STUDYING THE ROLE OF ENDOCANNABINOID SIGNALING IN REPRODUCTION

By

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INTRODUCTION

I. Lipid Signaling in Reproduction

Although the human population is growing rapidly, 15% of couples worldwide are infertile (1, 2), with infertility defined as the inability to conceive after one year of regular sexual intercourse. Infertility is a worldwide social and economic concern. Early pregnancy loss in humans often happens due to defects that occur before, during or immediately after implantation. In addition, the implantation rate remains disappointingly low in *in vitro* fertilization and embryo-transfer (IVF-ET) techniques which have overcome several causes of human infertility. Therefore, studying the physiological, genetic and molecular basis 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 on mice and other animal models combined with feasible experiments in humans will generate meaningful information to address these critical issues. Although details of many of the molecular interactions during the periimplantation events have not yet been defined, increasing evidence from gene expression and transgenic mouse studies reveal that synchronous development of preimplantation embryos to the blastocyst stage and differentiation of the uterus to the receptive stage are prerequisites for the initiation of implantation (3-5).

Over the past several years, molecular and genetic studies have provided evidence that lipid mediators, including prostaglandins (PGs) and lysophospholipids, are critical signaling molecules in coordinating events of early pregnancy (6-9). PGs, a major group of eicosanoid lipid mediators, are generated from arachidonic acid (AA), which is released from membrane phospholipids by phospholipase A_2 (PLA_2). AA thus released is transformed by cyclooxygenases (COXs) to PGH, which is then converted to various PGs by specific PG synthases (10). In mice, $PGF_{2\alpha}$ is mainly generated by COX-1 as a luteolytic hormone acting on the corpus luteum, which 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 PGs during pregnancy is further illustrated by poor fertility, resulting from deferred implantation, in mice lacking $cPLA_{2\alpha}$ (12). Lysophosphatidic acid (Lyso-PA) belongs to another group of lipid mediators, lysophospholipids. Lyso-PA influences a range of biological processes through its cell surface G protein-coupled receptors, LPA_{1-4} (32). Mice missing LPA_3, a G protein-coupled receptor of Lyso-PA, exhibit defects remarkably similar to defects in $cPLA_{2\alpha}$-deficient mice, such as deferred on-time implantation, retarded fetal development, embryo crowding, and sharing of one placenta by several embryos (12, 33). More intriguingly, the on-time implantation in LPA_3 deficient females is restored by PG supplementation (33). These studies suggest that appropriate lipid signaling is

indispensable for periimplantation events and that there is a cross-talk interaction between different lipid signaling pathways.

Recently, endocannabinoid signaling, mediated by another group of bioactive lipid molecules, has been highlighted as an important player in male and female reproductive systems. During the past 15 years, research from our laboratory demonstrated that the endocannabinoid system is present in the preimplantation embryo, oviduct and uterus, and revealed that appropriate endocannabinoid signaling is critical to preimplantation embryo development, their timely homing into the receptive uterus and on-time implantation (10-12). My work has added a new dimension to these studies in both male and female reproduction. The following chapters will illustrate these new findings.

II The Endocannabinoid System

Introduction

Marijuana, derived from the plant *Cannabis sativa*, has been used as a recreational drug for thousands of years due to its psychoactive effects including euphoria, sedation and analgesia. Because of its widespread use, research on the chemistry of *Cannabis* began decades ago (13). However, its major active component, Δ^9-tetrahydrocannabinol (Δ^9-THC), was not identified until 1964 (14). This discovery initiated a dramatic interest in cannabinoid research, further increased by the discovery and cloning of two types of cannabinoid receptors,

brain-type (CNR1) (15, 16) and spleen-type (CNR2) (17). Around the same time, several endogenous compounds targeting CNR1 and CNR2 were identified; they are collectively termed endocannabinoids. The two most studied endocannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). The structure of anandamide was first revealed in 1992 (18), while 2-AG was discovered in both the canine gut (19) and the rat brain (20) by two independent groups. All of the endocannabinoids, the enzymes responsible for their biosynthesis and degradation, and their receptors are collectively called the endocannabinoid system (Figure 1). Various aspects of the endocannabinoid system are described below.

AEA synthesis and degradation

It is widely accepted that anandamide is derived from the precursor N-arachidonoylphosphatidylethanolamine (NAPE) through its reaction with NAPE-hydrolyzing phospholipase D (NAPE-PLD) (21, 22), a member of the metallo-lactamase family with Ca^{2+} sensitive enzyme activity (23, 24). However, unaltered polyunsaturated NAE (N-acyl-ethanolamine) levels in NAPE-PLD deficient mice suggests other anandamide synthetic pathways (25). Recently, two other enzymatic routes were identified: 1) double deacylation of NAPE by a phospholipase/lysophospholipase B, α/β-hydrolase 4 (Abh4), to generate glycerophospho-NAE (GP-NAE) which is then cleaved by a phosphodiesterase to liberate anandamide (26), and 2) cleavage of NAPE by a phospholipase C to generate phosphoanandamide (pAEA), which is subsequently dephosphorylated

by a protein tyrosine phosphatase, PTPN22, to release anandamide (27). Although these pathways are found in both the CNS and peripheral tissues, the mechanisms by which these pathways can regulate and affect each other are still unknown.

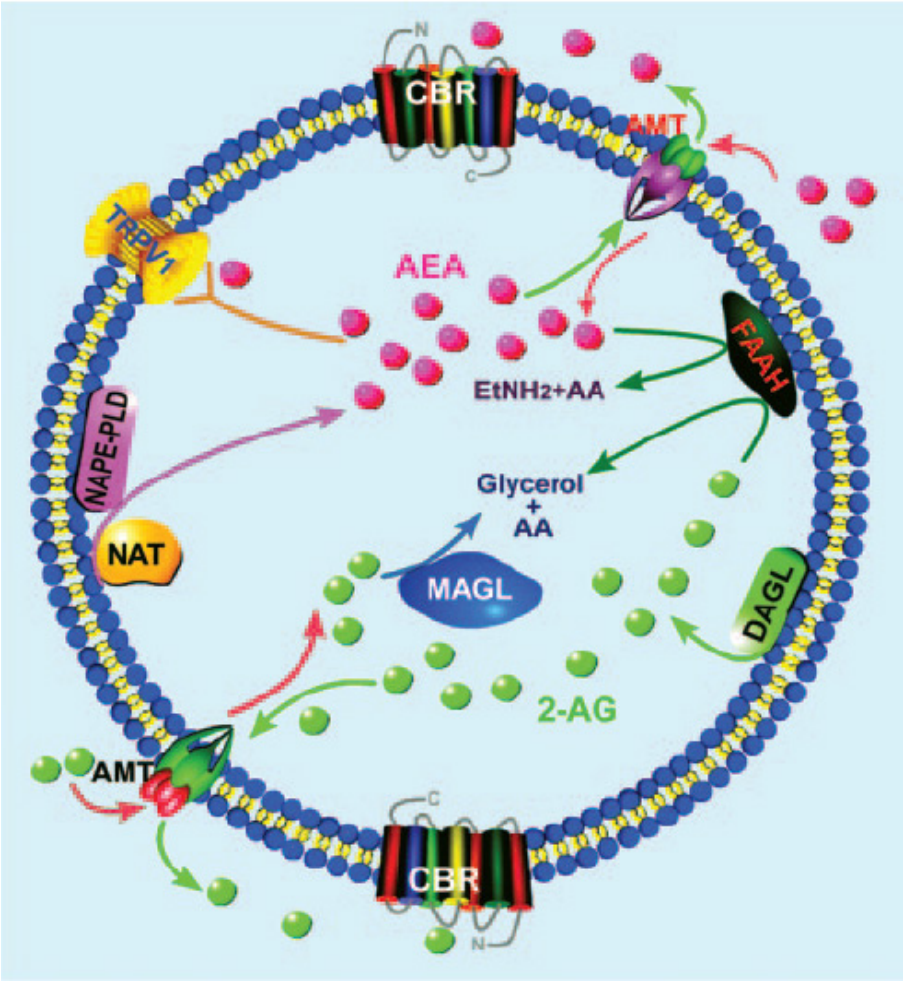

FIGURE 1. **The endocannabinoid system.** The synthesis of AEA from membrane *N*-arachidonoylphosphatidylethanolamines is catalyzed by the sequential activity of NAT and NAPE-PLD, which releases AEA and phosphatidic acid. AEA is transported in both directions through the cell membrane by a selective AMT and, once taken up, is hydrolyzed by FAAH to ethanolamine (EtNH2) and AA. The main targets of AEA are CNR1 and CNR2 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. The picture is adapted from Wang et al, Endo Rev, 27: 427-448, 2006.

Anandamide is degraded to ethanolamine and arachidonic acid (AA) by a membrane-bound fatty acid amide hydrolase (FAAH) (28, 29). FAAH can also hydrolyze other fatty acid amides, including 2-AG and the sleep-inducing substance oleamide (30). FAAH has been shown to be critical for regulating both the magnitude and duration of anandamide and other fatty acid amide signaling (31). Recently, a second membrane-associated fatty acid amide hydrolase has been found in humans and other primate genomes but not in that of the rodent (32).

The transport mechanism of anandamide, an uncharged hydrophobic molecule, across the plasma membrane is still under debate (33, 34). In the current models, the enzymes for the synthesis and degradation of endocannabinoids are thought to be located within the cell, so that the stimulation of cannabinoid receptors by endocannabinoids from the extracellular component requires these lipid mediators to cross the plasma membrane twice. While pharmacological and biochemical evidence points towards the existence of a specific anandamide transport protein using transporter inhibitors (35-38), no direct evidence for such

a transporter has been provided. Recently developed drugs have been shown to inhibit anandamide transport without affecting FAAH activity (39). However, chemical evidence shows that anandamide uptake is not reduced by putative transport inhibitors in FAAH knock-out cells, favoring the model that anandamide traverses the cell plasma membrane by simple diffusion (40). In addition, FAAH may not need a transporter to help reach its substrate anandamide (41).

2-AG synthesis and degradation

2-AG is derived from the precursor diacylglycerol by a membrane-bound sn1-diacylglycerol lipase (DAGL (42). To date, two isoforms of DAGL have been cloned, DAGLα and DAGLβ. The former is found mostly in the adult brain, while the latter is expressed in the developing brain (43). Like anandamide, 2-AG is produced on demand, but they differ in that anandamide often acts only as a partial agonist of cannabinoid receptors, while 2-AG acts as a full agonist. Interestingly, 2-AG's binding affinity to cannabinoid receptors is approximately 24 times less than that of anandamide but under most physiological conditions, 2-AG levels are much higher than anandamide (44). It still remains to be determined, therefore, how only a small percentage of 2-AG (10-20%) crosses the plasma membrane to interact with cannabinoid receptors (45).

2-AG accumulated in cells is degraded by either FAAH or a serine hydrolase, monoacylglycerol lipase (MAGL) (46). MAGL, a 33-KD protein, has been isolated, cloned and characterized in both rats and humans (46-48). Unlike FAAH, MAGL

is localized primarily in the cytosol, but not on the plasma membrane. Recently, using BV-2 cells, a mouse microglial cell line with MAGL activity, Muccioli et al. identified a novel protein which regulates 2-AG levels (49).

Cannabinoid receptors

Endocannabinoids, as well as plant-derived and synthetic cannabinoids, target cannabinoid receptors CNR1 and CNR2. Both CNR1 and CNR2 are G protein-coupled receptors with seven transmembrane domains. CNR1 is present mostly in the central nervous system and in some peripheral tissues including the heart, testis, liver, small intestine and uterus, while CNR2 is abundantly expressed in the spleen and several immune cells, including astrocytes (50-52). Both CNR1 and CNR2 are coupled with G proteins in the $G_{i/o}$ and G_q families, and the activation of cannabinoid receptors has different biological effects that are cell-type dependent. Signals mediated by cannabinoid receptors include regulation of Ca^{2+} channels (53-57), inhibition of adenylyl cyclase (16, 58), activation of phopholipase C (59) and stimulation of mitogen-activated protein kinases (MAPKs) including ERK, JNK and p38 (56, 60, 61).

In addition to CNR1 and CNR2, some evidence indicates the existence of other putative cannabinoid receptors (62). For example, it was shown that anandamide can protect murine neuroblastoma cells subjected to low serum-induced apoptosis by non-CNR1, non-CNR2 receptors (63). Furthermore, a novel cannabinoid receptor 3 (GPR55) has been reported (64, 65), which as of yet, is a

G protein-coupled orphan receptor. The physiological role of this receptor is not clearly understood.

Anandamide, but not 2-AG, can also activate receptors other than CNR1 and CNR2. One receptor that anandamide activates is the transient receptor potential vanilloid 1 (TRPV1) (66), a ligand-gated non-selective cationic channel. The binding of anandamide to its cytosolic binding site on TRPV1 triggers Ca^{2+} influx and eventual cytochrome c release (67, 68).

III. Endocannabinoids and Male Fertility

Introduction

The role of the endocannabinoid system in male fertility remains largely unexplored. Previous studies showed that rat testes are able to synthesize AEA (69), and there is evidence that long term exposure to THC induces male infertility (70). In addition, AEA is also present in human seminal plasma at nanomolar ranges (71), and human sperm fertility is influenced by AEA (72). Furthermore, components of the endocannabinoid system are detected in different cell types in the testis. CNR1 is present in leydig cells (73), which secret the main male hormone testosterone, whereas CNR2 is present in sertoli cells, which support spermatogenesis (74). Although some evidence indicates that testosterone secretion and sertoli cell function are both associated with AEA

levels in the testis, the underlying molecular mechanism of the association is still not known.

The endocannabinoid system in Sertoli cells

Sertoli cells are located within the seminiferous tubes. Before being released into the seminiferous tubes, sperm in different developmental stages have direct contact with sertoli cells, which regulate spermatogenesis by providing nutrients and hormonal signals. In mice, the expression of CNR2 on sertoli cells is consistent throughout the lifespan (74). However, the FAAH activity declines with age. Previous studies showed that sertoli cells undergo apoptosis after being exposed to AEA (74), and that this effect is mediated by receptors other than CNR1, CNR2 and TRPV1. In fact, CNR2 located on sertoli cells can protect the cells from apoptosis. FAAH is regulated by FSH, a hormone critical for fetal and early neonatal sertoli cell proliferation and for regulating spermatogenesis in adult males (75). Additionally, FAAH can modulate the pro-apoptotic effect of AEA on sertoli cells (74) indicating a close interplay between endocannabinoid signaling and hormones. Collectively, these results indicate that the endocannabinoid system is a new potential target to treat male infertility.

The endocannabinoid system in sperm

Endocannabinoid signaling has been shown to regulate sperm function in both invertebrates and mammals (72, 76-79). In sea urchins, sperm synthesize AEA (80) and AEA binds to CB receptors (77). Type-1 cannabinoid receptors and

FAAH are also found in the frog and rat (81). The endocannabinoid system in boars has been characterized by Dr. Maccarrone's group. Boar spermatozoa have the biochemical machinery to synthesize (NAPE-PLD), and degrade (AMT and FAAH) AEA (82). In addition, the targets of AEA, CNR1 and TRPV1 are present in boar spermatozoa. Only a limited number of studies have focused on understanding the role of the endocannabinoid system in sperm function using mouse models. There is evidence that CNR1 and FAAH are present in mouse spermatozoa (81). Another study shows that the biosynthetic and degrading enzymes of 2-AG, and CNR2 receptors are present in spermatogonia to spermatozoa during spermatogenesis in mice (83).

In mammals, ejaculated sperm must undergo a functional maturation, named "capacitation", to acquire the competence to fertilize an egg. During this biological process, intracellular calcium signaling is elevated and hyperactivated sperm motility is facilitated. These events are accompanied by changes in protein components on sperm heads that induce the acrosome reaction, enabling sperm to bind to the zona pellucida (ZP). Although previous studies show that this process involves modifications of intracellular ions (84, 85), plasma membrane fluidity (86), metabolism, and motility (87), the molecular signaling pathways and the local regulatory mechanism that allows capacitation to progress remain poorly understood. Recent evidence suggests that endocannabinoid signaling is involved in spermatogenesis and capacitation. In mouse spermatogenesis, while 2-AG likely plays an important role in promoting the meiotic progression of germ

cells by activating CNR2 receptors (83), endocannabinoid signaling through CNR1 helps keep sperm quiescent in the epididymis. A previous report showed that the percentage of motile sperm retrieved from the caput is lower than the sperm from the cauda, but the percentage of motile sperm within the caput is increased in the absence of CNR1 (88). Endocannabinoid signaling is also involved in sperm capacitation. In boars activation of CNR1 by an AEA-stable analog methanandamide (mAEA), inhibits capacitation (and hence the ability of sperm cells to react to ZP (zona pellucida) proteins with acrosome exocytosis) through a cAMP-dependent pathway, although CNR1 had no effect on spontaneous acrosome reaction (82). It was also noted that once the capacitation is completed, AEA stabilizes the acrosome membranes by activating TRPV1, thus reducing spontaneous acrosome reaction. In addition, AEA has been shown to reduce human sperm motility and capacitation-induced acrosome reaction (89). The effects can be blocked by a CNR1 antagonist SR141716, suggesting that the activity of AEA on sperm function requires CNR1 activation (89).

IV. Endocannabinoids and Female Reproduction

Periimplantation events

The beginning of a new life starts with the fertilization of an egg by a sperm (90, 91). The fertilized egg, now termed an embryo, develops inside the oviduct undergoing several mitotic divisions leading to a compacted ball of cells termed a

morula (Figure 2). The morula travels towards the uterus and, at the same time, a small cavity appears marking the beginning of the blastocyst stage. A blastocyst consists of two cell populations, the inner cell mass (ICM) which forms the embryo proper, and the outer layer of trophectoderm cells which generates the placenta and extraembryonic membranes (5, 92-94).

It is only at the blastocyst stage that an embryo makes its first physical and physiological contact with the uterine endometrium to begin the process of implantation (Figure 2). A reciprocal interaction between the blastocyst and receptive uterus is essential for successful implantation. For instance, the uterus can only accept the blastocyst for attachment when it is in the receptive state. It is only then that the uterus is able to support blastocyst growth, attachment, and subsequent implantation (3, 5, 95, 96). Although the genetic and molecular basis for implantation is not yet clearly understood, gene expression studies and genetically engineered mouse models have shown that various signaling pathways are involved in the implantation process with endocannabinoid signaling being a key player (3-5, 97).

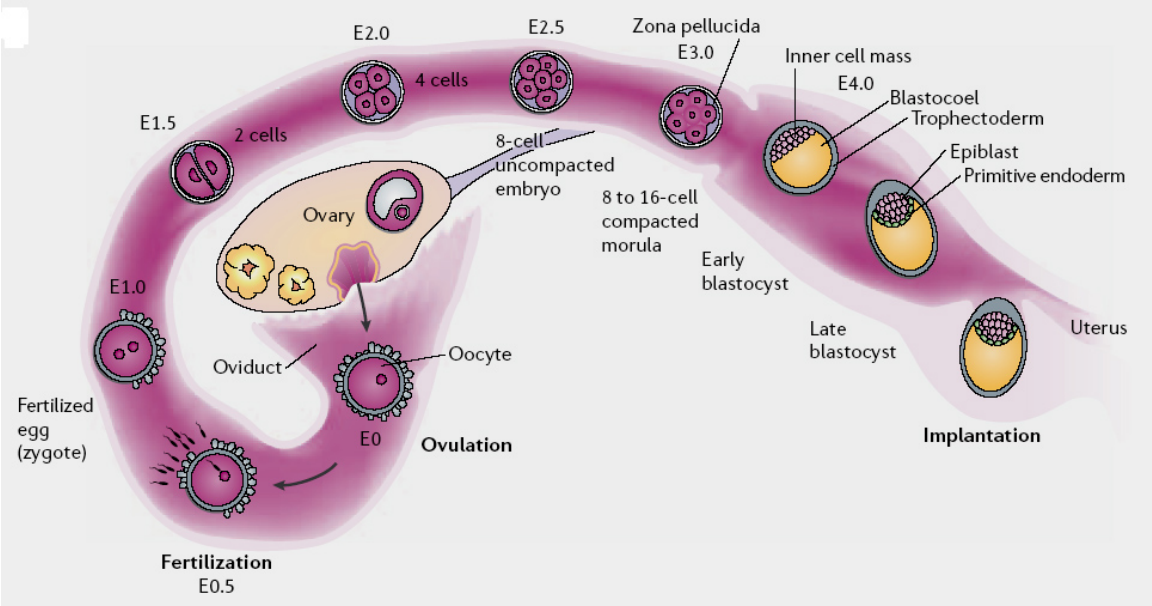

FIGURE 2. **Preimplantation embryo development and implantation in mice.** Following fertilization in the oviduct, the embryo undergoes several rounds of mitotic cell division, ultimately forming a ball of cells called a morula. At the late morula stage, the embryo enters the uterine lumen and transforms into a blastocyst that contains a cavity (called a blastocoel) with two distinct cell populations, the inner cell mass (ICM) and the trophectoderm (the progenitor of trophoblast cells). Before implantation, the blastocyst escapes from its outer shell (the zona pellucida) and differentiates to produce additional cell types — the epiblast and the primitive endoderm. At this stage, the trophectoderm attaches to the uterine lining to initiate the process of implantation. E, embryonic day.

Endocannabinoid signaling in the mouse female reproductive system

Both CNR1 and CNR2 receptors are expressed in the female mouse reproductive system. In preimplantation embryos, CNR1 is expressed from the late 2-cell through the blastocyst stage, whereas CNR2 is present from the 1-cell stage. CNR1 is localized primarily in the mural trophectoderm and intermittently

in the polar trophectoderm, but not in the inner cell mass (58). However, CNR1, but not CNR2, is also found in both the oviduct and uterus (12, 98).

Not only are the receptors present in these tissues, but also the enzymes necessary for the synthesis and degradation of anandamide. For example, NAPE-PLD is present in the nucleus and cytoplasm of preimplantation embryos from the 1-cell stage to the blastocyst stage and FAAH is present from the 2-cell stage to the blastocyst stage. Notably, FAAH is uniquely found in outer cell layers of morulae and in the trophectoderm of blastocysts. In the oviduct, NAPE-PLD levels are higher in the isthmus compared to levels in the ampullary region, whereas FAAH shows the reverse pattern, being higher in the ampullary region (11, 99). This inverse relationship holds true for FAAH and NAPE-PLD following implantation, in that NAPE-PLD levels are higher in inter-implantation sites on days 5 and 7 (day 1 = presence of vaginal plug) where FAAH levels are low, and NAPE-PLD levels are lower in implantation sites where FAAH levels are higher (100).

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

the interimplantation sites its lower level of expression is restricted to the luminal and glandular epithelia (100).

