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Jekyll and Hyde: Two Faces of Cannabinoid Signaling in Male and Female Fertility

Haibin Wang, Sudhansu K. Dey, and Mauro Maccarrone

Departments of Pediatrics, Cell and Developmental Biology, and Pharmacology (H.W., S.K.D.), Division of Reproductive and Developmental Biology, Vanderbilt University Medical Center, Nashville, Tennessee 37232; Department of Biomedical Sciences (M.M.), University of Teramo, Teramo 64100, Italy; and Mondino-Tor Vergata Neuropharmacology Center (M.M.), University of Rome Tor Vergata, 00133 Rome, Italy

Mammalian reproduction is a complicated process designed to diversify and strengthen the genetic complement of the offspring and to safeguard regulatory systems at various steps for propagating procreation. An emerging concept in mammalian reproduction is the role of endocannabinoids, a group of endogenously produced lipid mediators, that bind to and activate cannabinoid receptors. Although adverse effects of cannabinoids on fertility have been implicated for years, the mechanisms by which they exert these effects were not clearly understood. With the identification of cannabinoid receptors, endocannabinoid ligands, their key synthetic and hydrolytic pathways, and the generation of mouse models missing cannabinoid receptors, a wealth of information on the significance of cannabinoid/endocannabinoid signaling in spermatogenesis, fertilization, preimplantation embryo development, implantation, and postimplantation embryonic growth has been generated. This review focuses on various aspects of the endocannabinoid system in male and female fertility. It is hoped that a deeper insight would lead to potential clinical applications of the endocannabinoid signaling as a target for correcting infertility and improving reproductive health in humans. (*Endocrine Reviews* 27: 427–448, 2006)

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Abbreviations: AA, Arachidonic acid; AdR, adrenergic receptor; AEA, *N*-arachidonoylethanolamine (also known as anandamide); 2-AG, 2-arachidonoylglycerol; AMT, AEA membrane transporter; CB1, brain-type cannabinoid receptor; CB2, spleen-type cannabinoid receptor; COX, cyclooxygenase; $cPLA_2\alpha$, cytosolic $PLA_2\alpha$; DAG, diacylglycerol; DAGL, DAG lipase; E_2, 17β-estradiol; FAAH, fatty acid amide hydrolase; ICM, inner cell mass; INF-γ, interferon-γ; LIF, leukemia inhibitory factor; LPA, lysophosphatidic acid; MAGL, monoacylglycerol lipase; NAPE, *N*-acylphosphatidylethanolamine; NAT, *N*-acyltransferase; NK, natural killer; NO, nitric oxide; P_4, progesterone; PG, prostaglandin; PL, phospholipase; Th1, type 1 T-helper; Th2, type 2 T-helper; THC, Δ9-tetrahydrocannabinol; TRPV1, transient receptor potential vanilloid 1 (vanilloid receptor); ZP, zona pellucida.

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I. Lipid Signaling in Reproduction

SEXUAL PROCREATION IS initiated by interactions between a sperm and an egg leading to fertilization (1–4). The fertilized egg (embryo) undergoes several mitotic cell divisions, ultimately producing the blastocyst with two distinct cell types: the inner cell mass (ICM) and the trophectoderm (5–9). The nurturing of an offspring within the body and production of a live birth is an enduring task, requiring safeguard regulatory systems at various critical steps. Despite success in producing embryos and initiating embryonic development outside the womb by *in vitro* fertilization and embryo transfer, there is still a significant knowledge gap in understanding the mechanisms by which a successful pregnancy is achieved. A deeper insight into these processes will help to generate new ideas and concepts for improving fertility and pregnancy-associated health issues in humans. It is difficult to define the hierarchical landscape of the molecular pathways during human pregnancy, because of experimental difficulties and ethical restrictions on research with human embryos. It is hoped that experiments on mice and other animal models that bear certain reproductive similarities with humans combined with those feasible experiments in humans would generate meaningful information to address this critical issue. Over the past several years, molecular and genetic studies have provided evidence that lipid mediators serve as important signaling molecules in coordinating a series of events during early pregnancy.

Under pathophysiological conditions when a cell is activated in response to a stimulus, membrane phospholipids generate numerous lipid-signaling molecules, such as eicosanoids and lysophospholipids. Prostaglandins (PGs), one of the major group of eicosanoid lipid mediators, are generated from arachidonic acid (AA), which is released from membrane phospholipids by phospholipase (PL)A_2. AA thus released is transformed by cyclooxygenases (COXs) to PGH, which is then converted to various PGs by specific PG synthases (10). Distinct expression profiles of cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$), COX-1, and COX-2 in the ovary and uterus at different stages of pregnancy implicate their differential functions (11–14). In mice, COX-1-derived $PGF_{2\alpha}$, as a luteolytic hormone acting on the corpus luteum, is critical for the onset of parturition (15–18), whereas PGI_2 and PGE_2 generated by COX-2 are essential for ovulation, fertilization, implantation, and decidualization (11, 13, 19–22). The role of PG during pregnancy is further illustrated by poor fertility, resulting from deferred implantation, in mice lacking $cPLA_2\alpha$ (12). Collectively, these studies in mice establish the importance of lipid signaling through the $cPLA_2\alpha$-COX axis during early pregnancy (23, 24). Observations of COX-2 expression in the periovulatory ovary and uterus during implantation as well as delayed follicular rupture and increased incidence of miscarriages upon pharmacological inhibition of COX further suggest that $cPLA_2\alpha$-COX-derived PG signaling is also operative in human ovulation and pregnancy maintenance (25–29). Moreover, PGE_2 and $PGF_{2\alpha}$ are also thought to play important roles in human parturition by directly acting on the myometrium (30, 31).

Another example of the critical role of lipid signaling in reproduction is the influence of lysophosphatidic acid (LPA), a small lipid molecule belonging to the lysophospholipid group. LPA influences a range of processes through its cell-surface G protein-coupled receptors, LPA_{1-4} (32). A recent study in mice shows that LPA_3 is expressed in the uterine luminal epithelium, with peak expression occurring during the periimplantation period. Its expression overlaps with $cPLA_2\alpha$ and COX-2 at the site of blastocyst implantation (33). More importantly, mice missing LPA_3 exhibit remarkably similar defects as $cPLA_2\alpha$-deficient mice, such as deferred on-time implantation, retarded fetal development, embryo crowding, and sharing of one placenta by several embryos (12, 33). This study adds another genetic link between lipid signaling and female fertility. Restoration of on-time implantation in LPA_3-deficient females by PG supplementation further suggests a fundamental interaction between LPA-LPA_3 and $cPLA_2\alpha$-COX-2-PG signaling pathways (33). These findings establish a new concept that a short delay in the attachment of blastocysts to the uterine surface during early pregnancy adversely affects later developmental processes (24, 34, 35).

Increasing evidence points toward the pathophysiological significance of endocannabinoids, another group of bioactive lipid-signaling molecules, in both female and male fertility. These endogenous cannabinoid ligands mimic the action of natural cannabis compound Δ^9-tetrahydrocannabinol (THC) in many aspects of central and peripheral functions, including reproductive events (23, 36–51). THC is thought to account for the majority of the reproductive hazards in marijuana users (Table 1). For example, chronic marijuana use is associated with decreased plasma testosterone levels (52), reduced sperm counts, and impotency in men (52–56). In women, the chronic use of marijuana is often associated with fetal abnormalities and early pregnancy termination (57–66). In addition, early studies indicate that embryotoxicity and specific teratological malformation in animal models are correlated with exposure to natural cannabis extracts during pregnancy (67–73). With the characterization of cannabinoid receptors and their ligands endocannabinoids, the key synthetic and hydrolytic pathways of endocannabinoids, and the generation of gene mutation mouse models, a large body of molecular, genetic, and physiological evidence has been generated that supports key roles of cannabinoid ligand-receptor signaling in spermatogenesis, fertilization, preimplantation embryo development, implantation, and postimplantation embryonic growth. This review focuses on the roles of the endocannabinoid system in male and female fertility. A better understanding of this field will help in developing strategies for potential clinical applications of the endocannabinoid-targeted drugs as the next generation of therapeutics to treat human infertility.

II. The Endocannabinoid System

A. Introduction

Two main molecular targets of THC (Fig. 1), the psychoactive component of *Cannabis sativa*, are brain-type (CB1) and spleen-type (CB2) cannabinoid receptors (74–78). Over the last few years, a number of endogenous ligands for CB receptors have been identified; they are collectively called endocannabinoids. They are amides, esters, and ethers of long-chain polyunsaturated fatty acids, isolated from brain and peripheral tissues (79–82). Two arachidonate derivatives, *N*-arachidonoylethanolamine (AEA, know as anandamide) and 2-arachidonoylglycerol (2-AG) (Fig. 1), are the endocannabinoids whose biological activity has been best characterized to date (75, 81, 83–85). Also 2-AG-ether (noladin ether), an ether-type endocannabinoid (Fig. 1), is now included in the cohort of these lipid mediators (86), but its actual physiological relevance is still debatable (87). More recently, *O*-arachidonoylethanolamine (virodhamine), an "inverted AEA" (Fig. 1), has been identified and shown to behave as a partial agonist and a full agonist for CB1 and CB2, respectively (88). In addition, *N*-oleoylethanolamine, *N*-palmitoylethanolamine, and *N*-stearoylethanolamine are considered endocannabinoid-like molecules and may have an "entourage effect", *i.e.*, they may potentiate the activity of AEA or 2-AG by inhibiting their degradation (82, 89, 90).

