Cannabinoid/Endocannabinoid Signaling Impact on Early Pregnancy Events

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Contents

Abstract It has been known for decades that marijuana and its major psychoactive component Δ^9-tetrahydrocannabinol (THC) alter both male and female reproductive functions in humans and laboratory animals. The discovery of cannabinoid-like molecules (endocannabinoids), anandamide (AEA) and 2-arachidonylglycerol (2AG), as well as G-protein-coupled cannabinoid/endocannabinoid receptors CB_1 and $CB_{2,}$ created an opportunity to study the adverse and beneficial effects of cannabinoids/endocannabinoids on fertility using molecular, physiological and genetic approaches. In fact, studies to explore the significance of cannabinoid/endocannabinoid signaling in reproduction have revealed some intriguing physiological roles in early pregnant events. This review summarizes some aspects of these signaling molecules in preimplantation and implantation biology utilizing genetically engineered mouse models.

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D. Kendall and S. Alexander (eds.), *Behavioral Neurobiology of the Endocannabinoid System*, Current Topics in Behavioral Neurosciences 1,
DOI: 10.1007/978-3-540-88955-7_10,

Keywords Endocannaibnoids • CB1 • CB2 • Pregnancy • Implantation • Uterus • Oviduct

1 Introduction

Although the human population is growing rapidly, 15% of couples worldwide are infertile (Abma et al. 1997; Thonneau et al. 1991), with infertility defined as the inability to conceive after one year of regular sexual intercourse. Infertility is still a worldwide social and economic concern. Early pregnancy loss in humans often happens due to defects that occur before, during or immediately after implantation. Although in vitro fertilization and embryo transfer (IVF-ET) approaches have overcome several barriers of human infertility, the implantation rate still remains disappointingly low. Therefore, studying physiological, genetic and molecular bases of implantation is important. However, it is difficult to define the hierarchical landscape of molecular pathways during human pregnancy because of experimental difficulties and ethical restrictions on research with human embryos. It is hoped that experiments in mice and other animal models combined with feasible experiments in humans will generate meaningful information to address this critical issue. Although details of many of the molecular interactions during the peri-implantation events have not yet been defined, increasing evidence from gene expression and transgenic mouse studies reveals that synchronous development of the preimplantation embryo to the blastocyst stage and differentiation of the uterus to the receptive stage are prerequisites for the initiation of implantation (Dey et al. 2004; Paria et al. 2002; Wang and Dey 2006).

Over the past several years, molecular and genetic studies have provided evidence that lipid mediators are critical signaling molecules in coordinating events of early pregnancy (Shah and Catt 2005; Song et al. 2002; Wang and Dey 2005; Ye et al. 2005). Among these signaling pathways, endocannabinoid signaling has recently been highlighted as an important player in directing preimplantation development of embryos and their timely homing into the receptive uterus for implantation. This review highlights various aspects of the endocannabinoid system in female fertility. It is hoped that a deeper insight will lead to potential clinical applications, perhaps targeting the endocannabinoid signaling pathway to correct infertility and improve women's reproductive health. This article reviews the endocannabinoid system and its roles in peri-implantation biology primarily in genetically engineered mouse models.

2 Endocannabinoid Systems

Marijuana, derived from the plant *Cannabis sativa*, is widely used for its psychoactive effects, including euphoria and analgesia. Although it has been used recreationally for thousands of years, studies regarding the chemistry of *Cannabis* were

initiated just decades ago (Mechoulam and Hanus 2000). In 1964, Δ^9-tetrahydrocannabinol (Δ^9-THC) was identified as the major active component of marijuana (Gaoni and Mechoulam 1964), stimulating research on marijuana. In the early 1990s, research on marijuana was further boosted by the discovery and cloning of two cannabinoid receptors, brain-type (CB_1) (Devane et al. 1988; Matsuda et al. 1990) and spleen-type (CB_2) (Munro et al. 1993). At around the same time, several endogenous ligands were identified that target CB_1 and CB_2, and subsequently they were termed endocannabinoids. The two most studied endocannabinoids are *N*-arachidonoylethanolamine, commonly known as anandamide (AEA) (Devane et al. 1992) and 2-arachidonoylglycerol (2AG) (Mechoulam et al. 1995; Sugiura et al. 1995). Also see "The Life Cycle of the Endocannabinoids: Formation and Inactivation" in the chapter by Stephen P.H. Alexander and David A. Kendall, this volume, for a more detailed description.

