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Aspects of endocannabinoid signaling in periimplantation biology

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Abstract

Physiological roles of endocannabinoids, a group of endogenously produced cannabinoid-like lipid molecules that activate G-protein coupled cannabinoid receptors, are being increasingly appreciated in female reproduction. Adverse effects of cannabinoids on female fertility have been suspected for decades; however, underlying molecular and genetic bases by which they exert these effects were not clearly understood. The discovery of cannabinoid receptors (CB1 and CB2), endocannabinoid ligands (anandamide and 2-acylglycerol) as well as their key synthetic and hydrolytic pathways has helped to better understand the roles of cannabinoid/endocannabinoid signaling in preimplantation embryo development, oviductal embryo transport, embryo implantation and postimplantation embryonic growth. This review focuses on various aspects of the endocannabinoid system in female fertility based on studies that used knockout mouse models. The information generated from studies in mice is likely to shed deeper insight into fertility regulation in women.

Keywords

Anandamide; preimplantation embryo; cannabinoid receptor; uterus; oviduct; implantation; mouse

Introduction

Marijuana, derived from the plant *Cannabis sativa*, has been recreationally used for thousands of years due to its psychoactive effects including euphoria, sedation and analgesia. Because of its widespread use, research on the chemistry of *Cannabis* began decades ago [1]. However, its major active component, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), was not identified until 1964 [2]. This discovery initiated a dramatic interest in cannabinoid research, further increased by the discovery and cloning of two types of cannabinoid receptors, brain-type (CB1) [3,4] and spleen-type (CB2) [5]. Around the same time, several endogenous compounds targeting CB1 and CB2 were identified and collectively termed endocannabinoids. Among them, the two most since studied endocannabinoids are anandamide (AEA) and 2-AG with the structure of anandamide first revealed in 1992 [6] and 2-AG discovered by two independent groups in

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canine gut [7] and rat brain [8]. Various aspects of the endocannabinoid system in the context of female reproduction are discussed in this review article.

AEA synthesis and degradation

It is widely accepted that anandamide is derived from the precursor N-arachidonoylphosphatidylethanolamine (NAPE) through its reaction with NAPE-hydrolyzing phospholipase D (NAPE-PLD) [9,10], a member of the metallolactamase family with Ca^{2+} sensitive enzyme activity [11,12]. However, unaltered polyunsaturated NAE (N-acyl-ethanolamine) levels in NAPE-PLD deficient mice suggests other anandamide synthetic pathways [13]. Recently, two other enzymatic routes were identified: 1) double deacylation of NAPE by a phospholipase/lysophospholipase B, α/β -hydrolase 4 (Abh4), to generate glycerophospho-NAE (GP-NAE) which is then cleaved by a phosphodiesterase to liberate anandamide [14], and 2) cleavage of NAPE by a phospholipase C to generate phosphoanandamide (pAEA), which is subsequently dephosphorylated by a protein tyrosine phosphatase, PTPN22, to release anandamide [15]. Although these pathways are found in both the CNS and peripheral tissues, the mechanism(s) of how these pathways can regulate and affect each other are still unknown.

Anandamide is degraded to ethanolamine and arachidonic acid (AA) by a membrane-bound fatty acid amide hydrolase (FAAH) [16,17]. FAAH can also hydrolyze other fatty acid amides, including 2-AG and the sleep-inducing substance oleamide [18]. FAAH has been shown to be critical for regulating both the magnitude and duration of anandamide and other fatty acid amide signaling [19]. Recently, a second membrane-associated fatty acid amide hydrolase was found in humans and other primate genomes but not in that of the rodent [20].

The transport mechanism of anandamide, an uncharged hydrophobic molecule, across the plasma membrane is still under debate [21,22]. In the current models, the enzymes for the synthesis and degradation of endocannabinoids are thought to be located within the cell, so that the stimulation of cannabinoid receptors from the extracellular component by endocannabinoids requires endocannabinoids to cross the plasma membrane twice. While pharmacological and biochemical evidence points towards the existence of a specific anandamide transport protein using transporter inhibitors [23-26], no direct evidence for such a transporter has been provided. Recently developed drugs have been shown to inhibit anandamide transport without affecting FAAH activity [27]. However, chemical evidence shows that anandamide uptake is not reduced by putative transport inhibitors in FAAH knock-out cells, favoring the model that anandamide traverses cell plasma membrane by simple diffusion [28]. In addition, FAAH may not need a transporter to help reach its substrate anandamide [29].