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

Endocannabinoid signaling and preimplantation embryo development

Development of preimplantation embryos to blastocysts is critical for achieving implantation competency. Delayed development leads to defective implantation or implantation failure and, consequently, compromised pregnancy (5). Embryos exposed to high levels of endocannabinoids, as well as to plant-derived and synthetic cannabinoids, show retarded development. For example, high levels of anandamide causes blastocysts to have a reduced number of trophectoderm cells and decreases the rate of zona-hatching (101, 102). It has also been shown that anandamide, 2-AG, THC and WIN55212-2 (a synthetic cannabinoid agonist) arrest development of two-cell embryos to blastocysts (Figure 3) (58, 103). This arrested development, however, can be rescued by SR141716A and AM251 (CNR1 selective antagonists), but not by SR144528 (a CNR2 specific antagonist). Furthermore, a CNR2 agonist, AM663, fails to affect embryo development (103). These studies collectively indicate that endocannabinoids and cannabinoids mediate their effects on preimplantation embryos through CNR1.

The availability of *Cnr1*$^{-/-}$ and *Cnr2*$^{-/-}$ mouse models greatly expanded the field of endocannabinoid research. Using these mice, it was observed that *Cnr1*$^{-/-}$ and *Cnr1*$^{-/-}$ *Cnr2*$^{-/-}$ embryos recovered from oviducts on day 3 and from uteri on day 4 of pregnancy show aberrant development compared with wild-type embryos (104).

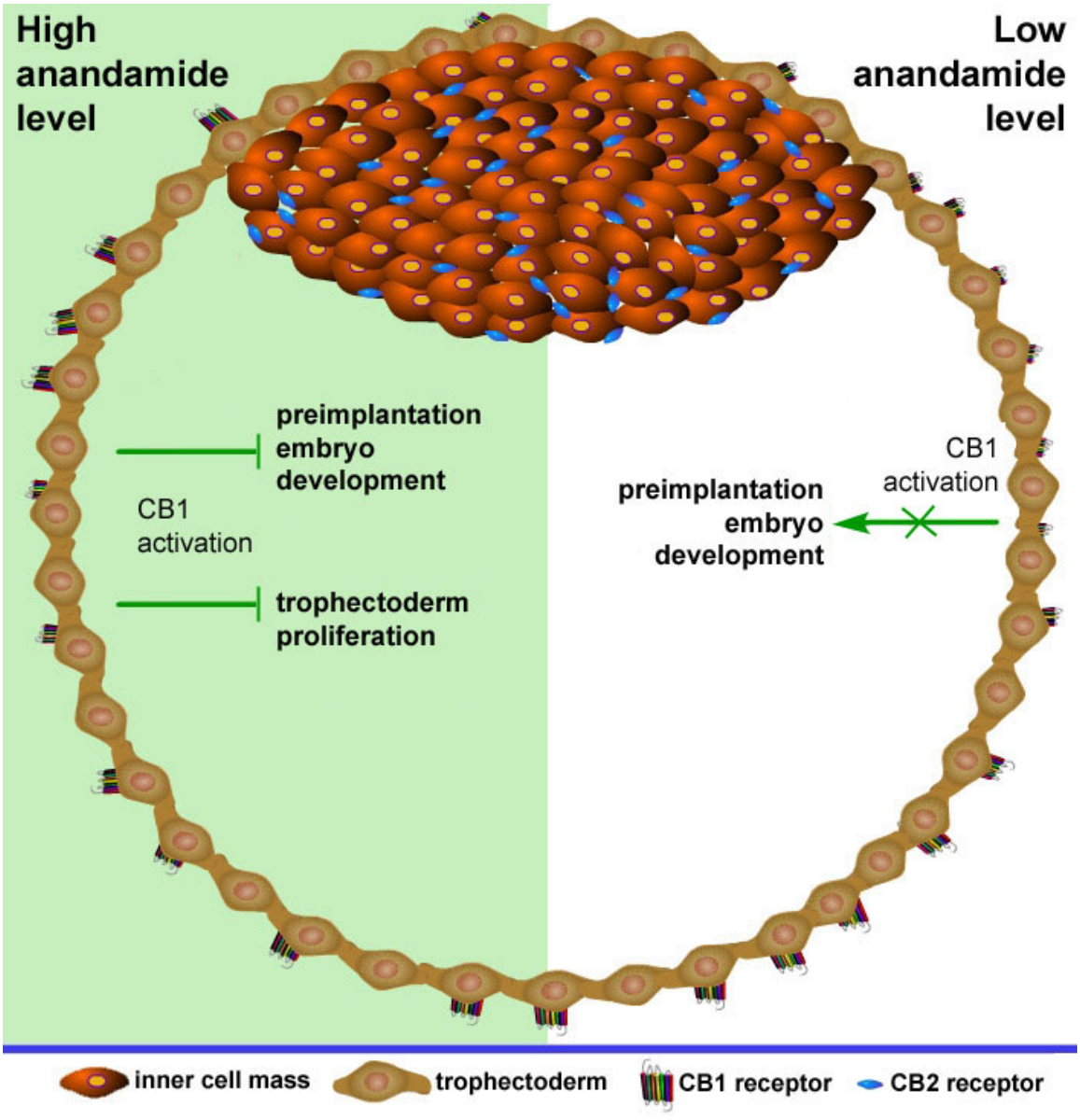

FIGURE 3. **Cannabinoid signaling in preimplantation embryo development.**

At a higher level of anandamide (28nm), cannabinoid signaling mediated by CNR1 inhibits trophectoderm proliferation and preimplantation embryo development. Blockage of endocannabinoid signaling also causes retarded preimplantation embryo development.

Interestingly, heterozygous embryos recovered from *Cnr1*$^{-/-}$ females mated with wild-type males showed normal embryo development (12). This finding prompted the hypothesis that while high cannabinoid levels can arrest early embryonic development, appropriate cannabinoid signaling under normal physiological conditions is beneficial to normal embryo development. This idea was then confirmed by in vitro embryo culture experiments, showing that low levels of anandamide (7 nM) promoted trophoblast differentiation and growth, while higher levels (28 nM) inhibited such development (105). The normal development of heterozygous embryos in a *Cnr1* deficient environment indicated that embryonic *Cnr1* receptors, but not oviductal *Cnr1* receptors, directed proper early embryonic development (10). It has been shown previously that most (79%) 2-cell wild-type embryos fail to develop to the blastocyst stage in the presence of excess anandamide. In contrast, more than 80% of *Cnr1*$^{-/-}$ or *Cnr1*$^{-/-}$*Cnr2*$^{-/-}$ double mutant embryos develop into blastocysts in the presence of similar levels of anandamide. However, in vitro development of *Cnr2*$^{-/-}$ embryos, like wild-type embryos, was severely compromised in the presence of anandamide (104). These results lend genetic support to the previous conclusion that CNR1, but not CNR2, respond to cannabinoids to govern embryonic development.

Interestingly, *Cnr2*$^{-/-}$ embryos collected from the oviduct on day 3 and uterus on day 4 also show some aberrant development (104), indicating that CNR2 does play some role in preimplantation embryo development. Recent observations of CNR2 expression in embryonic stem cells identified by microarray analysis (106) together with its absence in trophoblast stem cells (107), suggest that CNR2 expression is restricted to the inner cell mass of blastocysts. Thus, it is conceivable that CNR2 may play a role in ICM cell development and therefore, development of the embryo proper.

Collectively, cannabinoid signaling regulates preimplantation embryo development, with the current model implicating its mediation by the CNR1 receptor. Although the role of CNR2 remains puzzling, it seems to function as a low level cannabinoid signaling gatekeeper for preimplantation embryo development.

Endocannabinoid signaling and oviductal-uterine embryo transport

In parallel with preimplantation development, embryos are transported from the oviduct into the uterus. Embryos enter the uterus at the late morula stage and, coincident with this transport, a cavity appears marking the early blastocyst stage. The embryo only achieves implantation competency at the blastocyst stage. Thus, a successful implantation depends on the normal and timely transport of embryos from the oviduct into the uterus (Figure 4a). Although there is no evidence for implantation of embryos in the mouse oviduct, embryos can implant

in the human oviduct (Fallopian tube). Thus, a dysfunctional regulation of oviductal-uterine transport results in oviductal retention of embryos and can cause ectopic pregnancy in women (108, 109).

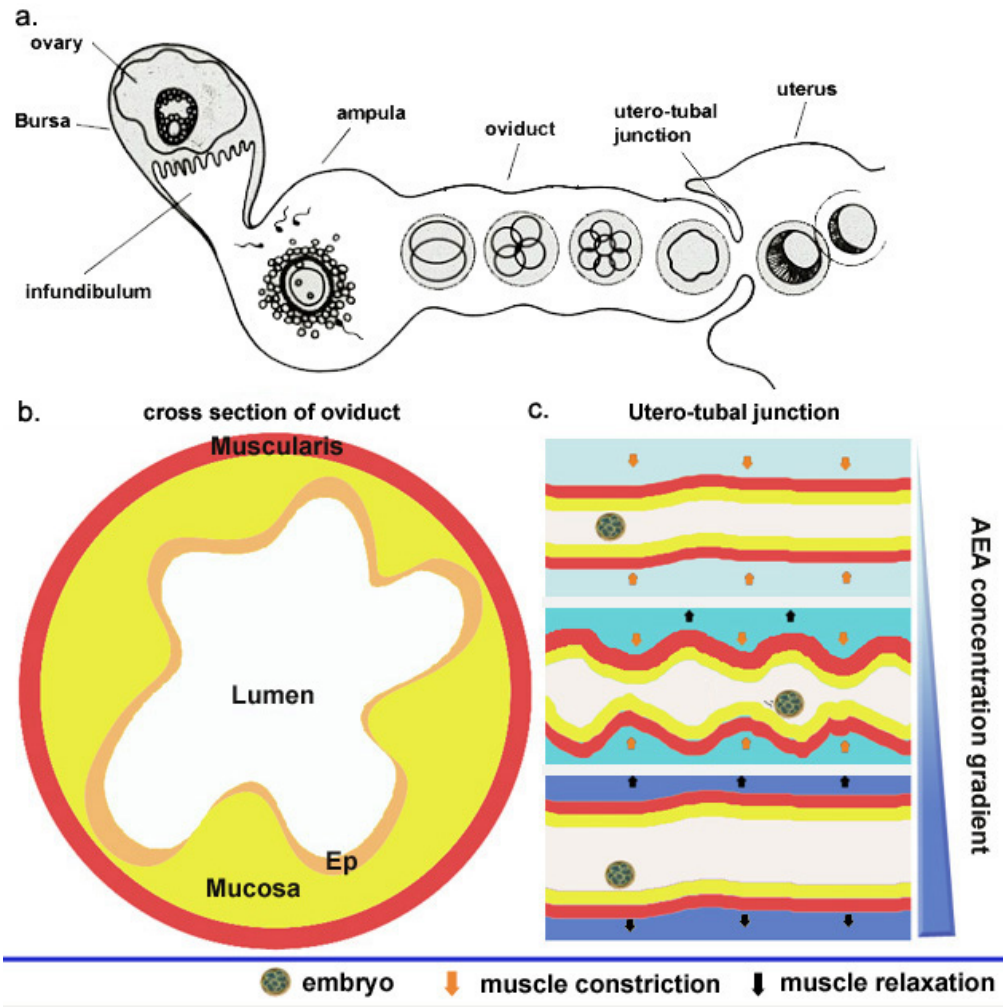

FIGURE 4. **a. Murine oviduct transport of preimplantation embryo.** An egg is fertilized in the ampula of the oviduct. Through cell division, it becomes a 2-cell, 4-cell, and 8-cell embryo, and eventually forms a morula. The morula passes through the utero-tubal junction to enter the uterus. **b. Schematic picture of cross section of the oviduct**. Ep, epithelium. **c. Muscularis movement of utero-tubal junction at different concentrations of AEA.** In the absence of AEA, NE causes muscle constriction, resulting in a narrow lumen. In the higher

concentration of AEA, cannabinoid signaling through CNR1 reduces the secretion of NE, thus relaxing muscle cells, resulting in a bigger lumen. The appropriate concentration of AEA triggers the muscle relaxation bit by bit to make a wave of the lumen, which is conducive for the embryo to enter uterus. Part a. is adapted from “Manipulating the Mouse Embryo; A Laboratory Manual”, page 497, 1994, Hogan, B et al.

Studies have shown that *Cnr1*$^{+/-}$ embryos have normal preimplantation development in *Cnr1*$^{-/-}$ oviducts. Interestingly, about 40% of the *Cnr1*$^{-/-}$ mothers still show pregnancy loss (12, 104). The observation that some *Cnr1*$^{-/-}$ females, mated with wild type males, did not have embryos in the uterus when flushed on the morning of day 4 of pregnancy suggested oviductal retention of embryos. *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ mice also show oviductal retention, but wild-type and *Cnr2*$^{-/-}$ mice do not, suggesting oviductal retention results specifically from the absence of *Cnr1*. This can be explained by the expression of *Cnr1* in the mouse oviduct. This same study showed that all of the oviduct-trapped embryos were morphologically and physiologically healthy, because they were able to implant in day 4 pseudopregnant uteri upon transfer, suggesting again that oviductal retention is due to maternal, but not embryonic defects. This speculation was further confirmed by reciprocal embryo transfers between *Cnr1*$^{-/-}$ and wild-type female mice. Only *Cnr1*$^{-/-}$ recipients displayed oviductal retention of embryos, irrespective of embryonic genotypes (12). In addition, wild-type mice with pharmacologically inhibited CNR1, but not CNR2, also show high rates of embryo retention in the oviduct. Notably, *Faah*$^{-/-}$ mice, which have higher oviductal anandamide levels, and wild-type mice exposed to THC or

methanandamide (an anandamide analog) also show oviduct embryo retention (11). All of 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 CNR1 in the oviduct regulates normal embryo oviductal transport.

It is known that the transport of an embryo through the oviduct is aided by a wave of oviduct muscle movement, ultimately controlled by the sympathetic nervous system (110). Stimulation of β2 adrenergic receptors (β2-AR) causes sphincter muscle relaxation, whereas stimulation of α1 adrenergic receptors (α1-AR) produces muscle contraction. It has been shown that reciprocal stimulation of these two receptors causes a wave of contractility and relaxation, which is conducive to the passage of embryos from the oviduct to the uterus (110, 111). It has also been found that exposure of wild-type oviducts to either an α1-AR agonist or a β2-AR antagonist causes embryos to be retained in the oviduct. In addition, *Cnr1* expression in the muscularis (Figure 4b) of the oviduct is colocalized with α1 and β2 adrenergic receptors, and oviductal nerve terminals in *Cnr1*$^{-/-}$ mice have increased releases of norepinephrine (NE) (12). These observations lead to speculation that CNR1-mediated endocannabinoid signaling is functionally coupled to adrenergic signaling to regulate oviductal motility, and the oviductal muscularis is predominantly in a contraction phase in the absence of CNR1. In contrast, high levels of endocannabinoid signaling, in either *Faah*$^{-/-}$ mice with naturally higher anandamide levels or wild-type mice exposed to

excessive natural or synthetic cannabinoid ligands, cause the oviductal muscularis to shift to a relaxation phase, thus impairing oviductal embryo transport to the uterus (Figure 4c).

In conclusion, the spatiotemporal expression of NAPE-PLD and FAAH in the oviduct creates an appropriate endocannabinoid tone, mediated by CNR1, to ultimately regulate the release of NE. Then, through the sympathetic nervous system, this signaling controls oviduct muscle contraction, consequently regulating oviduct-uterine embryo transport.

Endocannabinoid signaling and implantation

Attachment of the embryo to the luminal epithelium of the maternal uterus is a crucial step in mammalian reproduction. As the embryo travels into the uterus and differentiates into a blastocyst, the uterine cells undergo proliferation and differentiation to achieve a receptive state to accept the blastocyst for implantation. It is thought that blastocyst activation and uterine receptivity are two distinct events in the process of implantation (96), coordinated by estrogen and progesterone (112). Implantation can only occur when the blastocyst becomes implantation competent and the uterus achieves the receptive phase. The first attachment reaction occurs between the trophectoderm of the blastocyst and the uterine luminal epithelium.

Our laboratory has provided evidence that lower levels of AEA and CNR1 are beneficial for implantation. AEA levels have been measured in both receptive and nonreceptive uteri, with the former having a lower level of AEA compared with the latter (102). AEA levels are also critical in regulating preimplantation embryo development. In vitro experiments show that natural, synthetic or endogenous cannabinoids can inhibit preimplantation embryo development and blastocyst zona-hatching in culture, whereas blastocysts exposed to low levels of AEA show accelerated trophoblast differentiation and outgrowth (58, 102, 103). 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 (104). Immunostaining of CNR1 confirms that CNR1 is lower in activated blastocysts, but higher in dormant blastocysts (56, 104). Collectively, these results show that coordinated down-regulation of blastocyst CNR1 and uterine AEA levels are important for both blastocyst activation and uterine receptivity, two events critical for successful implantation.

To further address the underlying mechanism(s) by which differential uterine AEA levels are created, the expression profiles of NAPE-PLD and FAAH were examined in the uterus. Higher levels of *Nape-pld* mRNA and NAPE-PLD activity are found in nonreceptive uteri and in interimplantation sites, whereas both

mRNA and protein levels are lower in implantation sites and receptive uteri (99, 100). It is interesting that FAAH expression and activity show an inverse relationship. Higher FAAH expression and activity are observed at implantation sites and in the receptive uteri. Evidence points toward the possibility that the implanting blastocyst exerts an inhibitory effect on uterine *Nape-pld* expression, and upregulates uterine FAAH activity by releasing a lipid "FAAH activator" (99, 113). These observations suggest a potential role of the implanting embryo in regulating uterine AEA levels, perhaps to serve as a protective mechanism against exposure to detrimental levels of AEA. Regardless of its control, it is obvious that tight regulation of AEA plays an important role in implantation.

Other studies demonstrate that endocannabinoid signaling mediated by CNR1 on the embryo is coupled with different downstream signaling pathways, depending on the concentration of AEA. For instance, it has been shown that AEA-induced stimulatory and inhibitory effects in blastocyst function are mediated by ERK and Ca^{2+} signaling pathways. For example, while AEA at a low concentration (7 nM) activates ERK signaling via CNR1, higher AEA levels (28nM) fail to activate ERK, but instead inhibit Ca^{2+} mobilization (56). These results, combined with evidence that women with elevated peripheral AEA levels have spontaneous pregnancy loss (114, 115), demonstrate that endocannabinoid signaling is at least one of the pathways determining the fate of implantation and ultimately successful pregnancy.

With this introduction of the role of endocannabinoid signaling in male and female reproduction, I present my work on endocannabinoid signaling in fertilization and placentation in the next two chapters.

CHAPTER II

GENETIC LOSS OF *Faah* COMPROMISES MALE FERTILITY

Abstract

Marijuana is the most commonly used elicit drug. Although there is some indication that reproductive functions in males are impaired in chronic marijuana users, the genetic evidence and underlying causes remain largely unknown. Here we show that genetic loss of *Faah*, which encodes fatty acid amide hydrolase (FAAH), results in elevated levels of anandamide, an endocannabinoid, in the male reproductive system, leading to compromised fertilizing capacity of sperm. This defect is rescued by superimposing deletion of cannabinoid receptor 1 (*Cnr1*). Retention of *Faah*$^{-/-}$ sperm on the egg zona-pellucida provides evidence that sperm's capacity to penetrate the zona barrier is dampened by elevated anandamide levels. Collectively, the results show that aberrant endocannabinoid signaling via CNR1 impairs normal sperm function. Besides unveiling a new regulatory mechanism of sperm function, this study has clinical significance in male fertility.

Introduction

There is some evidence that male fertility in humans is negatively regulated by long-term exposure to marijuana extracts (reviewed in (10)). The major psychoactive component of marijuana is Δ^9-tetrahydrocannabinol (THC). Although *in vitro* experiments have shown that THC exerts adverse effects on sperm function (reviewed in (116)), there is still no *in vivo* or genetic evidence that cannabinoids impair male fertility. After THC was identified in 1964 (14), research on cannabinoids exploded with the discovery and cloning of two G-protein coupled cannabinoid receptors, brain-type *Cnr1* encoding CNR1 (15, 16) and spleen-type *Cnr2* encoding CNR2 (17). Around the same time, several endogenous lipid molecules targeting CNR1 and CNR2 were identified, collectively called endocannabinoids. The two most studied endocannabinoids are *N*-arachidonoylethanolamide (known as anandamide) and 2-arachidonoylglycerol (2-AG) (18-20). Anandamide levels are regulated by a balance between the rate of its synthesis and degradation. Anandamide was thought to be produced primarily from *N*-arachidonoylphosphatidylethanolamine (NAPE) by NAPE-hydrolyzing phospholipase D (NAPEPLD) (23). However, genetic studies in NAPEPLD deficient mice (25) and the recent identification of other anandamide synthetic pathways (26, 27) have revealed that regulation of anandamide synthesis is more complex than previously thought. Anandamide is degraded to ethanolamine and arachidonic acid by a membrane-bound fatty acid amide hydrolase (FAAH) (28, 29). Although FAAH can hydrolyze other endocannabinoids including 2-AG (30), studies in *Faah*$^{-/-}$ mice show that FAAH is

a major player in regulating the magnitude and duration of anandamide signaling (27, 117).

Sperm undergo a long journey to acquire fertilization capacity (118-120). Through the process of spermatogenesis, spermatogonia differentiate into highly polarized sperm which then undergo maturation in the epididymis before capacitation, acquiring motility in the female reproductive tract. After traveling through the uterine lumen and reaching ovulated eggs in the oviduct ampulla, capacitated sperm navigate through cumulus cells surrounding the egg to contact the zona-pellucida (zona), the outermost membrane of the egg. Upon binding to the zona, sperm undergo a Ca^{++}-dependent exocytotic event known as the acrosome reaction which is essential for their zona penetration and homing into the periviteline space. After a sperm binds to an egg plasma membrane, the two gametes unite, resulting in egg activation, pronuclear formation and syngamy. Each step in the process is essential for successful fertilization.

There are reports that endocannabinoids and their receptors are present in testes and sperm of invertebrates and vertebrates, including sea urchins, frogs, rats, mice, boars and humans (89). This conserved expression across species suggests that endocannabinoid signaling plays important roles in male reproduction. *In vitro* studies also showed that endocannabinoid signaling inhibits capacitation of boar sperm in a cAMP-dependent pathway and prevents the acrosome reaction (82), and that anandamide reduces human sperm motility by quenching mitochondrial activity (89). However, there is no *in vivo* genetic evidence of endocannabinoid signaling impacting male reproductive functions.

In this study, we used gene targeted mice for *Faah* to mimic the conditions of long-term exposure to marijuana to explore roles of cannabinoid/endocannabinoid signaling in male fertility.

Materials and Methods

Mice

Targeted deletion of *Faah, Cnr1* or *Cnr2* in mice (129/SvJ-C57BL/6J) has previously been described (117, 121, 122). Double mutants for *Faah*/*Cnr1* or *Faah*/*Cnr2* were generated using appropriate breeding strategies. Adult WT, $Faah^{-/-}$, $Faah^{-/-}/Cnr1^{-/-}$ and $Faah^{-/-}/Cnr2^{-/-}$ mice were housed at the Institutional Animal Care Facility according to NIH and institutional guidelines. Experiments were conducted on mice between 3 and 4 months of age. Testes and epididymis from $Faah^{-/-}$ and WT males were processed for anandamide measurement and in situ hybridization.