B. Metabolic routes

1. AEA synthesis and degradation. It is now widely accepted that AEA is produced by a transacylase-phosphodiesterase-mediated synthesis, starting from the precursor *N*-arachidonoylphosphatidylethanolamine (NArPE). The latter compound originates from the transfer of AA from the *sn*-1 position of 1,2-*sn*-di-arachidonoyl- phosphatidylcholine to phosphatidylethanolamine, catalyzed by a calcium-depen-

TABLE 1. Comparative adverse impacts of marijuana usage on reproductive events

	Animal models	Human
Male fertility	Blocks hypothalamic GnRH release, decreasing serum levels of FSH (266) and LH (144, 266–274)	Decreases serum LH (52, 296, 297) and testosterone levels (52)
	Inhibits prolactin secretion (144, 275–277)	Induces gynecomastia (298)
	Decreases testicular testosterone production (266, 270, 272, 278–283)	Decreases spermatogenesis and mobility (oligospermia), induces sperm anomalies, and blocks acrosome reaction (52–56)
	Causes testis lesions (284–287), reduces weights of testes and accessory reproductive organs (279, 281, 288, 289) and even induces demasculinization (290)	
	Disrupts normal spermatogenesis (153, 154, 286–289, 291–293) and reduces fertilization (146, 147, 149, 150, 152, 171)	
	Reduces copulatory behavior (144, 294, 295)	
Female fertility	Blocks hypothalamic GnRH release, thus decreasing serum levels of FSH (299–301) and LH (299–308)	Suppresses or increases serum LH levels in a menstrual stage-specific manner (328, 329)
	Inhibits prolactin secretion (277, 300, 309, 310)	Inhibits prolactin secretion (330, 331)
	Causes impaired ovarian function with reduced progesterone secretion (280, 311, 312)	Increases serum testosterone level (331)
	Disrupts normal reproductive cycle and ovulation (300, 306, 311–315)	Disrupts menstrual cycle (331)
	Delays sexual maturation (316, 317)	Poor oocyte retrieval rate when undergoing IVF treatment (66)
	Facilitates sexual behavior (318–320)	Causes intrauterine fetal growth restriction (57, 58)
	Exerts embyrotoxicity and inhibits early embryo development (68–73)	Increases the incidence of preterm birth (59, 61, 63, 64) and prematurity with low fetal birth weight (59–66)
	Induces implantation failure (191)	Induces greater difficulty at delivery (332–334)
	Increases incidence of miscarriage, stillbirths and term pregnancy failure (312, 321–323)	
	Reduces fetal birth weight (323–325)	
	Delays the onset of parturition (326)	
	Inhibits milk ejections during lactation (327)	

IVF, *In vitro* fertilization.

dent *N*-acyltransferase [*trans*-acylase (NAT)] (81, 91). NArPE is then cleaved by a recently characterized *N*-acylphosphatidylethanolamine (NAPE)-specific phospholipase D (NAPE-PLD), which belongs to the zinc metallo-hydrolase family of the β-lactamase fold (92) and releases AEA and phosphatidic acid. The biological activity of AEA at CB receptors is terminated by its removal from the extracellular space, which occurs through a two-step process: cellular uptake by a high-affinity transporter, followed by intracellular degradation by a fatty acid amide hydrolase (FAAH), *N*-arachidonoylethanolamine amidohydrolase (EC 3.5.1.4) (93, 94). Several properties of a selective AEA membrane transporter (AMT) have been characterized, although its molecular structure remains unknown (95–98). In fact, there is controversy regarding the existence of endocannabinoid transporters, and the mechanism by which AEA is taken up by cells is currently being debated (99, 100). The uptake of AEA has the features of a facilitated transport; it is dependent on the concentration, time, and temperature, and independent of external Na^+ ions or ATP hydrolysis. However, the molecular and genetic identity of AMT still remains unknown (100, 101). In particular, the relationship between AMT and FAAH is still under debate, because FAAH may not need a transporter to get in contact with AEA (102), and AMT perhaps exports, rather than imports, AEA across the plasma membrane (103). Increasing pharmacological, biochemical, and morphological evidence seems to favor for the existence of an AMT different from FAAH (96, 97). The development of new drugs able to inhibit AMT selectively without affecting FAAH corroborates this speculation (104). However, it has recently been suggested that AEA uptake is driven by nonprotein-mediated diffusion and is regulated by its degree of hydrolysis by FAAH in specific cell types (105). The target of some of the novel transport inhibitors recently developed seems not to be the membrane transporter, but rather FAAH or an uncharacterized intracellular component that delivers AEA to FAAH (105). Once taken up by cells, AEA is a substrate for FAAH that breaks the amide bond and releases AA and ethanolamine (106). Mammalian FAAH is a membrane-bound enzyme with a globular shape. It has 28 α-helices and 11β-sheets, which account for approximately 53 and 13% of the whole protein structure, respectively (102). This enzyme uses an unusual serine-serine-lysine (S241-S217-K142) catalytic triad (106).

Together with AEA and congeners, CB and non-CB receptors, NAT, NAPE-PLD, AMT, and FAAH, along with the enzymes that metabolize 2-AG (see *Section II.B.2*), constitute the "endocannabinoid system" (80, 82, 107–111). This system is schematically depicted in Fig. 2.

2. 2-AG synthesis and degradation. 2-AG acts as a potent and full agonist for both CB1 and CB2 and, like AEA, is not stored in intracellular compartments but is produced on demand. The biosynthetic pathway of 2-AG provides for rapid hy-

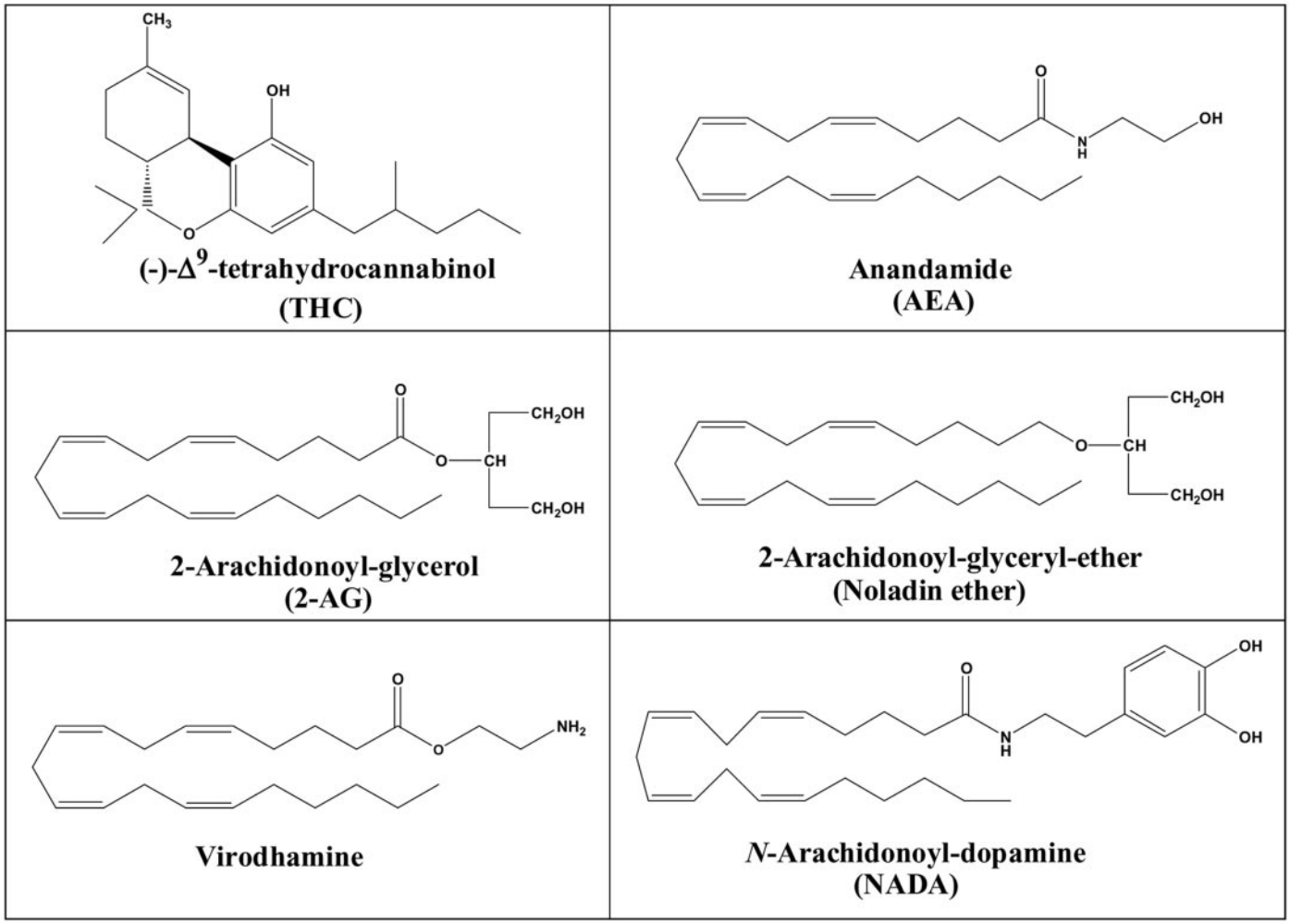

FIG. 1. Chemical structures of natural and endogenous cannabinoids.

drolysis of inositol phospholipids by a specific PL, PLC; this enzyme generates diacylglycerol (DAG), which is converted to 2-AG by a *sn*-1-DAG lipase (85). Recently, two *sn*-1-specific DAG lipases (DAGL) responsible for 2-AG synthesis have been cloned by confronting human genome with *Penicillium* DAGL sequence (112). These two isoforms (α and β) have molecular masses of 120 and 70 kDa, respectively, with four transmembrane domains and are members of the serine-lipase family with serine and aspartic acid (S443-D495) participating in the catalytic triad. The α isoform is predominant in the adult brain, whereas the β isoform is expressed in developing brain (112).