2-AG synthesis and degradation

2-AG is derived from the precursor diacylglycerol by a membrane-bound sn1-diacylglycerol lipase (DAGL [30]. To date, two isoforms of DAGL have been cloned, DAGL α and DAGL β . The former is found mostly in the adult brain, while the latter is expressed in the developing brain [31]. Like anandamide, 2-AG is produced on demand, but they differ in that anandamide often acts only as a partial agonist of cannabinoid receptors, while 2-AG acts as a full agonist. Interestingly, the binding affinity of 2-AG to cannabinoid receptors is approximately 24 times less than that of anandamide but under most physiological conditions, 2-AG levels are much higher than anandamide [32]. It still remains to be determined, therefore, how only a small percentage of 2-AG (10-20%) crosses the plasma membrane to interact with cannabinoid receptors [33].

After 2-AG is accumulated in cells, it can then be degraded by either FAAH or a serine hydrolase, monoacylglycerol lipase (MAGL) [34]. MAGL, a 33-KD protein, has been isolated, cloned and characterized in both rats and humans [34-36]. Unlike FAAH, MAGL is localized primarily in the cytosol, but not on the plasma membrane. Recently, Muccioli et al. identified a novel protein in BV-2 cells, a mouse microglial cell line, that has MAGL activity, and regulates 2-AG levels [37].

Cannabinoid receptors

Endocannabinoids, as well as plant-derived and synthetic cannabinoids, target cannabinoid receptors CB1 and CB2. Both CB1 and CB2 are G protein-coupled receptors with seven transmembrane domains. CB1 is present mostly in the central nervous system and in some peripheral tissues including heart, testis, liver, small intestine and uterus, while CB2 is abundantly expressed in spleen and several immune cells, including astrocytes [38-40]. Both CB1 and CB2 are coupled with G proteins in the $G_{i/o}$ and G_q families, and the activation of cannabinoid receptors has different biological effects that are cell-type dependent. Signals mediated by cannabinoid receptors include regulation of Ca^{2+} channels [41-45], inhibition of adenylyl cyclase [4,46], activation of phospholipase C [47] and stimulation of mitogen-activated protein kinases (MAPKs) including ERK, JNK and p38 [44,48,49].

In addition to CB1 and CB2, some evidence indicates the existence of other putative cannabinoid receptors [50]. For example, it was shown that anandamide can protect murine neuroblastoma cells subjected to low serum-induced apoptosis by non-CB1, non-CB2 receptors [51]. Furthermore, a novel cannabinoid receptor 3 (GPR55) has been reported [52, 53], which as of yet, is a G protein-coupled orphan receptor. However, physiological role of this receptor is not clearly understood.

Anandamide, but not 2-AG, can also activate receptors other than CB1 and CB2. One receptor that anandamide activates is the transient receptor potential vanilloid 1 (TRPV1) [54], a ligand-gated non-selective cationic channel. The binding of anandamide to its cytosolic binding site of TRPV1 triggers Ca^{2+} influx and eventual cytochrome c release [55,56].

Periimplantation events

The beginning of a new life starts with the fertilization of an egg by sperm [57,58]. The fertilized egg, now termed embryo, develops inside the oviduct undergoing several mitotic divisions eventually to a compacted ball of cells termed morula (Fig. 1). The morula travels towards the uterus and at the same time, a small cavity appears marking the beginning of the blastocyst stage. A blastocyst consists of two cell populations, the inner cell mass (ICM) which forms the embryo proper, and the outer layer of trophoblast cells which generates the placenta and extraembryonic membranes [59-62]. It is only at the blastocyst stage that an embryo makes its first physical and physiological contact with the uterine endometrium to begin the process of implantation (Fig. 1). A reciprocal interaction between the blastocyst and receptive uterus is essential for successful implantation. For instance, the uterus can only accept the blastocyst for attachment when it is in the receptive state. It is only then that the uterus is able to support blastocyst growth, attachment, and subsequent implantation [60,63-65]. Although the genetic and molecular basis for implantation is still not yet clearly understood, gene expression studies and genetically engineered mouse models have shown that a range of signaling pathways are involved in the implantation process with endocannabinoid signaling one of the key players [60,65-67].