Western blotting

Tissue samples were homogenized in lysis buffer [150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, and 50 mmol/L Tris (pH 8)] containing proteinase and phosphatase inhibitors. The lysates were centrifuged at 9,880 x *g* for 10 min at 4°C. Supernatants (25 µg) were boiled for 5 min in SDS sample buffer. Samples were run on 10% SDS-PAGE gels under reducing conditions and transferred onto nitrocellulose membranes. Membranes were blocked with 10% carnation milk in TBST and probed with antibodies against mouse FAAH (1:1000;

custom made by Ben Cravatt's lab (117)), CNR1 (1:2000) (123), CNR2 (1:250; Cayman), β-actin (ACTB) (1:100; Santa Cruz Biotechnology), overnight at 4°C. After thorough washings, blots were incubated in peroxidase-conjugated donkey/anti-goat IgG (1:2000) or donkey/anti-rabbit IgG (1:2000; Jackson ImmunoResearch Lab, Inc.), followed by washings. Protein signals were detected using chemiluminescent reagents (Amersham).

Immunohistochemistry

Immunostaining in Bouin's solution-fixed paraffin embedded sections (6 μm) was performed using antibodies specific to FAAH (1:200) (117), CNR1 (1:200) (123), or CNR2 (1:250; Cayman) following antigen retrieval in citrate buffer (pH 6.0) for 10 minutes in an autoclave. A Histostain-Plus (DAB) kit (Zymed) was used to visualize the antigen. Reddish brown deposits indicate sites of positive immunostaining.

Immunofluorescence

Sperm were isolated from the epididymis of mature WT males and thoroughly washed in PBS. Sperm were fixed with 1% formaldehyde at room temperature for 15 min. After blocking in 1% BSA/PBST, sperm were incubated with CNR1 antibody (1:200; ~500ng/ml IgG)(123) with or without blocking peptide overnight at 4°C. After thorough washings, secondary antibodies conjugated with Cy3 (Jackson ImmunoResearch Laboratories Inc.) were used to detect

immunofluorescence signaling. SYTO13 green fluorescence dye (Invitrogen) was used for nuclear staining.

Anandamide assay

Testes and sperm (100 mg) were pooled separately from 5 WT or *Faah*$^{-/-}$ mice in each group (n = 3–6) and were assayed for anandamide as previously described (12). Briefly, the pre-weighed samples were homogenized in ethyl acetate with 0.5% acetic acid. $^{2}H_{8}$-labeled anandamide was added as an internal standard to a mortar immediately prior to homogenization. The homogenate was centrifuged and the supernatant was dried, reconstituted in chloroform and purified on a silica-based solid-phase extraction cartridge. The eluent was dried, reconstituted in 1:8 of aqueous silver acetate to methanolic silver acetate and analyzed by reverse phase positive-ion ESI-HPLC-MS-MS. Quantification was performed by stable isotope dilution against the octadeuterated internal standard.

In situ hybridization

Frozen sections (12 μm) were hybridized with ^{35}S-labeled cRNA probes for mouse *Cnr1* or *Cnr2* as previously described (124). Sections hybridized with sense probes served as negative controls and showed no positive signals.

***In vitro* fertilization (IVF)**

IVF was performed as previously described (125). Briefly, WT females were superovulated by intraperitoneal injections of 5 IU pregnant mare serum

gonadotropin (PMSG, Sigma) followed by injections of 5 IU human chorionic gonadotropin (hCG, Sigma) 48 h later. Cumulus–oocyte complexes (COCs) were collected from the oviduct ampulla 12–14 h post-hCG injection and placed in 100 µl droplets of HTF medium (Chemicon, Temecula, CA, USA). In most IVF experiments, zona-intact eggs were used. In some IVF experiments zona-free eggs were used. COCs were treated with hyaluronidase (Sigma) and cumulus-free eggs were then exposed to acidic Tyrode's solution and passed through a pipette several times until zona pellucidae were dissolved. Eggs were washed three times in HTF medium and incubated for >1 hour to allow surface proteins to recover (126). Sperm were collected from the cauda of the epididymis and placed into 400 ml HTF medium to allow capacitation for 2.5 h in a humidified 5% CO_2 incubator at 37°C. Sperm (~1.2–1.5 $X10^6$ sperm/ml) were then co-incubated with eggs to allow fertilization. After 6 h, sperm were removed and putative zygotes placed in 200 µl drops of KSOM (potassium simplex optimized medium; Chemicon) and incubated in a humidified 5% CO_2 incubator at 37^oC. The cleavage rate (two-cell stage) after 24 h was used as an index of fertilization. Formation of blastocysts at 120 h indicated developmental potential of fertilized embryos.

Evaluation of sperm-zona binding in IVF

After sperm were incubated with eggs for 2 hours in IVF experiments, eggs were removed and attached sperm were stained with propidium iodide and FITC conjugated antibody specific to IZUMO, generated in Okabe's laboratory (127).

Analysis of acrosome reaction by flow cytometry

WT and *Faah*$^{-/-}$ caudal sperm were incubated in the HTF medium with anti-IZUMO antibody conjugated with FITC to monitor spontaneous acrosome reaction by flow cytometry at 30 minutes intervals for up to 3 h. Sperm were stained with propidium iodide (10 µg/ml) 2 minutes before flow cytometry analysis. Viable sperm were selected by propidium iodide staining, while acrosomal reacted sperm were identified by anti-IZUMO antibody staining (127).

Evaluation of sperm motility

After capacitation 20 µl of media containing sperm (2 X10^6 sperm/ml) was placed on a pre-warmed slide under a cover slip for 30 and 90 minutes. Sperm motility was recorded in 12 frames per second for 20 seconds at a resolution of 640 X 512 pixels. The total travel distance and linear travel distance (linear distance from the starting point to end point) and travel time were measured by Nikon Nis-elements' object tracking function. The curvilinear velocity was calculated from the total distance traveled divided by the travel time. The linear velocity was calculated from the linear travel distance divided by the travel time, whereas linearity was calculated from the linear velocity divided by the curvilinear velocity.

Results

Faah$^{-/-}$ males have compromised fertility

We have previously shown that FAAH is a key metabolic regulator of anandamide levels in mice (117) and that FAAH deficiency results in higher anandamide levels in the female reproductive tract, impairing normal oviductal embryo transport and embryo development (27). In the course of these studies, analysis of breeding results showed that litter sizes generated by mating wild-type (WT) females with *Faah*$^{-/-}$ males are 13% smaller than those resulting from breeding WT females with WT males (Table 1). These results suggested that FAAH deficiency compromises male fertility.

Table 1. Reproductive performance of *Faah*$^{-/-}$ males

Genotype		No. of litters examined*	Average litter size (mean ± SEM)
♀	♂		
WT	**WT**	30	8.1±0.4
	***Faah*$^{-/-}$**	23	7.0±0.4†
***Faah*$^{-/-}$**	**WT**	21	6.3±0.2
	***Faah*$^{-/-}$**	39	4.1±0.3‡

Litter sizes were recorded from indicated crossing strategies. *Litters were produced using different males. †*P*=0.06. *P* value was generated by unpaired t-test of litter sizes produced from breedings of WT females by *Faah*$^{-/-}$ males compared with those generated from breedings of WT females by WT males. ‡*P<0.001. P* value was generated by unpaired t-test of litter sizes from breeding *Faah*$^{-/-}$ females with *Faah*$^{-/-}$ males compared to litter sizes from breeding *Faah*$^{-/-}$ females with WT males.

This is further evident from our findings of significantly reduced litter sizes generated by *Faah*$^{-/-}$ females mated with *Faah*$^{-/-}$ males compared to those generated by breeding *Faah*$^{-/-}$ females with WT males (Table 1). These breeding results prompted us to further examine the fertility of *Faah*$^{-/-}$ males. We used WT females mated with either *Faah*$^{-/-}$ or WT males. Females were killed on the morning of day 2 of pregnancy and oviducts flushed to record fertilized (2-cell embryos) and unfertilized eggs. We observed that WT females mated with *Faah*$^{-/-}$ males had significantly reduced numbers of fertilized eggs compared with those recovered from WT females mated with WT males. In addition, the number of WT females yielding fertilized eggs was lower (Figure 5 a & b). These data clearly corroborate the breeding data that FAAH deficiency impairs male fertility.

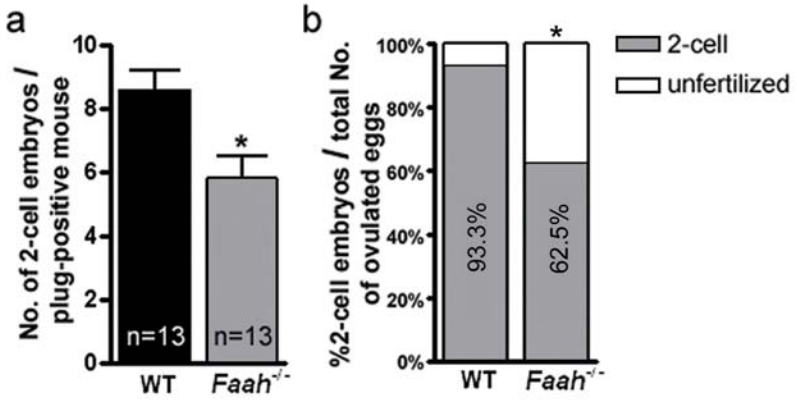

FIGURE 5. **FAAH deficiency impairs sperm fertility**. (a) Number of 2-cell embryos per plug-positive WT females mated with WT or *Faah*$^{-/-}$ males. Numbers of plug-positive mice (n) used are shown within the bars. *P < 0.05, unpaired *t*-test. (b) Percent 2-cell embryos and unfertilized eggs retrieved from the same groups. Thirteen mice were used in each group. *P < 0.01, chi square test.

Collectively, our findings show that *Faah*$^{-/-}$ sperm under perform even in the WT female reproductive tract and that function of null sperm is further compromised in the *Faah*$^{-/-}$ female reproductive tract. These observations provide evidence that paternal FAAH deficiency is a cause for compromised fertility.

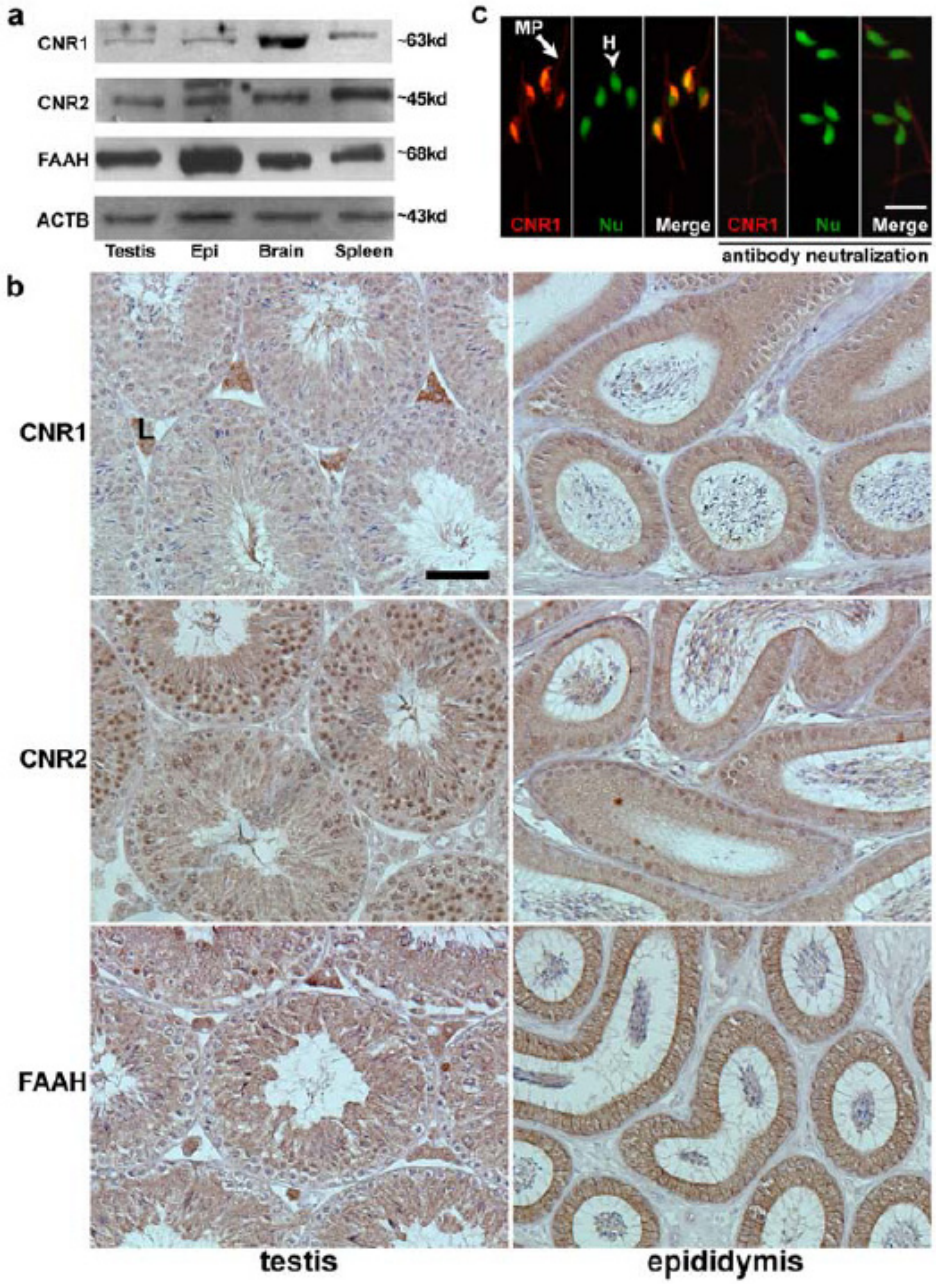

FIGURE 6**. FAAH and CB receptors are expressed in the male reproductive tract**. (a) Western blotting of CNR1, CNR2 and FAAH in the WT testis and epididymis. Brain tissue extracts served as positive controls for CNR1 and FAAH, while spleen tissue samples as positive controls for CNR2. ACTB is a loading control. Epi, epididymis. (b) Immunolocalization of CNR1, CNR2 and FAAH in the testis and epididymis. Bar, 50 μm. L, Leydig cells. (c) CNR1 immunostaining (red) in sperm (left 3 panels); sperm exposed to CNR1 antibody pre-absorbed with an antigenic peptide (right 3 panels). In each group, CNR1 staining, nuclear staining and merged pictures were showed from left to right. Nuclei were counter-stained with Cyto-13 (green). H (arrowhead) indicates sperm head, while MP (arrow) indicates sperm midpiece. Nu, nuclear. Bar, 10 μm.

Endocannabinoid signaling is present in the male reproductive system

The extent and duration of anandamide signaling via CNR1 and/or CNR2 is mainly regulated by FAAH (117). Thus, we examined the expression of CNR1, CNR2 and FAAH in the testis and epididymis to study potential roles of anandamide in regulating male fertility. Western blotting analysis showed that FAAH, CNR1 and CNR2 are present in both the testis and epididymis of WT mice (Figure 6a). We next examined cell - specific localization of FAAH and CB receptors in the testis and epididymis of WT mice by immunohistochemistry (Figure 6b). We found that while CNR1 was present in Leydig cells and epididymal epithelial cell surfaces, testicular spermatocytes and spermatids showed modest positive staining. In contrast, CNR2 was localized in spermatocytes and Sertoli cells encircling spermatocytes/spermatids in the testis. In the epididymis, epithelial cell surfaces were decorated with CNR2 immunostaining, whereas signals were undetectable in interstitial cells. FAAH

was present in spermatocytes and spermatids, while spermatogonia had little or no positive signals. Sertoli cells and Leydig cells also showed positive staining of FAAH. The localization of FAAH was clearly evident on cell surfaces of the epididymal epithelium. The antibody specificity was

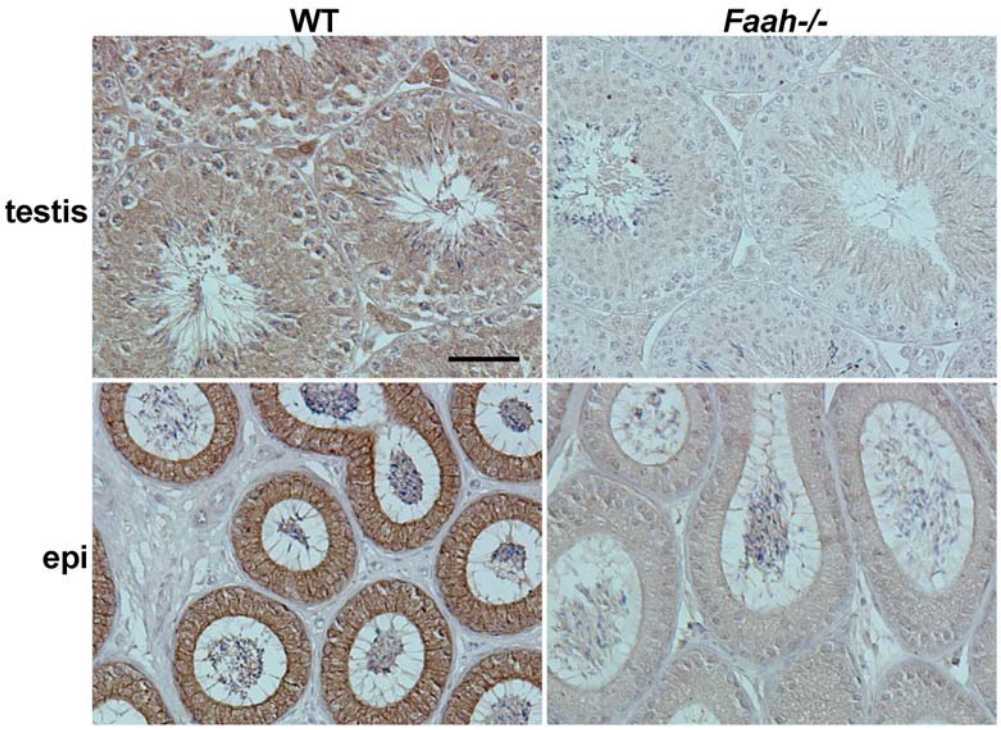

FIGURE 7**. FAAH protein is absent in *Faah*$^{-/-}$ testis and epididymis.** Immunostaining of FAAH in the testis and epididymis of WT and *Faah*$^{-/-}$ mice. Epi, epididymis. Bar, 50μm.

confirmed using *Faah*$^{-/-}$ tissues (Figure 7). The presence of FAAH on both the testis and epididymis suggests that endocannabinoid levels in these tissues are tightly regulated by FAAH.

The presence of CNR1 and CNR2 on sperm was also examined by immunofluorescence. As shown in Figure 6c, CNR1 immunofluorescence is

primarily noted in anterior regions of sperm heads, the site of the acrosomal sac, but also in the midpiece. CNR1 is undetectable in the principle and end piece of sperm tails. Sperm incubated with CNR1 antibody pre-absorbed with an antigenic peptide showed that while the signal in the anterior region of sperm heads is specific, the signal is non-specific in the midpiece (Figure 6c). Interestingly, CNR2 was undetectable in sperm (Figure 8). Our findings of the presence of FAAH, CNR1 and CNR2 in the testis and epididymis, and that of FAAH and CNR1 in sperm, would suggest that endocannabinoid signaling plays a role in spermatogenesis and sperm maturation.

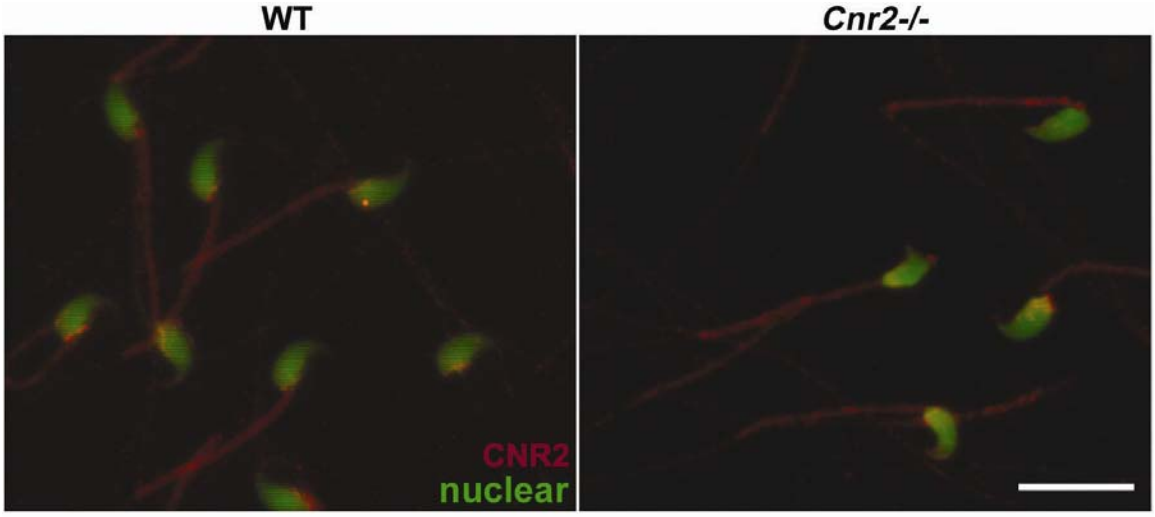

FIGURE 8**. CNR2 is undetectable in sperm.** Non-specific fluorescence (red) was noted in the midpiece of both WT and Cnr2-/- sperm. Bar, 10μm.

FAAH deficiency elevates anandamide levels in the testis and epididymis

To provide genetic evidence for FAAH's function in the male reproductive system, we measured anandamide and 2-AG levels in the testis and epididymis of WT

and *Faah*$^{-/-}$ mice using reverse-phase high-performance liquid chromatography/tandem mass spectrometry. As shown in Figure 9a, the testis and epididymis from *Faah*$^{-/-}$ mice had significantly increased anandamide levels, suggesting that FAAH is a primary enzyme that regulates anandamide turnover in these tissues. Higher testicular anandamide levels in *Faah*$^{-/-}$ males corroborate our previous observation (128). However, levels of 2-AG in the testis and epididymis were comparable between *Faah*$^{-/-}$ and WT males (Figure 9a). These results are consistent with our previous data in the uterus, showing unaltered 2-AG levels in the absence of FAAH (27).