2.1 AEA Synthesis and Degradation

It is widely accepted that AEA is derived from the precursor *N*-arachidonoylphosphatidylethanolamine (NAPE) through its reaction with NAPE-hydrolyzing phospholipase D (NAPE-PLD) (Natarajan et al. 1982, 1984), a member of the metallo-lactamase family with Ca^{2+}–sensitive enzyme activity (Okamoto et al. 2004; Ueda et al. 2001). However, unaltered polyunsaturated NAE (N-acyl-ethanolamine) levels in NAPE-PLD deficient mice suggest that other AEA synthetic pathways also contribute to levels of AEA (Leung et al. 2006). 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 AEA (Simon and Cravatt 2006), and (2) cleavage of NAPE by phospholipase C to generate phosphor-AEA (pAEA) which is subsequently dephosphorylated by a protein tyrosine phosphatase (PTPN22) to release AEA (Liu et al. 2006). Although these pathways are found in both the CNS and peripheral tissues, mechanism(s) by which these pathways are regulated and affect each other are still unknown.

AEA signaling through CB receptors occurs through a two-step process. AEA is first taken up by the cell through an AEA membrane transporter (AMT) and then degraded intracellularly by the fatty acid amide hydrolase (FAAH) (Cravatt et al. 1996; Giang and Cravatt 1997). It is to be noted, however, that the existence of endocannabinoid transporters is still under debate (Glaser et al. 2005; Mechoulam and Deutsch 2005). The current models suggest that enzymes for the synthesis and degradation of endocannabinoids are localized within the cell. This means that stimulation of cannabinoid receptors by endocannabinoids from the extracellular component requires them to cross the cell membrane twice. This concept is controversial, based on research on the transporter. In fact, the uptake of AEA has features similar to facilitated transport, dependent on concentration, time, and temperature, and independent of external Na^+ ions or ATP hydrolysis (Mechoulam

and Deutsch 2005). The development of new drugs that inhibit AMT selectively without affecting FAAH corroborates this speculation (Ortega-Gutierrez 2005). However, FAAH may not need a transporter to contact AEA for its degradation (Bracey et al. 2002). It is suggested that AEA uptake instead is driven by non-protein mediated diffusion and is regulated by its degree of hydrolysis by FAAH (Kaczocha et al. 2006). Along this same tenet, it is thought that the target of some of these recently developed transport inhibitors is an uncharacterized intracellular component that delivers AEA to FAAH (Kaczocha et al. 2006).

After AEA is accumulated within the cell, it is degraded to ethanolamine and arachidonic acid (AA) by FAAH (Cravatt et al. 1996; Giang and Cravatt 1997). Mammalian FAAH is a membrane-bound enzyme with a globular shape. It has 28 α-helices and 11 β-sheets, which account for approximately 53 and 13% of the whole protein structure, respectively (Bracey et al. 2002). This enzyme uses an unusual serine–serine–lysine (S241–S217–K142) catalytic triad (McKinney and Cravatt 2005). FAAH can also hydrolyze other endocannabinoids including 2AG and the sleep-inducing substance, oleamide (McKinney and Cravatt 2005). FAAH has also been shown to be critical for regulating both the magnitude and duration of AEA and other fatty acid amide signaling (Cravatt and Lichtman 2002). Recently, a second membrane-associated fatty acid amide hydrolase was found in human and other primate genomes but not in that of rodents (Wei et al. 2006).

2.2 2AG Synthesis and Degradation

2AG was discovered by two independent groups, with one group identifying it in the canine gut and the other in the rat brain (Mechoulam et al. 1995; Sugiura et al. 1995). 2AG is derived from the precursor diacylglycerol by a membrane-bound sn1-diacylglycerol lipase (DGL) (Moriyama et al. 1999). To date, two isoforms of DGL have been cloned: DGLα and DGLβ. The α and β isoforms have molecular masses of 120 and 70 kDa, respectively, with four transmembrane domains, and they are members of the serine lipase family with serine and aspartic acid (S443–D495) participating in the catalytic triad. DGLα is mainly expressed in the adult brain, whereas DGLβ is expressed in the developing brain (Bisogno et al. 2003). Like AEA, 2AG is produced as necessary, but these two endocannabinoids differ in that AEA often acts only as a partial agonist of cannabinoid receptors, while 2AG acts as a full agonist. Interestingly, the binding affinity of 2AG to cannabinoid receptors is approximately 24 times less than that of AEA, but under most physiological conditions, 2AG levels are much higher than AEA (Sugiura et al. 2006). It still remains to be determined, therefore, how only a small percentage of 2AG (10–20%) crosses the plasma membrane to interact with cannabinoid receptors (Bisogno et al. 1997).

Like AEA, the termination of pharmacological effects of 2AG requires it to be transported into the intracellular compartment. It is proposed that the 2AG membrane transporter is the same as AMT (Beltramo and Piomelli 2000). In fact, 2AG

accumulation is directly reduced by an AMT inhibitor, AM404, and indirectly reduced by high concentrations of AA (Beltramo and Piomelli 2000). After 2AG accumulates in cells, it can then be degraded by either FAAH or a serine hydrolase, monoacylglycerol lipase (MGL) (Goparaju et al. 1999). MGL, a 33-kD protein, has been isolated, cloned and characterized in both rats and humans (Dinh et al. 2002; Goparaju et al. 1999; Ho et al. 2002). Unlike FAAH, MGL is localized primarily in the cytosol, but not on the plasma membrane. Recently, Muccioli et al. identified a novel protein in a mouse microglial cell line that has MGL activity and regulates 2AG levels (Muccioli et al. 2007).

