphibian *Taricha granulosa*, *cb1* has been sequenced[55]; it is highly expressed in the brain and it shows the same characteristics reported for mammalian CB receptors. In *Xenopus laevis,*[56] CB1 has been found in the spinal cord in regions strongly involved in spinal analgesia, suggesting that endocannabinoids might participate in the control of pain sensitivity. In the frog, *Rana esculenta*, evidence of CB1 expression in both central nervous system and testis has recently been provided, thus confirming the high conservation degree of the cannabinergic system in vertebrates.[57] CB1 cDNA from frog brain and testis has been cloned and, because of the presence of nucleotide differences in brain and testis, the genomic DNA sequences from the same tissues of the same pool of animals were determined. The changes observed did not affect the predicted amino acid sequence except for the 70 and 408 positions. The presence of polymorphic sites was excluded because these studies were carried out on tissues collected during the annual reproductive cycle from the same pool of animals. However, this finding is particularly intriguing because different cDNA sequences in brain and testis led to different mRNA folding, which may affect the mRNA's stability and viability (Fig. 3).[58] Comparable events occur in fish and mammals. In particular, in *F. rubripes* changes in the 241 and 463 positions affect amino acid composition. Whether we are in the presence of posttranslational modifications needs further investigation. The importance of these findings increases when considering that, in humans, *cb1* nucleotide changes are often associated with behavioral/neurological diseases.[59,60]

In avian species, binding sites have been found in the zebra finch *Taeniopygia guttata.*[61] Furthermore, a cDNA fragment coding part of

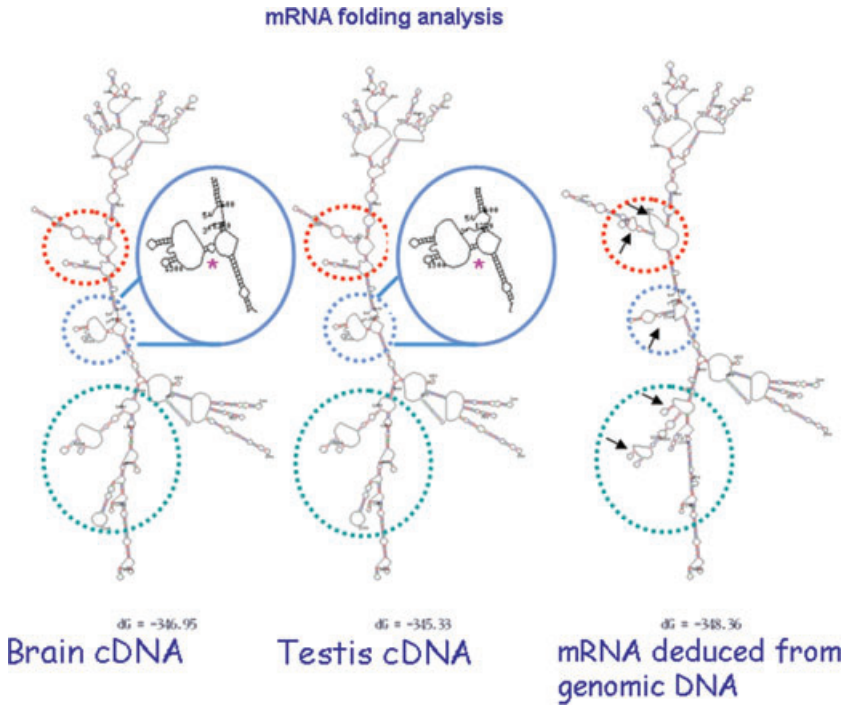

Figure 3. Both brain and testis mRNA secondary structure is affected by nucleotide changes, and these modifications might alter its stability. (In color in *Annals* online.)

a CB1-like protein has been amplified. Interestingly, CB1 was expressed in the regions of the brain involved in song learning, thus suggesting a "new" role for ECS in vocal development.

The reported absence of binding sites in lamprey brain[51] is very intriguing, this animal being one of the very few representatives of primitive vertebrates (the agnathans). There are two possible explanations: CBs may have developed after the divergence of agnathans from the lineage that gave rise to the vertebrate classes, but CBs are already present in urochordates and cephalochordates; or lamprey may have lost, for unknown reasons, CB coding genes. However, lamprey genome analysis has not been carried out to date.