The pharmacological effects of 2-AG depend on its life span in the extracellular space, which in turn is limited by a rapid transport through the membrane. It is proposed that the 2-AG membrane transporter is the same as AMT (113). In fact, 2-AG accumulation is reduced by an AMT inhibitor, AM404, and indirectly by high concentrations of AA (113). The effect of AM404 is due to the inhibition of AMT, but not due to FAAH activity, because the level of 2-AG remains unaltered in the presence of two strong FAAH inhibitors, URB597 and AM374 (114).

Once accumulated in the cell, 2-AG can be degraded by FAAH (115), but FAAH is not the only enzyme responsible for its metabolism. In fact, mice lacking FAAH are unable to metabolize AEA but can still hydrolyze 2-AG (116). An enzyme responsible for 2-AG degradation, monoacylglycerol lipase (MAGL), has been isolated from the porcine brain (115) and cloned and characterized in rat (117) and human brain (118). Rat brain MAGL is a 33-kDa protein and, unlike FAAH, is localized in the cytosol (117). MAGL and DAGL are members of the endocannabinoid system as shown in Fig. 2.

C. Molecular targets and signaling pathways

1. Cannabinoid receptors. AEA and 2-AG differentially activate cannabinoid receptors. CB1 are most abundant in the central nervous system but are present in peripheral tissues including the heart, uterus, embryo, testis, liver, small intestine, and peripheral cells like lymphocytes. CB2 are predominantly expressed in astrocytes, spleen, and immune cells (23, 74–76, 111, 119). Both CB1 and CB2 belong to the rhodopsin family of G protein-coupled seven *trans*-membrane spanning receptors. They show 44% overall identity, with 68% identity within the transmembrane regions, and are coupled mainly to the $G_{i/o}$ family of G proteins (74). Signal transduction

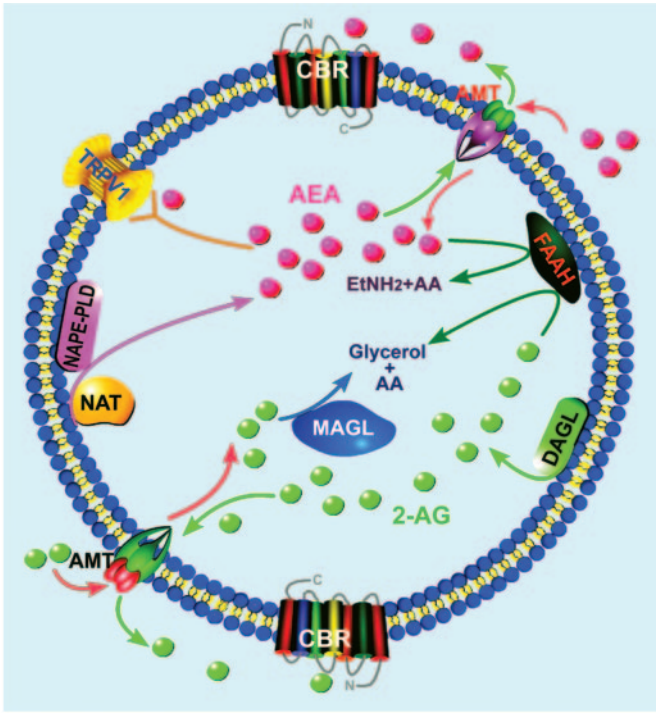

FIG. 2. The endocannabinoid system. The synthesis of AEA from membrane *N*-arachidonoylphosphatidylethanolamines is catalyzed by the sequential activity of NAT and NAPE-PLD, which releases AEA and phosphatidic acid. AEA is transported in both directions through the cell membrane by a selective AMT and, once taken up, is hydrolyzed by FAAH to ethanolamine ($EtNH_2$) and AA. The main targets of AEA are CB1 and CB2 receptors (CBR), showing an extracellular binding site, and type-1 vanilloid receptors (TRPV1), showing an intracellular binding site. 2-AG is also released from membrane lipids through the activity of DAGL. 2-AG can also be hydrolyzed by FAAH or, more importantly, by MAGL, releasing glycerol and AA. The transport of 2-AG across the cell membrane may be mediated by AMT or a related transporter, and CBR (but not TRPV1) is the target of this endocannabinoid.

pathways regulated by CB-coupled $G_{i/o}$ proteins include the inhibition of adenylyl cyclase (69, 77), regulation of ionic currents (inhibition of voltage-gated L, N, and P/Q-type Ca^{2+} channels as well as activation of K^+ channels) (120–125), the activation of focal adhesion kinase (126) and MAPK (125, 127, 128). In addition, activation of CB1 or inhibition of CB2 alters nitric oxide (NO) synthase (74, 119). However, a recent report shows that WIN55,212–2, an aminoalkylindole cannabinoid agonist, increases intracellular Ca^{++} via CB1 coupling to Gq/11 G proteins (129), suggesting diversity of CB1 signaling pathways. Furthermore, there is some evidence that endocannabinoids induce a biological activity via other CB receptors, like a purported CB3 (GPR55) receptor (130–132). GPR55 is an orphan G protein-coupled receptor that has low sequence homology (10–15%), compared to that of CB1 or CB2, and is expressed in the testis at approximately a 15-fold higher level than in the brain (130, 131). GPR55 does not appear to couple with G_i or G_s proteins, suggesting that it activates different signal transduction pathways from those executed by CB1 and CB2 (130, 131). Among the non-CB receptors, the type-1 vanilloid receptor [now called transient receptor potential vanilloid 1 (TRPV1)] has attracted great interest as a new molecular target of AEA.

2. Vanilloid receptors. TRPV1 is a six-*trans*-membrane spanning protein with intracellular N and C terminals and a pore-loop between the fifth and sixth transmembrane helices (133). TRPV1 is a ligand-gated and nonselective cationic channel that is activated by molecules derived from plants, such as capsaicin (the pungent component of "hot" red peppers) and resinferatoxin, and also by stimuli like heat and protons (134). In the last few years, a number of studies have suggested a physiological role for AEA as a TRPV1 agonist, leading to the concept that AEA, besides being an endocannabinoid, is also a true "endovanilloid" (135, 136). In contrast, 2-AG is unable to bind to and activate TRPV1 (134, 136). The interaction of AEA with TRPV1 occurs at a cytosolic binding site (135, 137), triggering activation of nonselective ion channels, activation of protein kinases, increased intracellular Ca^{++} concentration, mitochondrial uncoupling, and release of cytochrome c (138, 139). TRPV1 is expressed in peripheral sensory fibers (134) and in several nuclei of the central nervous system (140), suggesting the existence of central endogenous agonists for its activation. In this context, *N*-arachidonoyldopamine, an endogenous capsaicin-like substance (Fig. 1), has been identified; it activates TRPV1 with high potency and is also a potent cannabimimetic compound (141). Collectively, there seems to be an overlap between the endogenous cannabinoid system and the vanilloid system.

Activation of the different molecular targets by AEA or 2-AG leads to several biological activities, some of which are summarized in Table 2. It appears that virtually all central and peripheral systems in mammals are affected by endocannabinoids. Among the peripheral activities of AEA, the regulation of reproduction is an emerging interest (23, 36–49). The effects of AEA are under metabolic control, so that within a very narrow concentration range AEA regulates blastocyst function and implantation (125, 142, 143). A more detailed account of this regulation is described in the following sections.

III. Endocannabinoids and Male Fertility

A. Introduction

Although there is evidence that chronic administration of THC to animals induces male impotency (144) and reduces testosterone secretion, sperm production, motility, and viability as well as acrosome reaction and fertilization (51, 145–158), a role for the endocannabinoid system in male fertility is still largely unexplored. Recent reports show that *N*-acylethanolamines are present in human reproductive fluids at low nanomolar ranges (49), and that AEA influences human sperm functions (48, 146). For example, *in vitro* studies demonstrate that the AEA congener *N*-palmitoylethanolamine affects the time-course of capacitation of human spermatozoa by modulating their membrane properties (159, 160). Furthermore, the rat testis is able to synthesize AEA (161), and human seminal plasma contains AEA (49). The presence of CB1 in Leydig cells (162) and its association with testosterone secretion have also been observed in mice (163). There is evidence that THC alters Sertoli cell function (164), although the underlying molecular mechanism is not known.

TABLE 2. Effects of endocannabinoids and congeners in the central nervous and peripheral systems

Central nervous system	Peripheral systems
Thalamus, hypothalamus, hippocampus	Cardiovascular system
Control of pain initiation	Profound decrease in blood pressure (hypotension) and heart rate (bradycardia)
Control of wake/sleep cycles	Induction of hypotension during hemorrhagic shock or endotoxic shock
Control of thermogenesis	Vasodilation
Control of food intake	Platelet aggregation
Impairment of working memory	Immune system
Impairment of memory consolidation	Alteration of synthesis and secretion of ILs
Inhibition of long-term potentiation	Down-regulation of rat mast cell activation
Inhibition of glutamatergic transmission	Stimulation of hematopoietic cell growth
Basal ganglia, striatum, globus pallidus	Inhibition of leukemia inhibitory factor (LIF) release
Control of psychomotor disorders	Inhibition of neutrophil recruitment
Interference with dopaminergic transmission	Digestive tract
Inhibition of γ-aminobutyric acid (GABA)ergic transmission	Inhibition of peristalsis
Potentiation of γ-aminobutyric acid (GABA)-mediated catalepsy	Inhibition of intestinal motility
Cortex, cerebellum, spinal cord	Liver
Blockade of *N*-methyl-D-aspartate (NMDA) receptors	Control of lipogenesis and peripheral energy balance
Control of tremor and spasticity	
Retina	
Control of scotopic vision	

Because Sertoli cells are involved in the regulation of germ cell development by providing nutrients and hormonal signals needed for spermatogenesis, their ability to bind and degrade AEA seems important in controlling the spermatogenic output. Because AEA can serve as a proapoptotic factor (138), it may also be involved in the survival and death of Sertoli cells. In the same context, the possible interplay of AEA with FSH could be of interest, because FSH dramatically impacts fetal and early neonatal Sertoli cell proliferation and is critical for regulating spermatogenesis in adult males (165). These aspects of Sertoli cell biology with relation to endocannabinoid signaling are further elaborated below.