2.3 Cannabinoid Receptors

Endocannabinoids, as well as plant-derived and synthetic cannabinoids, target cannabinoid receptors CB_1 and CB_2. They are G protein-coupled receptors with seven transmembrane domains. CB_1 is present mostly in the central nervous system and in some peripheral tissues including heart, testis, liver, small intestine and uterus, while CB_2 is abundantly expressed in astrocytes, spleen and several immune cells (Howlett et al. 2002; McAllister and Glass 2002; Pertwee and Ross 2002). CB_1 and CB_2 show 44% overall identity and both are coupled with G proteins in the $G_{i/o}$ and G_q families. Activation of each CB receptor subtype has different biological effects with most being cell-type dependent. Signal transduction pathways regulated by CB receptors include inhibition of adenylyl cyclase (Matsuda et al. 1990; Paria et al. 1995), regulation of Ca^{2+} channels (Caulfield and Brown 1992; Gebremedhin et al. 1999; Lauckner et al. 2005; Mackie and Hille 1992; Wang et al. 2003), activation of phospholipase C (Zoratti et al. 2003) and stimulation of mitogen-activated protein kinases (MAPKs) including ERK, JNK and p38 (Bouaboula et al. 1995; Murphy and Blenis 2006; Wang et al. 2003).

Some evidence indicates the existence of other putative cannabinoid receptors in addition to CB_1 and CB_2 (Baker et al. 2006). For example, it was shown that AEA can protect murine neuroblastoma cells subjected to low serum-induced apoptosis by non-CB_1, non-CB_2 receptors (Matas et al. 2007). Furthermore, a novel cannabinoid receptor 3 (GPR55) has been reported (McPartland et al. 2006; Sawzdargo et al. 1999), which, as yet, is a G protein-coupled orphan receptor. However, the physiological role of this receptor is not clearly understood.

AEA, but not 2AG, can also activate receptors other than CB_1 and CB_2. One receptor that AEA activates is the transient receptor potential vanilloid 1 (TRPV1) (Van Der Stelt and Di Marzo 2004), a ligand-gated non-selective cationic channel. TRPV1 can also be activated by molecules derived from plants, such as capsaicin (the pungent component of “hot” red peppers) and resinferatoxin, and also by stimuli including heat and low PH (Protons). Some recent studies suggest a physiological role for AEA as a TRPV1 agonist. The binding of AEA to the cytosolic binding site of TRPV1 triggers Ca^{2+} influx and eventual cytochrome c release (De Petrocellis et al. 2001; Maccarrone and Finazzi-Agro 2003).

