REPRODUCTION
REVIEW

Endocannabinoid system and pregnancy

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Abstract

The endocannabinoid system (eCS), is a complex system, comprising the main endogenous ligands anandamide and 2-arachidonoyl glycerol, the cannabinoid receptors CB1 and CB2 and the biosynthetic and degrading enzymes. Cumulative evidence shows that the eCS plays an important role in reproduction, from egg fertilization to parturition. Therefore, alterations in this system, either by recreation/therapeutic use of cannabis or deregulation of the endogenous cannabinoids, might lead to adverse pregnancy outcomes, including retardation in embryo development, poor blastocyst implantation, inhibition of decidualization, miscarriage and compromised placentation. Nevertheless, the molecular mechanisms by which the eCS participates in different stages of pregnancy remain poorly understood. In this review, we will examine the evidence from animal and human studies to support the role of the eCS in implantation, early-to-late pregnancy and placentation as well as the difficulties of targeting this system for treatment of female infertility.

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The endocannabinoid system

Humans have been consuming cannabis since prehistory, not only for religious/spiritual or hedonic purposes but also for its purported medicinal effects. However, for a long time, research on active principles of cannabis remained elusive. It was not until the mid-1960s that Mechoulam and Gaoni (1965) isolated and characterized the main psychoactive component of *Cannabis sativa*, Δ^9-tetrahydrocannabinol (THC). Due to its lipophilic nature, it was first believed that THC exerted its psychoactive effects by nonspecific mechanisms. However, in the late 1980s, Devane and coworkers (1988) identified a specific binding site for cannabinoids in the rat brain; a couple of years later, Matsuda and coworkers (1990) cloned the first cannabinoid receptor, which is now known as type 1 cannabinoid receptor (*Cb1*). Shortly after this discovery, a second cannabinoid receptor was identified by sequence homology and was named as type 2 cannabinoid receptor (*Cb2*) (Munro *et al.* 1993). Initially, it was presumed that CB1 was expressed mainly in the central nervous system (CNS) and CB2 in the periphery. However, today it is well established that both receptors are expressed in the CNS and peripheral tissues. CB1 and CB2 belong to the superfamily of 7-transmembrane G protein-coupled receptors (GPCRs). Activation of both receptors results in the inhibition of cyclic AMP (Matsuda *et al.* 1990, Slipetz *et al.* 1995), activation of mitogen-activated protein kinase (MAPKs) signaling pathways (Bouaboula *et al.* 1995, 1999, Sánchez *et al.* 1998, Wang *et al.* 2003, Correa *et al.* 2005, 2009, Franklin & Carrasco 2013), regulation of Ca^{2+} channels and inwardly rectifying K^+ currents (Caulfield & Brown 1992, Mackie & Hille 1992, Gebremedhin *et al.* 1999, Begg *et al.* 2003, Wang *et al.* 2003) and activation of phospholipase C (Zoratti *et al.* 2003). Cumulative evidence suggests the existence of a putative third type of cannabinoid receptor ('CB3'), called GPR55 (Ryberg *et al.* 2007, Sharir & Abood 2010). Interestingly, and adding complexity to the pharmacology of cannabinoids, it has been shown that these compounds might elicit response in other receptors, such as transient receptor potential vanilloid 1 (TRPV1), peroxisome proliferator-activated receptors (PPARs) and GPR119 (Huang *et al.* 2002, Sun *et al.* 2006, Syed *et al.* 2012).

The first endogenous ligand for cannabinoid receptors, arachidonylethanolamide (AEA), was isolated in 1992 by the efforts of Dr. Mechoulam's lab, and it was named 'anandamide' (Devane *et al.* 1992). Not long after that, a second endogenous ligand, 2-arachidonoyl glycerol (2-AG), was discovered (Mechoulam *et al.* 1995, Sugiura *et al.* 1995). AEA and 2-AG are the best characterized and most studied endogenous cannabinoids or 'endocannabinoids'. Nonetheless, many other endocannabinoids have been identified, such as *O*-arachidonoyl ethanolamine (virodhamine) (Porter 2002), 2-arachidonoyl glycerol ether (noladin ether) (Hanus *et al.* 2001) and *N*-arachidonoyl-amino acids such as *N*-arachidonoyl-dopamine (NADA)

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(Huang *et al.* 2002). However the physiological role of these and other naturally occurring endocannabinoids have not been extensively studied.