B. The endocannabinoid system in Sertoli cells

In mice, Sertoli cells have the biochemical machinery to bind and degrade AEA at various developmental stages (4 to 24 d) (166). For example, immature Sertoli cells express functional CB2 on their surface, but the receptor level does not fluctuate much during aging (166). Instead, FAAH activity declines age-dependently due to reduced transcription, and so does the uptake of AEA through AMT. A typical AMT was found to uptake AEA in Sertoli cells and, like AMT of other human peripheral cells, this uptake was significantly increased by NO donors (166). This effect might be relevant *in vivo*, because NO plays roles in regulating male fertility (167, 168). In particular, NO regulates the contribution of Sertoli cells to fertility and to inflammation-mediated infertility (169). A faster removal of AEA from the extracellular space might be the rationale for some effects of NO.

An interesting observation is that AEA can force Sertoli cells to undergo apoptosis and that this process is more evident upon aging (166). The proapoptotic effect of AEA is not mediated by CB1, CB2, or TRPV1. Instead, CB2 expressed by Sertoli cells have a protective role against the harmful effects of AEA, and so does FSH. In fact, FSH dose-dependently inhibits apoptosis, and this event is correlated with increased FAAH activity (166). The finding that the endocannabinoid system is operative in Sertoli cells opens up a new perspective to the understanding and treatment of male infertility. In particular, the observation that FAAH modulates the biological effects of AEA on Sertoli cells and that this FAAH-mediated control is under hormonal regulation constitutes a concept that AEA hydrolysis is an important checkpoint in human fertility.

C. The endocannabinoid system in sperm

AEA signaling is implicated in regulating sperm functions required for fertilization in invertebrates and mammals, including humans (48, 145–152). It has been shown in sea urchin (*Strongylocentrotus purpuratus*) that sperm synthesizes AEA (170) and AEA binds to CB receptors and reduces fertilizing capacity of sperm (48, 146–152, 171). Unlike lower animals, ejaculated sperm from mammals must undergo functional maturation for fertilizing an egg. Sperm acquire fertilization competence because they reside in the female genital tract where, after a series of physiological changes, they become "capacitated", *i.e.*, able to fertilize an egg (1–3). Capacitation consists of changes occurring at two sites: 1) on the sperm head that enables it to bind to the zona pellucida (ZP) and induces acrosome reaction; and 2) in the flagellum, where hyperactivated sperm motility is facilitated. The control of this crucial process involves modifications of intracellular ions (172, 173), plasma membrane fluidity (174), metabolism, and motility (175). However, the sequence of these changes and local regulatory mechanisms that allow capacitation to progress as sperm become closer to an oocyte still remains poorly understood. Recently, AEA has been shown to reduce human sperm motility by reducing mitochondrial activity (176). In addition, AEA inhibits capacitation-induced acrosome reaction, and its effects are prevented by the CB1 antagonist SR141716 (176). These data led to the suggestion that the activity of AEA on sperm function requires CB1 activation. We investigated whether sperm cells of boar (*Sus scropha*) are able to bind and metabolize AEA and whether

this endocannabinoid modulates their function. Boar sperm biology in some aspects resembles that of humans, and boar spermatozoa are available in a greater amount than human spermatozoa. Thus, boar sperm were used as a model to fully characterize the endocannabinoid system (177–179).

Boar spermatozoa have the biochemical machinery to bind (CB1 and TRPV1), synthesize (NAPE-PLD), and degrade (AMT and FAAH) AEA (180). It was also shown that activation of CB1 by an AEA-stable analog, methanandamide, inhibits capacitation and hence the ability of sperm cells to react to ZP proteins with acrosome exocytosis through a cAMP-dependent pathway, although CB1 was ineffective on spontaneous acrosome reaction (180). It was also noted that once the capacitation is completed, AEA stabilizes the acrosome membranes by activating TRPV1, thus reducing spontaneous acrosome reaction. These results are schematically depicted in Fig. 3.

Taken together, sperm function seems to be regulated by endocannabinoids that exert a dual stage-dependent effect. On one hand, AEA, present in both seminal plasma and uterine fluids, may prevent premature capacitation in freshly ejaculated sperm via a CB1-mediated mechanism for traveling along the uterine tract without any fertilizing potential. Conversely, a few hours later when sperm have reached the oviduct (a condition that corresponds to *in vitro* capacitation), this inhibitory brake becomes less stringent. It is speculated that spermatozoa are exposed to a progressively reduced concentration of AEA in the proximal female genital tract (49), and sperm capacitation occurs as a consequence of release from CB1 inhibition. The observation that the endocannabinoid system is operative in sperm adds a new dimension to the intricate endocannabinoid network regulating mammalian fertility. Overall, these findings present new perspectives to the understanding and treatment of male fertility problems.

IV. Endocannabinoids and Female Fertility: Embryo Implantation

A. Introduction

The onset of a new life begins with fertilization of a mature oocyte with capacitated sperm (1–4). The one-cell fertilized zygote, now termed embryo, undergoes several mitotic cell divisions, eventually forming the blastocyst with two distinct cell populations, the ICM and a layer of trophectoderm cells surrounding the ICM (7–9). The embryo proper is derived exclusively from the ICM, whereas the placenta and extraembryonic membranes are generated from cells contributed by the trophectoderm (181, 182). A two-way interaction between the blastocyst and the maternal uterine luminal epithelium initiates the process of implantation. A considerable amount of early pregnancy loss occurs due to either preimplantation embryonic death or implantation failure resulting from asynchronous embryonic development and/or failure of the uterus to differentiate to the receptive stage (183, 184). Understanding the mechanism of preimplantation embryonic development and implantation in the uterus is a fundamen-

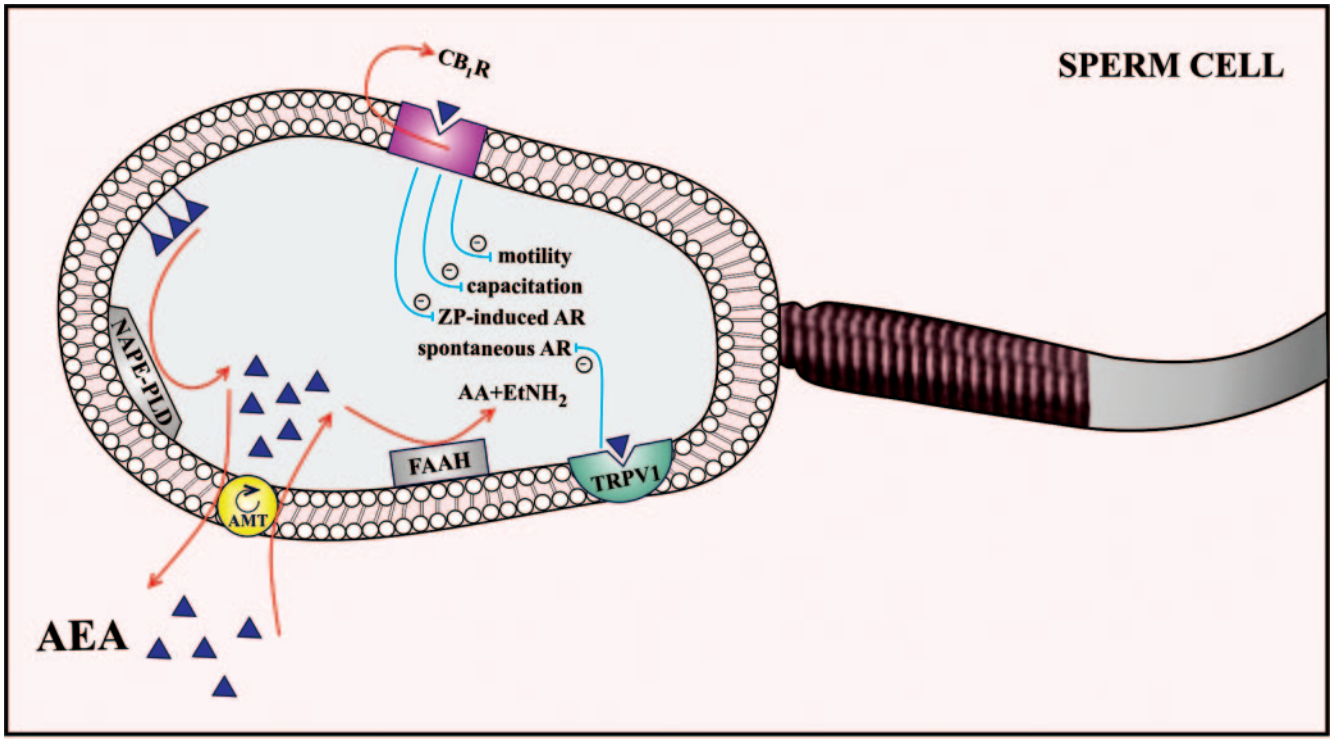

FIG. 3. The endocannabinoid system in sperm function. Binding of AEA to the extracellular site of CB1 (CB1R) leads to inhibition of sperm motility, capacitation, and ZP-induced acrosomal reaction (AR), without affecting spontaneous AR. In contrast, binding of a lipoxygenase-catalyzed oxygenation of arachidonylethanolamide to the intracellular site of vanilloid receptors (TRPV1) inhibits spontaneous AR. Sperm also possess the AMT, the AEA-synthesizing phospholipase D (NAPE-PLD), and the AEA-hydrolyzing FAAH. FAAH cleaves AEA into ethanolamine ($EtNH_2$) and AA.

tal challenge to reproductive biologists with the goal of alleviating the problems of human infertility and ensuring the birth of quality offspring. Such knowledge will also help in developing novel contraceptive approaches to restrict rapidly growing world population.