The activity of FAAH, the enzyme responsible for AEA degradation, has been measured in invertebrates, such as *H. vulgaris*, *P. lividus*, in the locust *Schistocerca gregaria*,[46] and *C. intestinalis*.[62] As stated above for ligands, the presence of FAAH activity is not synonymous with ECS presence; in fact, it was originally found in liver cells where its role is, at the moment, unrelated to cannabinoid signaling. As a result of the cloning and sequencing of the mammalian FAAH gene,[63] the search for related genes in nonmammalian models is possible. The presence of FAAH-related peptides has been considered in *D. melanogaster* and *C. elegans;* however, we do not know yet if these proteins are functional orthologues of the mammalian FAAH. It is possible that FAAH-like proteins of invertebrates are simply related members of a larger family of enzymes sharing a particular "cleavage" activity.

All the results listed above allow some conclusions. First, it is clear that ligands and receptors have developed separately, the ligand being present in several invertebrate species. Furthermore, it is possible that such molecules do not need receptors to function but they use membrane proteins or, alternatively, they can disrupt membrane architecture inducing

cell response. Lastly, in the absence of CB receptors, AEA and 2-AG might play other roles; in insects, for example, 2-AG plays a defense function against organisms expressing CB1. Plants produce "entourage compounds" able to activate CB-like responses even in the absence of CB receptors and endocannabinoids.[64]

The ECS is Also Present in the Testis

We will focus on the male reproductive system where the presence of ECS has been demonstrated in several cell types. Exhaustive recent reviews on endocannabinoids and female reproduction are readily available.[22,65] Spermatogenesis, the process whereby spermatozoa (SPZ) are formed, is a complex process that includes mitotic phases, meiotic divisions, and differentiation stages. These events are driven by pituitary hormones and by a network of locally produced signals. Once produced, SPZ progress to the epididymis where they undergo additional maturation, acquiring the motility necessary to reach and fertilize the egg. As a result of the complex organization of mammalian testis,[66–68] the use of lower vertebrates as well as KO animals provided the opportunity to study local cell-to-cell communications and factors involved.[67,68]

Endocannabinoids are synthesized by the gonads. Rat testis produces AEA,[14] germ and Sertoli cells are sources of AEA, and both mammalian and nonmammalian SPZ produce endocannabinoids.[69,70] Indeed, CB1 KO mice showed a low testosterone secretion in an *in vitro* study.[71] Recently, Maccarrone *et al.*[72] reported that isolated immature Sertoli cells from 4- to 24-day-old mice have an ECS consisting of the biochemical machinery to synthesize, transport, degrade, and bind both AEA and 2-AG. Controversial results have been obtained in isolated Sertoli cells between 4 and 16 days as they do not express CB1.[73] Using morphological and biochemical approaches, the presence of CB1 has been demonstrated in the tubular and interstitial compartment of prepuberal and adult rat testis.[74] In particular, in the tubular area CB1 is present in round spermatids (rSPT; 31 days), in elongating SPT (35–41 days), and in SPZ where it is localized in the region adjacent to the acrosome and in the tail. The expression of CB1 mRNA from 7 days onward, showing a marked decrease between 14 and 31 days and a significant increase at 35 days, correlates with the appearance of rSPT. A decrease is also observed at 41 days followed by a significant increase from 60 days onward that might be correlated with the increase of Leydig cell numbers in adults. In Sertoli cells, CB1 appears concomitantly with the expression in SPT (41 days).

In the rat interstitial compartment, CB1 appeared from 14 days onward in spindle-shaped cells, which are committed to differentiate to adult Leydig cells (ALC).[74] These cells pass through a progenitor Leydig cell stage before their transformation into round-shaped cells with numerous lipid droplets and abundant smooth endoplasmic reticulum (immature Leydig cell, by 28 days). A role for CB1 in this system was looked for when the immunopositivity of these cells for 3β-hydroxysteroid dehydrogenase (3β-HSD), the classic marker for Leydig cells, has been determined. Immature Leydig cells divide once (around 41 days) before their differentiation into ALC. At this stage, CB1 levels are very scarce and their mitotic index increases. In fact, at 41 days only immature Leydig cells not expressing CB1 are mitotically active (Fig. 4). Furthermore, because in CB1 KO mice few Leydig cells are present, this further suggests that the ECS is involved in ALC differentiation.[74]

A whole ECS has been characterized in the anuran amphibian *R. esculenta*.[57,58,70] In particular, the presence of ECS has been reported in the tubular compartment, as found in mouse and rat. Furthermore, the expression of CB1 and FAAH proteins has been studied in both germ cells and isolated SPZ.[70]