Endocannabinoids are not stored in vesicles but synthesized *de novo* upon demand via the hydrolysis of cell membrane lipid precursors (Fig. 1). The canonical biosynthetic pathway for AEA is the sequential action of *N*-acyltransferase (NAT) and *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (Di Marzo *et al.* 1994, Sugiura *et al.* 1996). For 2-AG, the main biosynthetic pathway involves the hydrolysis of phosphatidylinositol by phospholipase C (PLC), producing 1,2-diacylglycerol (DAG), which is then converted into 2-AG by a diacylglycerol lipase α/β (DAGL) (Prescott & Majerus 1983, Sugiura *et al.* 1995).

Once released, the endocannabinoids bind to CB1 and/or CB2 receptor to elicit their effect. Endocannabinoid-mediated signaling is terminated by their fast catabolism, mainly through the action of hydrolytic enzymes. AEA is rapidly degraded by the fatty acid amide hydrolase (FAAH) (Cravatt *et al.* 1996), whereas 2-AG is inactivated by the monoacylglycerol lipase (MAGL) (Dinh *et al.* 2002). However, alternative pathways have been described. Thus, AEA and 2-AG can be oxidized by the cyclooxygenase-2 (COX-2), distinct lipoxygenases (LOXs) or cytochrome P450 (CYP) into metabolic products, which produce biological effects by cannabinoid receptor-independent mechanisms (Yu *et al.* 1997, Burstein *et al.* 2000, Kozak *et al.* 2002, Urquhart *et al.* 2015). Again, there is a paucity of data on the pathophysiological role of these metabolic products and their mechanisms of action.

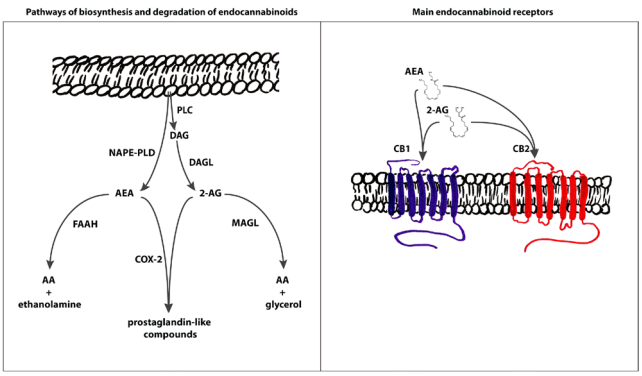

Figure 1 Schematic representation of components of the endocannabinoid system with the postulated pathways of biosynthesis and degradation of anandamide (AEA) and 2-arachidonylglycerol (2-AG), as well as their site of action. AA, arachidonic acid; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; COX-2, cyclooxygenase type 2; DAG, diacylglycerol; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAPE-PLD, N-acylphosphatidylethanolamine-specific phospholipase D; PLC, phospholipase C.

The endocannabinoid system in reproductive tissues

Clinical observations have reported that heavy cannabis users present altered parameters associated with a diminished fertility capacity and reproductive failure, such as decreased plasma testosterone levels, reduced sperm count, alterations in sperm motility and capacitation, embryotoxicity and fetal abnormalities as well as early pregnancy termination (reviewed by Wang *et al.* 2006*a*). Moreover, a reduced success rate of *in vitro* fertilization is reported in cannabis consumers (Klonoff-Cohen *et al.* 2006). These observations prompted researchers to investigate the presence and function of components of the eCS in both male and female reproductive tissues. Thus, it has been shown that *N*-acylethanolamines are present in rodent and human reproductive fluids (Schuel *et al.* 2002) and that the endometrium expresses higher levels of AEA than any other reproductive tissues (Das *et al.* 1995). Different lines of research have demonstrated the presence of the entire active eCS (NAPE-PLD, CB1, CB2 and FAAH) in rodent and in human ovarian tissue (El-Talatini *et al.* 2009*a*), oviduct (Wang *et al.* 2006*b*), uterus (Das *et al.* 1995, Paria *et al.* 2001, Scotchie *et al.* 2015) and testis (Cobellis *et al.* 2006, Battista *et al.* 2008). In male reproductive tissues, CB1 is expressed in germ cells, from spermatogonia to spermatozoa (Rossato *et al.* 2005), and Leydig cells (Gye *et al.* 2005), while CB2 is expressed in Sertoli cells (Maccarrone *et al.* 2003*a*). In female reproductive tissues, CB1 and CB2 receptors have been detected in oocytes at all stages of maturation, whereas NAPE-PLD and FAAH expressions are high in growing secondary and tertiary follicles and corpora lutea and albicantia (El-Talatini *et al.* 2009*b*). Furthermore, AEA levels in human follicular fluid correlates with oocyte maturation (El-Talatini *et al.* 2009*a*).