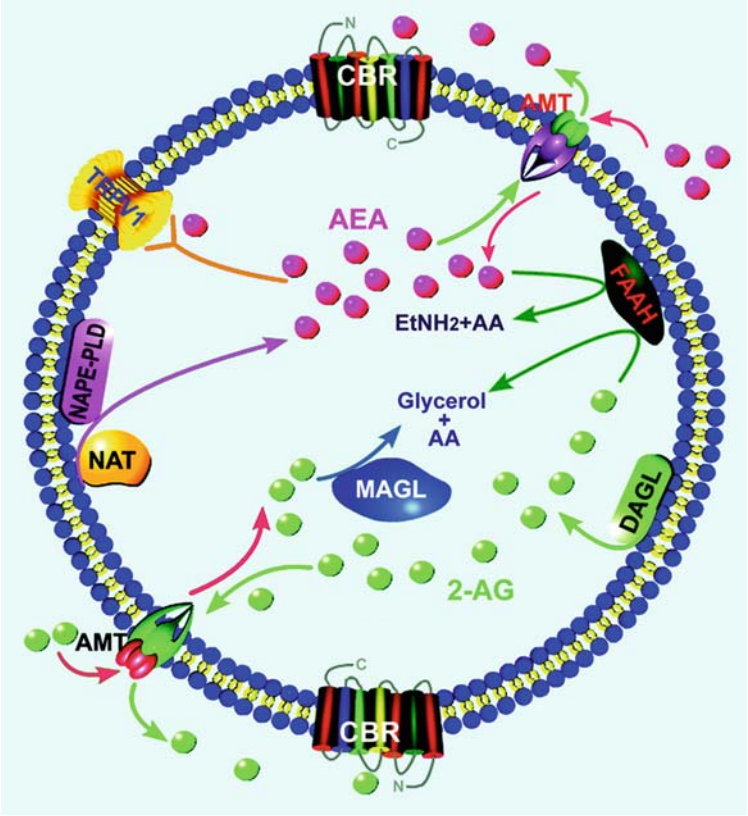

Fig. 1 The endocannabinoid system. Synthesis of AEA from membrane *N*-arachidonoylphosphatidylethanolamines is catalyzed by sequential activities of *N*-acyltransferase (NAT) and NAPE-PLD, which releases AEA and phosphatidic acid. AEA is transported in both directions through the cell membrane by a selective AMT and, once taken up, is hydrolyzed by FAAH to ethanolamine ($EtNH_2$) and AA. The main targets of AEA are CB_1 and CB_2 receptors (CBR) with extracellular binding sites, and type-1 vanilloid receptors (TRPV1) with intracellular binding sites. 2AG is also released from membrane lipids through the activity of DGL. 2AG can also be hydrolyzed by FAAH or more importantly by MGL, releasing glycerol and AA. The transport of 2AG across the cell membrane may be mediated by AMT or a related transporter, and CBR (but not TRPV1) is the target of this endocannabinoid. This figure is adapted from Wang et al. (2006a)

Endocannabinoid receptors and their ligands together with the synthesis and degradation enzymes collectively constitute the endocannabinoid system (Fig. 1).

3 Peri-implantation Events

Life begins with the fusion of two haploid gametes, an egg and a sperm (Evans and Florman 2002; Wassarman et al. 2001). The one-cell fertilized zygote, now termed an embryo, undergoes several mitotic cell divisions, eventually forming the blastocyst.

The blastocyst is comprised of two distinct cell populations, the inner cell mass (ICM) and an outer layer of trophectoderm cells (Rossant and Tam 2004; Wang and Dey 2006; Zernicka-Goetz 2005). The embryo proper is derived exclusively from the ICM, whereas the placenta and extraembryonic membranes are generated from cells contributed by the trophectoderm (Cross et al. 1994; Rossant 2004). During early pregnancy, another critical event occurs in parallel with preimplantation embryonic development – the embryos' timely transport 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 the late blastocyst stage. A two-way interaction between the blastocyst and maternal uterine luminal epithelium initiates the process of implantation (Dey et al. 2004; Paria et al. 2002; Wang and Dey 2006). Although the precise sequence and details of the molecular interactions involved in these processes are not clearly understood, increasing evidence from gene expression and transgenic mouse studies during the last two decades shows that coordinated integration of a range of paracrine, autocrine, and/or juxtacrine signaling pathways participates in embryo–uterine dialog during implantation (Carson et al. 2000; Dey et al. 2004; Paria et al. 2002; Red-Horse et al. 2004; Wang and Dey 2006). Among these, endocannabinoid signaling has recently been highlighted as an important player in directing preimplantation embryo development, the timely homing of embryos into a receptive uterus, and coordinating blastocyst activation and uterine receptivity for implantation.

3.1 Preimplantation Embryo Development

Development of preimplantation embryos to blastocysts is critical for achieving implantation competency. Their delayed development causes defective or failure of implantation, leading to compromised pregnancy (Wang and Dey 2006).

Endocannabinoid signaling occurs in preimplantation embryos, the oviduct and uteri. Both CB_1 and CB_2 are present in preimplantation embryos (Das et al. 1995; Paria et al. 1995, 2001; Wang et al. 2004), while only CB_1 is expressed in the oviduct and uterus. While CB_1 mRNA is detected from the 4-cell through the blastocyst stages, CB_2 is present from the 1-cell through the blastocyst stages (Paria et al. 1995). AEA binding sites are also evident in embryos at these stages. Notably, these binding sites are primarily located in outer cells of embryos at 8-cell, morula, and blastocyst stages. Dey's group has shown that AEA binds to a single class of high-affinity receptors on blastocysts. The presence of CB_1 mRNA correlates with CB_1 protein as detected by immunocytochemistry (Paria et al. 2001; Yang et al. 1996). Moreover, blastocyst CB_1 is biologically active, since both THC and AEA inhibit forskolin-stimulated cAMP formation in the embryo, and this inhibition is prevented by pertussis toxin pretreatment (Das et al. 1995; Paria et al. 1995). Recent observations of expression of CB_2 in early embryos and embryonic stem cells by microarray analysis (Sharov et al. 2003), and the absence of its expression in trophoblast stem cells derived from preimplantation blastocysts,

suggests that CB_2 expression is restricted to the ICM of blastocysts (Hamatani et al. 2004). Thus, while the role of CB_2 in early embryos remains unknown, the presence of functional CB_1 suggests that mouse embryos are potential targets of endocannabinoids and natural cannabinoids.

Embryos exposed to high levels of endocannabinoids, plant-derived and/or synthetic cannabinoids show retarded development. For example, high levels of AEA causes blastocysts to have a reduced number of trophectoderm cells and decreases the rate of zona-hatching (Schmid et al. 1997; Yang et al. 1996). Furthermore, AEA, 2AG, THC or WIN55212-2 (a synthetic cannabinoid agonist) arrests the development of two-cell embryos to blastocysts (Paria et al. 1995, 1998b). This developmental defect, however, is rescued by SR141716A or AM251 (synthetic CB_1-selective antagonists), but not by SR144528 (a CB_2-selective antagonist). Furthermore, a CB_2 agonist, AM663, fails to influence embryo development (Paria et al. 1998b). These studies collectively provide evidence that endocannabinoids or cannabinoids mediate their effects on preimplantation embryos via CB_1 (Fig. 2).