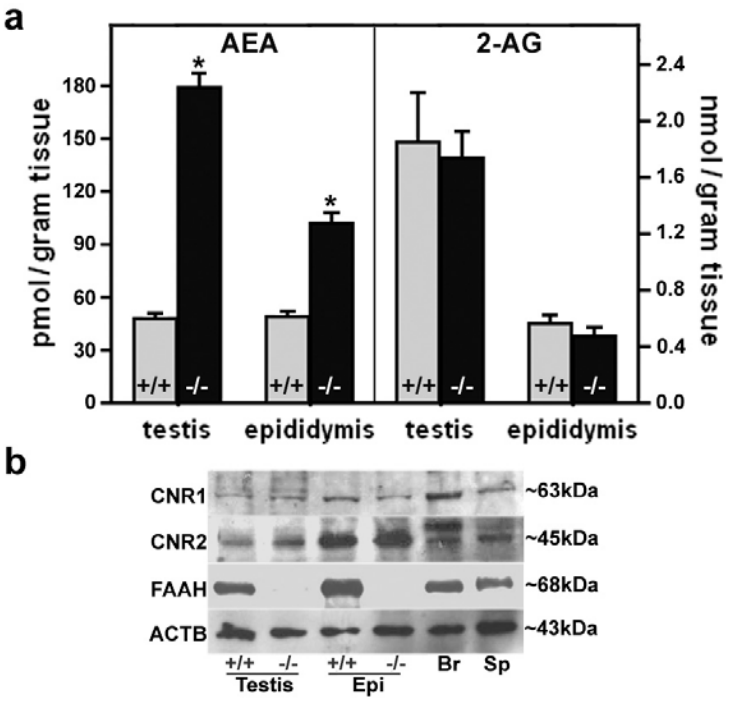

FIGURE 9. **FAAH deficiency elevates anandamide levels**. (a) Anandamide, but not 2-AG, levels in *Faah*$^{-/-}$ testis and epididymis were higher than those in WT (n=10; **P* < 0.05, unpaired *t*-test). (b) Western blotting of CNR1, CNR2 and FAAH in the testis and epididymis of WT and *Faah*$^{-/-}$ males. Brain and spleen samples served as positive controls, while ACTB served as a loading control. Epi, epididymis. Br, brain. Sp, spleen.

Higher anandamide levels in the *Faah*$^{-/-}$ testis and epididymis prompted us to speculate that reduced fertility in these males is due to persistent and/or elevated endocannabinoid signaling. However, it is possible that there is a negative feedback loop to downregulate the expression of CB receptors to counter the consequence of high anandamide levels. To address this possibility, we examined the status of CB receptors in the testis and epididymis of WT and *Faah*$^{-/-}$ mice by Western blotting. As shown in Figure 9b, levels of CNR1 and CNR2 protein in these tissues were comparable between *Faah*$^{-/-}$ and WT males. These results suggest that higher anandamide levels do not appreciably downregulate CNR1 or CNR2 expression. To further confirm that expression of CNR1 and CNR2 is not altered in *Faah*$^{-/-}$ males, in-situ hybridization and immunohistochemistry were performed. Expression patterns of both CNR1 and CNR2 were similar in WT and *Faah*$^{-/-}$ epididymis (data not shown). Collectively, the data suggest that the status of CB receptors is not altered by higher anandamide levels and that heightened signaling via CNR1 and/or CNR2 occurs in the presence of increased anandamide levels.

FAAH deficiency impairs sperm fertilizing capacity

Our *in vivo* breeding data led us to speculate that higher anandamide levels in males lacking FAAH results in their reduced fertility. To examine this, we first compared histology of the testis and epididymis as well as sperm morphology

between *Faah*$^{-/-}$ and WT males at the age of 3-4 months. To our surprise, no apparent histological abnormalities were observed in these tissues missing *Faah* (Figures 10). We next explored whether FAAH deficiency in males impairs sperm's fertilizing capacity by performing *in vitro* fertilization (IVF) experiments using *Faah*$^{-/-}$ or WT sperm with WT eggs. Sperm retrieved from the caudal epididymis were subjected to capacitation *in vitro* for two hours before placing them with eggs in culture. The fertilization rate was calculated by counting the number of 2-cell embryos developed on the second day after IVF. As shown in Table 2, sperm retrieved from WT males showed a 75% fertilization rate with 97% of 2-cell embryos developing to blastocysts. In contrast, *Faah*$^{-/-}$ sperm showed remarkably a reduced fertilization rate (42%), although development of fertilized eggs into blastocysts was comparable (89%) to those of WT (97%). These results suggest that the fertilizing capacity of *Faah*$^{-/-}$ sperm is compromised due to impairment in the male reproductive tract before ejaculation, since no exogenous anandamide was added to the capacitation medium.

Table 2. Higher anandamide levels impair sperm fertilizing capacity *in vitro* via CNR1.

Genotypes	No. of eggs used for IVF	% IVF rate (No. of 2-cell embryos/total No. of eggs used)	% development (No. of blastocysts /total no. of 2-cell embryos used)
WT	624	75 (466/624)	97 (450/466)
***Faah*$^{-/-}$**	528	42 (221/528)*	89 (197/221)
***Faah*$^{-/-}$/*Cnr1*$^{-/-}$**	177	70 (124/177)	93 (115/124)
***Faah*$^{-/-}$/*Cnr2*$^{-/-}$**	118	11 (13/118)*	84.6 (11/13)

IVF rates of WT eggs fertilized by sperm of different genotypes were recorded on day 2 post-fertilization. The rate of blastocyst formation from 2-cell embryos was evaluated on the 5th day of culture. Fertilizing capacity of *Faah*$^{-/-}$ or *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ sperm was significantly lower than WT sperm (*P<0.01; χ^2-analysis).

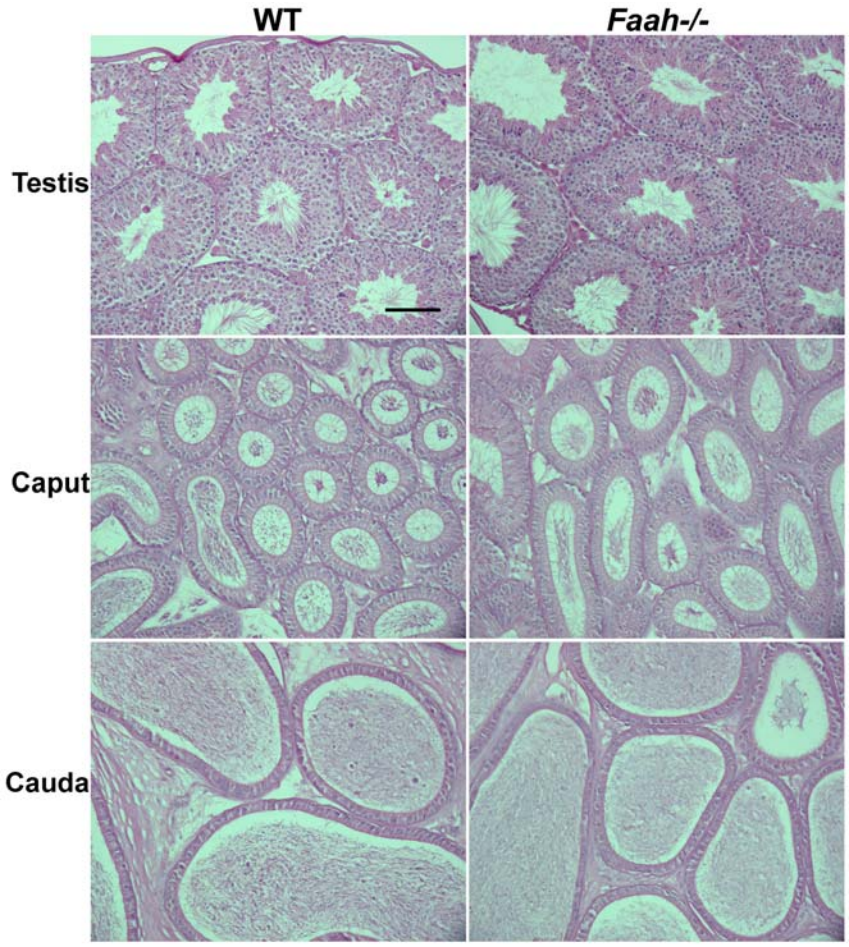

FIGURE 10**. The histology of *Faah*$^{-/-}$ testis and epididymis is comparable to those of WT mice.** H&E staining of the testis, caput and cauda sections from *Faah*$^{-/-}$ males show histology similar to WT males. Bar, 100µm.

Deletion of *Cnr1* reverses impaired fertilizing capacity of *Faah*$^{-/-}$ sperm

Sustained higher anandamide levels in the male reproductive tract lacking FAAH are capable of exerting endocannabinoid signaling either through CNR1, CNR2 or both. To address this question, we generated *Faah*$^{-/-}$/*Cnr1*$^{-/-}$ and *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ double mutant mice. We again performed IVF using sperm retrieved from *Faah*$^{-/-}$/*Cnr1*$^{-/-}$ or *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ males with eggs isolated from WT females. As shown in Table 2, sperm isolated from *Faah*$^{-/-}$/*Cnr1*$^{-/-}$ males exhibited a 70% fertilization rate with 93% of fertilized eggs developing to the blastocyst stage, but sperm isolated from *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ males showed a remarkably low fertilization rate (11%). These data show that in the absence of CNR1, *Faah*$^{-/-}$ sperm escape the deleterious effects of higher anandamide levels. It is interesting to note that *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ sperm had a more inferior fertilizing capacity than *Faah*$^{-/-}$ sperm. The results provide genetic evidence that higher anandamide levels work through CNR1 in the *Faah*$^{-/-}$ male reproductive tract to impair sperm fertilizing capacity.

***Faah*$^{-/-}$ sperm have poor zona-penetrating ability**

Our next objective was to see which step in the fertilization process is impaired in *Faah*$^{-/-}$ sperm. We first examined whether *Faah*$^{-/-}$ sperm can adhere to zona-pellucidae and if so, whether they can undergo acrosome reaction. IZUMO, a recently discovered protein, is not displayed on plasma membranes of acrosome-intact sperm (127). Following acrosome reaction, IZUMO is exposed and participates in sperm-egg fusion. Thus, only acrosome-reacted sperm are stained by the IZUMO antibody.

WT or *Faah*$^{-/-}$ sperm were incubated with WT eggs for 2 hours and then stained with propidium iodide to label cell nuclei. We found that after the 2-hour incubation most WT sperm detached from the zona surface (Figure 11a), whereas numerous *Faah*$^{-/-}$ sperm were still attached to the zona. Even after several washings, *Faah*$^{-/-}$ sperm remained adherent to the zona indicating good binding of *Faah*$^{-/-}$ sperm to the zona. These results suggested that most eggs were fertilized by WT sperm, but eggs incubated with *Faah*$^{-/-}$ sperm were still unfertilized. We then stained the sperm attached to eggs with the IZUMO antibody.

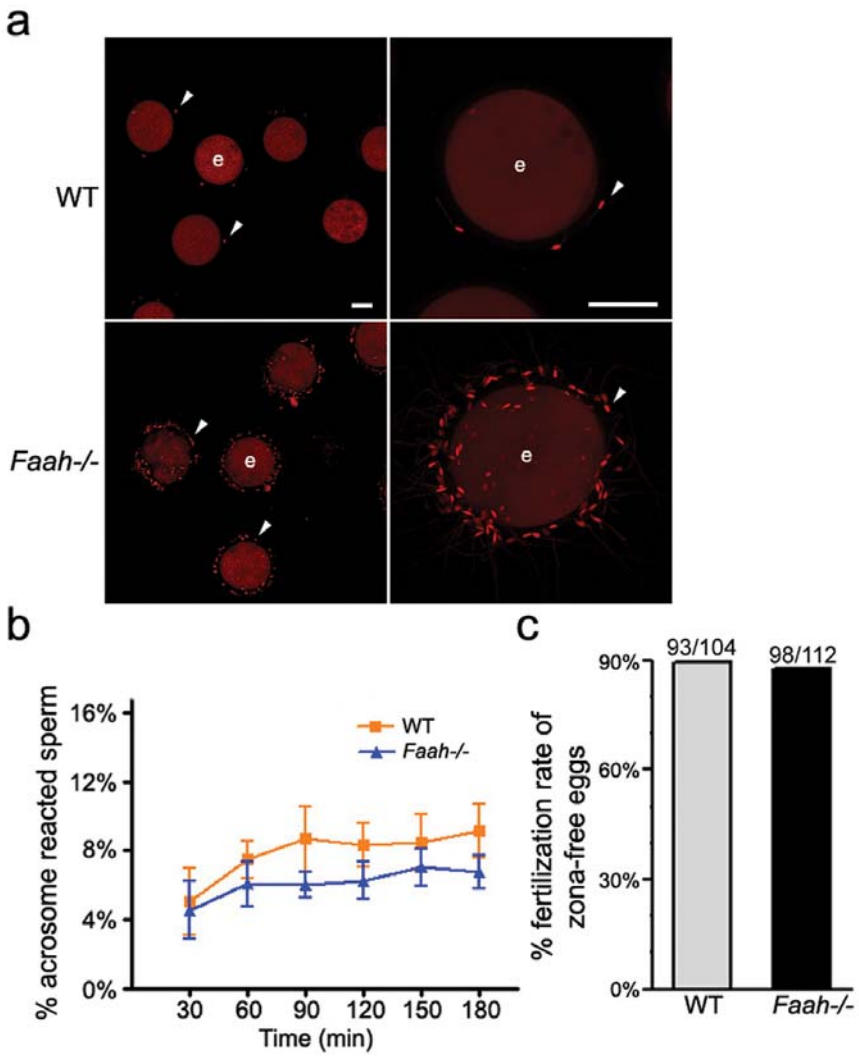

FIGURE 11. **Zona-penetrating capacity of *Faah*$^{-/-}$ sperm is inferior**. (a) Sperm-egg interactions using zona-intact WT eggs. After 2 hours of incubation with eggs, *Faah*$^{-/-}$ sperm were still attached to zona pellucidae. Bar, 40μm. Arrow heads indicate sperm on the zona-intact egg surface; e, egg. (b) Spontaneous acrosome reaction as assessed by flow cytometry. Rate (%) of acrosome-reacted WT and *Faah*$^{-/-}$ sperm at each time point was analyzed by flow cytometry as described in the methods and no statistically significant difference was noted between the two groups as analyzed by student t test. (c) IVF rates of zona-free WT eggs fertilized by WT or Faah$^{-/-}$ sperm. Numbers above the bars indicate the number of fertilized eggs/total zona-free eggs used for IVF.

Many *Faah*$^{-/-}$ sperm remaining on the zona surface showed positive signal by the IZUMO antibody (figure 12), indicating they underwent acrosome reaction. To further confirm that *Faah*$^{-/-}$ sperm undergo normal acrosome reaction, we examined the spontaneous acrosome reaction rate of *Faah*$^{-/-}$ sperm. The acrosome reaction, which occurs during sperm penetration through the zona, can also occur spontaneously without binding to the zona. Analysis of spontaneous acrosome reaction is used to assess the fertilizing ability of human (129) and mouse sperm (130). We compared the status and time-course of spontaneous acrosome reaction of WT and *Faah*$^{-/-}$ sperm in the fertilization medium by flow cytometry. While viable sperm were selected by propidium iodide staining, acrosome-reacted sperm were identified by IZUMO staining. As shown in figure 11b, the percentage of acrosome-reacted *Faah*$^{-/-}$ sperm is somewhat lower than that of WT acrosome-reacted sperm, but the difference is not statistically significant. Collectively, these data suggest that *Faah*$^{-/-}$ sperm can bind to the zona and undergo acrosome reaction, but still have difficulty fertilizing eggs.

The acrosome reaction is not the only prerequisite for zona penetration: sperm motility and acrosomal release of proteases are also involved in this process (120). To examine whether *Faah*$^{-/-}$ sperm can penetrate the zona successfully, we performed IVF using sperm from *Faah*$^{-/-}$ or WT mice incubated with zona-free WT eggs. To our surprise, *Faah*$^{-/-}$ sperm exhibited a comparable fertilizing capacity with WT sperm (Figure 11c), indicating that the zona is a major barrier for normal fertilization by *Faah*$^{-/-}$ sperm.

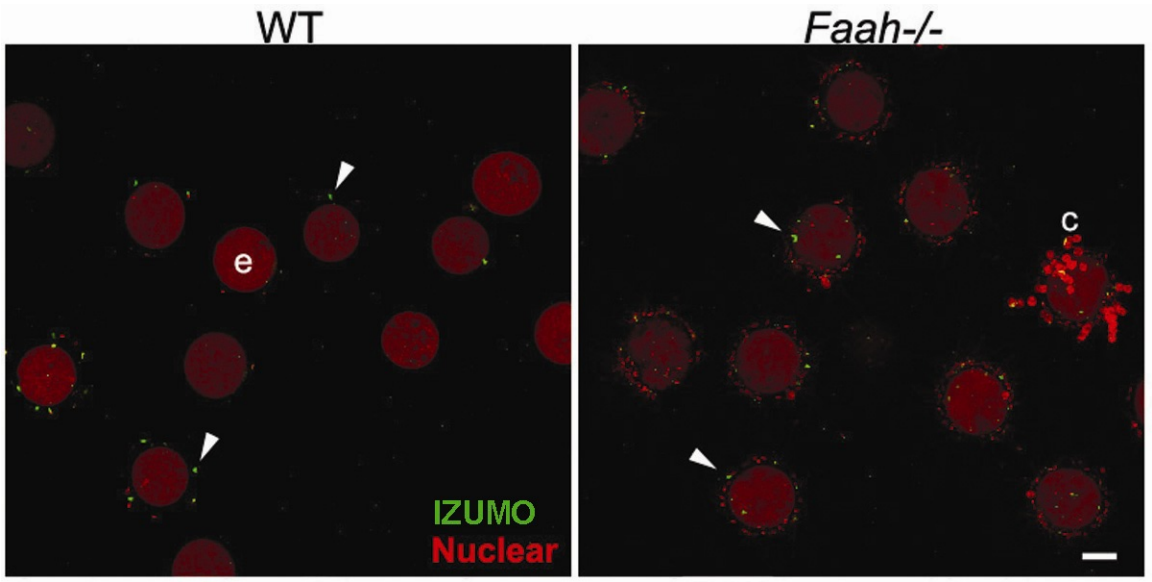

FIGURE 12**. Immunofluorescence of IZUMO in sperm.** After 2 hours of incubation with zona-intact WT eggs, sperm on the zona were stained with propidium iodide (red) and with an antibody to IZUMO (green). Arrowheads indicate acrosome-reacted sperm on eggs. Bar, 40μm. E, egg; c, cumulus cells.

Sperm motility is attenuated in *Faah*$^{-/-}$ males

It is generally accepted that robust sperm motility is an important component of normal male fertility (131), and that hyperactivated sperm motility is correlated with sperm's ability to fertilize zona-intact eggs (132). In a low viscosity medium, motility of hyperactivated sperm is characterized by asymmetrical flagellar bends with larger amplitude and curvature, and moving trajectories that are irregular and highly curved (133). We often observed sluggish motility of *Faah*$^{-/-}$ sperm when they were incubated in the capacitation medium. We speculated that the reduced zona-penetrating ability of *Faah*$^{-/-}$ sperm could be due to their reduced motility and/or hyperactivation. Therefore, we assayed motility of WT and *Faah*$^{-/-}$ sperm after capacitation for 30 and 90 minutes *in vitro*. In this measurement, the

curvilinear velocity was calculated from the total distance traveled divided by the travel time; this parameter indicates sperm's swimming ability. The linear velocity was calculated from the distance between the start and end points divided by the travel time. The linearity is the ratio of linear velocity over the curvilinear velocity; this is an indicator of straightness of sperm movement.

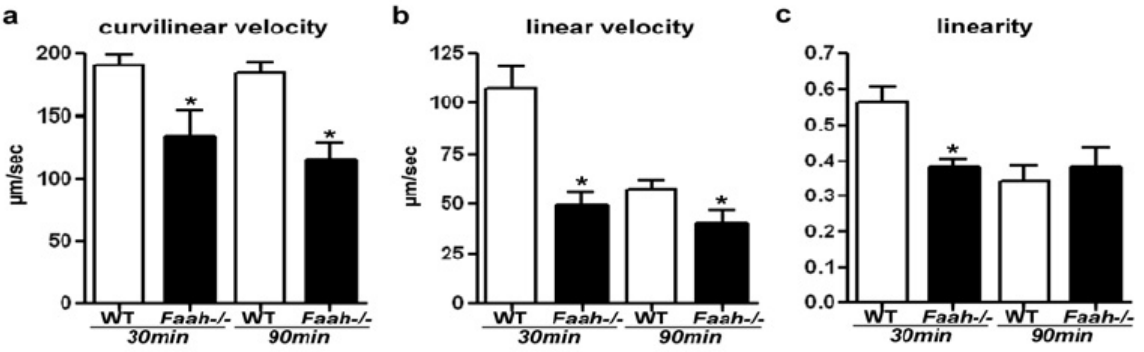

FIGURE 13**. Motility of *Faah*$^{-/-}$ sperm is inferior.** (a) Curvilinear velocity of WT and *Faah*$^{-/-}$ sperm. Curvilinear velocities of *Faah*$^{-/-}$ sperm were significantly lower than those of WT at 30 and 90 minutes of capacitation. (*P < 0.01, unpaired t-test) (b) Linear velocity of WT and *Faah*$^{-/-}$ sperm. Linear velocities of *Faah*$^{-/-}$ sperm were significantly lower than those of WT sperm (*P < 0.05, unpaired t-test). (c) Linearity of WT and *Faah*$^{-/-}$ sperm. Linearity of *Faah*$^{-/-}$ sperm was significantly lower than WT sperm at 30 minutes of capacitation. (*P < 0.05, unpaired t-test).

The movement of *Faah*$^{-/-}$ sperm was significantly slower than those of WT sperm both at 30 and 90 minutes of incubation in the capacitation medium (Figure 13a). The movement of WT sperm was primarily straight at 30 minutes of capacitation with symmetrical flagellar beats. After 90 minutes of capacitation, WT sperm showed a hyperactivated movement pattern, resulting in reduced linear velocity (Figure 13b) and linearity (Figure 13c); the curvilinear velocity was

not significantly changed (Figure 13a). However, *Faah*$^{-/-}$ sperm demonstrated irregular movement at 30 minutes of capacitation distinguished by low linear velocity and linearity (Figure 13b and 13c). Although their moving trajectories were erratic, the seemingly hyperactivated movement of *Faah*$^{-/-}$ sperm was not the consequence of harder beating of flagellum after capacitation, because the moving speed of *Faah*$^{-/-}$ sperm stayed at low levels. These results show that heightened anandamide signaling in the male reproductive tract compromises motility of *Faah*$^{-/-}$ sperm, leading to reduced zona-penetration and fertilization.

Discussion

Emerging evidence shows that endocannabinoid signaling plays critical roles in reproduction. Endocannabinoid signaling is operative in the oviduct, uterus and embryo, and aberrant endocannabinoid signaling adversely affects oviductal transport of embryos and their development (10). Consistent with our present findings, endocannabinoids and their receptors were reported to be present in the testis and sperm of both invertebrates and vertebrates (68, 69, 79, 82, 89, 134). However, our studies showing the presence of the endocannabinoid system in different regions along the male reproductive tract suggests that endocannabinoid signaling has diverse physiological functions. In this respect, Sertoli cells exposed to higher anandamide levels were shown to undergo apoptosis (74), and FAAH activity is regulated by follicle stimulating hormone in mouse sertoli cells (135). In addition, sperm fertility and acrosome reaction were

reported to be adversely affected if exposed *in vitro* to high anandamide levels (77, 89).