It is well established that plasma AEA levels fluctuate with the natural menstrual cycle, with the highest levels during the follicular phase (Lazzarin *et al.* 2004, El-Talatini *et al.* 2010). Interestingly, the expression and distribution of FAAH, NAPE-PLD, CB1 and CB2 in female reproductive tissues also vary with the menstrual cycle (reviewed in Di Blasio *et al.* 2013), suggesting that the eCS expression is under hormonal control. Indeed, progesterone and estrogen are shown to regulate the AEA levels by modulating the uterine expression of FAAH (Maccarrone *et al.* 2000*a*). Conversely, gonadotrophin secretion is regulated by endocannabinoids via activating CB1 receptors localized at the preoptic area of the hypothalamus (Park *et al.* 2004). At this level, cannabinoids negatively regulate the activity of gonadotrophin-releasing hormone (GnRH)-secreting neurons (Gammon *et al.* 2005). However, CB1 receptors are also present at the pituitary level, suggesting a localized effect of cannabinoids on luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Wenger *et al.* 1995).

A cannabinoid-induced downregulation of GnRH, LH and/or FSH secretion might explain the reduced plasma testosterone levels and decreased sperm count found in heavy marijuana consumers (Whan *et al.* 2006).

Despite the amount of data available, the precise role of the eCS in male and female fertility remains poorly understood and conflicting data have been reported, suggesting the need for further research of the physiological role of the eCS in reproductive organs (reviewed by Karasu *et al.* 2011). Here, we will focus on the physiological and pathological roles of the eCS throughout pregnancy.

The endocannabinoid system during implantation

Implantation is the very early stage of pregnancy at which the blastocyst adheres and invades the wall of the uterus. The process of implantation implies a highly synchronized and complex bidirectional communication between the conceptus and the luminal endometrial cells, and it could be divided in three stages: apposition, adhesion and penetration. On contact with the blastocyst, the underlying area of contact in the stroma becomes increasingly vascularized and edematized. This process is called 'primary decidualization reaction'.

The endocannabinoid system plays an important role during the process of implantation. Studies in mice have shown that a delicate balance between anandamide (AEA) synthesis (driven mainly by NAPE-PLD) and degradation (mainly by FAAH) is necessary to ensure an appropriate 'AEA tone' during implantation (Paria *et al.* 1996, Guo *et al.* 2005). Indeed, it has been shown that too high and too low levels of AEA are deleterious for pregnancy (Sun & Dey 2009). Accordingly, *in vitro* experiments have demonstrated that high levels of AEA inhibit the development of 2-cell embryos into blastocysts and the lysis of the zona pellucida (a process known as 'zona hatching') as well as the trophoblast differentiation (Schmid *et al.* 1997, Wang *et al.* 2006*b*). Conversely, *in vitro* low levels of AEA accelerate the trophoblast differentiation and outgrowth. Interestingly, CB2 is present as early as 1-cell stage through blastocyst stage, whereas *Cb1* mRNA is only detected from 4-cell stage onward (Paria *et al.* 1995).

On the maternal side, AEA and 2-AG are produced by the murine uterus, and their levels are tightly regulated by the NAPE-PLD/FAAH and DALGα/MAGL ratios respectively (Guo *et al.* 2005, Wang *et al.* 2007, Fonseca *et al.* 2010*a*). Thus, DALGα and MAGL are distributed during the implantation process in a way that minimizes the excessive exposure to high levels of 2-AG (Wang *et al.* 2007, Fonseca *et al.* 2010*b*). Nevertheless, the role of 2-AG during different stages of pregnancy is not completely understood. On the other hand, Guo and coworkers (2005) have shown that on days 1–4 of pregnancy in mice, NAPE-PLD is highly expressed in the luminal and glandular epithelium of the uterus compared with the stroma, suggesting that the epithelium is the major source of endocannabinoids before implantation. Nevertheless, on days 5–7 of pregnancy, NAPE-PLD is expressed only by luminal and glandular epithelial cells in the interimplantation sites together with a very low expression of this enzyme in the sites of blastocyst implantation. The opposite is true for uterine FAAH expression; its levels are higher at implantation sites and lower at interimplantations sites (Wang *et al.* 2007). As a result, higher levels of AEA are present in the interimplantation than at implantation sites (Paria *et al.* 2001, Guo *et al.* 2005) (Fig. 2). It has been hypothesized that the embryo plays a role in regulating the uterine endocannabinoid tone by releasing, a yet to be characterized, 'FAAH activator' that decreases the uterine NAPE-PLD/FAAH ratio, reducing AEA levels at the implantation site (Maccarrone *et al.* 2004). Accordingly, it has been shown that lysophosphatidic acid induces the expression of FAAH during the implantation window (day 5 of gestation) in rats (Sordelli *et al.* 2012*a*) and that the presence of the embryo is required for AEA action on uterine nitric oxide synthase activity (Sordelli *et al.* 2011). Moreover, to minimize the exposure to toxic levels of AEA, the blastocyst approaching implantation regulates its expression of CB1. Thus, it has been shown that blastocysts collected from the uterus on the morning of day 4 of pregnancy expressed higher levels of AEA