Endocannabinoid signaling in the murine female reproductive system

Both CB1 and CB2 receptor are expressed in the female mouse reproductive system. In preimplantation embryos, CB1 is expressed from the late 2-cell through the blastocyst stage, whereas CB2 is present from the 1-cell stage. CB1 is localized primarily in the mural trophoctoderm and intermittently in the polar trophoctoderm, but not in the inner cell mass [46]. However, CB1, but not CB2, is also found in both the oviduct and uterus [68,69].

Not only are the receptors present in these tissues, but also the enzymes necessary for the synthesis and degradation of anandamide. For example, NAPE-PLD is present in the nucleus and cytoplasm of preimplantation embryos from the 1-cell stage to blastocyst stage and FAAH, from the 2-cell stage to blastocyst. Notably, FAAH is uniquely found in outer cell layers of morulae and in the trophoctoderm of blastocysts. In the oviduct, NAPE-PLD levels are higher in the isthmus compared to levels in the ampullary region, whereas FAAH shows the reverse pattern, being higher in the ampullary region [70,71]. This inverse relationship holds true for FAAH and NAPE-PLD following implantation in that NAPE-PLD levels are higher in inter-implantation sites on days 5 and 7 (day 1 = presence of vaginal plug) where FAAH level is low, and NAPE-PLD levels are lower in implantation sites where FAAH levels are higher [72].

With respect to 2-AG synthesis, the dominant DAGL isoform, DAGL α , is almost undetectable on days 1 and 4 of pregnancy, but is markedly upregulated in the luminal epithelium of inter-implantation sites on days 5 and 7. MAGL is present at low levels in both the luminal and glandular epithelia on days 1 through 4 of pregnancy. On days 5 through 7, MAGL expression is induced in subepithelial stromal cells at the site of blastocyst attachment and in the embryo, whereas at the interimplantation sites, its lower level of expression is restricted to the luminal and glandular epithelia [72].

The tightly regulated spatiotemporal expression patterns of the key synthetic and hydrolytic enzymes and cannabinoid receptors create an appropriate endocannabinoid signaling conducive to successful early pregnancy events, including preimplantation embryo development and on-time implantation.

Endocannabinoid signaling and preimplantation embryo development

Development of preimplantation embryos to blastocysts is critical for achieving implantation competency. Delayed development leads to defective implantation or implantation failure, and consequently compromised pregnancy [60]. Embryos exposed to high levels of endocannabinoids, as well as to plant-derived and synthetic cannabinoids, show retarded development. For example, high levels of anandamide causes blastocysts to have a reduced number of trophoctoderm cells and decreases the rate of zona-hatching [73,74]. It has also been shown that anandamide, 2-AG, THC and WIN55212-2 (a synthetic cannabinoid agonist) arrest development of two-cell embryos to blastocysts (Fig 2) [46,75]. This arrested development, however, can be rescued by SR141716A and AM251 (CB1 selective antagonists), but not by SR144528 (a CB2 specific antagonist). Furthermore, a CB2 agonist, AM663, fails to affect embryo development [75]. These studies collectively indicate that endocannabinoids and cannabinoids mediate their effects on preimplantation embryos through CB1.