Although these *in vitro* studies suggested a role for endocannabinoids in male reproduction, our experiments were designed to see *in vivo* effects of sustained higher anandamide levels in the male reproductive tract on various aspects of sperm function. We used *Faah*$^{-/-}$ mice with higher anandamide levels as a model system to mimic the conditions of long-term exposure to marijuana to explore the role of cannabinoid/endocannabinoid signaling in male fertility. Results of our IVF experiments with *Faah*$^{-/-}$ sperm show a resemblance to reduced sperm fertilizing capacity and motility in marijuana users (136-138). Our findings of compromised fertilizing capacity of *Faah*$^{-/-}$ sperm *in vivo* and *in vitro* as well as their inability to recover in normal capacitating medium provide strong evidence that functional impairment of sperm exposed to higher anandamide levels *in vivo* persists for a prolonged period or becomes irreversible. Our results are clinically relevant since long-term *in vivo* exposure to marijuana is implicated in reduced male fertility (136-138).

IVF experiments using zona-free eggs is an established method to study cellular mechanisms of gamete adhesion and fusion (139). Using this strategy, here we have shown that *Faah*$^{-/-}$ sperm are capable of adhering to and fusing with zona-free eggs similar to WT sperm. The fact that the fertilization rate of *Faah*$^{-/-}$ sperm increased from 42% with zona-intact eggs to 90% with zona-free eggs suggests that the zona is a major barrier to *Faah*$^{-/-}$ sperm, since these null sperm display comparable spontaneous acrosomal reaction rates to WT sperm

as evident from IZUMO staining and flow cytometry analysis. We speculate that factors other than the acrosomal reaction weaken the sperm's penetrating capacity through the zona. Sperm motility and acrosomal enzymes are both involved in zona penetration (120). It is possible that contents released from the acrosomal sac lack appropriate protease activity required for penetration, or that *Faah*$^{-/-}$ sperm cannot acquire hypermotility following capacitation. Our results suggest that reduced motility is a contributing factor for the reduced zona-penetrating ability of *Faah*$^{-/-}$ sperm. However, other factors such as protease activity may also contribute to sperm's reduced capacity for zona penetration. It is interesting to note that *Faah*$^{-/-}$ sperm show asymmetric flagellar beats at 30 minutes of capacitation. We still do not know whether *Faah*$^{-/-}$ sperm show straight-forward moving trajectory before 30 minutes of capacitation. Although it would be interesting to know the motility of *Faah*$^{-/-}$ sperm right after they are placed in the capacitation medium, we were unable to acquire this information because of the time necessary for sperm manipulation and counting.

Reversal of the defects of FAAH deficiency in the absence of CNR1 suggests that anandamide signaling exerts its effects on sperm through CNR1. Since CNR1 is expressed in the testis, epididymis and sperm, it is not clear where and how CNR1-mediated signaling regulates sperm fertility. Since sperm display CNR1, it is possible that higher anandamide levels directly target sperm to alter their function. Alternatively, heightened signaling via CNR1 in the presence of higher anandamide levels in *Faah*$^{-/-}$ testis and epididymis perhaps changes the internal milieu to affect sperm maturation, affecting sperm fertility. Our findings

that *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ sperm show inferior fertilizing capacity compared to sperm deleted of *Faah*$^{-/-}$ only suggests that anandamide working via CNR2 is important for normal sperm fertility. Alternatively, in the absence of CNR2, higher levels of anandamide are exclusively available to CNR1 which may further enhance its adverse effects on sperm function. The latter speculation seems more probable, since homozygous crossings of *Cnr2*$^{-/-}$ mice have an average litter size of about 7 (n=6), whereas homozygous crossings of *Faah*$^{-/-}$/*Cnr2*$^{-/-}$ produces an average of 4 pups per litter (n=12). Although breeding data are confounded by maternal factors other than IVF results, this observation suggests that CNR2 has limited roles in sperm function under normal physiological anandamide levels.

The present investigation has high physiological significance, since sperm in *Faah*$^{-/-}$ males and those in chronic marijuana users are all subjected to enhanced cannabinoid/endocannabinoid signaling. Beneficial effects of anandamide in neurodegeneration, cancer, pain and anxiety (140-143) have prompted increasing interest and effort in developing FAAH inhibitors as novel therapeutic drugs. Thus, the adverse effects of anandamide should be carefully weighed against its beneficial effects. This study provides insights into male fertility regulation by endocannabinoid signaling, and may shed light on improving male fertility.

CHAPTER III

ENDOCANNABINOID SIGNALING DIRECTS DIFFERENTIATION OF TROPHOBLAST CELL LINEAGES AND PLACENTATION

Abstract

Placentation is critical for fetal development. Exposure to marijuana during pregnancy has adverse effects, but it is not known if the placenta is a target of cannabinoid/endocannabinoid signaling. Using mice as a model organism, we found that the endocannabinoid system is present in the ectoplacental cone and spongiotrophoblast cells. We also observed that aberrant endocannabinoid signaling confers premature trophoblast stem cell differentiation, and defective trophoblast development and invasion. These defects are reflected in retarded fetal development and elevated spontaneous pregnancy loss. Because the endocannabinoid system is conserved across species, including humans, our study suggests that endocannabinoid signaling is critical to placentation and pregnancy success in humans.

Introduction

In eutherians, the placenta is the sole bridge between the mother and fetus. While maternal resources filtered across the selective barrier of the placenta nourish and protect the conceptus, the placenta also reshapes the maternal physiology to facilitate fetal survival. Most placental functions are attributed to various trophoblast cell lineages: trophoblast giant cells, spongiotrophoblast, syncytiotrophoblast and glycogen trophoblast cells (144). The fact that all trophoblast cell types are derived from trophoblast stem (TS) cells suggest that differentiation of TS cells is a finely tuned process (145). An appropriate ratio of different trophoblast cell types is a prerequisite for normal placentation, and any aberration in trophoblast differentiation compromises normal placentation.

In mice, immediately after implantation, the mural trophectoderm penetrates the uterine stroma, forming primary trophoblast giant cells (TGC). The polar trophectoderm, which is adjacent to the inner cell mass, forms the extraembryonic ectoderm and ectoplacental cone (EPC), in which progenitor cells that differentiate into secondary trophoblast giant cells reside. Both primary and secondary giant cells undergo multiple rounds of endoreduplication, resulting in cells with polyploid nuclei (146, 147). The EPC and extraembryonic ectoderm cells remain diploid, and give rise to the spongiotrophoblast and labyrinth layers of the placenta (145). *In vivo*, the progenitor cells in the extraembryonic ectoderm and EPC generate chorionic trophoblasts or spongiotrophoblasts (SPT). However, *in vitro* TS cells primarily differentiate into TGC, leading to the concept

that while differentiation to the giant cell lineage is a default pathway, differentiation to other lineages requires distinct signals (94).

Endocannabinoid signaling is a critical regulator of various pregnancy events in mice and humans. Abnormal cannabinoid/endocannabinoid signaling leads to a series of defects that are reflected in embryo development, oviductal transport of embryos, implantation, parturition and early fetal loss in mice (12, 104, 148-151). There is evidence that the endocannabinoid system is also present in the human placenta (152, 153). However, the definitive role of endocannabinoid signaling in placentation remains undefined.

Endocannabinoid signaling is normally mediated by two G-protein coupled cannabinoid receptors, CNR1 (15, 16) and CNR2 (17). Several endogenous bioactive lipid molecules targeting CNR1 and CNR2 have been identified, collectively called endocannabinoids. To date, most work on endocannabinoid signaling has focused on the roles of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (18-20). AEA levels are regulated by a balance between the rate of its synthesis and degradation. Several AEA synthetic pathways have recently been identified (23, 26, 27). The presence of multiple pathways provides evidence that the regulation of AEA synthesis is complex. AEA is degraded to ethanolamine and arachidonic acid by a membrane-bound fatty acid amide hydrolase (FAAH) (28, 29). Although FAAH can hydrolyze other endocannabinoids including 2-AG (30), studies in *Faah*$^{-/-}$ mice show that FAAH is

the major player regulating the magnitude and duration of anandamide signaling (27, 117).

Cannabinoid receptors have been cloned from various species, including the sea urchin, fish, leech, cat, dog, bovine, mouse and human (154). The evolutionary conserved nature of the endocannabinoid system across species from invertebrates to vertebrates suggests its important biological functions. Studies indicate that mice and humans have many similarities regarding the regulation of endocannabinoid signaling and its physiological roles. For instance, successful implantation requires tightly regulated anandamide signaling in both mice and humans (10, 104, 155). The absence of CNR1 in mice causes oviductal retention of preimplantation embryos (156), while women with ectopic pregnancy have attenuated expression of CNR1 in the Fallopian tube (157).

Previous studies have shown the presence of the endocannabinoid system in the human placenta (158). However, the underlying mechanism of endocannabinoid signaling affecting pregnancy success is not known. In this study, we used a genetic approach to address this question. We found midgestational fetal loss in mice missing *Cnr1*. Further in vivo and in vitro studies show that CNR1 deficient TS cells fail to appropriately differentiate to normal placentation, leading to midgestational fetal demise and compromised pregnancy outcome.

Materials and Methods

Reagents

Antibodies to anti-CNR1 (N-15) antibody were purchased from Santa Cruz Biotechnology; anti-cytokeratin antibody was from DAKO Diagnostics; anti-phospho-AKT (Ser-473 or 308) was from Cell Signaling. Anti-FAAH antibody was a gift from Dr. Benjamin F. Cravatt (The Scripps Research Institute, CA). SR141716 and SR144528 were dissolved in ethanol and diluted to a final concentration with ethanol less than 0.1%.

Animals

Adult wild-type, *Cnr1* and *Faah* mutant mice on C57BL/6J genetic background generated as previously described (117, 159) were housed in the animal care facility of Cincinnati Children's Hospital Medical Center according to National Institutes of Health and institutional guidelines for laboratory animals. Female mice were mated with fertile males and were checked for vaginal plug at 0900h for confirmation of pregnancy (day 1). To examine the postimplantation development of embryos, mice were killed between 0900-1000h on the given day as described in the paper and implantation sites were weighed, then frozen or fixed for further study. Some implantation sites of day 12 and 14 were dissected out to weigh the placenta and fetus.

In situ and Northern hybridization

In situ hybridization and Northern blotting were performed as previously described (124). Antisense 35S-labeled or 32P-labeled cRNA probes produced from specific cDNA were used to detect the specific mRNA on frozen sections or membranes. The densities of Northern bands were quantified using Scion Image software.

Trophoblast Stem (TS) Cell Culture

Wild-type or *Cnr1*$^{-/-}$*Cnr2*$^{-/-}$ trophoblast stem cell (TS) lines were generated as previously reported (160). Cells were maintained in a proliferative state in media (TS maintaining media) containing 70% embryonic mouse fibroblast cells conditioned medium, 30% TS cell medium, 25ng/ml FGF4 and 1 μg/ml heparin, incubated in a humidified tissue culture incubator at 37˚C, 5% CO_2 atmosphere. To introduce TS cell spontaneous differentiation, cells were cultured in medium without conditioned medium, serum, FGF4 and heparin for up to 6 days with medium changed everyday.

BrdU (5-Bromo-2'-deoxyuridine) incorporation

Two hours before sacrifice, animals were injected intraperitoneally with BrdU reagent (catalog No. 00-0103, Invitrogen, CA) at 1ml/100 gram body weight. Implantation sites were fixed in 10% neutral buffered formalin, and embedded in paraffin. The Brdu signals on paraffin sections were detected using the BrdU staining kit (catalog No. 93-3943, Invitrogen, CA), following the manufacturer's protocol.

Proliferation assay

The proliferation assays of the wild-type or *Cnr1*$^{-/-}$*Cnr2*$^{-/-}$ TS cells were performed using the Cell Proliferation Assay kit (Promega). In brief, TS cells (0.5, 1, 2 X 10^3 cell/100 µl well) were suspended in TS maintaining media, seeded on to 96-well plates and incubated until analyzed. At different time points, mixed MTS/PMS solution (20µl/well) was added and incubated for 4 hours and then absorbance read at 490 nm under an ELISA plate reader (Bio-TEK). The proliferation assay was performed on counted cells seeded for 1 hour to determine the correlation coefficients of absorbance-cell number standard curves. The cell numbers at different time points were calculated by the coefficient using standard curves. The assays were performed in triplicate and the experiments were repeated three times.

Invasion assay

The invasion assays were performed in 24-well BD Biocoat Matrigel invasion chambers following the manufacturer's instructions. In brief, Matrigel inserts rehydrated with RPMI1640 medium were placed over the BD Falcon TC companion plate containing 0.75 ml/well TS maintaining media. Wild-type or *Cnr1*$^{-/-}$*Cnr2*$^{-/-}$ TS cells were suspended in TS maintaining media with different treatments but minus condition medium and serum at a density of 10 X 10^4 cell/ml. Then 0.5 ml cell suspensions were seeded within the Matrigel inserts immediately. After 22 or 44 hours incubation, the Matrigel and non-invading cells

are removed by cotton swab from the upper surface of the membrane of the inserts. The invaded cells at the lower surface of the membrane were then fixed with 10% neutral buffered formalin and stained with crystal violet. After air drying, the cells could be counted or a picture taken under an inverted microscope. The assays were performed in triplicate and the experiments were repeated three times.

Statistical Analysis

Comparison of means was performed using Student's *t* test. Data are shown as mean ± SEM.

Results

Genetic loss of *Cnr1* compromises pregnancy success in mice

Endocannabinoid signaling is a key player in regulating female fertility. Our previous studies have shown that about 30% of embryos are trapped in the oviduct of *Cnr1*$^{-/-}$ mice (12). In addition, our breeding data of *Cnr1*$^{-/-}$ females crossed with *Cnr1*$^{-/-}$ males show a 50% reduction in litter sizes compared to crosses between wild-type (WT) females and males (Figure 14a). This dramatic reduction in litter sizes suggests that approximately 20% of embryos are lost during gestation, considering that 30% of embryos are trapped in the oviduct. Moreover, 40% of plug-positive *Cnr1*$^{-/-}$ females did not produce any litters (12), underscoring the fact that embryo loss during gestation is even more severe.

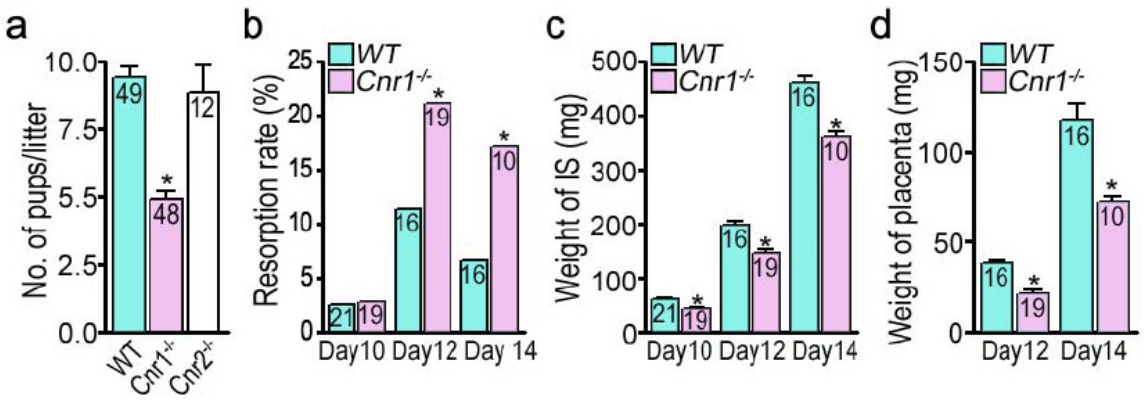

FIGURE 14. ***Cnr1*$^{-/-}$ mice have compromised placentation, embryo development and pregnancy outcome.** (a) *Cnr1*$^{-/-}$, but not *Cnr2*$^{-/-}$, mice have smaller litter size (unpaired *t*-test, **P*<0.05). (b) A higher rate of resorptions is seen in *Cnr1*$^{-/-}$ mice on days 12 and 14 of pregnancy (*F*-test, **P*<0.05). (c) *Cnr1*$^{-/-}$ implantation sites (IS) weigh less than those of WT at midgestation (unpaired *t*-test, **P*<0.05). (d) Average weights of *Cnr1*$^{-/-}$ placenta are less than those of WT on days 12 and 14 of pregnancy (unpaired *t*-test, **P*<0.05). Numbers within bars indicate numbers of dams examined.

In order to determine the stage when the sign of resorption is clearly evident, we examined the pregnancy status on days 10 through 14 in *Cnr1*$^{-/-}$ and WT females. We found that a higher rate of resorption occurs on days 12 and 14 of pregnancy in mutant females (Figure 14b). Furthermore, weights of implantation sites in *Cnr1*$^{-/-}$ females are less than those in WT mice (Figure 14c). Since placentation occurs during this period, we also recorded placental weights. Placental weights in *Cnr1*$^{-/-}$ mice are also significantly lower than those in WT mice (Figure 14d). These results prompted us to hypothesize that CNR1 deficiency causes placentation defects, and thus fetal death during midgestation.

Endocannabinoid system is present in midgestational placentas

To investigate the role of the endocannabinoid system in midgestation, we first measured the levels of the two most studied endocannabinoids, AEA and 2-AG, which target CNR1 evoking various biological functions. We found that the levels of both AEA and 2-AG are comparable between WT and *Cnr1*$^{-/-}$ females in day 12 uteri and placentas (Fig 15a). Furthermore, the expression pattern of CNR1 was examined by immunohistochemistry. We found that CNR1 positive cells are present in the EPC on day 10 of pregnancy. On day 14, CNR1 is expressed in the SPT layer which is derived from the EPC (Fig 15b). FAAH showed expression patterns similar to CNR1 (Fig 15c). The presence of endocannabinoid

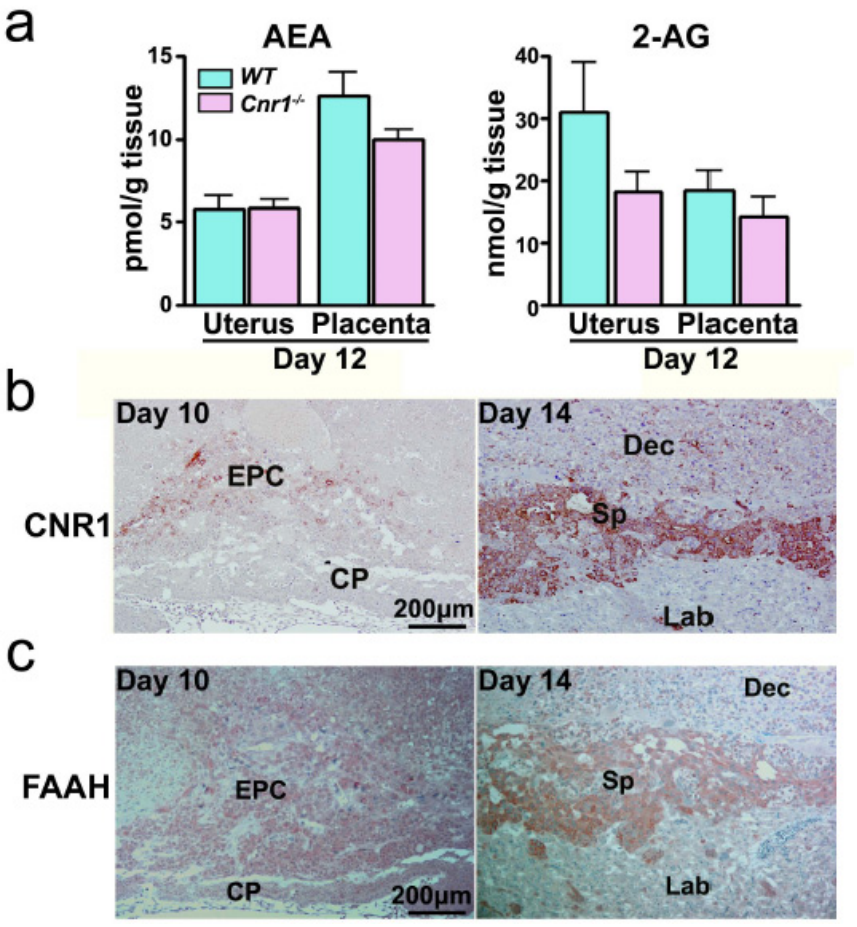

FIGURE 15**. Endocannabinoid system is present in midgestational placentas.** (a) Levels of AEA and 2-AG are comparable in WT and *Cnr1*$^{-/-}$ midgestational uteri and placentas. Uterine and placental samples were pooled separately from 5 pregnant WT or *Cnr1*$^{-/-}$ mice in each group (n = 4-5) (b) CNR1 is present in trophoblast cells in the EPC on day 10 of pregnancy and SPT layer on day 14 of pregnancy. (c) FAAH expression pattern is similar to CNR1 in midgestational placentas. Results are representative of at least 2 to 3 independent samples. EPC, ectoplacental cone; CP, chorionic plate; Dec, decidua basalis; Sp, spongiotrophoblast; Lab, labyrinth.

ligands, receptors and a key regulatory enzyme in midgestation placentas suggests that endocannabinoids play an important role in placentation. Additionally, the restricted expression of CNR1 and FAAH within the EPC, and thereafter in the SPT, led to the speculation that generation, differentiation, and/or maintenance of SPT cells is regulated by endocannabinoid signaling, and an aberration of this signaling, as in CNR1 deficiency, leads to abnormal placentation.

Spongiotrophoblast development is compromised in *Cnr1*$^{-/-}$ mice

To better understand the underlying cause of midgestational embryo loss in the absence of CNR1, we analyzed placental development in WT and *Cnr1* null mice. Placental lactogen I (*Pl1*), encoding the prolactin-like hormone, is specifically expressed in TGC (161), while trophoblast-specific protein α (*Tpbpa*, also known as *4311*) is explicitly expressed in SPT cells (162). In situ hybridization results show that *Pl1* expression in *Cnr1*$^{-/-}$ placentas on day 10 of pregnancy is comparable to that in WT mice (Figure 16a). However, *Tpbpa* expression is

appreciably reduced in *Cnr1*$^{-/-}$ placentas, providing evidence that the development of the SPT layer has been compromised. These results are consistent with Northern hybridization results, showing much higher *Tpbpa* expression in WT mice compared with *Cnr1*$^{-/-}$ mice on day 10 of pregnancy. *Pl1* expression levels are similar in *Cnr1*$^{-/-}$ and WT females on the same day (Figure 16b).