Development of the preimplantation embryo to the blastocyst stage and differentiation of the uterus to the receptive stage are basic requirements for the initiation of implantation in all species (5, 6, 8). Although the precise sequence and details of the molecular interactions involved in these processes have not yet been defined, increasing evidence from gene expression and transgenic mouse studies during the last two decades shows that coordinated integration of a range of signaling pathways in paracrine, autocrine, and/or juxtacrine manners participates in embryo-uterine dialogue during implantation (5, 6, 8, 185, 186). Among these signaling molecules, endocannabinoid signaling has recently been highlighted as an important player in directing preimplantation embryo development and the timely homing of embryos into a receptive uterus for implantation.

B. Endocannabinoids and preimplantation embryo development

THC, the major psychoactive component in marijuana, has been shown to exert a wide array of adverse effects on human health, including reproduction (23, 36–49). With the identification of CB1 and CB2 (77, 78) and two major endocannabinoids, AEA and 2-AG (83–85), as ligands for these receptors in the early 1990s, we and others using mice as an animal model have been pursuing experiments to explore the pathophysiological significance of cannabinoid/endocannabinoid ligand-receptor signaling during early pregnancy.

In mice, only CB1 is expressed in the oviduct and uterus, whereas both CB1 and CB2 are expressed in preimplantation embryos (69, 187–189). CB1 mRNA is primarily detected from the four-cell stage through the blastocyst stage, whereas CB2 is present from the one-cell through the blastocyst stage (69). Autoradiographic binding sites of [^{3}H]AEA are also evident from the one-cell through blastocyst stages. Interestingly, the majority of AEA binding sites are noted in outer cells of embryos at the eight-cell, morula, and blastocyst stages. Scatchard analysis of binding kinetics in d 4 blastocysts (d 1 = vaginal plug) showed that AEA binds to a single class of high-affinity receptors. The presence of CB1 mRNA and protein as detected by immunocytochemistry in preimplantation embryos correlates well with AEA binding sites (188, 190). Furthermore, blastocyst CB1 is biologically active, because both THC and AEA inhibit forskolin-stimulated cAMP formation, and this inhibition is prevented by pertussis toxin pretreatment (69, 187). The presence of biologically active CB1 in the blastocyst suggested that the mouse embryo is a potential target for both endocannabinoids and natural cannabinoids. In fact, synthetic (CP 55940, WIN 55212–2), natural (THC), or endogenous (AEA and 2-AG) cannabinoids arrest the development of two-cell embryos into blastocysts in culture (69, 191). A reduction in trophectodermal cell numbers is noted in those blastocysts that escape the developmental arrest in the presence of cannabinoid agonists (190). Furthermore, these adverse effects are reversed by simultaneous addition of selective antagonists to CB1 [SR 141716 (192)] with cannabinoid agonists (191), but not by a selective CB2 antagonist [SR 144528 (193)]. Recent observations of CB2 expression in early embryos and embryonic stem cells by microarray analysis (194), and the absence of its expression in trophoblast stem cells derived from preimplantation blastocysts (H. Wang and S. K. Dey, unpublished data) suggest that CB2 expression is restricted to blastocyst ICM cells. Collectively, these results suggest that cannabinoids mediate their actions on preimplantation embryos via CB1. The role of CB2 in the early embryo is yet to be defined.

With the availability of cannabinoid receptor knockout mice in the late 1990s (195, 196), the physiological relevance of cannabinoid receptor signaling during early embryo development was further examined. It was observed that *CB1*$-/-$, *CB2*$-/-$, or *CB1*$-/-\times$*CB2*$-/-$ double mutant embryos recovered from the oviduct on d 3 and from the uterus on d 4 of pregnancy show asynchronous development compared with wild-type embryos (188, 189). This impaired *in vivo* embryo development is rescued by mating *CB* mutant females with wild-type males, producing all heterozygous embryos in a mutant maternal environment. This indicates that embryonic CB receptors, but not maternal factors, direct early synchronous embryonic development (189). These studies also provide genetic evidence that CB1 and CB2 are critical for preimplantation embryo development, although asynchronous development of *CB2*$-/-$ embryos still remains puzzling. Because CB2 is expressed in the embryonic stem cells, but not in trophectoderm-derived trophoblast stem cells, it is conceivable that CB2 plays a role in specifying pluripotent ICM cell lineage during blastocyst formation. To ascertain whether embryos deficient in cannabinoid receptors respond to endocannabinoids *in vitro*, two-cell wild-type or mutant embryos were cultured in the presence or absence of AEA. Although a comparable development of wild-type and mutant embryos was observed in the absence of AEA in culture, *CB1*$-/-$ and *CB1*$-/-\times$*CB2*$-/-$ mutant embryos, but not *CB2*$-/-$ or wild-type embryos, were resistant to the inhibitory action of AEA (188). This observation reinforces the tenet that CB1 is the functional receptor for ensuring normal embryo growth and differentiation to blastocysts. Collectively, these studies provide pharmacological, molecular, and genetic evidence that the preimplantation embryo is indeed a target for cannabinoid ligand-receptor signaling.

C. Endocannabinoids and oviductal embryo transport

During early pregnancy, another critical event occurring in parallel with preimplantation embryonic development is the timely transport of 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 achieve implantation competency, escape from the ZP, and implant into the receptive uterus. Thus, normal oviductal embryo transport is one of the prerequisites for on-time implantation in the uterus, whereas a dysfunctional regulation of this process resulting from oviductal embryo retention may increase the incidence of pregnancy failure or cause tubal pregnancy in humans.

During the course of our study over the past several years exploring the potential physiological roles of endocannabinoid signaling during early pregnancy, we have consistently observed that approximately 40% of *CB1−/−* mice show pregnancy loss (188, 189). Because these mutant mice have normal ovulation and fertilization when compared with wild-type mice (189), we initially thought that asynchronous embryo development could be the major cause of this pregnancy loss. We speculated that normal pregnancy in *CB1−/−* mice would be restored by mating mutant females with wild-type males to generate all heterozygous embryos with normal preimplantation growth. However, we still observed that about 40% of *CB1−/−* mothers did not yield any embryos in the uterus when examined in the midmorning on d 4 of pregnancy, suggesting that a maternal, but not embryonic, loss of CB1 is the determining cause for pregnancy failure.

To determine the underlying cause for this pregnancy failure, we examined oviductal embryo transport in *CB1−/−*, *CB2−/−*, and *CB1−/−×CB2−/−* double mutant females. No embryos were found to be trapped in the oviducts in wild-type or *CB2−/−* mice, which was expected because only CB1 is expressed in the mouse oviduct and uterus. However, a substantial number of *CB1−/−* and *CB1−/−×CB2−/−* mice showed impaired oviductal transport with retention of embryos at the morula and blastocyst stages within the oviduct on d 4. These trapped embryos appeared morphologically normal and implanted upon transfer into d 4 pseudopregnant receptive uteri, suggesting that they remained implantation-competent. These results indicate that maternal expression of CB1 in the reproductive tracts plays a fundamental role in ensuring normal oviduct to uterine transport of embryos, and its deficiency results in embryo retention in the oviduct for an extended period, causing reduced fertility in *CB1−/−* mice. This observation was confirmed in our reciprocal embryo transfer experiments between *CB* mutant and wild-type mice. Indeed, only *CB1−/−* recipients show oviductal embryo retention and implantation failure, irrespective of the genotypes of donor embryos (189). We further observed that wild-type pregnant mice treated with a CB1-selective antagonist (SR141716) exhibit impaired embryo transit through the oviduct, but this defect did not occur in mice treated with vehicle or a CB2-selective antagonist (SR144528). Interestingly, wild-type females exposed to a stable AEA analog (methanandamide) or natural THC showed pregnancy loss with embryos retained in the oviduct (189). These observations demonstrate that aberrant cannabinoid signaling, either silenced or enhanced, impairs embryo transport. This suggests that there is an endocannabinoid tone mediated via CB1 in the oviduct that regulates normal embryo transport into the uterus for implantation. Collectively, these observations provide evidence that whereas embryonic CB1 primarily contributes to normal embryo development, oviductal CB1 directs timely oviductal transport of embryos. Although there is no evidence of ectopic pregnancy in animals other than humans, these findings may have clinical importance because embryo retention in the fallopian tube as a result of dysfunctional muscular contraction is one cause of ectopic pregnancy in women (197, 198). The ability of trapped blastocysts within *CB1−/−* oviducts to implant after transfer into wild-type pseudopregnant mouse uteri suggests that similar embryo retention in the fallopian tube would result in ectopic pregnancy in women.

Previous studies have established that the journey of the embryo from the oviductal isthmus into the uterus in rodents is aided by a wave of regulated contraction and relaxation of the oviduct muscularis. It is thought that the sympathetic neuronal circuitry, under the direction of ovarian hormones, coordinates the "closing and opening" of the sphincter at the isthmus-uterine junction, thereby regulating the timely passage of embryos from the oviduct into the uterus (199, 200). During pregnancy, rising progesterone (P_4) levels from the newly formed corpus luteum decrease the turnover rates and thus the levels of noradrenaline, a ligand with higher affinity for the α-adrenergic receptor (AdR) than the β-AdR at the adrenergic nerve endings (201). In contrast, the sensitivity of the β-AdR is increased in the circular muscle of the oviduct isthmus under P_4 dominance, causing muscle relaxation and facilitating embryo transport through the oviduct (199). Observations of embryo retention within the oviduct in wild-type females after exposure to an α1-AdR agonist phenylephrine and/or a β2-AdR antagonist butoxamine (189) led us to speculate that CB1-mediated endocannabinoid signaling is functionally coupled to adrenergic signaling to regulate oviductal motility conducive to embryo transport. Indeed, CB1 expression is colocalized with that of α1- and β2-AdRs in mouse oviductal muscularis at the isthmus region, and the β-AdR agonist isoproterenol restores normal embryo transport in *CB1−/−* mice (189). Experiments with *in vitro* [^{3}H]noradrenaline release provided further evidence that genetic or pharmacological loss of oviductal CB1 increases noradrenaline release from the adrenergic nerve terminals, maintaining a smooth muscle contractile tone through α-AdR, and thereby impeding oviductal embryo transport. In contrast, exposure to excessive natural or synthetic cannabinoid ligands leads to predominant relaxation phase of the oviductal muscularis due to attenuated noradrenaline release, impairing embryo transport (189). Collectively, these findings reinforce the concept that aberrant cannabinoid signaling, arising from either silencing or amplification, impedes the highly coordinated oviductal smooth muscle contraction and relaxation critical to embryo transport during early pregnancy. The potential mechanism creating this endocannabinoid tone in the oviduct during early pregnancy remains to be explored. A differential regional expression of NAPE-PLD and FAAH with higher expression of NAPE-PLD in the isthmus and FAAH in the ampulla may contribute to generate an appropriate level of AEA conducive to preimplantation embryo growth and transportation (H. Wang and S. K. Dey, unpublished data).