The availability of *CB1*^{-/-} and *CB2*^{-/-} mouse models greatly expanded the field of endocannabinoid research. Using these mice, it was observed that *CB1*^{-/-} and *CB1*^{-/-}*CB2*^{-/-} embryos recovered from oviducts on day 3 and from uteri on day 4 of pregnancy show aberrant development compared with wild-type embryos [76]. Interestingly, heterozygous embryos recovered from *CB1*^{-/-} females mated with wild-type males showed normal embryo development [69]. This finding prompted the hypothesis that while high cannabinoid levels

can arrest early embryonic development, appropriate cannabinoid signaling under normal physiological conditions is beneficial to normal embryo development. This idea was then confirmed by in vitro embryo culture experiments, showing that low levels of anandamide (7 nM) promoted trophoblast differentiation and growth, while higher levels (28 nM) inhibited such development [77]. The normal development of heterozygous embryos in CB1 deficient environment indicated that embryonic CB1 receptors, but not oviductal CB1 receptors, directed proper early embryonic development [78]. It has been shown previously that most (79%) 2-cell wild-type embryos fail to develop to the blastocyst stage in the presence of excess anandamide. In contrast, more than 80% of *CB1*^{-/-} or *CB1*^{-/-}*CB2*^{-/-} double mutant embryos develop into blastocysts in the presence of similar levels of anandamide. However, in vitro development of *CB2*^{-/-} embryos, like wild-type embryos, was severely compromised in the presence of anandamide [76]. These results lend genetic support to the previous conclusion that CB1, but not CB2, respond to cannabinoids to govern embryonic development.

Interestingly, *CB2*^{-/-} embryos collected from the oviduct on day 3 and uterus on day 4 also show some aberrant development [76], indicating that CB2 does play some role in preimplantation embryo development. Recent observations of CB2 expression in embryonic stem cells by microarray analysis [79] together with its absence in trophoblast stem cells [80], suggest that CB2 expression is restricted to the inner cell mass of blastocysts. Thus, it is conceivable that CB2 may play a role in ICM cell development and therefore, development of the embryo proper.

Collectively, cannabinoid signaling can regulate preimplantation embryo development, with the current model implicating its mediation by CB1 receptor. Although the role of CB2 remains puzzling, it seems to function as a low level cannabinoid signaling gatekeeper for preimplantation embryo development.

Endocannabinoid signaling and oviductal-uterine embryo transport

In parallel with preimplantation embryo development, embryos are transported from the oviduct into the uterus. Embryos enter the uterus at the late morula stage and coincident with this transport, a cavity appears marking the early blastocyst stage. The embryo only achieves implantation competency at the blastocyst stage. Thus, a successful implantation depends on normal and timely transport of these embryos from the oviduct into the uterus (Fig. 3a). Although there is no evidence for implantation of embryos in the mouse oviduct, embryos can implant in the human oviduct (Fallopian tube). Thus, a dysfunctional regulation of oviductal-uterine transport results in oviductal retention of embryos, and thus can cause ectopic pregnancy in women [81,82].

Studies have shown that *CB1*^{+/-} embryos have normal preimplantation development in *CB1*^{-/-} oviducts. Interestingly, however, about 40% of the *CB1*^{-/-} mothers still show pregnancy loss [69,76]. The observation that some *CB1*^{-/-} females, mated with wild type males, did not have embryos in the uterus when flushed on the morning of day 4 of pregnancy suggested oviductal retention of embryos. *CB1*^{-/-}/*CB2*^{-/-} mice also show oviductal retention, but wild-type and *CB2*^{-/-} mice do not, suggesting oviductal retention results specifically from the absence of CB1. This can be explained by the expression pattern of cannabinoid receptors in the oviduct, since CB1 is present in murine oviducts. This same study showed that all the trapped embryos are morphologically and physiologically healthy, because they can implant in day 4 pseudopregnant uteri, suggesting again that oviductal retention is due to maternal, but not embryonic defects. This speculation was further confirmed by reciprocal embryo transfer between *CB1*^{-/-} and wild-type female mice. Only *CB1*^{-/-} recipients displayed oviductal retention of embryos, irrespective of embryonic genotypes [69]. In addition, wild-type mice with pharmacologically inhibited CB1, but not CB2, also show high rate of embryo retention

in the oviduct. Notably, *FAAH*^{-/-} mice, which have higher oviductal anandamide levels, and wild-type mice exposed to THC or methanandamide (anandamide analog) also show oviduct embryo retention [71]. All these observations suggest that the regulation of oviduct-uterine transport is not simply an up-or-down regulation of endocannabinoid signaling. Instead, it suggests that a finely regulated endocannabinoid tone mediated by CB1 in the oviduct regulates normal embryo oviductal transport.