The availability of gene targeted *cnr1* and *cnr2* mouse models has greatly expanded the field of endocannabinoid research. It was observed that $CB_1^{-/-}$ and $CB_1^{-/-}/CB_2^{-/-}$ embryos recovered from oviducts (day 3) and uteri (day 4) of pregnant mice show asynchronous development compared with wild-type embryos (Paria et al. 2001). Interestingly, heterozygous embryos recovered from $CB_1^{-/-}$

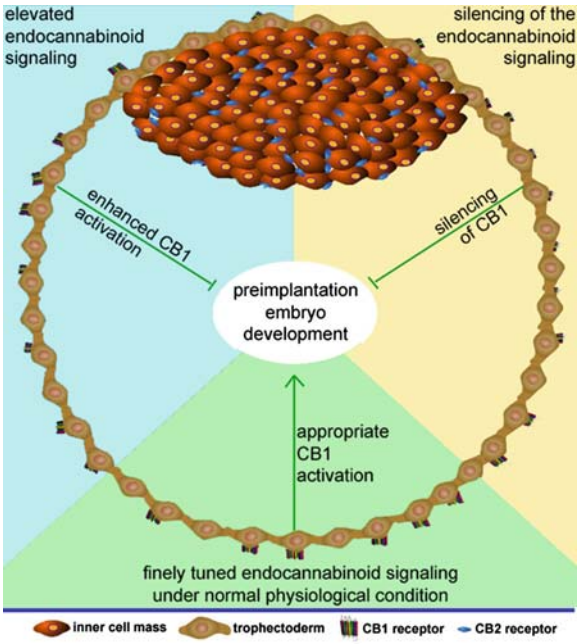

Fig. 2 Cannabinoid signaling in preimplantation embryo development. Both exaggerated or absent cannabinoid/endocannabinoid signaling mediated by CB_1 leads to aberrant preimplantation embryo development. This figure is adapted from Sun and Dey (2008)

females mated with wild-type males showed normal embryo development (Wang et al. 2004). These findings also imply that embryonic CB_1 receptors, but not oviductal (maternal) CB_1 receptors, direct appropriate early embryonic development (Wang et al. 2006a). Furthermore, normal development of heterozygous null embryos suggests that even one copy of CB_1 is sufficient for normal development. These findings prompted the hypothesis that appropriate endocannabinoid signaling is necessary for embryo development.

In vitro embryo culture experiments showed that most 2-cell wild-type embryos fail to develop to the blastocyst stage in the presence of excess AEA. However, low levels of AEA (7 nM) promoted trophoblast differentiation and growth, while higher levels (28 nM) inhibited such development (Wang et al. 1999). In contrast, more than 80% of $CB_1^{-/-}$ or $CB_1^{-/-}/CB_2^{-/-}$ double mutant embryos develop into blastocysts in the presence of similar levels of AEA. Interestingly, in vitro development of $CB_2^{-/-}$ embryos, like wild-type embryos, was severely compromised in the presence of AEA (Paria et al. 2001). These results lend genetic support that CB_1, but not CB_2, responds to cannabinoids to govern embryonic development.

Interestingly, $CB_2^{-/-}$ or $CB_1^{-/-}/CB_2^{-/-}$ embryos collected from the oviduct on day 3 and uterus on day 4 also show asynchronous development (Paria et al. 2001), indicating that CB_2 apparently has some role in preimplantation embryo development. The significance of this finding is not fully understood. However, recent observations of CB_2 expression in embryonic stem cells by microarray analysis (Sharov et al. 2003) together with its absence in trophoblast stem cells (Wang and Dey, unpublished data) suggest that CB_2 expression is restricted to the inner cell mass (ICM), pointing toward a role of CB_2 in ICM cell development and thus development of the embryo proper. Collectively, cannabinoid signaling can regulate preimplantation embryo development, with the current model implicating its effects mediated via CB_1 receptors. However, the role of CB_2 receptors in embryo development remains puzzling.