D. Biphasic endocannabinoid sensor in blastocyst implantation

Upon a successful journey through the oviduct, the embryo at the late morula or early blastocyst stage encounters a new microenvironment in the uterus for its attainment of implantation competency. It is thought that blastocyst activation and uterine receptivity are two distinct events in the

process of implantation (202). In mice, ovarian P_4 and 17β-estradiol (E_2) are the primary factors that coordinate these two events (5). Under P_4 priming, the closure of the uterine lumen occurs and coincides with the escape of the blastocyst from the ZP, bringing the blastocyst trophectoderm in close contact with the uterine luminal epithelium (blastocyst apposition). Superimposition of the P_4-primed uterus with preimplantation ovarian E_2 secretion and its catechol metabolite, 4-hydroxy-17β-estradiol (4-OH-E_2), produced from primary E_2 in the uterus, differentially regulate uterine preparation and blastocyst activation, respectively. For example, the primary ovarian E_2, via its interaction with nuclear E_2 receptors, participates in the preparation of the P_4-primed uterus to the receptive state in an endocrine manner, whereas its metabolite 4-OH-E_2, mediates blastocyst activation for implantation in a paracrine manner (203). These coordinated actions of P_4 and E_2 set up the window of implantation. The first event in the process of implantation is the attachment of the blastocyst trophectoderm with the uterine luminal epithelium that occurs within a narrow time frame when an intimate two-way dialogue occurs between the implantation-competent blastocyst and the receptive uterus. In mice, this attachment reaction is initiated around 2400 h of d 4 of pregnancy (204). However, elimination of preimplantation E_2 secretion by ovariectomy on the morning of d 4 results in implantation failure with blastocyst dormancy within the quiescent uterine lumen (205, 206). This condition is referred to as delayed implantation and can be maintained for many days by continued P_4 treatment. However, implantation with blastocyst activation is rapidly initiated by a single injection of E_2 in the P_4-primed uterus (205, 206). This physiologically relevant delayed implantation model has been widely used to identify signaling pathways mediating embryo-uterine cross-talk during blastocyst implantation. Using normal pregnancy and delayed implantation model, we have elucidated a unique association of endocannabinoid-CB1 signaling in embryo-uterine interactions during implantation.

As stated earlier, natural, synthetic, or endogenous cannabinoids can dramatically inhibit preimplantation embryo development and blastocyst zona-hatching in culture (69, 191, 207). This observation correlates well with higher levels of AEA in the nonreceptive uterus (142, 188, 207). On the other hand, lower levels of AEA in the receptive uterus and at the implantation site suggest that regulated AEA levels are conducive to normal embryo development and implantation. There is evidence that cannabinoid effects are differentially executed, depending on the embryonic stage and cannabinoid levels in the uterine environment. Blastocysts exposed in culture to low levels of AEA exhibit accelerated trophoblast differentiation and outgrowth, whereas inhibition of trophoblast differentiation is observed at higher doses of AEA (143, 208), suggesting dual functions of AEA depending on its local concentration (143, 207). Thus, uterine AEA levels are critical in regulating the "window" of implantation by synchronizing trophoblast differentiation and uterine preparation to the receptive state.

To gain further insight into the underlying causes of these biphasic effects of AEA, the status of anandamine binding in preattachment and attachment-competent blastocysts immediately before implantation on d 4 of pregnancy was studied. Similar expression studies were also performed using dormant and estrogen-activated blastocysts. The results showed that normal blastocysts collected in the early morning of d 4 have higher levels of AEA binding, but this binding remarkably declines in blastocysts recovered on d 4 in late afternoon before the attachment reaction (188). These observations suggest that down-regulation of AEA binding to the blastocyst is important for achieving implantation competence. Similarly, dormant blastocysts also show increased levels of AEA binding sites, but this binding significantly decreases by 12 h after termination of dormancy by an E_2 injection (188). The immunoreactive CB1 protein parallels AEA binding in dormant and activated blastocysts (125, 188). These results collectively suggest that coordinated down-regulation of blastocyst CB1 and uterine AEA levels in the receptive uterus are important for implantation. It is interesting to note that the peripheral AEA levels remain relatively low during implantation, whereas the levels increase before and during parturition in humans (209).

Increasing evidence suggests that the bioeffectiveness of AEA depends on its concentration in the extracellular space, which is regulated by its synthesis by NAPE-PLD, its transport across the plasma membrane, and its degradation by FAAH (92–98, 210). To further address the underlying mechanism by which differential uterine AEA levels are spatiotemporally established under different pregnancy status, we examined the expression profiles of NAPE-PLD and FAAH in the mouse uterus during early pregnancy. Correlating with higher levels of AEA in the nonreceptive uterus and interimplantation sites, higher levels of *Nape-pld* mRNA and NAPE-PLD activity were detected in these tissues compared with the implantation site and receptive uterus (142). It is interesting to note that the implanting blastocyst exerts an inhibitory effect on uterine *Nape-pld* expression (142). There is also evidence that blastocysts can up-regulate uterine FAAH activity by releasing a lipid "FAAH activator" (211). 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. This is further confirmed by the observation of higher FAAH expression and activity in the implanting embryo (212, 213). Therefore, the differential and dynamic expression and activity of NAPE-PLD and FAAH in the embryo and uterus create optimal levels of AEA beneficial to blastocyst activation and uterine receptivity for implantation. This tight regulation of AEA synthesis and hydrolysis in the pregnant uterus further indicates that endocannabinoid ligand-receptor signaling plays an important role in implantation.

These studies clearly establish the concept that whereas lower levels of AEA and CB1 are beneficial for implantation, higher levels are detrimental. Using the delayed implantation mouse model, we have provided further evidence that AEA at low concentrations confers blastocyst competency to implantation via CB1 (125), whereas experimentally elevated natural or synthetic cannabinoid levels interfere with implantation. These findings are consistent with our previous observations of stimulation and inhibition of trophoblast growth at low and high AEA levels, respectively (143). To reveal the underlying mechanism of this biphasic AEA action

in blastocyst implantation, we further explored the potential signaling pathways that are coupled with CB1 under different AEA concentrations. We found that AEA-induced stimulatory and inhibitory influences on blastocyst function and implantation are executed by different signal transduction pathways: the ERK and Ca^{++} signaling pathways. AEA at a low concentration activates ERK signaling in dormant blastocysts via CB1. In contrast, at higher AEA levels, it fails to achieve ERK activation, but instead inhibits Ca^{++} mobilization (125). This finding provided for the first time a potential "cannabinoid sensor" mechanism to influence crucial steps during early pregnancy. An association of spontaneous pregnancy loss with elevated peripheral AEA levels in women (214, 215) is consistent with the observations in mice (see *Section V*). These findings in mice and humans reinforce the concept that endocannabinoid signaling is at least one of the pathways determining the fate of embryo implantation. In this regard, there is evidence that activation of CB1 inhibits human decidualization and promotes apoptosis of decidual cells *in vitro* (216), thus adding a new role of endocannabinoids in human pregnancy. The possible physiological consequence of the different signaling pathway triggered by AEA through GPR55 (130, 131, 217) deserves further investigation. In addition, the pathophysiological impact of other endocannabinoids or "endocannabinoid-like" compounds (218) on various reproductive events warrants further investigation.

Taken together, these studies demonstrate that under normal physiological conditions, endocannabinoid signaling through CB1 is crucial to various female reproductive events that include development of embryos, their oviductal transport, and ultimately their homing and implantation in the receptive uterus; conversely, an aberration in endocannabinoid signaling, either silenced or enhanced, derails these processes (Fig. 4). These observations add a new dimension to the concern that the adverse effects of maternal use of cannabinoids on offspring may be seeded very early in pregnancy. There is now evidence that defective implantation creates an adverse ripple effect during the subsequent course of pregnancy both in humans and mice (12, 33, 35). Therefore, our findings in mice raise a cautionary note for women of reproductive ages regarding chronic abuse or medicinal consumption of marijuana or other endocannabinoid system-oriented drugs. More importantly, they raise caution against the use of CB1 antagonists to treat obesity in humans.

V. Endocannabinoids and Female Fertility: Immunoregulation

A. Th1/Th2 cytokines and fertility

There is evidence for the role of peripheral lymphocytes in embryo implantation and successful pregnancy in humans (219). In fact, normal gestation is based on an early immunological adaptation that involves peripheral T lymphocytes in pregnant women (40, 219, 220). These cells produce type 1 T-helper (Th1) and type 2 T-helper (Th2) cytokines, which have opposite effects on trophoblast growth, as schematically depicted in Fig. 5. Th2 cytokines (IL-3, IL-4, and IL-10) favor blastocyst implantation and successful pregnancy by promoting trophoblast growth either directly or indirectly through the inhibition of natural killer (NK) cell activity and the stimulation of natural suppressor cells. Conversely, Th1 cytokines [IL-2, IL-12, and interferon-γ (INF-γ)] impair gestation by causing a direct damage to the trophoblast through stimulation of NK cells and secretion of TNF-α by macrophages. The latter cells play a role in this network, but several aspects of their contribution to the balance of Th1 and Th2 cytokines remain to be elucidated. The trophoblast stimulates the release of profertility Th2 cytokines from T lymphocytes (so-called "Th2 bias") through IL-4, whereas the antifertility Th1 bias is signaled by IL-2. In addition, P_4 plays a role in this network, and in fact it induces a Th2 bias by binding to the intracellular P_4 receptor in T cells (219, 221).