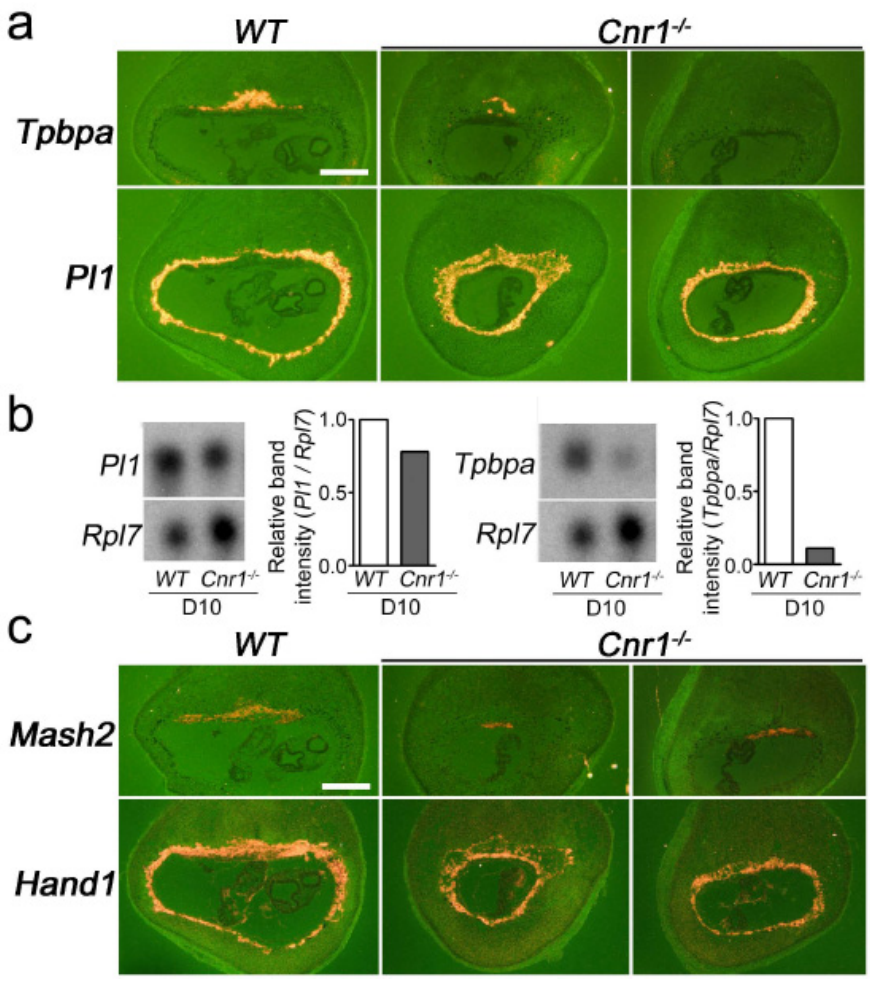

FIGURE 16. ***Cnr1*$^{-/-}$ spongy layer is developmentally compromised.** (a) While numbers of *Tpbpa* positive cells are considerably lower in *Cnr1*$^{-/-}$ placentas, *Pl1* expression in *Cnr1*$^{-/-}$ placentas on day 10 is comparable to that in WT placentas.

Scale bar: 500 μm. (b) Northern blotting results using RNA samples from WT and *Cnr1*$^{-/-}$ implantation sites on day 10 of pregnancy show that *Tpbpa*, but not *Pl1*, levels are reduced. Levels of *Tpbpa* and *Pl1* mRNAs were normalized and quantified against *Rpl7* (a house-keeping gene) band intensities using the same membrane. (c) Levels of *mash2* transcript were reduced in *Cnr1*$^{-/-}$ placentas, but *Hand1* levels were comparable in WT and *Cnr1*$^{-/-}$ placentas. Scale bar: 500 μm.

Secondary TGCs are differentiated from precursor cells present in the EPC and later in the SPT layer. The terminal differentiation of precursor cells is regulated by a reciprocal interaction between two basic helix-loop-helix transcription factor genes, *Hand1* and *Mash2* (163), and the expression of these genes overlap in the EPC and the SPT. *Mash2* maintains giant cell precursors in an undifferentiated state (164), whereas *Hand1* promotes differentiation into TGCs (165). The compromised SPT layer in *Cnr1*$^{-/-}$ mice may arise from misregulated expression of *Hand1* and *Mash2*. Therefore, we examined the expression of these two genes at the implantation site on day 10 by in situ hybridization. We observed similar *Hand1* expression patterns and signal intensity in both TGC and SPT cells in WT and *Cnr1*$^{-/-}$ mice (Figure 16c). However, the population of *Mash2* positive cells was reduced in *Cnr1*$^{-/-}$ placentas. These results offer two hypotheses: first, the deficiency of *Cnr1* in the EPC suppresses the proliferation of spongiotrophoblast cells, and thus reduces the population of *Tpbpa* positive trophoblast cells, or the lack of CNR1 in trophoblast precursor cells promotes their differentiation to TGC but not SPT cells.

Trophoblast cell proliferation is stifled in *Cnr1*$^{-/-}$ EPC

To test the hypothesis that CNR1 deficiency inhibits trophoblast cell proliferation in the EPC, cell proliferation was examined by BrdU incorporation at the implantation sites on day 9 in *Cnr1*$^{-/-}$ and WT mice. SPT cells are derived from trophoblast cells in the EPC

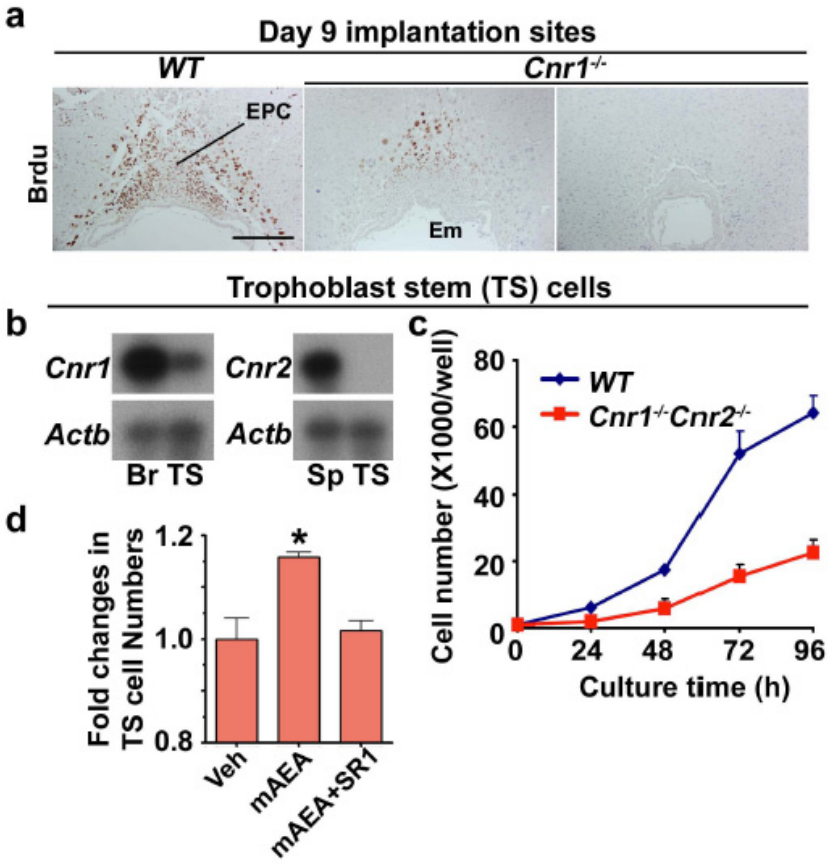

FIGURE 17**. Compromised proliferation of trophoblast cells missing *Cnr1*.** (a) BrdU incorporation results show that *Cnr1*$^{-/-}$ TS cells in the EPC proliferate at a much slower rate compared to WT TS cells. EPC, ectoplacental cone; Em, embryo. Scale bar: 200μm. (b) RT-PCR analysis coupled with Southern blotting shows that *Cnr1*, but not *Cnr2*, is expressed in WT TS cells. RNA samples from brain (Br) and spleen (Sp) were used as positive controls for *Cnr1* and *Cnr2*,

respectively. TS, trophoblast stem cells. (c) Proliferation of *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells is much slower than WT TS cells *in vitro* (n=3). (d) Treatment with mAEA increases WT TS cell proliferation *in vitro*, and this effect is suppressed by a CNR1 specific inhibitor, SR1 (n=3; unpaired *t*-test, **P*<0.05).

(166), which show rapid proliferation on day 9 of pregnancy. In WT mice, increased proliferation was observed in the EPC (Figure 17a), TGC and mesometrial decidual cells. However, the number of BrdU positive cells was considerably reduced in *Cnr1*$^{-/-}$ EPC and TGC. Although many *Cnr1*$^{-/-}$ embryos showed retarded development (Figure 17a, right panel), some *Cnr1*$^{-/-}$ embryos showing development comparable to WT embryos (Figure 17a, middle panel) also had fewer BrdU positive cells in EPC and TGC. Similar results were observed in *Cnr1*$^{-/-}$ day 11 implantation sites (Figure 18). Reduced proliferation of *Cnr1*$^{-/-}$ TS (Figure 17a), which later differentiate into SPT, may have resulted in compromised SPT development.

To complement our observations *in vivo*, proliferation of TS cells were assessed *in vitro* by MTS proliferation assays, in which the cell number is reflected by the absorbance of colored product in the presence of a substrate (167). Because *Cnr2* is not expressed in TS cells (Figure 17b), TS cells deficient in both *Cnr1* and *Cnr2* were used to substitute for *Cnr1*$^{-/-}$ TS cells in *in vitro* experiments. Standard curves for *Cnr1*$^{-/-}$*Cnr2*$^{-/-}$ and WT TS cells were generated from the absorbance values against known numbers of WT and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells. To assess cell proliferation, the same numbers of *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ and WT TS

cells were seeded in multiple wells, and cell numbers were counted using standard curves on given time points shown in Figure 17c. *Cnr1*$^{-/-}$*Cnr2*$^{-/-}$ TS cell proliferation was remarkably slower than WT TS cells. To confirm that the effect is mediated by CNR1, we further tested WT TS cell proliferation in the presence

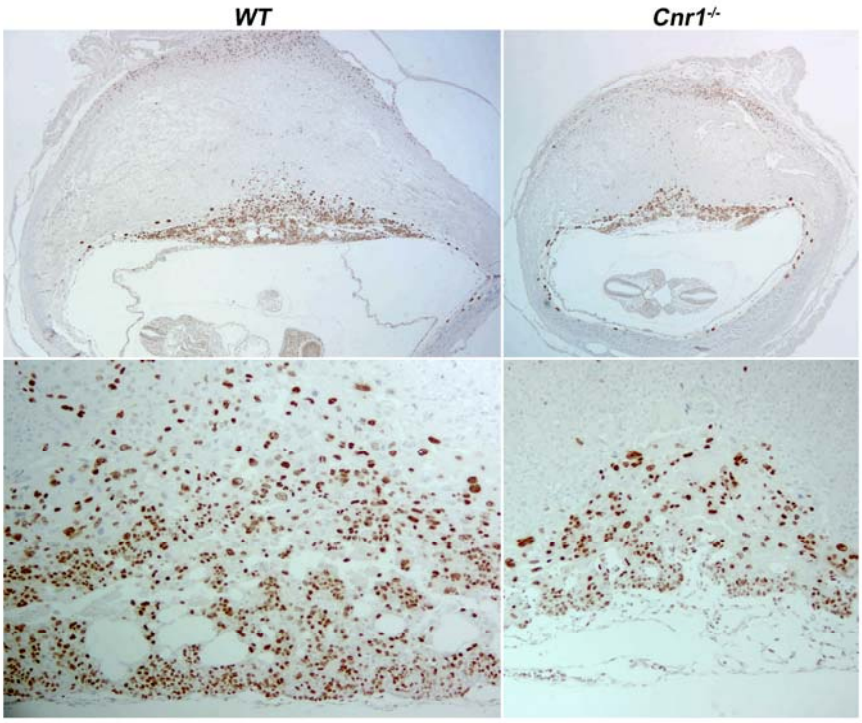

Figure 18. **Proliferation of *Cnr1*$^{-/-}$ trophoblast cells is compromised.** In day 11 (D11) implantation sites, the number of BrdU positive cells in *Cnr1*$^{-/-}$ EPCs is much less than in WT EPC. Results are representative of at least 2 to 3 independent samples.

of methanandamide (mAEA), a stable analog of AEA, or a vehicle (control). Compared to the control group, mAEA-treated WT TS cells proliferated at a significantly faster rate (Figure 17d), but were inhibited by SR141716 (SR1), a CNR1 selective antagonist, suggesting AEA promotes TS proliferation via CNR1.

SPT cell proliferation is tightly regulated by the PI3K-pAKT signaling pathway (168). In addition, cannabinoid signaling via CNR1 can regulate AKT activation by the $G_{i/o}$/PI3K pathway (169, 170). We speculated that

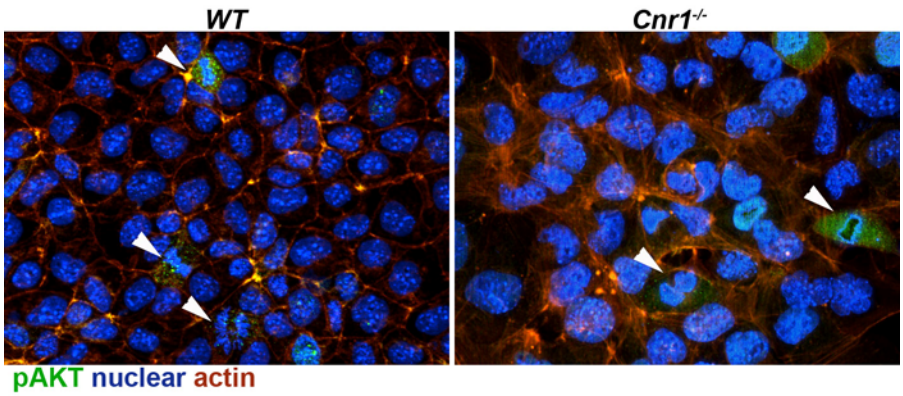

FIGURE 19. **pAKT is localized in TS cells undergoing division.** Immunofluorescence results show that pAKT is localized in TS cells in the M phase of the cell cycle (white arrowheads). TS cells were cultured in TS cell media as described in methods. Results are representative of 2 to 3 independent samples. Scale bar: 50μm.

endocannabinoid signaling via CNR1 would regulate trophoblast cell proliferation by altering AKT activation. Our immunofluorescence results show that pAKT is localized in TS cells undergoing division (Figure 19), suggesting pAKT plays a role in TS cell proliferation. To test whether CNR1-mediated signaling involves AKT signaling in placentation, pAKT was localized by immunohistochemistry in day 10 placentas. Most trophoblast cells in the WT EPC were positive for pAKT, whereas only sporadic trophoblast cells in the *Cnr1*$^{-/-}$ EPC were pAKT positive (Figure 20a). These results provide evidence that CNR1 deficiency suppresses AKT activation, thus downregulating trophoblast cell proliferation in the EPC and

SPT. To see whether AKT activation is mediated by the PI3K pathway, WT and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells were treated with PI3K inhibitor LY294002 and pAKT levels were examined by Western blotting (Figure 20b). LY294002 was effective in attenuating PI3K activity in WT and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells. Because CNR1 deficiency confers compromised TS cell proliferation both *in vivo* and *in vitro* (Figure 20a and 20c), we speculated that the blockage of PI3K in WT TS cells should mimic the effect seen in CNR1 deficiency. Indeed, addition of LY294002 significantly slowed down proliferation of WT and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells in a concentration dependent manner (Figure 20c). Collectively, the results show that CNR1 mediated signaling via the PI3K-AKT pathway is critical for appropriate TS cell proliferation.

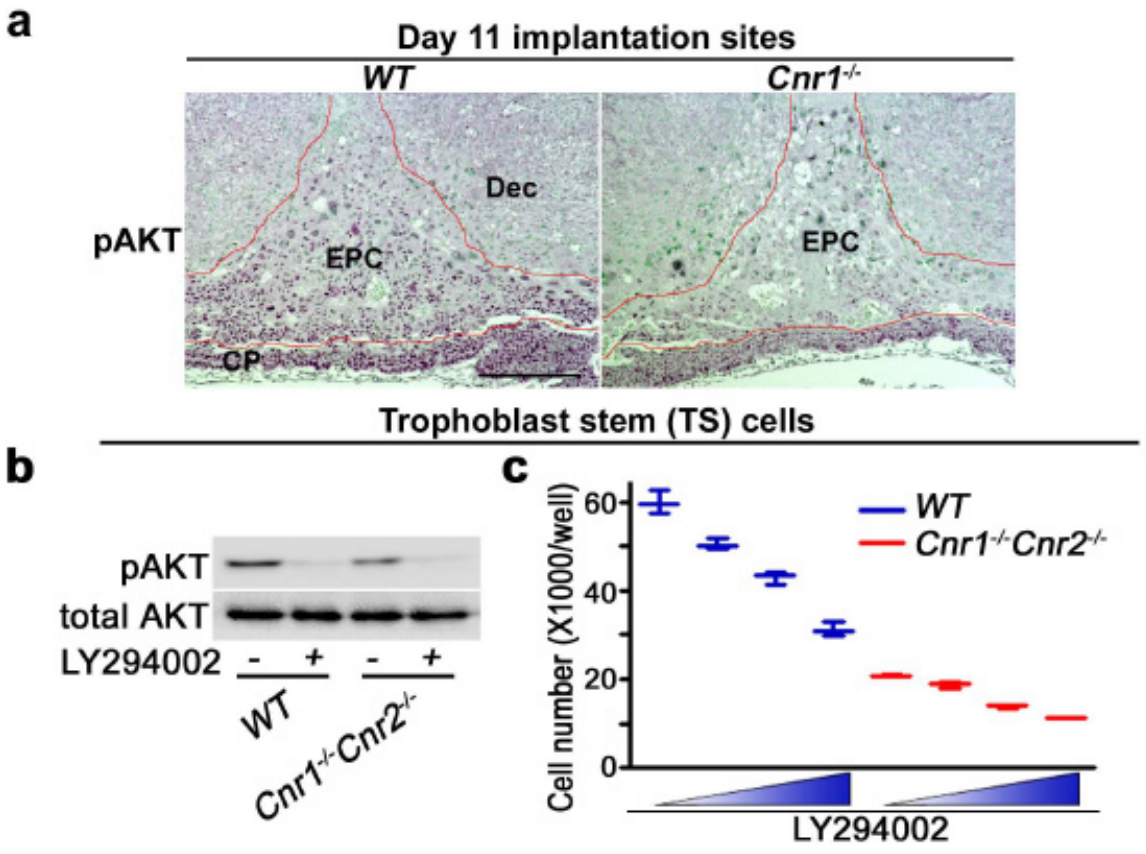

FIGURE 20. **Endocannabinoid signaling regulates TS cell proliferation via PI3K/AKT signaling.** (a) *Cnr1*$^{-/-}$ placentas have less pAKT positive cells in the EPC as indicated by pAKT immunostaining. EPC, ectoplacental cone. Dec, decidua basalis. CP, chorionic plate. Results are representative of at least 2 to 3 independent samples. Scale bar: 200μm. (b) Levels of pAKT in TS cells were reduced by PI3K inhibitor LY294002. WT TS cells have higher pAKT levels compared to *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells. Total AKT served as a loading control. (c) LY294002 attenuated TS cell proliferation in a concentration dependent manner. TS cells of both phenotypes were treated with 0, 2, 20, 40μM LY294002 for 72 hours (n=3).

CNR1 deficient TS cells are biased to differentiate into trophoblast giant cells

Since the SPT in the *Cnr1*$^{-/-}$ placenta is compromised, we examined whether the TS cell fate is biased in the absence of CNR1 in addition to its role in cell proliferation. *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells cultured in the presence of FGF4 and mouse embryonic fibroblast (MEF) condition medium displayed dramatic morphological changes. *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells were of irregular shape, bigger with large nuclei, and had numerous stress fibers (Figure 21a). Cytoskeleton reorganization and larger cell size indicate that TS cells are prone to differentiate into TGC (171). *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells resemble those in a differentiated state, forming loose and chaotic cell colonies (Figure 21a). Furthermore, spontaneous differentiation was induced in WT and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells by culturing them in the absence of

FGF4 and MEF condition medium. The trophoblast giant cell and spongy cell markers were examined by Northern blotting. As shown in figure 21b, although WT and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells differentiate into trophoblast giant cells, as evident by the expression of *Pl1* gene, the SPT marker *Tpbp* was only observed in WT trophoblast cells. These results indicate that endocannabinoid

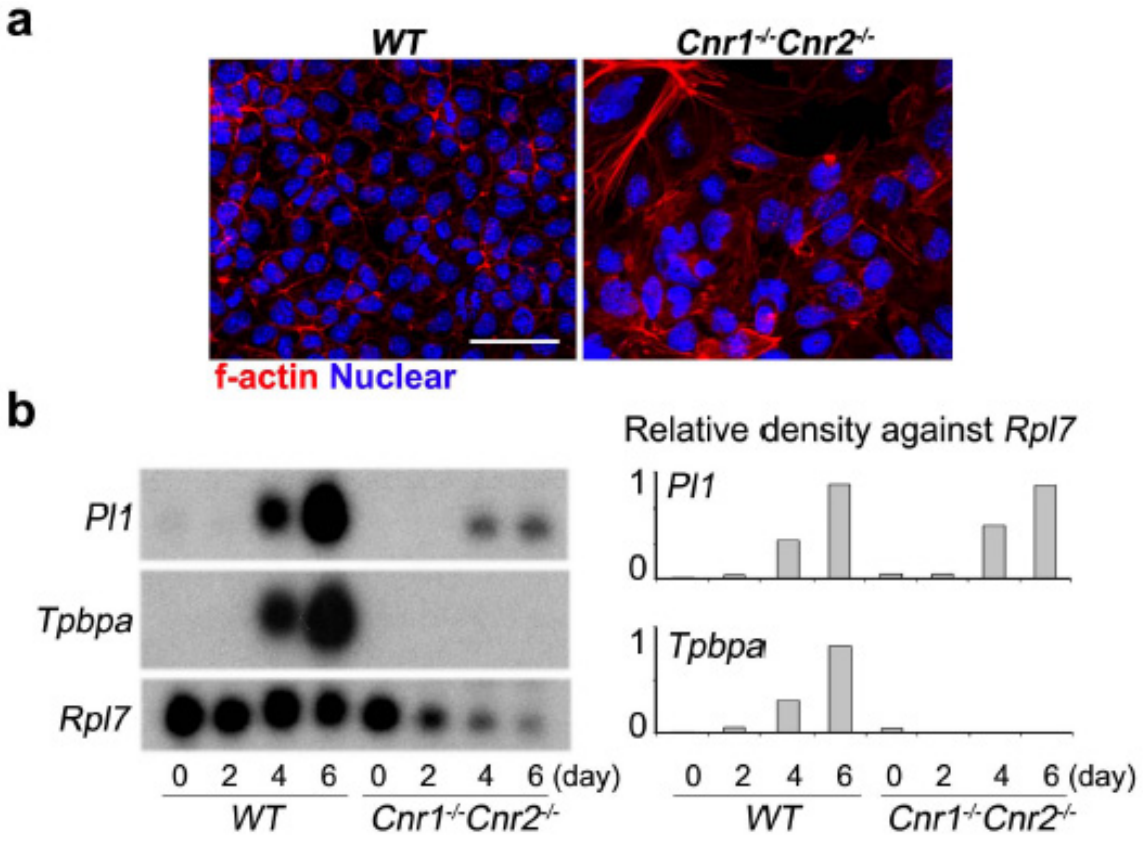

FIGURE 21. ***Cnr1*$^{-/-}$ TS cells are more prone to differentiate into TGC.** (a) Under similar culture conditions, *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells have more stress fibers, as evident from increased f-actin immunofluorescence staining. Results are representative of at least 2 to 3 independent samples. Scale bar: 50μm. (b) Northern blotting results from spontaneous differentiation experiments show that WT TS cells expressed both Pl1 and *Tpbpa*, whereas *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells expressed only *Pl1,* suggesting that most *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$*TS* cells had differentiated

into TGCs. Levels of *Tpbpa* and *Pl1* mRNAs were normalized and quantified against *Rpl7* using the same membrane.

signaling is required to maintain the appropriate TS cell state; the absence of CNR1 signaling suppresses differentiation of TS cells into SPT cells, but promotes differentiation to TGC.