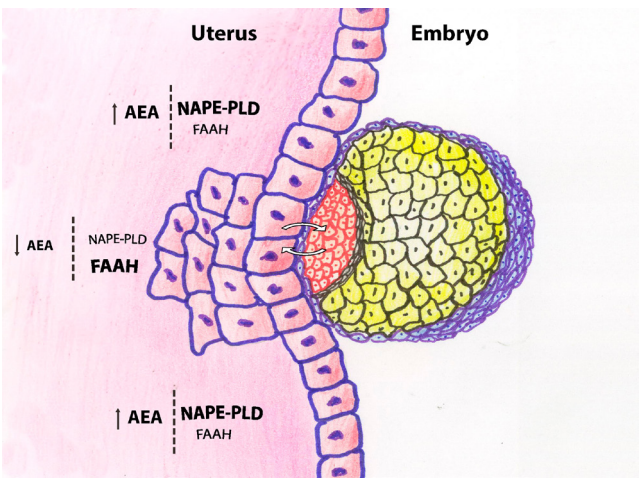

Figure 2 Schematic representation of the feto–maternal interface and the role of the endocannabinoid signaling during implantation according to rodent and human studies (adapted and modified from Fonseca *et al.* 2013*a*). The process of implantation requires a highly synchronized and complex bidirectional communication between the conceptus and the endometrium. The endocannabinoid system (eCS) plays an important role during the process of implantation. A delicate balance between anandamide (AEA) synthesis (driven mainly by NAPE-PLD) and degradation (mainly by FAAH) is necessary to ensure an appropriate 'AEA tone' during implantation. The implantation site presents low levels of AEA by highly expressing FAAH together with a low expression of NAPE-PLD. In contrast, the adjacent tissue presents high levels of AEA by expressing high levels of NAPE-PLD and low levels of FAAH.

binding sites compared with blastocysts recovered on the evening of day 4, before the attachment reaction (Paria *et al.* 2001). It has been reported that activated blastocysts show higher expression of CB1 than dormant blastocysts (Wang & Dey 2005). Thus, AEA levels are tightly regulated in human pregnancy, showing no variations during the first and second trimesters (Lam *et al.* 2008). Conversely, elevated serum levels of AEA are found in women with nonviable first-trimester pregnancy compared with those in women with viable pregnancies (Taylor *et al.* 2011). Similarly, Habayeb and coworkers (2008*a*,*b*) found higher plasma levels of AEA in women prone to miscarriage compared with those in the normal birth group. Contrariwise, Tong and coworkers (2012) found no differences between the plasma levels of AEA of asymptomatic women at 6–10 weeks of gestation who miscarried and those who did not. Nevertheless, Maccarrone and coworkers (2003*a*,*b*) have shown that high levels of AEA are correlated with low levels of progesterone, which is associated with implantation failure and/or abnormal development of the feto–maternal interface that eventually leads to miscarriage. Indeed, progesterone has been shown to stimulate FAAH activity in human peripheral lymphocytes (Maccarrone *et al.* 2001*a*), with these cells playing a crucial role during human embryo implantation. Lipopolysaccharide (LPS), the main component of Gram-negative bacteria frequently associated with maternal infection and pregnancy loss, not only reduces FAAH activity and increases the production of AEA in human lymphocytes (Maccarrone *et al.* 2001*b*) and murine macrophages (Liu *et al.* 2003), but also causes a serum progesterone withdrawal in 7-day pregnant mice (Aisemberg *et al.* 2013) – changes that were associated with miscarriage. Data from our laboratory have shown that progesterone supplementation to LPS-treated pregnant mice restores FAAH activity in murine peripheral mononuclear cells (Wolfson *et al.* 2013). Accordingly, Maccarrone and coworkers (2000*b*) reported that FAAH activity in human peripheral lymphocytes was lower in women who had spontaneous miscarriages than in those who did not. In fact, increased AEA levels are also found in the peripheral blood of women with ectopic pregnancy together with a reduced FAAH activity in peripheral lymphocytes (Gebeh *et al.* 2013). Similarly, women subjected to *in vitro* fertilization (IVF) or intracytoplasmatic sperm injection (ICSI) and become pregnant show low levels of serum AEA at the time of implantation in comparison with those who did not (El-Talatini *et al.* 2009*a*). Furthermore, *Faah*$^{-/-}$ mice, which have higher oviductal AEA levels also show impaired embryo transport through the oviduct (Wang *et al.* 2006*b*). This seems to be due to maternal and not embryonic defects because the embryo can implant in day 4 pseudopregnant uteri (Wang *et al.* 2004, Sun & Dey 2008). Interestingly, *Faah*$^{-/-}$ embryo exhibits retarded development (Wang *et al.* 2006*b*, Xie *et al.* 2012).