3.2 Oviductal–Uterine Embryo Transport

In parallel with preimplantation embryo development, embryos transit from the oviduct to the uterus. The oviduct consists of an ampulla and isthmus and is connected to the uterus through the utero-tubal junction. The ampulla is lined with many more ciliated cells than the isthmus, while the isthmus possesses a thicker muscular layer because of their distinct functions (Gaddum-Rosse and Blandau 1976). In mice, embryos transit rapidly though the oviduct ampulla due to the forward-moving beating of the cilia present on the epithelial cell surface. Once they reach the ampulla–isthmus junction, they reside at the isthmus for approximately 3 days. Then the embryos are propelled through the utero-tubal junction by a wave of regulated contraction and relaxation of the isthmus smooth muscle (Halbert et al. 1976). Embryos enter the uterus at the late morula stage, and coincident with this transport a cavity appears in the embryo, 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 embryos from the oviduct to the uterus. Although there is no evidence for implantation of embryos in the mouse oviduct, human embryos can implant in the human oviduct (Fallopian tube). A dysfunctional regulation of oviductal–uterine transport results in oviductal retention of embryos, and thus can lead to ectopic pregnancy in women (Farquhar 2005; Pisarska et al. 1998).

In the mouse oviduct, CB_1, not CB_2, is detected (Das et al. 1995; Wang et al. 2004). Both NAPE-PLD and FAAH are also present in the oviduct. NAPE-PLD levels are higher in the isthmus compared to the ampullary region, whereas FAAH shows the reverse pattern, being higher in the ampullary region (Guo et al. 2005; Wang et al. 2006b). This spatially different expression pattern of key enzymes in AEA regulation suggests that endocannabinoid signaling has a physiological role in the oviduct.

Studies from our group have shown that almost half of $CB_1^{-/-}$ mice show pregnancy loss. These mice, however, have normal ovulation and fertilization (Paria et al. 2001; Wang et al. 2004). It was initially thought that asynchronous embryo development is a contributor to this pregnancy loss. Based on this assumption, normal pregnancy in $CB_1^{-/-}$ mice would be restored by mating mutant females with wild-type males to generate all heterozygous embryos with normal preimplantation growth, since $CB_1^{+/-}$ embryos have normal preimplantation development in $CB_1^{-/-}$ oviducts. However, almost half of $CB_1^{-/-}$ mothers still showed pregnancy loss (Paria et al. 2001; Wang et al. 2004). This suggested that maternal CB_1, and not embryonic CB_1, is the cause for pregnancy failure.

Further investigation found that pregnancy failure in $CB_1^{-/-}$ females was attributed to oviductal retention of embryos (Fig. 3a). $CB_1^{-/-}$/ $CB_2^{-/-}$ mice also show oviductal retention, but wild-type and $CB_2^{-/-}$ mice do not, suggesting that oviductal retention results from the lack of CB_1. This is consistent with the expression pattern of CB receptors in the oviduct, in that CB_1 is present in murine oviducts, but not CB_2. This same study showed that all the trapped embryos in the oviduct are morphologically and physiologically healthy, because they can implant when transferred into day 4 pseudopregnant uteri, again confirming that oviductal retention is due to lack of maternal CB_1. This was further confirmed by reciprocal embryo transfer between $CB_1^{-/-}$ and wild-type female mice. Only $CB_1^{-/-}$ recipients displayed oviductal retention of embryos, irrespective of embryonic genotypes (Wang et al. 2004). In addition, wild-type mice with pharmacologically inhibited CB_1, but not CB_2, also show high rate of embryo retention in the oviduct. Notably, $FAAH^{-/-}$ mice, which have higher oviductal AEA levels, and wild-type mice exposed to THC or meth-AEA (a stable AEA analog) also show oviductal retention of embryos (Wang et al. 2006b). 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 CB_1 in the oviduct regulates normal embryo transport through the oviduct (Fig. 3b).

It is known that the transport of embryos through the oviduct is aided by a wave of movements in the oviduct muscle that is controlled by the sympathetic nervous