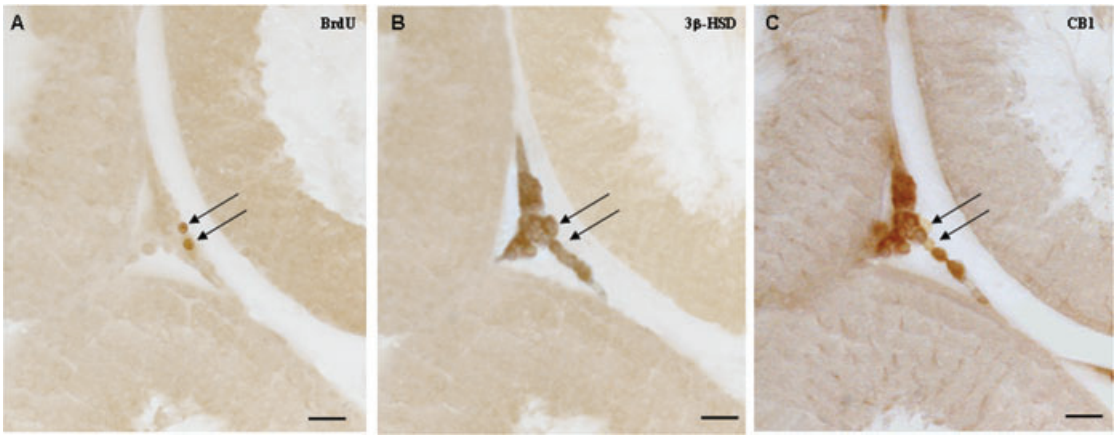

Figure 4. Immunostaining of consecutive rat testis (41 days) sections with bromodeoxyuridine (**A**), 3β-hydroxysteroid dehydrogenase (3β-HSD) (**B**), and CB1 (**C**). Two proliferating Leydig cells (arrows, panel A), identified by 3β-HSD (arrows, panel B), are not stained by anti-CB1 (arrows, panel C). Scale 20 μ. (In color in *Annals* online.)

What Is the Role of the Endocannabinoids in the Control of Reproductive Function?

Hypothalamus–Pituitary Axis

It is well accepted that endocannabinoids modulate reproduction at multiple levels.[22] Centrally, they depress the secretion of the anterior pituitary hormones thyroid-stimulating hormone, luteinizing hormone (LH), growth hormone, and prolactin[75–77] while they do not have a clear effect on follicle-stimulating hormone secretion. The sites of cannabinoid action have been described either at the pituitary or at the hypothalamic level.[78,79] In mammal and amphibian anterior pituitary, CB1 activity is present, in particular in lactotrophs and gonadotrophs.[79,80] The effect of AEA has been demonstrated *in vitro* in dispersed rat pituitary cells[81]; AEA regulates prolactin, LH,[71] adrenocorticotropic hormone,[82] and GH[81] secretion. Furthermore, endocannabinoids modify copulatory behavior.[22]

Recently, CB1 has been cloned in *R. esculenta*[58] and its involvement in the control of male reproduction has been determined.[70,83] During the annual reproductive cycle, CB1 mRNA shows an expression profile in the brain, but a direct link with reproduction is still lacking. It is known that AEA decreases gonadotropin-releasing hormone (GnRH) release in rats and that immortalized GnRH neurons synthesize endocannabinoids and express CB1.[84] Using these data and *R. esculenta* as an experimental model, a link between AEA and GnRH has been investigated. By the use of double-labeling immunofluorescence, the relationship between CB1 and GnRH in several brain areas controlling reproduction has been found. GnRH-I and CB1 signals are in close contiguity, and GnRH neurons expressing CB1 represent a subpopulation in the septal and preoptic area. GnRH neurons project their axons to the vascular zone of the median eminence. The expression pattern of both messengers shows opposite profiles in the brain areas (telencephalon and diencephalon) mainly involved in GnRH release and the control of reproduction. Other data on the inhibitory effect of AEA on GnRH-I expression and GnRH agonist inhibitory effect on GnRH-I mRNA synthesis, with a consequent increase in CB transcription, further support the morphofunctional anatomical link and provide an explanation for the reciprocal

relationship between the ECS and GnRH neuronal activity.[57,58]

Sperm Activity

The first reports concerning AEA effects on fertilization appeared in 1987 when Schuel *et al.*[40] demonstrated the ability of THC to block the acrosome reaction, naturally stimulated by a ligand present in the egg jelly coat, impairing fertilization in sea urchin SPZ.[85] The blocking of the acrosome reaction might be a result of AEA binding, which affects the opening of ion channels; in fact, the use of ionomycin and nigerin, which open Ca^{+2} and K^{+} ion channels, induces the acrosome reaction artificially in SPZ pretreated with AEA.