Regarding the expression of cannabinoid receptor during the implantation stage, it is shown that both CB1 and CB2 are expressed in nonpregnant human endometrium (Taylor *et al.* 2010) as well as in rat endometrium during early pregnancy (Fonseca *et al.* 2009). A reduced expression of CB1 in fallopian tubes is associated with ectopic pregnancy (Horne *et al.* 2008). It has been proposed that CB1 plays an important role in embryo transport because *Cb1*$^{-/-}$ and *Cb1*$^{-/-}$*Cb2*$^{-/-}$ but not *Cb2*$^{-/-}$ mice show oviductal retention (Wang *et al.* 2004), suggesting that the lack of CB1 is responsible for this phenomenon. Moreover, wild-type mice pharmacologically treated with CB1 antagonist, but not CB2 antagonists, also show high rates of embryo retention in the oviduct.

The endocannabinoid system during early pregnancy

Once the blastocyst comes in contact with the endometrial layer of the uterus, the endometrial stromal cells undergo a series of morphological changes (known as decidualization), which prepare the uterus for trophoblast invasion.

In humans, decidualization happens in the normal menstrual cycle during the luteal phase (Salamonsen *et al.* 2003). Interestingly, during the luteal phase, the levels of plasma AEA are lower than those in the follicular phase (Habayeb *et al.* 2004), which suggest that steroid hormones are involved in the regulation of AEA levels in human pregnancy. However, in rodents, decidualization only occurs when the blastocyst is in close contact with the endometrium (Fonseca *et al.* 2012), with stromal cells proliferating and differentiating into decidual cells. Abnormalities during the process of decidualization are associated with increased risk of pregnancy complications, miscarriage, preeclampsia, fetal growth restriction and preterm labor (Fonseca *et al.* 2013*a*).

Several studies have provided evidence of the expression of CB1, CB2, NAPE-PLD and FAAH in decidua of women with viable pregnancies (Habayeb *et al.* 2004) as well as in rodents (Fonseca *et al.* 2009, 2010*a*, Taylor *et al.* 2011) suggesting the role of the endocannabinoid system in the process of decidual differentiation and remodeling. Fonseca *et al.* (2009) have shown that CB1 expression is markedly upregulated during midpregnancy in rats, a period of maximal decidual development. After reaching its maximum development, the decidua undergoes regression with apoptosis playing a crucial role (Gu *et al.* 1994, Dai *et al.* 2000). Pharmacological activation of CB1 receptor with the synthetic cannabinoid WIN 55,212-2 inhibits the induction of human decidual cell differentiation and induces apoptosis by a cAMP-dependent mechanism (Moghadam *et al.* 2005). Thus, AEA and 2-AG were