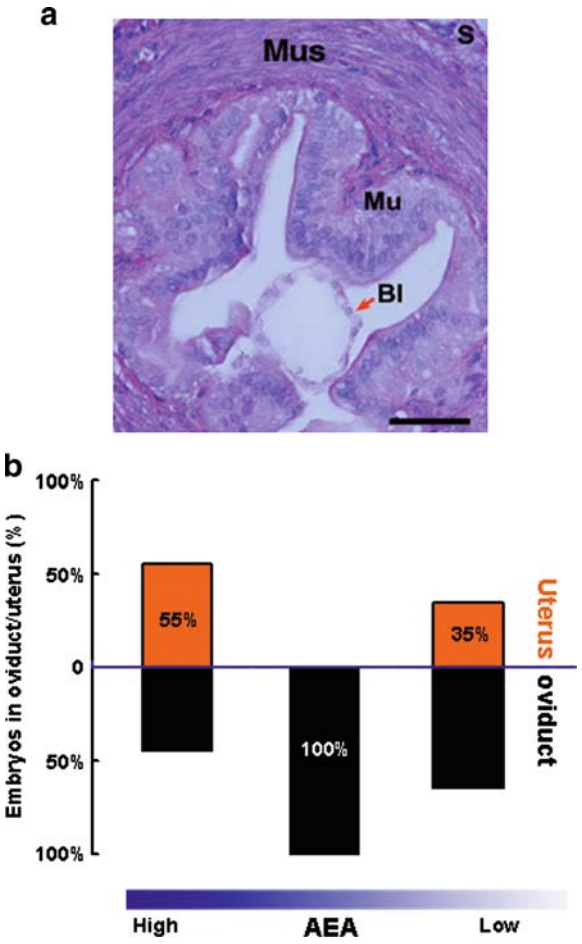

Fig. 3 Impaired oviductal embryo transport. (**a**) A representative histological section of a day 7 pregnant *Cnr1*$^{-/-}$ oviduct showing a trapped blastocyst (arrow) at the oviduct isthmus. Bl, blastocyst; Mus, muscularis; Mu, mucosa; S, serosa. Bar, 100 μm. (**b**) Percentage of embryos recovered from oviducts or uteri at different AEA levels. Panel **a** is adapted from Wang et al. (2004); and panel **b** is reproduced from Wang et al. (2004, 2006b)

system (Heilman et al. 1976). Stimulation of β_2-adrenoceptors (β_2-AR) causes muscle relaxation, whereas stimulation of α_1-AR confers 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 (Heilman et al. 1976; Howe and Black 1973). In this respect, exposure of wild-type oviducts to either an α_1-AR agonist or a β_2-AR antagonist leads to oviductal retention of embryos. In addition, CB_1 expression in the muscularis of the oviduct is colocalized with α_1- and β_2-adrenoceptors, and $CB_1^{-/-}$ oviducts show increased release of norepinephrine (NE) (Wang et al. 2004).

These observations provide evidence that CB_1-mediated endocannabinoid signaling is coupled to adrenergic signaling to regulate oviductal motility, and that the oviductal muscularis is predominantly in a contraction phase in the absence of CB_1. In contrast, heightened endocannabinoid signaling, in either $FAAH^{-/-}$ mice with naturally higher AEA 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.

In conclusion, the spatiotemporal expression of NAPE-PLD and FAAH in the oviduct creates an appropriate endocannabinoid tone, executed by CB_1 receptors to regulate the release of NE. Silencing or enhanced endocannabinoid/cannabinoid signaling impedes the highly coordinated oviductal smooth muscle contraction and relaxation through the sympathetic nervous system, consequently regulating the transit of embryos from the oviduct to the uterine lumen.

3.3 Implantation

Attachment of the embryo to the luminal epithelium of the 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 (implantation competency) and uterine receptivity are two distinct events in the process of implantation (Paria et al. 1993). The attainment of implantation competency of the blastocyst and uterine receptivity are primarily coordinated by the ovarian steroid hormones, estrogen and progesterone (Paria et al. 1998a). Progesterone has been shown to be essential for implantation and pregnancy maintenance in all mammals studied, whereas the requirement for ovarian estrogen is species-specific. In mice, under progesterone priming, closure of the uterine lumen occurs and coincides with the escape of the blastocyst from the zona pellucida, bringing the blastocyst trophectoderm into close contact with the uterine luminal epithelium. Superimposition of the progesterone-primed uterus with preimplantation ovarian estrogen and its catechol metabolite, 4-hydroxy-17β-estradiol (4-OH-E_2) differentially regulate uterine preparation and blastocyst activation, respectively. Estrogen, via its interaction with nuclear estrogen receptors, participates in the preparation of the progesterone-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 (Paria et al. 1998a). These coordinated actions of progesterone and estrogen are crucial for the regulation of the window of implantation.

One major step in the process of implantation is the attachment of the blastocyst trophectoderm with the uterine luminal epithelium. This occurs within a narrow time frame concurrent with an intimate two-way dialog that occurs between the implantation-competent blastocyst and the receptive uterus. In mice, this attachment reaction is initiated around midnight on day 4 of pregnancy (Das et al. 1994).

However, elimination of preimplantation estrogen secretion by ovariectomy on the morning of day 4 results in implantation failure with blastocyst dormancy within the quiescent uterine lumen (McLaren 1971; Yoshinaga and Adams 1966). This condition is referred to as delayed implantation and can be maintained for many days by continued progesterone treatment. However, implantation with blastocyst activation is rapidly initiated by a single injection of estrogen (McLaren 1971; Yoshinaga and Adams 1966). This physiologically relevant delayed implantation model has been widely used to identify signaling pathways mediating embryo–uterine cross-talk during implantation. Endocannabinoid signaling has also recently been shown to participate in embryo–uterine interactions during implantation.

Our group has found that lower levels of AEA and CB_1 receptors are beneficial for implantation. AEA levels have been measured in both receptive and nonreceptive uteri, with the former having lower levels of AEA compared with the latter (Schmid et al. 1997). In vitro experiments also show that natural, synthetic or endogenous cannabinoids inhibit preimplantation embryo development and blastocyst zona-hatching in culture, whereas blastocysts exposed to low levels of AEA show accelerated trophoblast differentiation and outgrowth (Paria et al. 1995, 1998b; Schmid et al. 1997). 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 (Paria et al. 2001). Immunostaining confirmed that CB_1 is lower in activated blastocysts compared to dormant blastocysts (Paria et al. 2001; Wang et al. 2003). Collectively, these results show that the coordinated down-regulation of blastocyst CB_1 and uterine AEA levels are critical in regulating the "window" of implantation by synchronizing trophoblast differentiation and uterine preparation to the receptive state.