Cnr1 deficient trophoblast cells show shallow invasion

The maternal and fetal vascular systems are brought into close proximity through placentation, and trophoblast cells directly tap into the maternal vascular system (172). Thus, trophoblast cell invasiveness is critical for successful placentation. Cyclooxygenase-2 (COX2) is expressed in invading trophoblast cells with the initiation of placentation. Immunolocalization shows that *Cnr1*$^{-/-}$ EPC has reduced COX2 expression compared to WT EPC on day 10 of pregnancy (Figure 22b), suggesting that *Cnr1*$^{-/-}$ EPC cells are less invasive. Western blotting results are consistent with immunohistochemistry signals (Figure 22c). The invasion of glycogen trophoblast cells (GTC) into the decidua basalis is another indicator of trophoblast invasiveness. These cells, derived from the SPT on day 12, continue to express caudal type homeobox 2 (CDX2) (144). We examined CDX2 expression in the placenta on day 14. In WT females, an abundant number of CDX2-positive glycogen trophoblast cells invade into the decidual basalis, while only a sporadic number with shallow invasion were found beyond the TGC

border in *Cnr1*$^{-/-}$ mice (Figure 22a). The results suggest that the trophoblast invasive ability is compromised in the absence of CNR1.

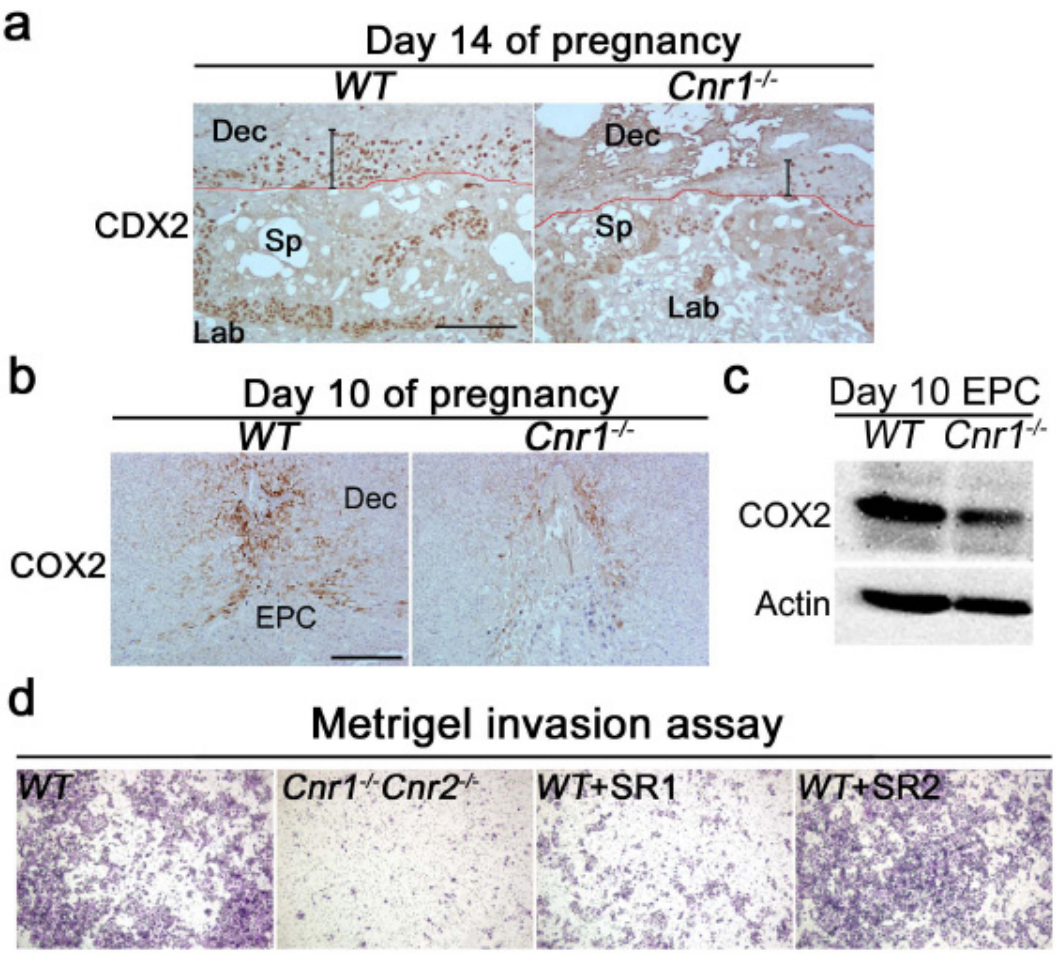

FIGURE 22. ***Cnr1***$^{-/-}$ **trophoblast cells are less invasive.** (a) Glycogen trophoblast cell invasion into the decidua basalis on day 14 of pregnancy in *Cnr1*$^{-/-}$ mice is shallow as shown by CDX2 immunostaining. Scale bar: 100μm. (b) In *Cnr1*$^{-/-}$ mice, the number of COX2- positive cells, invading into the maternal decidua around the ectoplacental cone, is low. Scale bar: 200μm. (c) Western blotting of COX2 levels in extracts of isolated EPC from day 10 WT and *Cnr1*$^{-/-}$ implantation sites. (d) In *in vitro* invasion assays, numbers of *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells successfully penetrating through the matrigel are much lower than those of WT TS cells. Invasive capacity of WT TS cells is attenuated by a CNR1

antagonist SR1 (7nM), indicating that CNR1 is critical for normal TS cell invasion. Results are representative of at least 2 to 3 independent samples.

Matrigel invasion assays were used to study the invasive ability of TGC (173). Using this assay, we examined the effects of endocannabinoid signaling on TS cell invasive behavior *in vitro*. Only a limited number of *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ TS cells were able to penetrate through the matrigel membrane after 44 hrs in culture (Figure 22d), suggesting that CNR1 deficiency suppresses the invasive ability of trophoblast cells. In addition, WT TS cell invasiveness was blocked by the CNR1-selective antagonist SR1, but not SR144528 (SR2), a CNR2 antagonist. The results show that the silencing of CNR1 dampens trophoblast invasion.

Discussion

To support fetal survival and growth, a functional placenta requires an appropriate and balanced amount of different trophoblast cells. Using the CNR1 deficient mouse, we show that endocannabinoid signaling, which presents in the EPC and thereafter in the spongiotrophoblast layer, plays roles in both trophoblast cell growth and differentiation. The ablation of CNR1-mediated endocannabinoid signaling inhibits TS cell proliferation, differentiation into SPT cells, and trophoblast invasiveness. Consequently, defective placentation and fetal development lead to increased rates of resorption in *Cnr1*$^{-/-}$ mice.

The spongy layer is located between the outer secondary TGCs and inner labyrinth layer. The function of the spongiotrophoblast layer is still not clearly

understood. It is thought to function as structural support for the developing labyrinth which is populated by maternal and fetal capillaries (172). Studies in *Mash2* deficient mice have revealed that all of the labyrinth, SPT and TGC layers are affected in the absence of Mash2 (174), even though *Mash2* is specifically expressed within the spongy layer. This indicates that a well-developed spongiotrophoblast layer is required for normal placentation. We found that CNR1 is expressed in the EPC and later in SPT layers. The CNR1 deficient placenta has a reduced *Tpbp*-positive SPT layer with unaffected TGCs. Thus, the compromised SPT development leads to abnormal placentation and consequently resorption of the feto-placental unit.

The expression of *Hand1* and *Mash2* overlaps in the ectoplacental cone and spongiotrophoblast layer in which *Tpbpa*-positive trophoblast precursor cells reside. The coordinated antagonistic effects of *Hand1* and *Mash2* regulate the differentiation of the precursor cells into secondary TGCs (163). However, a recent cell lineage tracing study shows that not all secondary TGCs are derived from *Tpbpa* positive cells, indicating that the cell fate of some TGCs is decided before *Tpbpa* expression (175). In vitro spontaneous differentiation experiments with *Cnr1*$^{-/-}$*/Cnr2*$^{-/-}$ TS cells show that *Tpbpa* is barely detected, indicating that most, if not all, TS cells differentiate into TGC cells in the absence of CNR1. It is possible that deficiency of CNR1 directly promotes differentiation of TS cells into TGCs *in vitro*, without going through the transition phase of *Tpbpa* positive precursor cells. Alternatively, the reduced *Tpbpa*-positive cells in *Cnr1*$^{-/-}$ mice

could be the consequence of reduced TS cell proliferation. To dissect out the role of CNR1 in TS cell proliferation from its role in TS cell differentiation, cell proliferation was examined using FGF4 and MEF-condition medium to keep the TS cells undifferentiated. We found that TS cell division is coupled with upregulated pAKT, and deficiency of CNR1 dampens TS cell proliferation by downregulating pAKT levels. These results are consistent with previous studies in other systems where CNR1 is coupled with Gi/o and activates AKT signaling by turning on 1-Phosphatidylinositol 3-Kinase (169, 170, 176).

In the MTS cell proliferation assay used to examine TS cell proliferation, the basic principle is that the mitochondria reductase in viable cells can turn phenazine methosulfate (PMS) and MTS into water-soluble formazan, giving a purple color. The absorbance (maximum at 490-500 nm) of this colored solution proportionally reflects the number of viable cells (167). Two standard curves were generated using the given number of WT or *Cnr1*$^{-/-}$*/Cnr2*$^{-/-}$ TS cells. Both curves were linear, validating that the colorimetric readings are proportional to cell numbers. Intriguingly, the slope of the curve for *Cnr1*$^{-/-}$*/Cnr2*$^{-/-}$ TS cells is greater than that of the WT TS cells, indicating *Cnr1*$^{-/-}$*/Cnr2*$^{-/-}$ TS cells have more mitochondria reductase than WT TS cells. These results demonstrate that *Cnr1*$^{-/-}$*/Cnr2*$^{-/-}$ TS cells are more metabolically active than WT TS cells.

Endocannabinoid signaling has been shown to be present in human and rat placentas (158, 177). The conserved presence of the endocannabinoid system in

placenta indicates its role in placentation. However, it was not clear how endocannabinoid signaling regulates placentation. Using the mouse model, we show here that endocannabinoid signaling is capable of interfering with the fate of mouse TS cell differentiation, and that aberrant endocannabinoid signaling causes defective placental development and higher incidence of fetal resorptions in midgestation. Our studies on endocannabinoid signaling in the mouse placenta may shed some light on potential endocannabinoid studies in humans.

CHAPTER IV

CLOSING REMARKS

More and more lipid molecules are being revealed as indispensable components of different biological signaling pathways. Some of these lipid mediators are found to be critical in coordinating events of early pregnancy. Our laboratory has demonstrated that appropriate endocannabinoid signaling is critical to preimplantation embryo development, their timely homing into the receptive uterus and on-time implantation (10-12). My work has added new dimensions to the role of endocannabinoid signaling by extending its role in sperm function in the context of fertilization in male reproduction and placentation in female reproduction.

In males, the components of the endocannabinoid system are found in the testis and epididymis. To study the effect of marijuana in reproductive functions in chronic marijuana users, I have characterized male reproductive phenotypes in mice with genetic loss of *Faah*. In these mice, the levels of anandamide in the testis and epididymis are elevated, mimicking long-term exposure to marijuana. The aberrant endocannabinoid signaling compromises the fertilizing capacity of sperm. Retention of *Faah*$^{-/-}$ sperm on the egg zona-pellucida provides evidence that sperm's capacity to penetrate the zona barrier is dampened. Furthermore, we showed this defect is rescued by superimposing deletion of CNR1, indicating

the endocannabinoid signaling on sperm is mediated by CNR1. Men who smoke marijuana frequently have significantly less seminal fluid, a lower total sperm count and their sperm behave abnormally, all of which may affect fertility adversely. My studies may provide therapeutic targets to improve male fertility.

In females, our lab's previous studies demonstrated the roles of endocannabinoid signaling in preimplantation embryo development and oviductal transportation. My work expands our understanding to midgestation in pregnancy. Previous studies in our lab showed the endocannabinoid system was present in embryos, and my work revealed that the expression of CNR1 and FAAH is retained in trophoblast stem cells and spongy cells during placentation. In addition, the endocannabinoids are also actively synthesized in placenta. Using mice deficient of CNR1, we showed endocannabinoid signaling via CNR1 is required for TS cells to appropriately differentiate into spongiotrophoblast and trophoblast giant cells. CNR1 deficient TS cells are biased to differentiate into trophoblast giant cells. In addition, CNR1 mediated endocannabinoid signaling via the PI3K-AKT pathway also regulates TS cell proliferation.

Genetically manipulated mouse models studied to date are not totally infertile, indicating the role of endocannabinoid signaling is complimentary to fertility regulation. However, my work, together with previous studies, demonstrates endocannabinoid signaling has various roles in different physiological processes in both male and female reproduction. The fact that it is present in different

animal species further proves that evolutionarily conserved lipid signaling is important for reproduction. Further studies are needed to reveal additional functions of endocannabinoid signaling in reproduction and other physiological processes.

REFERENCES

1. Thonneau P, Marchand S, Tallec A, Ferial ML, Ducot B, Lansac J, Lopes P, Tabaste JM, Spira A 1991 Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988-1989). Human reproduction (Oxford, England) 6:811-816
2. Abma JC, Chandra A, Mosher WD, Peterson LS, Piccinino LJ 1997 Fertility, family planning, and women's health: new data from the 1995 National Survey of Family Growth. Vital and health statistics:1-114
3. Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T, Wang H 2004 Molecular cues to implantation. Endocrine reviews 25:341-373
4. Paria BC, Reese J, Das SK, Dey SK 2002 Deciphering the cross-talk of implantation: advances and challenges. Science (New York, NY 296:2185-2188
5. Wang H, Dey SK 2006 Roadmap to embryo implantation: clues from mouse models. Nature reviews 7:185-199
6. Wang H, Dey SK 2005 Lipid signaling in embryo implantation. Prostaglandins & other lipid mediators 77:84-102
7. Shah BH, Catt KJ 2005 Roles of LPA3 and COX-2 in implantation. Trends in endocrinology and metabolism: TEM 16:397-399
8. Song H, Lim H, Paria BC, Matsumoto H, Swift LL, Morrow J, Bonventre JV, Dey SK 2002 Cytosolic phospholipase A2alpha is crucial [correction of A2alpha deficiency is crucial] for 'on-time' embryo implantation that directs subsequent development. Development (Cambridge, England) 129:2879-2889
9. Ye X, Hama K, Contos JJ, Anliker B, Inoue A, Skinner MK, Suzuki H, Amano T, Kennedy G, Arai H, Aoki J, Chun J 2005 LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. Nature 435:104-108
10. Wang H, Dey SK, Maccarrone M 2006 Jekyll and hyde: two faces of cannabinoid signaling in male and female fertility. Endocrine reviews 27:427-448
11. Wang H, Xie H, Guo Y, Zhang H, Takahashi T, Kingsley PJ, Marnett LJ, Das SK, Cravatt BF, Dey SK 2006 Fatty acid amide hydrolase deficiency limits early pregnancy events. The Journal of clinical investigation 116:2122-2131
12. Wang H, Guo Y, Wang D, Kingsley PJ, Marnett LJ, Das SK, DuBois RN, Dey SK 2004 Aberrant cannabinoid signaling impairs oviductal transport of embryos. Nature medicine 10:1074-1080
13. Mechoulam R, Hanus L 2000 A historical overview of chemical research on cannabinoids. Chemistry and physics of lipids 108:1-13
14. Gaoni Y, Mechoulam R 1964 Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. Journal of the American Chemical Society 86:1646-1647
15. Devane WA, Dysarz FA, 3rd, Johnson MR, Melvin LS, Howlett AC 1988 Determination and characterization of a cannabinoid receptor in rat brain. Molecular pharmacology 34:605-613

16. Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI 1990 Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561-564
17. Munro S, Thomas KL, Abu-Shaar M 1993 Molecular characterization of a peripheral receptor for cannabinoids. Nature 365:61-65
18. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R 1992 Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science (New York, NY 258:1946-1949
19. Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, et al. 1995 Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochemical pharmacology 50:83-90
20. Sugiura T, Kudo N, Ojima T, Mabuchi-Itoh K, Yamashita A, Waku K 1995 Coenzyme A-dependent cleavage of membrane phospholipids in several rat tissues: ATP-independent acyl-CoA synthesis and the generation of lysophospholipids. Biochimica et biophysica acta 1255:167-176
21. Natarajan V, Reddy PV, Schmid PC, Schmid HH 1982 N-Acylation of ethanolamine phospholipids in canine myocardium. Biochimica et biophysica acta 712:342-355
22. Natarajan V, Schmid PC, Reddy PV, Schmid HH 1984 Catabolism of N-acylethanolamine phospholipids by dog brain preparations. Journal of neurochemistry 42:1613-1619
23. Okamoto Y, Morishita J, Tsuboi K, Tonai T, Ueda N 2004 Molecular characterization of a phospholipase D generating anandamide and its congeners. The Journal of biological chemistry 279:5298-5305
24. Ueda N, Liu Q, Yamanaka K 2001 Marked activation of the N-acylphosphatidylethanolamine-hydrolyzing phosphodiesterase by divalent cations. Biochimica et biophysica acta 1532:121-127
25. Leung D, Saghatelian A, Simon GM, Cravatt BF 2006 Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. Biochemistry 45:4720-4726
26. Simon GM, Cravatt BF 2006 Endocannabinoid biosynthesis proceeding through glycerophospho-N-acyl ethanolamine and a role for alpha/beta-hydrolase 4 in this pathway. The Journal of biological chemistry 281:26465-26472
27. Liu J, Wang L, Harvey-White J, Osei-Hyiaman D, Razdan R, Gong Q, Chan AC, Zhou Z, Huang BX, Kim HY, Kunos G 2006 A biosynthetic pathway for anandamide. Proceedings of the National Academy of Sciences of the United States of America 103:13345-13350
28. Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB 1996 Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384:83-87
29. Giang DK, Cravatt BF 1997 Molecular characterization of human and mouse fatty acid amide hydrolases. Proceedings of the National Academy of Sciences of the United States of America 94:2238-2242

30. McKinney MK, Cravatt BF 2005 Structure and function of fatty acid amide hydrolase. Annual review of biochemistry 74:411-432
31. Cravatt BF, Lichtman AH 2002 The enzymatic inactivation of the fatty acid amide class of signaling lipids. Chemistry and physics of lipids 121:135-148
32. Wei BQ, Mikkelsen TS, McKinney MK, Lander ES, Cravatt BF 2006 A second fatty acid amide hydrolase with variable distribution among placental mammals. The Journal of biological chemistry 281:36569-36578
33. Mechoulam R, Deutsch DG 2005 Toward an anandamide transporter. Proceedings of the National Academy of Sciences of the United States of America 102:17541-17542
34. Glaser ST, Kaczocha M, Deutsch DG 2005 Anandamide transport: a critical review. Life sciences 77:1584-1604
35. Oddi S, Bari M, Battista N, Barsacchi D, Cozzani I, Maccarrone M 2005 Confocal microscopy and biochemical analysis reveal spatial and functional separation between anandamide uptake and hydrolysis in human keratinocytes. Cell Mol Life Sci 62:386-395
36. Moore SA, Nomikos GG, Dickason-Chesterfield AK, Schober DA, Schaus JM, Ying BP, Xu YC, Phebus L, Simmons RM, Li D, Iyengar S, Felder CC 2005 Identification of a high-affinity binding site involved in the transport of endocannabinoids. Proceedings of the National Academy of Sciences of the United States of America 102:17852-17857
37. Ligresti A, Morera E, Van Der Stelt M, Monory K, Lutz B, Ortar G, Di Marzo V 2004 Further evidence for the existence of a specific process for the membrane transport of anandamide. The Biochemical journal 380:265-272
38. Hillard CJ, Jarrahian A 2003 Cellular accumulation of anandamide: consensus and controversy. British journal of pharmacology 140:802-808
39. Ortega-Gutierrez S 2005 Therapeutic perspectives of inhibitors of endocannabinoid degradation. Current drug targets 4:697-707
40. Kaczocha M, Hermann A, Glaser ST, Bojesen IN, Deutsch DG 2006 Anandamide uptake is consistent with rate-limited diffusion and is regulated by the degree of its hydrolysis by fatty acid amide hydrolase. The Journal of biological chemistry 281:9066-9075
41. Bracey MH, Hanson MA, Masuda KR, Stevens RC, Cravatt BF 2002 Structural adaptations in a membrane enzyme that terminates endocannabinoid signaling. Science (New York, NY 298:1793-1796
42. Moriyama T, Urade R, Kito M 1999 Purification and characterization of diacylglycerol lipase from human platelets. Journal of biochemistry 125:1077-1085
43. Bisogno T, Howell F, Williams G, Minassi A, Cascio MG, Ligresti A, Matias I, Schiano-Moriello A, Paul P, Williams EJ, Gangadharan U, Hobbs C, Di Marzo V, Doherty P 2003 Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. The Journal of cell biology 163:463-468
44. Sugiura T, Kishimoto S, Oka S, Gokoh M 2006 Biochemistry, pharmacology and physiology of 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand. Progress in lipid research 45:405-446

45. Bisogno T, Sepe N, Melck D, Maurelli S, De Petrocellis L, Di Marzo V 1997 Biosynthesis, release and degradation of the novel endogenous cannabimimetic metabolite 2-arachidonoylglycerol in mouse neuroblastoma cells. The Biochemical journal 322 (Pt 2):671-677
46. Goparaju SK, Ueda N, Taniguchi K, Yamamoto S 1999 Enzymes of porcine brain hydrolyzing 2-arachidonoylglycerol, an endogenous ligand of cannabinoid receptors. Biochemical pharmacology 57:417-423
47. Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D 2002 Brain monoglyceride lipase participating in endocannabinoid inactivation. Proceedings of the National Academy of Sciences of the United States of America 99:10819-10824
48. Ho SY, Delgado L, Storch J 2002 Monoacylglycerol metabolism in human intestinal Caco-2 cells: evidence for metabolic compartmentation and hydrolysis. The Journal of biological chemistry 277:1816-1823
49. Muccioli GG, Xu C, Odah E, Cudaback E, Cisneros JA, Lambert DM, Lopez Rodriguez ML, Bajjalieh S, Stella N 2007 Identification of a novel endocannabinoid-hydrolyzing enzyme expressed by microglial cells. J Neurosci 27:2883-2889
50. Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG 2002 International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacological reviews 54:161-202
51. Pertwee RG, Ross RA 2002 Cannabinoid receptors and their ligands. Prostaglandins, leukotrienes, and essential fatty acids 66:101-121
52. McAllister SD, Glass M 2002 CB(1) and CB(2) receptor-mediated signalling: a focus on endocannabinoids. Prostaglandins, leukotrienes, and essential fatty acids 66:161-171
53. Caulfield MP, Brown DA 1992 Cannabinoid receptor agonists inhibit Ca current in NG108-15 neuroblastoma cells via a pertussis toxin-sensitive mechanism. British journal of pharmacology 106:231-232
54. Mackie K, Hille B 1992 Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proceedings of the National Academy of Sciences of the United States of America 89:3825-3829
55. Gebremedhin D, Lange AR, Campbell WB, Hillard CJ, Harder DR 1999 Cannabinoid CB1 receptor of cat cerebral arterial muscle functions to inhibit L-type Ca2+ channel current. The American journal of physiology 276:H2085-2093
56. Wang H, Matsumoto H, Guo Y, Paria BC, Roberts RL, Dey SK 2003 Differential G protein-coupled cannabinoid receptor signaling by anandamide directs blastocyst activation for implantation. Proceedings of the National Academy of Sciences of the United States of America 100:14914-14919
57. Lauckner JE, Hille B, Mackie K 2005 The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. Proceedings of the National Academy of Sciences of the United States of America 102:19144-19149