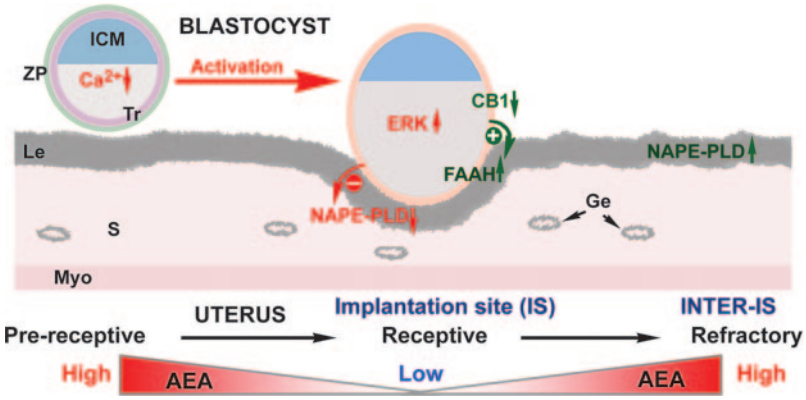

FIG. 4. Endocannabinoid signaling in blastocyst activation and implantation. Evidence suggests that regulated levels of endocannabinoids, primarily AEA, in the receptive uterus and CB1 in activated blastocysts, are beneficial for implantation, whereas higher levels are detrimental to this process. This 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 Ca^{2+} channel activity via CB1. Uterine AEA levels conducive to implantation are primarily regulated by the coordinated expression and activity of *N*-acylphosphatidylethanolamine-hydrolyzing PLD (NAPE-PLD) that generates AEA and by FAAH that degrades AEA in the uterus during early pregnancy. In addition, the implanting blastocyst down-regulates uterine NAPE-PLD expression, but enhances uterine FAAH activity via releasing a putative FAAH activator, thus contributing to rapid turnover of AEA at the implantation site. Ge, Glandular epithelium; IS, implantation site; INTER-IS, interimplantation site; Le, luminal epithelium; Myo, myometrium; S, stroma; Tr, trophectoderm.

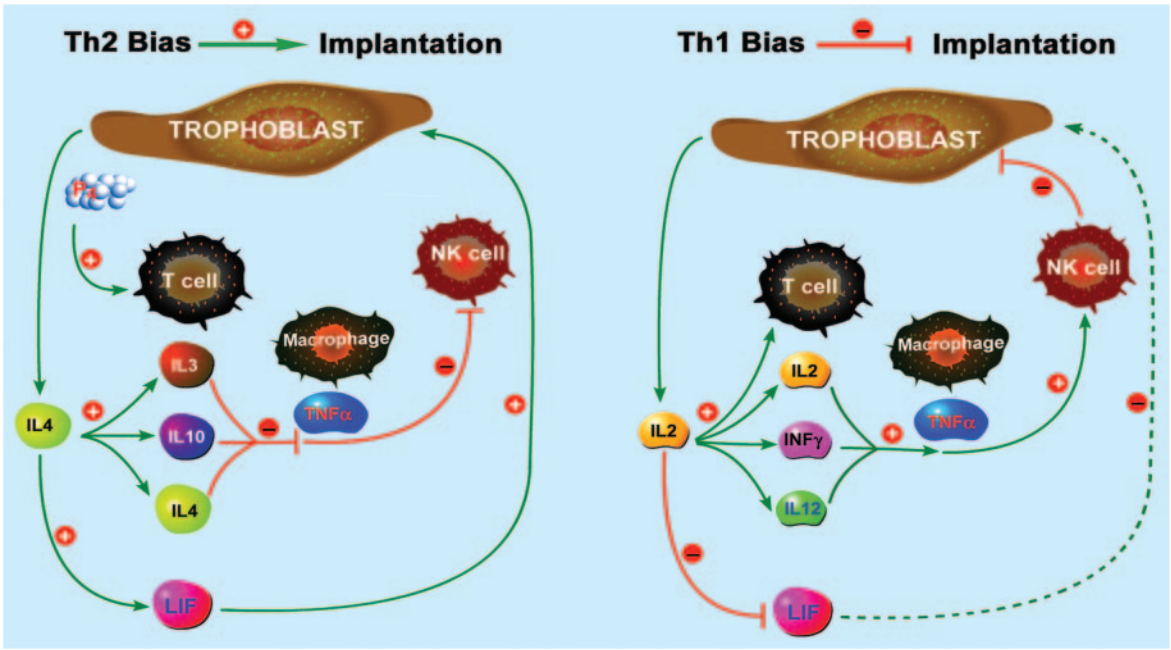

FIG. 5. Th1/Th2 cytokines in human fertility. Th2 cytokines (IL-3, -4, and -10) are released by T cells and favor blastocyst implantation by promoting trophoblast growth through inhibition of NK cell activity. Conversely, Th1 cytokines (IL-2, IL-12, and INF-γ), also released by T cells, impair gestation by damaging the trophoblast through stimulation of NK cells. Macrophages contribute to NK activity by secreting TNFα. The trophoblast induces a Th2 bias through IL-4, whereas IL-2 stimulates the release of Th1 cytokines. Also, P_4 induces the Th2 bias by binding to T lymphocytes. Finally, these cells release LIF under positive or negative control of IL-4 or IL-2, respectively.

The resulting hormone-cytokine network is a key element at the fetal-maternal interface, and a defect in its integrity may result in fetal loss (219, 221–224). Additionally, T lymphocytes produce leukemia inhibitory factor (LIF), which favors embryo implantation and survival (221–224). IL-4 stimulates, whereas IL-2 inhibits LIF release (Fig. 5). Clinical observations that women with unexplained recurrent abortions have reduced expression of LIF production suggest that this cytokine is indeed critical for implantation and pregnancy maintenance in humans (219, 220, 225). In this context, P_4-induced Th2 bias has been found to stimulate LIF release from T lymphocytes (Fig. 5), and IL-4 has been shown to mediate this P_4 effect (219).

B. The endocannabinoid system in lymphocytes of pregnant women

The FAAH activity is known to regulate the endogenous tone and the biological activity of AEA *in vivo* (226). In this respect, lymphocyte FAAH has been shown to influence pregnancy outcome by regulating AEA level at the fetal-maternal interface (40), which appears to interfere with the lymphocyte-dependent cytokine network. Thus, decreased activity and expression of FAAH in peripheral lymphocytes is associated with pregnancy loss and may serve as an early (<8 wk gestation) marker of human spontaneous abortion; AMT activity and cannabinoid binding are not altered (214, 227). Interestingly, defective FAAH in maternal lymphocytes is also associated with failure to achieve an ongoing pregnancy after *in vitro* fertilization and embryo transfer (215). Therefore, it seems that FAAH, but not AMT or cannabinoid receptors, is important in lymphocyte-mediated regulation of the hormone-cytokine network at the fetal-maternal interface in natural and medically-assisted gestation. In addition, analysis of the lymphocyte-endocannabinoid system during human ovulatory cycles has shown the highest FAAH activity and the lowest AEA concentrations on d 21 of the cycle (Table 3), a period that temporally coincides with the putative window of uterine receptivity for implantation (228); binding to CB1 and activities of AMT and NAPE-PLD were similar in T cells at all stages of the ovulatory cycle (Table 3).

C. FAAH as a molecular integrator of fertility signals

FAAH expression in T lymphocytes has been shown to be regulated by Th1/Th2 cytokines: IL-4 and IL-10 enhance

TABLE 3. The endocannabinoid system in human lymphocytes during the ovulatory cycle

Parameter	Day 7	Day 14	Day 21
CB1 binding (cpm per mg protein)	20,000 ± 2,030 (100%)	20,000 ± 2,050 (100%)	17,400 ± 1,795 (87%)
FAAH activity (pmol/min·mg protein)	115 ± 12 (100%)	46 ± 5 (40%)[a]	253 ± 22 (220%)[a]
AMT activity (pmol/min·mg protein)	50 ± 5 (100%)	43 ± 4 (86%)	45 ± 5 (90%)
NAPE-PLD activity (pmol/min·mg protein)	130 ± 15 (100%)	117 ± 12 (90%)	130 ± 15 (100%)
AEA content (pmol per mg protein)	2.15 ± 0.20 (100%)	3.76 ± 0.35 (175%)[b]	1.29 ± 0.14 (60%)[b]

[a] $P < 0.01$ *vs.* d 7.
[b] $P < 0.05$ *vs.* d 7 ($P > 0.05$ in all other cases).