described as proapoptotic lipids in primary rat decidual cell cultures (Fonseca *et al.* 2009, 2010*b*). Therefore, an 'endocannabinoid tone' needs to be tightly maintained to avoid the toxic effects on the changing decidua. Low levels of AEA induce morphological and molecular changes characteristic of an apoptotic cell death, whereas higher concentrations are associated with an increased release of lactate dehydrogenase, characteristic of a necrotic process (Fonseca *et al.* 2009). Interestingly, these effects were reversed by blocking CB1, but not CB2 or TRPV1. Activation of CB1 results in *de novo* synthesis of ceramide and signaling via p38 MAPK phosphorylation, which leads to mitochondrial stress and ROS production and subsequently, apoptosis (Fonseca *et al.* 2013*b*). The fact that increased levels of AEA exert toxic effects on decidua via activation of CB1 receptor is corroborated by our observation that *Cb1*$^{-/-}$ mice are resistant to LPS-induced early embryo loss and presented a greater number of pups per litter (Wolfson *et al.* 2015). Conversely, Horne and coworkers (2008) have found that women with ectopic pregnancy show low decidual expression of CB1, and Wang and coworkers (2004) reported impaired oviductal transport in *Cb1*$^{-/-}$ mice. Therefore, a lack of expression of CB1 might be beneficial in protecting the pregnancy from inflammation but might predispose to other pathologies. The role of CB2 on decidual cells is, however, much less clear. It has been reported that high concentrations of AEA induces, via a CB2-dependent mechanism, inhibition of proliferation of the human choriocarcinoma cell line BeWo, a model of first-trimester trophoblast (Trabucco *et al.* 2009). We have shown that high levels of AEA stimulate nitric oxide (NO) synthesis on murine decidua in response to LPS and that these effects were abrogated by blocking either CB1 or CB2 (Vercelli *et al.* 2009). Moreover, AEA and its nonhydrolyzable analog, methanandamide, modulate the LPS-induced synthesis of prostaglandins in uterine explants from pregnant mice (Vercelli *et al.* 2012). Furthermore, LPS administration to 7-day pregnant mice induces a decrease in decidual FAAH activity (Wolfson *et al.* 2015). Our observations point toward the endocannabinoid system as a mediator of the deleterious effects of LPS in reproductive events.

As it has been previously mentioned, FAAH is the main catabolic enzyme for the degradation of AEA into arachidonic acid and ethanolamine, providing a source for prostaglandin synthesis. As decidual FAAH might be downregulated during maternal infections (Wolfson *et al.* 2015), AEA could be a direct substrate to COX-2 oxidative metabolism to produce prostaglandin-like compounds called prostaglandin-ethanolamides or 'prostamides' (PMs). Indeed, Almada and coworkers (2015) have recently shown that AEA is metabolized into prostamide-E2 (PME2) in rat decidual cells when FAAH is inhibited. PME2 mimics the proapoptotic actions of AEA on decidual cells (Almada *et al.* 2015). Prostamides are up to 3-fold higher in AEA-treated tissues from *Faah*$^{-/-}$-mice compared with their wild-type counterparts (Weber *et al.* 2004). These findings provide evidence that low FAAH activity resulting in increased prostamide concentrations might have a deleterious impact on pregnancy.

Interestingly, the interaction between AEA and COX-2 seems to be far more complex because several reports indicate that AEA is capable of modulating prostaglandin production. Thus, in the amnion, AEA acts via CB1 to increase PGE_2 levels (Mitchell *et al.* 2008), whereas it exerts opposite effects on uterine PGE_2 and $PGF_{2\alpha}$ biosynthesis, by inhibiting PGE_2 production and increasing $PGF_{2\alpha}$ levels (Vercelli *et al.* 2012). Similarly, COX-2-derived prostaglandins mediate the inhibitory effect of AEA on NOS activity in the receptive rat uterus (Sordelli *et al.* 2012*b*). The endocannabinoid system appears to be at the center of an intricate biochemical cross talk that further complicates the understanding of its role in the physiology and pathology of pregnancy.

Alterations in endocannabinoid signaling are associated with early pregnancy loss, although disparate results have been reported. No differences were found in the expression of NAPE-PLD in the trophoblast, mesenchymal core or placental decidua from spontaneous miscarriages compared with placentae from elective surgical termination (Taylor *et al.* 2011). Contrarily, Trabucco and coworkers (2009) have found a 2-fold increase in *Nape-pld* mRNA expression in the first trimester (weeks 9–12) placentae from elective abortions than in that from spontaneous miscarriage. This observation would suggest that before a miscarriage, there are low levels of AEA in placenta, which is in stark contrast to the reports that women at high risk of miscarriage present high plasma levels of AEA (Habayeb *et al.* 2004, 2008*a*, Taylor *et al.* 2011). On the other hand, Tong and coworkers (2012) have failed to find differences in plasma AEA levels between women with normal pregnancy and women who subsequently miscarried. Conversely, it has been found that FAAH is more abundant in the decidual stromal cells of women with recurrent miscarriage than in the placenta from normal pregnancies (Chamley *et al.* 2008). No differences, however, have been found in FAAH expression on trophoblast cells from women with normal pregnancy and recurrent miscarriage (Chamley *et al.* 2008). Collectively, these observations suggest that the expression of the endocannabinoid system is time and tissue specific and that alterations in this system are associated with early pregnancy loss.