It is known that the transport of an embryo through the oviduct is aided by a wave of oviduct muscle movement, ultimately controlled by the sympathetic nervous system [83]. Stimulation of β 2 adrenergic receptors (β 2-AR) causes sphincter muscle relaxation, whereas stimulation of α 1 adrenergic receptor (α 1-AR) produces muscle contraction. It has been shown that reciprocal stimulation of these two receptors causes a wave of contractility and relaxation, which is conducive to the passage of embryos from the oviduct to the uterus [83,84]. It has also been found that exposure of wild-type oviducts to either an α 1-AR agonist or a β 2-AR antagonist causes embryos to be retained in the oviduct. In addition, CB1 expression in the muscularis (Fig. 3b) of the oviduct is colocalized with α 1 and β 2 adrenergic receptors, and oviductal nerve terminals in *CB1*^{-/-} mice have increased release of norepinephrine (NE) [69]. All of these observations lead to the speculation that CB1-mediated endocannabinoid signaling is functionally coupled to adrenergic signaling to regulate oviductal motility, and the oviductal muscularis is predominantly in a contraction phase in the absence of CB1. In contrast, high levels of endocannabinoid signaling, in either *FAAH*^{-/-} mice with naturally higher anandamide levels or wild-type mice exposed to excessive natural or synthetic cannabinoid ligands, cause the oviductal muscularis to shift to a relaxation phase, thus impairing oviductal embryo transport to the uterus (Fig. 3c).

In conclusion, the spatiotemporal expression of NAPE-PLD and FAAH in the oviduct creates an appropriate endocannabinoid tone, mediated by CB1 to ultimately regulate the release of NE. Then through the sympathetic nervous system, this signaling controls oviduct muscle action, consequently regulating oviduct-uterine embryo transport.

Endocannabinoid signaling and implantation

Attachment of the embryo to the luminal epithelium of the maternal uterus is a crucial step in mammalian reproduction. As the embryo travels into the uterus and differentiates into a blastocyst, the uterine cells undergo proliferation and differentiation to achieve a receptive state to accept the blastocyst for implantation. It is thought that blastocyst activation and uterine receptivity are two distinct events in the process of implantation [64], coordinated by estrogen and progesterone [85]. Implantation can only occur when the blastocyst becomes implantation competent and the uterus achieves the receptive phase. The first attachment reaction occurs between the trophectoderm of the blastocyst and the uterine luminal epithelium.

We have provided evidence that lower levels of AEA and CB1 are beneficial for implantation. AEA levels have been measured in both receptive and nonreceptive uteri, with the former having a lower level of AEA compared with the latter [74]. AEA levels are also critical in regulating preimplantation embryo development. In vitro experiments show that natural, synthetic or endogenous cannabinoids can inhibit preimplantation embryo development and blastocyst zona-hatching in culture, whereas blastocysts exposed to low levels of AEA show accelerated trophoblast differentiation and outgrowth [46,74,75]. In vivo experiments show that wild-type blastocysts collected from the uterus on the early morning of day 4 of pregnancy have higher levels of AEA binding, and this binding remarkably declines in blastocysts recovered on the evening of day 4 prior to implantation. These observations suggest that implantation competency requires downregulation of AEA binding to the blastocyst [76]. Immunostaining of CB1 confirms that CB1 is lower in activated blastocysts, but higher in

dormant blastocysts [44,76]. Collectively, these results show that coordinated down-regulation of blastocyst CB1 and uterine AEA levels are important for both blastocyst activation and uterine receptivity, two events critical for successful implantation (Fig. 4).

To further address the underlying mechanism(s) by which differential uterine AEA levels are created, the expression profiles of NAPE-PLD and FAAH were examined in the uterus. Higher levels of *Nape-pld* mRNA and NAPE-PLD activity is found in nonreceptive uteri and in interimplantation sites, whereas both mRNA and protein levels are lower in implantation sites and receptive uteri [70,72]. It is interesting that FAAH expression and activity show an inverse relationship. Higher FAAH expression and activity are observed at implantation sites and in the receptive uteri. Evidence points toward the possibility that the implanting blastocyst exerts an inhibitory effect on uterine *Nape-pld* expression, and upregulates uterine FAAH activity by releasing a lipid “FAAH activator” [70,86]. These observations suggest a potential role of the implanting embryo in regulating uterine AEA levels, perhaps to serve as a protective mechanism against exposure to detrimental levels of AEA. Regardless of its control, it is obvious that tight regulation of AEA plays an important role in implantation.