FAAH activity, whereas IL-2 and INF-γ attenuate its activity (227). In particular, IL-4, known to mediate favorable effects of P_4 on pregnancy (221, 229), also partially mediates the effect of P_4 on FAAH expression. Unlike FAAH, little alteration is noted for AMT, NAT, and NAPE-PLD activities and CB1 expression in intact lymphocytes by this steroid (227, 230). P_4 also modulates the effects of THC on sexual receptivity (227). It appears that this effect of P_4 occurs through increased level of the transcription factor Ikaros, which in turn increases *FAAH* gene expression by binding to a specific sequence in the promoter region (230). Profertility Th2 cytokines potentiate the activation of FAAH by P_4, whereas antifertility Th1 cytokines have the opposite effect (227). Leptin is also an important regulator of fertility (231) and immune response (232), and leptin knockout (*ob/ob*−/−) mice are infertile (231). Leptin, too, enhances *FAAH* gene transcription through a signal transducer and activator of transcription 3-mediated up-regulation of the *FAAH* promoter (233). Leptin alone or synergistically with P_4 reduces AEA levels in T cells, without affecting the other players in the endocannabinoid system (233). Overall, the up-regulation of lymphocyte FAAH by profertility signals strengthen the speculation that this enzyme affects human fertility by modulating the AEA levels. Also, uterine FAAH is regulated by sex hormones (234), but the implications and molecular details of this regulation are still elusive. Nonetheless, FAAH activity seems to be important for embryo-uterine cross-talk, because mouse blastocysts release a soluble "FAAH activator", which contributes to AEA disposal at the implantation site (211). The initial biochemical characterization of this activator shows that it is neutralized by lipase activity, whereas PLA_2, PLC or PLD, DNAse I, or RNAse A are ineffective. Additionally, the FAAH activator does not resemble platelet-activating factor, leukotriene B_4, or PGs known to be present in blastocysts (211); its molecular identity is under investigation. In addition, there is molecular evidence that uterine NAPE-PLD is a major player in regulating the levels of AEA in the uterus during early pregnancy (142). In fact, NAPE-PLD activity was higher at the interimplantation site and lower at the implantation site, and E_2 and P_4 down-regulated its expression (142). Thus, P_4 seems to up-regulate lymphocyte FAAH activity, whereas it down-regulates uterine NAPE-PLD expression. This would suggest that regulated AEA levels are critical to successful implantation and pregnancy establishment. On a final note, it is also possible that interplay between endocannabinoids and eicosanoids (prostanoids, leukotrienes, or lipoxins) contributes to immunoregulation of fertility, but this speculation awaits experimental support.

VI. Endocannabinoids and Clinical Implications

Implications of the endocannabinoid system working via cannabinoid and vanilloid receptors in many central and peripheral aspects of human pathophysiology have been proposed. We describe here the endocannabinoid signaling pathways that impact both male and female fertility with the hope of designing and developing endocannabinoid-oriented drugs for the treatment of infertility. In this respect, the biphasic roles of endocannabinoid signaling in fertilization, preimplantation embryo development, oviductal embryo transport, and implantation have major clinical implications (69, 125, 142, 143, 146, 148, 180, 187–189, 191, 207, 211–213, 215, 227, 230, 234, 235). The incidence of spontaneous abortion, the most common adverse outcome of pregnancy, associated with considerable sufferings and medical costs (236, 237), is increased by cigarette smoking and the use of illicit drugs (238, 239). It is interestingly to note that the early stages (< 8 wk) of spontaneous abortion in humans are associated with decreased activity and expression of FAAH in maternal T lymphocytes and increased blood levels of AEA. This correlation of down-regulation of FAAH in women who are prone to miscarriage appears specific, because other members of the endocannabinoid system are not affected (214, 215, 227). This suggests that high FAAH activity with low AEA levels are among the factors that are important for successful pregnancy and raises concerns about marijuana use by pregnant women.

We suggest that FAAH is an important player in coordinating hormone-cytokine-endocannabinoid networks critical to reproduction. Thus, the FAAH level may serve as a marker to monitor the early stages (<8 wk) of human gestation. Because low FAAH levels correlate with pregnancy failure, it is possible that drugs that enhance FAAH activity may improve human fertility. Regulating endocannabinoid levels via metabolic pathways could be an advantage over the use of CB receptor agonists and antagonists to eliminate their psychotropic effects (109, 240–242). However, the development and use of selective FAAH activators as therapeutics for fertility regulation may significantly reduce or eliminate AEA signaling via CB receptors and increase the incidence of reproductive disturbances (188, 189). Furthermore, FAAH regulates the levels of several other endogenous endocannabinoid-like compounds whose functions are not yet known, leaving open the question of the biological consequences of their enhanced degradation upon treatment with FAAH activators. Other enzymes that catalyze the hydrolysis of *N*-palmitoylethanolamine (243) and hydrolysis (117) and synthesis (112) of 2-AG have recently been identified. It remains to be seen how these pathways contribute to the overall endocannabinoid tone relevant to reproductive functions. In addition, the development and use of FAAH activators calls for attention to "the other side of the coin," because there is effort to develop FAAH inhibitors (rather than activators) as novel therapeutic agents for the treatment of pain (226), neurodegenerative disorders (111), cancer (244, 245), and anxiety (246).

VII. Conclusions and Future Direction

Mammalian reproduction is a complex process, involving spermatogenesis, oogenesis, fertilization, preimplantation embryo development, timely passage of embryos through the oviduct, and their implantation in the uterus, eventually establishing a functional placenta for successful pregnancy. Each step in this process requires spatiotemporally regulated various networks of endocrine, paracrine, juxtacrine, and autocrine modulators. Although previous and prevailing

studies have placed much emphasis on the roles of cannabinoids/endocannabinoids on neuronal functions (109, 111, 247–249), there were sporadic reports of adverse effects of cannabinoids on pregnancy outcome, including retarded embryo development and pregnancy failure. Emerging evidence now points toward important roles of the endocannabinoid system in mammalian reproduction. In this review, we present molecular, genetic, physiological, and pharmacological evidence that cannabinoid/endocannabinoid signaling is functionally operative in both male and female reproductive events.

With respect to male reproductive functions, endocannabinoids show biphasic roles in regulating Sertoli cell apoptosis via differential receptor signaling mechanisms. Furthermore, we describe here a stage-dependent effect of AEA on sperm function in that it prevents premature capacitation via a CB1 to ensure normal transit of sperm through the uterus to oviduct. It is tempting to suggest that such an inhibitory brake may become less stringent when sperm reaches the oviduct, the site of fertilization. Activation of CB1 by AEA (extracellular signaling) leads to inhibition of sperm motility, capacitation, and ZP-induced but not spontaneous acrosome reaction. In contrast, binding of AEA to TRPV1 receptors (intracellular signaling) inhibits spontaneous acrosome reaction.

Upon fertilization, one-cell embryos initiate cell division and undergo preimplantation development. As described above, endocannabinoid signaling through CB1 is crucial to various female reproductive events that include development of embryos, their oviductal transport, and ultimately their homing and implantation in the receptive uterus; conversely, an aberration in endocannabinoid signaling, either silenced or enhanced, derails these processes. Furthermore, decreased activity and expression of FAAH in maternal T lymphocytes and resulting increased AEA levels are associated with spontaneous abortion in women. These findings add a new dimension to the concern that the adverse effects of maternal use of cannabinoids on offspring may be seeded very early in pregnancy, thus raising a cautionary note for women of reproductive ages regarding chronic abuse or medicinal consumption of marijuana or other endocannabinoid system-oriented drugs. More importantly, they raise caution against the use of CB1 antagonists to treat obesity in humans. Further in-depth investigation is warranted to better understand pathophysiological significance of the cannabinoid/endocannabinoid signaling pathway in mammalian reproduction.

Synchronous development of the preimplantation embryo to the blastocyst stage before implantation is one of the prerequisites for normal "on-time" implantation. We have observed that either silencing or amplifying CB signaling leads to asynchronous preimplantation embryo development (188, 189). However, the underlying molecular mechanisms remain unknown. Recently, global gene expression profiles during preimplantation mouse development analyzed by microarray techniques have generated a comprehensive data set covering nearly all mouse genes during early embryogenesis (194, 250–256). Therefore, strategies comparing global gene expression, proteomic, and/or lipidomic profiles between normal and defective embryos may help to identify novel genes and gene products regulated by endocannabinoid signaling in the periimplantation embryo development.

Furthermore, it is crucial to gather in-depth information on how the endocannabinoid signaling is differentially regulated in peripheral and central systems. It is especially an important concern for developing endocannabinoid system-oriented drugs for selectively targeting central or peripheral tissues to avoid adverse effects in unrelated tissue types. Therefore, more efforts should be directed to explore in detail the tissue- and cell-specific effects of endocannabinoid signaling using conditional transgenic mouse models. In fact, there is a recent study showing functional dissociation of FAAH between central and peripheral tissues using brain tissue-selective mutant mice (257). On the other hand, efforts should also be directed to elucidate the common link that encompasses the central and peripheral endocannabinoid signaling. For example, increasing evidence suggests that AEA-CB1 signaling exerts a regulatory role on hypothalamic neuronal activity for the pulsatile release of GnRH (GnRH) (258–260), a central hormone that controls reproductive performances in both males and females.

Although a wealth of knowledge on the roles of lipid mediators including endocannabinoids, LPA and PGs, and protein signaling molecules, such as growth factors, cytokines, homeotic genes, and transcription factors in embryo-uterine interactions during implantation, has been generated (5, 6, 8, 23, 36, 183), their hierarchical blueprint in directing uterine and embryonic functions during pregnancy remains to be deciphered. We need to understand whether these pathways function independently or in parallel, or converge to a common signaling pathway to establish a network of lipid-protein signaling cross-talk between the embryo and uterus that is necessary for implantation. In this respect, another area of attention in endocannabinoid research has evolved from the finding that the COX and lipoxygenase enzymes can use AEA and 2-AG as substrates to generate novel PGs and hydroxy-endocannabinoids (261–264), suggesting a close link between endocannabinoids and eicosanoids (265). The potential function and signaling pathways of these metabolic products in reproductive events remain to be determined.

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Address all correspondence and requests for reprints to: S. K. Dey, Division of Reproductive and Developmental Biology, Departments of Pediatrics and Cell & Developmental Biology and Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37232. E-mail: sk.dey@vanderbilt.edu; or M. Maccarrone, Department of Biomedical Sciences, University of Teramo, 64100 Teramo, Italy. E-mail: mmaccarrone@unite.it

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