Endocannabinoid system during placentation

Once the peak phase of decidualization ends, placentation begins to establish maternal–fetal circulation. The placenta requires a proper number and distribution of the different trophoblastic cells to perform its physiological functions. Changes in trophoblast

proliferation, differentiation and/or distribution lead to an abnormal placentation.

The major endocannabinoids AEA and 2-AG are detected in murine placentae. Indeed, *Faah*$^{-/-}$ mice, but not *Cb1*$^{-/-}$ mice, show high levels of placental AEA, supporting the hypothesis that FAAH is the major metabolic gatekeeper of AEA levels. Immunohistochemical analysis has shown that FAAH and CB1 are expressed on ectoplacental cone (EPC) on day 10 of pregnancy and the spongiotrophoblast cells (SPT) layer on day 14 (Sun *et al.* 2010). Moreover, CB1 and FAAH are shown to be expressed in human amniotic epithelial cells, chorionic cytotrophoblasts (Park *et al.* 2003) and syncytiotrophoblasts (Habayeb *et al.* 2008*b*). AEA metabolic enzymes are also expressed during human placental development (Chamley *et al.* 2008, Taylor *et al.* 2011), and the whole eCS is shown to be present in murine placentae (Sun *et al.* 2010). Different lines of research have provided evidence that altered expression of NAPE-PLD, CB1 and FAAH in the first-trimester placentae and high levels of AEA are associated with an endangered pregnancy outcome (Park *et al.* 2003, Trabucco *et al.* 2009, Meccariello *et al.* 2014). Altered levels of AEA influence trophoblast invasion (Sun *et al.* 2010) and proliferation (Xie *et al.* 2012). Furthermore, higher plasma levels of AEA are detected in women with nonviable pregnancies (Taylor *et al.* 2011). However, in rats, at the peak of decidua development, high NAPE-PLD activity increases AEA levels, which are involved in decidua remodeling (Fonseca *et al.* 2013*a*,*b*, 2014). Notwithstanding, FAAH activity prevails during placentation (Fonseca *et al.* 2014), giving further support to the hypothesis that this enzyme is the key regulator of AEA levels (Fig. 3).

Placentation is shown to be compromised in *Cb1*$^{-/-}$ mice. These animals show lower placental weight on days 12 and 14 than WT mice (Sun *et al.* 2010) and show a retarded development of the SPT layer. Abnormal endocannabinoid signaling affects the proliferation of trophoblast progenitor cells in the EPC, which later differentiate into SPT cells (Sun & Dey 2012). *Cb1*$^{-/-}$ trophoblast progenitor cell proliferation is much reduced, whereas *Faah*$^{-/-}$ trophoblast progenitor cell proliferation is only mildly reduced (Sun & Dey 2012). Accordingly, trophoblast stem cells (TSCs) from WT mice treated with methanandamide show a faster proliferation rate, which is attenuated by the selective antagonism of CB1 (Sun *et al.* 2010). In contrast, other reports show that AEA not only prevents BeWo trophoblast cell proliferation in a dose-dependent manner (Habayeb *et al.* 2008*b*) but also induces apoptosis (Costa *et al.* 2015). Similar to AEA, Costa and coworkers (2014) have described the expression of 2-AG metabolic enzymes in human cytotrophoblasts and in BeWo cells. Furthermore, 2-AG has been shown to induce apoptosis in these cells via a CB2 receptor-dependent mechanism (Costa *et al.* 2014).