Other studies demonstrate that endocannabinoid signaling mediated by CB1 on the embryo is coupled with different downstream signaling pathways, depending on the concentration of AEA. For instance, it has been shown that AEA-induced stimulatory and inhibitory effects in blastocyst function are mediated by ERK and Ca^{2+} signaling pathways. For example, while AEA at a low concentration (7 nM) activates ERK signaling via CB1, higher AEA levels (28nM) fail to activate ERK, but instead inhibit Ca^{2+} mobilization [44]. These results, combined with evidence that women with elevated peripheral AEA levels have spontaneous pregnancy loss [87,88], demonstrate that endocannabinoid signaling is at least one of the pathways determining the fate of implantation and ultimately successful pregnancy.

Closing remarks

Under normal physiological conditions, endocannabinoid signaling through CB1 is crucial to various female reproductive events, including preimplantation embryo development, oviductal transport, and ultimately implantation in the uterus. Either silenced or enhanced endocannabinoid signaling derails these processes. These studies not only provide new molecular mechanisms governing periimplantation events, but also raise caution against the use of CB1 antagonists to treat obesity in humans.

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Figure 1. Preimplantation embryo development and implantation in mice

Following fertilization in the oviduct, the embryo undergoes several rounds of mitotic cell division, ultimately forming a ball of cells termed morula. At the late morula stage, the embryo enters the uterine lumen and transforms into a blastocyst that contains a cavity (blastocoel) with two distinct cell populations, the inner cell mass (ICM) and the trophectoderm (the progenitor of trophoblast cells). Before implantation, the blastocyst escapes from its outer shell (the zona pellucida) and differentiates to produce additional cell types — the epiblast and the primitive endoderm. At this stage, the trophectoderm attaches to the uterine lining to initiate the process of implantation. E, embryonic day. The figure is adapted from REF 60 (2006) Wang et al.

Figure 2. Cannabinoid signaling in preimplantation embryo development

Both exaggerated or absence of cannabinoid/endocannabinoid signaling mediated by CB1 leads to aberrant preimplantation embryo development.

Figure 3. a. A cartoon of oviductal transport of preimplantation embryos in mice

Ovulated eggs are fertilized in the ampulla of the oviduct. Fertilized eggs through successive cell divisions form morulae. Morulae pass through the utero-tubal junction to enter into the uterine lumen. b. A schematic diagram of a cross-section of the oviduct. Ep, epithelium. c. A proposed scheme of contraction-relaxation waves of oviductal muscularis at the utero-tubal region influenced by varying concentrations of AEA. In the absence of AEA, increased release of norepinephrine (NE) produces muscular contractions, resulting in narrowing of the lumen. At higher concentrations of AEA, endocannabinoid signaling through CB1 reduces the release of NE, thus relaxing the muscularis, widening the lumen. Thus, appropriate endocannabinoid signaling creates waves of contraction-relaxation, moving forward embryos from the oviduct into the uterine lumens. The panel a of this figure is adapted from "Manipulating the Mouse Embryo; A Laboratory Manual", page 497, 1994, Hogan, B et al.

Figure 4. Endocannabinoid signaling in blastocyst activation and implantation

Evidence suggests that regulated levels of AEA in the receptive uterus and CB1 in activated blastocysts are beneficial for implantation, whereas higher levels are detrimental to this process. This biphasic role of AEA is further supported by findings that AEA within a very narrow range regulates blastocyst activation and implantation by differentially modulating ERK signaling and Ca²⁺ channel activity via CB1. Ge, Glandular epithelium; IS, implantation site; INTER-IS, interimplantation site; Le, luminal epithelium; Myo, myometrium; S, stroma; Tr, trophoblast. This figure is adapted from REF 78, 2006, Wang et al.