Mammalian and nonmammalian vertebrate SPZ display a true ECS having a suggested role that is a main role during the journey of SPZ to the site of fertilization. A direct involvement in the capacitation process has been demonstrated by the use of CB1 antagonists.[86] Furthermore, AEA reduces, in a dose-dependent manner, human sperm motility by reducing mitochondrial activity and, at higher concentrations, by decreasing sperm viability.[86,87] Experiments carried out on boar SPZ (*Sus scropha*) demonstrated that CB receptors are involved in the control of the acrosome reaction, which takes place when SPZ recognizes and binds to zona pellucida proteins allowing gamete fusion. TRPV1 in SPZ seems to regulate the spontaneous acrosome reaction that might cause its precocious activations. It is possible that CB receptor activation has a role in the prevention of "polyspermy" and/or a precocious and useless acrosome reaction. This control is displayed by endocannabinoids, which will drive these events in a correct space/time pattern.[69]

To verify a link between ECS and reproduction, Cobellis *et al.*[70] used the anuran amphibian *R. esculenta* in looking for the existence of both FAAH and CB1 proteins during the annual reproductive cycle. Nonmammalian vertebrates are very useful in reproductive studies; in fact, numerous species are seasonal breeders and spermatogenesis occurs in cysts consisting of Sertoli cells enveloping clusters of germ cells at the same stage of maturation. During the annual reproductive cycle, spermatogenesis progression is regulated by endocrine and local products, and this allows the possibility of having in each period of the year a defined well-known population of cysts. Consequently, this model represents a powerful tool for delineating molecular signaling involved in the control of spermatogenesis. In addition, as environmental factors (photoperiod or temperature) influence reproductive functions, it is possible, by modifying animal storage conditions, to induce or block some reproductive functions.[67,88]

During the annual reproductive cycle of *R. esculenta*, the expression of both FAAH and CB1 is concomitant with the appearance of SPT (during September–October); SPT are scarcely represented in other stages of spermatogenesis. The demonstration that endocannabinoids might affect reproduction in amphibians has also been provided.[70] In particular, the step involving SPZ motility acquisition, occurring during the journey from the cloacal fluid to the aquatic environment, has been shown to be critical. Indeed, when SPZ are in the cloaca, CBs are able to block SPZ motility. Once released in the aquatic environment, the dilution effect allows SPZ to move.[70] In mice, which display internal fertilization, research has concentrated on the epididymis where SPZ(s) move from the head to the tail to acquire their correct motility at the right time and space.[89] In particular, in CB1 KO mice the percentage of motile SPZ in the head is significantly higher compared to normal mice.

Taken together, these results let us focus attention on the ECS as a new target system involved in the regulation of male fertility, opening additional paths to be followed to solve male fertility problems.

Concluding Remarks

It is clear that an endocannabinoid signaling system is present in the phylogenetic tree after the divergence of protostomian/

deuterostomian. The findings in *H. vulgaris,* the only protostomian expressing such a system, need to be confirmed by a biomolecular approach. It is possible that in invertebrates, instead of a classical ECS, there are molecules able to bind cannabinoid-like binding sites present on plasma membranes. These proteins, although able to bind endocannabinoids, could not be considered receptors. In fact, as a consequence of binding, the complex might function by disrupting membrane architecture. The finding of CB in urochordates and cephalochordates and the absence in invertebrates support the hypothesis that urochordates, cephalochordates, and chordates share a common ancestor. Furthermore, these findings provide insights into the ancestral functions of CB receptors prior to the emergence of CB1 and CB2 receptors in vertebrates. As a consequence, the conclusion drawn by Salzet *et al.*[90] about the conservation of the ECS throughout evolution from celoenterates to man needs to be revised.

As far as the involvement of the ECS in male fertility, it is clear that its engagement is at multiple levels. Furthermore, the ECS displays a different mode of action depending on the species as a consequence of different reproductive strategies. For example, in amphibians showing external fertilization, a dilution mechanism evolved to allow the acquisition of SPZ motility. In mammals displaying internal fertilization, the same mechanism might be present in the epididymis where the modulation of endocannabinoid signaling regulates this process.

No one knows if, when, or how the endocannabinoid story will end; more likely it will be another never-ending story. A possible end, if we can ever consider real end points in research, will come from genome sequences, with perhaps new, exciting, working hypotheses to demonstrate.

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Conflicts of Interest

The authors declare no conflicts of interest.

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