Interestingly, *Cb1*$^{-/-}$ and *Faah*$^{-/-}$ placentae show an abnormal differentiation of trophoblast precursor cells, which are more prone to differentiate into trophoblast giant cells (TGCs) in detriment of the spongiotrophoblast cells (Sun *et al.* 2010). Reciprocal embryo transfer experiments between *Cb1*$^{-/-}$ and WT mice have shown that there is a lack of CB1 in the embryo that provokes this biased trophoblast differentiation (Sun *et al.* 2010). Pharmacological blockade of CB1 receptor on days 8 and 9 of pregnancy, when placentation begins, further confirms the previous observation. Indeed, treatment with SR14716A (a CB1 antagonist) reduces the number of SPT cells in WT placentae. Furthermore, invasive capacity of the trophoblast cells of the maternal decidual zone to reshape and redirect the maternal blood vessels to support embryo growth is critical for a successful pregnancy (Cross *et al.* 2002). It has been shown that the invasion of glycogen trophoblast cells

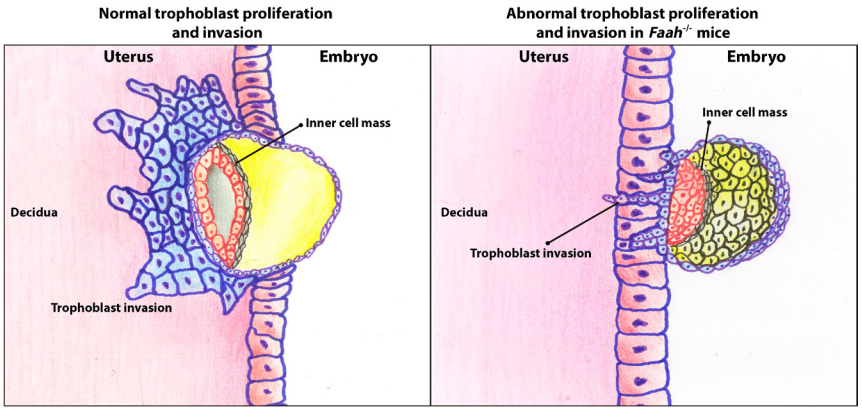

Figure 3 Schematic representation of the feto–maternal interface and the role of the endocannabinoid signaling during trophoblast proliferation and invasion according to studies in FAAH-deficient mice (adapted and modified from Fonseca *et al.* 2013*a*). Trophoblast proliferation and invasion are compromised in *Faah*$^{-/-}$ mice suggesting an important role of the endocannabinoid system in these processes. Placentae from FAAH$^{-/-}$ mice show an abnormal differentiation of trophoblast precursor cells, which are more prone to differentiate into trophoblast giant cells (TGCs) in detriment of the spongiotrophoblast cells (SPT). This biased trophoblast differentiation compromises the invasive capacity of these cells to reshape and redirect the maternal blood vessels to support embryo growth, representing a risk for the pregnancy outcome.

(GTC) into the *decidua basalis* is highly reduced in *Cb1*$^{-/-}$ mice compared with WT mice (Sun *et al.* 2010). These observations further support the hypothesis that an appropriate endocannabinoid signaling is required for the correct placental development and excessively high levels of endocannabinoids are toxic to trophoblast cells, therefore represents a risk factor for the first-trimester miscarriages (Habayeb *et al.* 2008*b*).

Final remarks

With the increasing number of countries decriminalizing, and even legalizing, the recreational use of cannabis, there is a critical need for understanding the effects of this drug in female fertility and the involvement of the eCS in the pathophysiology of the different stages of pregnancy. This is particularly important in the case of women seeking pregnancy who consume cannabis for medical reasons (such as treatment of nausea and vomiting, reduction of symptoms associated with some neurological diseases, reduction of intraocular pressure in glaucoma and treatment of depression and pain). The evidence presented here shows that alterations in the eCS lead to poor pregnancy outcomes: poor blastocyst implantation, inhibition of decidualization, retardation in embryo development, miscarriage and compromised placentation. Although targeting the endocannabinoid system represents an attractive pharmacological strategy to maintain a healthy pregnancy, the intrinsic promiscuity of this system makes it difficult for the development of therapeutic drugs and/or to predict their effects. Overall, the expression/activity of the several components of the endocannabinoid system (NAPE-PLD, CB1, CB2 and FAAH) must be tightly regulated to keep it at physiological levels at every stage of pregnancy, from implantation to parturition, to prevent the negative effects during this period and to assure a healthy pregnancy. As onset of labor approaches, AEA levels increase dramatically. In any case, the enzyme FAAH seems to be the 'metabolic checkpoint' of endocannabinoid activity. Future research efforts should be directed to understand the complex interaction of this system.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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