Concurrent with this tenet, higher levels of *nape-pld* mRNA and NAPE-PLD activity are found in nonreceptive uteri and in interimplantation sites, compared to implantation sites and receptive uteri (Guo et al. 2005; Wang et al. 2007). It is interesting that FAAH expression and activity show the inverse relationship: higher FAAH expression and activity are observed at implantation sites and in receptive uteri. Some evidence points to 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" (Guo et al. 2005; Maccarrone et al. 2004). 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.

Because of the biphasic action of AEA during embryo implantation, several studies have begun to unravel the underlying mechanism(s) for this by delineating potential signaling pathways coupled with CB_1 (Fig. 4). These studies have found that, under different AEA concentrations, endocannabinoid signaling mediated by embryonic CB_1 is coupled with specific downstream signaling pathways.

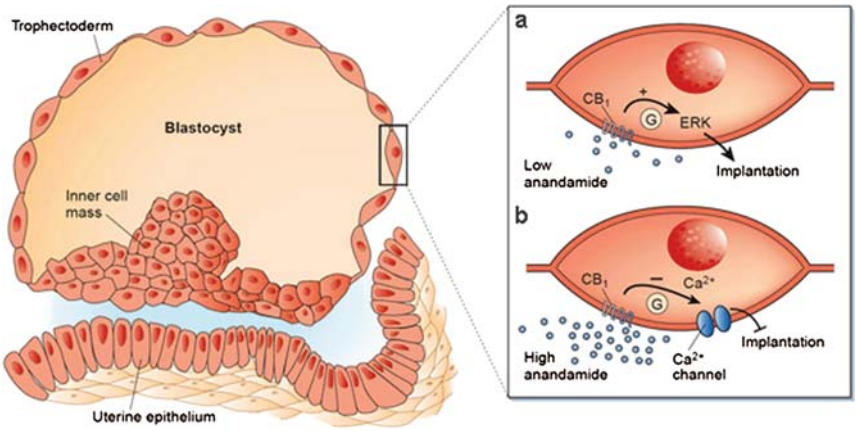

Fig. 4 Influence of cannabinoid/endocannabinoid signaling in embryo implantation in mice. (**a**) At low concentrations, anandamide activates CB_1 displayed on the surface of trophectoderm cells, stimulating ERK and facilitating implantation; (**b**) at higher concentrations, anandamide engages a second CB_1-dependent pathway, which inhibits the activity of voltage-operated N-type calcium channels, reduces calcium entry, and blocks implantation. CB_1 indicates brain-type cannabinoid receptor. This figure is adapted from Piomelli (2004)

For instance, AEA induces stimulatory and inhibitory effects on blastocyst function through ERK and Ca^{2+} signaling pathways, respectively. While AEA at a low concentration (7 nM) activates ERK signaling via CB_1, higher AEA levels (28 nM) fail to activate ERK, but instead inhibit Ca^{2+} mobilization (Wang et al. 2003).

4 Conclusion

In this review, we present molecular, genetic, physiological, and pharmacological studies describing roles of cannabinoid/endocannabinoid signaling that is operative during early pregnancy events. Studies in mouse models demonstrate that under normal physiological conditions, endocannabinoid signaling through CB_1 is crucial to development of embryos and their oviductal transport, as well as their homing and implantation in the receptive uterus. Either silenced or overwhelming endocannabinoid signaling derails these processes. A considerable amount of early pregnancy loss occurs due to either preimplantation embryonic death or implantation failure resulting from asynchronous embryonic development and failure of the uterus to differentiate to the receptive stage (Wilcox et al. 1988). Therefore, our findings in mice raise concerns not only for women of reproductive age who chronically abuse marijuana, but for those who use marijuana or other endocannabinoid system-oriented drugs for medicinal purposes. In addition, studies described here raise caution against the use of CB_1 antagonists to treat obesity in humans,

since there is evidence that women with elevated peripheral AEA levels have spontaneous pregnancy loss (Maccarrone et al. 2000, 2002). Future studies need to be directed towards endocannabinoids' roles in placentation and parturition, since early pregnancy often influences the later developmental processes, ultimately determining the success of pregnancy.

Acknowledgements The authors thank Susanne Tranguch for her critical reading of the manuscript. This work was supported in part by NIH grants DA06668, HD12304, and P01-CA-77,839. S. K. Dey is a recipient of Method to Extend Research in Time (MERIT) Awards from the National Institute on Drug Abuse (NIDA) and the National Institute of Child Health and Human Development (NICHD).

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