58. Paria BC, Das SK, Dey SK 1995 The preimplantation mouse embryo is a target for cannabinoid ligand-receptor signaling. Proceedings of the National Academy of Sciences of the United States of America 92:9460-9464
59. Zoratti C, Kipmen-Korgun D, Osibow K, Malli R, Graier WF 2003 Anandamide initiates Ca(2+) signaling via CB2 receptor linked to phospholipase C in calf pulmonary endothelial cells. British journal of pharmacology 140:1351-1362
60. Bouaboula M, Poinot-Chazel C, Bourrie B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G, Casellas P 1995 Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. The Biochemical journal 312 (Pt 2):637-641
61. Murphy LO, Blenis J 2006 MAPK signal specificity: the right place at the right time. Trends in biochemical sciences 31:268-275
62. Baker D, Pryce G, Davies WL, Hiley CR 2006 In silico patent searching reveals a new cannabinoid receptor. Trends in pharmacological sciences 27:1-4
63. Matas D, Juknat A, Pietr M, Klin Y, Vogel Z 2007 Anandamide protects from low serum-induced apoptosis via its degradation to ethanolamine. The Journal of biological chemistry 282:7885-7892
64. Sawzdargo M, Nguyen T, Lee DK, Lynch KR, Cheng R, Heng HH, George SR, O'Dowd BF 1999 Identification and cloning of three novel human G protein-coupled receptor genes GPR52, PsiGPR53 and GPR55: GPR55 is extensively expressed in human brain. Brain Res Mol Brain Res 64:193-198
65. McPartland JM, Matias I, Di Marzo V, Glass M 2006 Evolutionary origins of the endocannabinoid system. Gene 370:64-74
66. Van Der Stelt M, Di Marzo V 2004 Endovanilloids. Putative endogenous ligands of transient receptor potential vanilloid 1 channels. European journal of biochemistry / FEBS 271:1827-1834
67. De Petrocellis L, Bisogno T, Maccarrone M, Davis JB, Finazzi-Agro A, Di Marzo V 2001 The activity of anandamide at vanilloid VR1 receptors requires facilitated transport across the cell membrane and is limited by intracellular metabolism. The Journal of biological chemistry 276:12856-12863
68. Maccarrone M, Finazzi-Agro A 2003 The endocannabinoid system, anandamide and the regulation of mammalian cell apoptosis. Cell death and differentiation 10:946-955
69. Sugiura T, Kondo S, Sukagawa A, Tonegawa T, Nakane S, Yamashita A, Waku K 1996 Enzymatic synthesis of anandamide, an endogenous cannabinoid receptor ligand, through N-acylphosphatidylethanolamine pathway in testis: involvement of Ca(2+)-dependent transacylase and phosphodiesterase activities. Biochemical and biophysical research communications 218:113-117
70. Murphy LL, Gher J, Steger RW, Bartke A 1994 Effects of delta 9-tetrahydrocannabinol on copulatory behavior and neuroendocrine responses of male rats to female conspecifics. Pharmacol Biochem Behav 48:1011-1017
71. Schuel H, Burkman LJ, Lippes J, Crickard K, Forester E, Piomelli D, Giuffrida A 2002 N-Acylethanolamines in human reproductive fluids. Chem Phys Lipids 121:211-227
72. Schuel H, Burkman LJ 2005 A tale of two cells: endocannabinoid-signaling regulates functions of neurons and sperm. Biol Reprod 73:1078-1086

73. Gye MC, Kang HH, Kang HJ 2005 Expression of cannabinoid receptor 1 in mouse testes. Arch Androl 51:247-255
74. Maccarrone M, Cecconi S, Rossi G, Battista N, Pauselli R, Finazzi-Agro A 2003 Anandamide activity and degradation are regulated by early postnatal aging and follicle-stimulating hormone in mouse Sertoli cells. Endocrinology 144:20-28
75. Orth JM, McGuinness MP, Qiu J, Jester WF, Jr., Li LH 1998 Use of in vitro systems to study male germ cell development in neonatal rats. Theriogenology 49:431-439
76. Hall W, Solowij N 1998 Adverse effects of cannabis. Lancet 352:1611-1616
77. Schuel H, Burkman LJ, Lippes J, Crickard K, Mahony MC, Giuffrida A, Picone RP, Makriyannis A 2002 Evidence that anandamide-signaling regulates human sperm functions required for fertilization. Mol Reprod Dev 63:376-387
78. Schuel H, Chang MC, Berkery D, Schuel R, Zimmerman AM, Zimmerman S 1991 Cannabinoids inhibit fertilization in sea urchins by reducing the fertilizing capacity of sperm. Pharmacol Biochem Behav 40:609-615
79. Schuel H, Goldstein E, Mechoulam R, Zimmerman AM, Zimmerman S 1994 Anandamide (arachidonylethanolamide), a brain cannabinoid receptor agonist, reduces sperm fertilizing capacity in sea urchins by inhibiting the acrosome reaction. Proceedings of the National Academy of Sciences of the United States of America 91:7678-7682
80. Bisogno T, Ventriglia M, Milone A, Mosca M, Cimino G, Di Marzo V 1997 Occurrence and metabolism of anandamide and related acyl-ethanolamides in ovaries of the sea urchin Paracentrotus lividus. Biochim Biophys Acta 1345:338-348
81. Cobellis G, Cacciola G, Scarpa D, Meccariello R, Chianese R, Franzoni MF, Mackie K, Pierantoni R, Fasano S 2006 Endocannabinoid system in frog and rodent testis: type-1 cannabinoid receptor and fatty acid amide hydrolase activity in male germ cells. Biol Reprod 75:82-89
82. Maccarrone M, Barboni B, Paradisi A, Bernabo N, Gasperi V, Pistilli MG, Fezza F, Lucidi P, Mattioli M 2005 Characterization of the endocannabinoid system in boar spermatozoa and implications for sperm capacitation and acrosome reaction. J Cell Sci 118:4393-4404
83. Grimaldi P, Orlando P, Di Siena S, Lolicato F, Petrosino S, Bisogno T, Geremia R, De Petrocellis L, Di Marzo V 2009 The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis. Proceedings of the National Academy of Sciences of the United States of America 106:11131-11136
84. Fukami K, Yoshida M, Inoue T, Kurokawa M, Fissore RA, Yoshida N, Mikoshiba K, Takenawa T 2003 Phospholipase Cdelta4 is required for Ca2+ mobilization essential for acrosome reaction in sperm. J Cell Biol 161:79-88
85. Wennemuth G, Babcock DF, Hille B 2003 Calcium clearance mechanisms of mouse sperm. J Gen Physiol 122:115-128
86. Gadella BM, Harrison RA 2000 The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. Development 127:2407-2420
87. Suarez SS, Ho HC 2003 Hyperactivated motility in sperm. Reprod Domest Anim 38:119-124

88. Ricci G, Cacciola G, Altucci L, Meccariello R, Pierantoni R, Fasano S, Cobellis G 2007 Endocannabinoid control of sperm motility: the role of epididymus. Gen Comp Endocrinol 153:320-322
89. Rossato M, Ion Popa F, Ferigo M, Clari G, Foresta C 2005 Human sperm express cannabinoid receptor Cb1, the activation of which inhibits motility, acrosome reaction, and mitochondrial function. The Journal of clinical endocrinology and metabolism 90:984-991
90. Evans JP, Florman HM 2002 The state of the union: the cell biology of fertilization. Nature cell biology 4 Suppl:s57-63
91. Wassarman PM, Jovine L, Litscher ES 2001 A profile of fertilization in mammals. Nature cell biology 3:E59-64
92. Rossant J, Tam PP 2004 Emerging asymmetry and embryonic patterning in early mouse development. Developmental cell 7:155-164
93. Rossant J 2004 Lineage development and polar asymmetries in the peri-implantation mouse blastocyst. Seminars in cell & developmental biology 15:573-581
94. Cross JC, Werb Z, Fisher SJ 1994 Implantation and the placenta: key pieces of the development puzzle. Science (New York, NY 266:1508-1518
95. Yoshinaga K 1988 Uterine receptivity for blastocyst implantation. Annals of the New York Academy of Sciences 541:424-431
96. Paria BC, Huet-Hudson YM, Dey SK 1993 Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. Proceedings of the National Academy of Sciences of the United States of America 90:10159-10162
97. Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M, Fisher SJ 2004 Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. The Journal of clinical investigation 114:744-754
98. Das SK, Paria BC, Chakraborty I, Dey SK 1995 Cannabinoid ligand-receptor signaling in the mouse uterus. Proceedings of the National Academy of Sciences of the United States of America 92:4332-4336
99. Guo Y, Wang H, Okamoto Y, Ueda N, Kingsley PJ, Marnett LJ, Schmid HH, Das SK, Dey SK 2005 N-acylphosphatidylethanolamine-hydrolyzing phospholipase D is an important determinant of uterine anandamide levels during implantation. The Journal of biological chemistry 280:23429-23432
100. Wang H, Xie H, Sun X, Kingsley PJ, Marnett LJ, Cravatt BF, Dey SK 2007 Differential regulation of endocannabinoid synthesis and degradation in the uterus during embryo implantation. Prostaglandins & other lipid mediators 83:62-74
101. Yang ZM, Paria BC, Dey SK 1996 Activation of brain-type cannabinoid receptors interferes with preimplantation mouse embryo development. Biol Reprod 55:756-761
102. Schmid PC, Paria BC, Krebsbach RJ, Schmid HH, Dey SK 1997 Changes in anandamide levels in mouse uterus are associated with uterine receptivity for embryo implantation. Proceedings of the National Academy of Sciences of the United States of America 94:4188-4192
103. Paria BC, Ma W, Andrenyak DM, Schmid PC, Schmid HH, Moody DE, Deng H, Makriyannis A, Dey SK 1998 Effects of cannabinoids on preimplantation mouse

embryo development and implantation are mediated by brain-type cannabinoid receptors. Biol Reprod 58:1490-1495
104. Paria BC, Song H, Wang X, Schmid PC, Krebsbach RJ, Schmid HH, Bonner TI, Zimmer A, Dey SK 2001 Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation. The Journal of biological chemistry 276:20523-20528
105. Wang J, Paria BC, Dey SK, Armant DR 1999 Stage-specific excitation of cannabinoid receptor exhibits differential effects on mouse embryonic development. Biol Reprod 60:839-844
106. Sharov AA, Piao Y, Matoba R, Dudekula DB, Qian Y, VanBuren V, Falco G, Martin PR, Stagg CA, Bassey UC, Wang Y, Carter MG, Hamatani T, Aiba K, Akutsu H, Sharova L, Tanaka TS, Kimber WL, Yoshikawa T, Jaradat SA, Pantano S, Nagaraja R, Boheler KR, Taub D, Hodes RJ, Longo DL, Schlessinger D, Keller J, Klotz E, Kelsoe G, Umezawa A, Vescovi AL, Rossant J, Kunath T, Hogan BL, Curci A, D'Urso M, Kelso J, Hide W, Ko MS 2003 Transcriptome analysis of mouse stem cells and early embryos. PLoS biology 1:E74
107. Wang H, Dey SK unpublished data.
108. Pisarska MD, Carson SA, Buster JE 1998 Ectopic pregnancy. Lancet 351:1115-1120
109. Farquhar CM 2005 Ectopic pregnancy. Lancet 366:583-591
110. Heilman RD, Reo RR, Hahn DW 1976 Changes in the sensitivity of adrenergic receptors in the oviduct during early gestation in the rabbit. Fertility and sterility 27:426-430
111. Howe GR, Black DL 1973 Autonomic nervous system and oviduct function in the rabbit. I. Hormones and contraction. Journal of reproduction and fertility 33:425-430
112. Paria BC, Lim H, Wang XN, Liehr J, Das SK, Dey SK 1998 Coordination of differential effects of primary estrogen and catecholestrogen on two distinct targets mediates embryo implantation in the mouse. Endocrinology 139:5235-5246
113. Maccarrone M, DeFelici M, Klinger FG, Battista N, Fezza F, Dainese E, Siracusa G, Finazzi-Agro A 2004 Mouse blastocysts release a lipid which activates anandamide hydrolase in intact uterus. Molecular human reproduction 10:215-221
114. Maccarrone M, Valensise H, Bari M, Lazzarin N, Romanini C, Finazzi-Agro A 2000 Relation between decreased anandamide hydrolase concentrations in human lymphocytes and miscarriage. Lancet 355:1326-1329
115. Maccarrone M, Bisogno T, Valensise H, Lazzarin N, Fezza F, Manna C, Di Marzo V, Finazzi-Agro A 2002 Low fatty acid amide hydrolase and high anandamide levels are associated with failure to achieve an ongoing pregnancy after IVF and embryo transfer. Molecular human reproduction 8:188-195
116. Rossato M, Pagano C, Vettor R 2008 The cannabinoid system and male reproductive functions. Journal of neuroendocrinology 20 Suppl 1:90-93
117. Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, Lichtman AH 2001 Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. Proceedings of the National Academy of Sciences of the United States of America 98:9371-9376

118. Nixon B, Aitken RJ, McLaughlin EA 2007 New insights into the molecular mechanisms of sperm-egg interaction. Cell Mol Life Sci 64:1805-1823
119. Okabe M, Cummins JM 2007 Mechanisms of sperm-egg interactions emerging from gene-manipulated animals. Cell Mol Life Sci 64:1945-1958
120. Primakoff P, Myles DG 2002 Penetration, adhesion, and fusion in mammalian sperm-egg interaction. Science (New York, NY 296:2183-2185
121. Zimmer A, Zimmer AM, Hohmann AG, Herkenham M, Bonner TI 1999 Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. Proceedings of the National Academy of Sciences of the United States of America 96:5780-5785
122. Jarai Z, Wagner JA, Varga K, Lake KD, Compton DR, Martin BR, Zimmer AM, Bonner TI, Buckley NE, Mezey E, Razdan RK, Zimmer A, Kunos G 1999 Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. Proceedings of the National Academy of Sciences of the United States of America 96:14136-14141
123. Twitchell W, Brown S, Mackie K 1997 Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. Journal of neurophysiology 78:43-50
124. Das SK, Wang XN, Paria BC, Damm D, Abraham JA, Klagsbrun M, Andrews GK, Dey SK 1994 Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. Development 120:1071-1083
125. Matsumoto H, Ma W, Smalley W, Trzaskos J, Breyer RM, Dey SK 2001 Diversification of cyclooxygenase-2-derived prostaglandins in ovulation and implantation. Biol Reprod 64:1557-1565
126. Yamagata K, Nakanishi T, Ikawa M, Yamaguchi R, Moss SB, Okabe M 2002 Sperm from the calmegin-deficient mouse have normal abilities for binding and fusion to the egg plasma membrane. Developmental biology 250:348-357
127. Inoue N, Ikawa M, Isotani A, Okabe M 2005 The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. Nature 434:234-238
128. Cravatt BF, Saghatelian A, Hawkins EG, Clement AB, Bracey MH, Lichtman AH 2004 Functional disassociation of the central and peripheral fatty acid amide signaling systems. Proceedings of the National Academy of Sciences of the United States of America 101:10821-10826
129. Ohashi K, Saji F, Kato M, Tsutsui T, Tomiyama T, Tanizawa O 1995 Acrobeads test: a new diagnostic test for assessment of the fertilizing capacity of human spermatozoa. Fertility and sterility 63:625-630
130. Inoue N, Ikawa M, Nakanishi T, Matsumoto M, Nomura M, Seya T, Okabe M 2003 Disruption of mouse CD46 causes an accelerated spontaneous acrosome reaction in sperm. Molecular and cellular biology 23:2614-2622
131. Turner RM 2006 Moving to the beat: a review of mammalian sperm motility regulation. Reproduction, fertility, and development 18:25-38
132. Boatman DE, Robbins RS 1991 Bicarbonate: carbon-dioxide regulation of sperm capacitation, hyperactivated motility, and acrosome reactions. Biology of reproduction 44:806-813

133. Suarez SS, Katz DF, Owen DH, Andrew JB, Powell RL 1991 Evidence for the function of hyperactivated motility in sperm. Biology of reproduction 44:375-381
134. Wenger T, Ledent C, Csernus V, Gerendai I 2001 The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. Biochemical and biophysical research communications 284:363-368
135. Rossi G, Gasperi V, Paro R, Barsacchi D, Cecconi S, Maccarrone M 2007 Follicle-stimulating hormone activates fatty acid amide hydrolase by protein kinase A and aromatase-dependent pathways in mouse primary Sertoli cells. Endocrinology 148:1431-1439
136. Kolodny RC, Masters WH, Kolodner RM, Toro G 1974 Depression of plasma testosterone levels after chronic intensive marihuana use. The New England journal of medicine 290:872-874
137. Hembree WC, Nahas GG, Zeidenberg P, Huang HFS 1979 Changes in human spermatozoa associated with high dose marihuana smoking. In: Nahas GG, Paton WDM eds. Marihuana, Bilogical Effects. Pergamon, New York: Oxford; 429-439
138. Nahas GG, Frick HC, Lattimer JK, Latour C, Harvey D 2002 Pharmacokinetics of THC in brain and testis, male gametotoxicity and premature apoptosis of spermatozoa. Human psychopharmacology 17:103-113
139. Evans JP 2002 The molecular basis of sperm-oocyte membrane interactions during mammalian fertilization. Human reproduction update 8:297-311
140. Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D 2003 Modulation of anxiety through blockade of anandamide hydrolysis. Nature medicine 9:76-81
141. Lichtman AH, Shelton CC, Advani T, Cravatt BF 2004 Mice lacking fatty acid amide hydrolase exhibit a cannabinoid receptor-mediated phenotypic hypoalgesia. Pain 109:319-327
142. Bifulco M, Di Marzo V 2002 Targeting the endocannabinoid system in cancer therapy: a call for further research. Nature medicine 8:547-550
143. Guzman M 2003 Cannabinoids: potential anticancer agents. Nature reviews 3:745-755
144. Adamson SL, Lu Y, Whiteley KJ, Holmyard D, Hemberger M, Pfarrer C, Cross JC 2002 Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. Dev Biol 250:358-373
145. Cross JC, Baczyk D, Dobric N, Hemberger M, Hughes M, Simmons DG, Yamamoto H, Kingdom JC 2003 Genes, development and evolution of the placenta. Placenta 24:123-130
146. Varmuza S, Prideaux V, Kothary R, Rossant J 1988 Polytene chromosomes in mouse trophoblast giant cells. Development 102:127-134
147. Zybina EV, Zybina TG 1996 Polytene chromosomes in mammalian cells. Int Rev Cytol 165:53-119
148. Paria BC, Wang H, Dey SK 2002 Endocannabinoid signaling in synchronizing embryo development and uterine receptivity for implantation. Chemistry and physics of lipids 121:201-210
149. Wang H, Xie H, Dey SK 2008 Loss of cannabinoid receptor CB1 induces preterm birth. PLoS ONE 3:e3320

150. Zuckerman B, Frank DA, Hingson R, Amaro H, Levenson SM, Kayne H, Parker S, Vinci R, Aboagye K, Fried LE, et al. 1989 Effects of maternal marijuana and cocaine use on fetal growth. N Engl J Med 320:762-768
151. McCance-Katz EF 1991 The consequences of maternal substance abuse for the child exposed in utero. Psychosomatics 32:268-274
152. Habayeb OM, Taylor AH, Bell SC, Taylor DJ, Konje JC 2008 Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. Endocrinology 149:5052-5060
153. Trabucco E, Acone G, Marenna A, Pierantoni R, Cacciola G, Chioccarelli T, Mackie K, Fasano S, Colacurci N, Meccariello R, Cobellis G, Cobellis L 2009 Endocannabinoid system in first trimester placenta: low FAAH and high CB1 expression characterize spontaneous miscarriage. Placenta 30:516-522
154. Onaivi ES, Leonard CM, Ishiguro H, Zhang PW, Lin Z, Akinshola BE, Uhl GR 2002 Endocannabinoids and cannabinoid receptor genetics. Prog Neurobiol 66:307-344
155. Habayeb OM, Bell SC, Konje JC 2002 Endogenous cannabinoids: metabolism and their role in reproduction. Life Sci 70:1963-1977
156. Gupta RA, Wang D, Katkuri S, Wang H, Dey SK, DuBois RN 2004 Activation of nuclear hormone receptor peroxisome proliferator-activated receptor-delta accelerates intestinal adenoma growth. Nature medicine 10:245-247
157. Horne AW, Phillips JA, 3rd, Kane N, Lourenco PC, McDonald SE, Williams AR, Simon C, Dey SK, Critchley HO 2008 CB1 expression is attenuated in Fallopian tube and decidua of women with ectopic pregnancy. PLoS ONE 3:e3969
158. Park B, Gibbons HM, Mitchell MD, Glass M 2003 Identification of the CB1 cannabinoid receptor and fatty acid amide hydrolase (FAAH) in the human placenta. Placenta 24:990-995
159. Zimmer A, Zimmer AM, Hohmann AG, Herkenham M, Bonner TI 1999 Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice
Proceedings of the National Academy of Sciences of the United States of America 96:5780-5785
160. Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J 1998 Promotion of trophoblast stem cell proliferation by FGF4. Science 282:2072-2075
161. Faria TN, Ogren L, Talamantes F, Linzer DI, Soares MJ 1991 Localization of placental lactogen-I in trophoblast giant cells of the mouse placenta. Biol Reprod 44:327-331
162. Lescisin KR, Varmuza S, Rossant J 1988 Isolation and characterization of a novel trophoblast-specific cDNA in the mouse. Genes Dev 2:1639-1646
163. Scott IC, Anson-Cartwright L, Riley P, Reda D, Cross JC 2000 The HAND1 basic helix-loop-helix transcription factor regulates trophoblast differentiation via multiple mechanisms. Mol Cell Biol 20:530-541
164. Guillemot F, Nagy A, Auerbach A, Rossant J, Joyner AL 1994 Essential role of Mash-2 in extraembryonic development. Nature 371:333-336
165. Riley P, Anson-Cartwright L, Cross JC 1998 The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. Nat Genet 18:271-275

166. Cross JC 2005 How to make a placenta: mechanisms of trophoblast cell differentiation in mice--a review. Placenta 26 Suppl A:S3-9
167. Cory AH, Owen TC, Barltrop JA, Cory JG 1991 Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun 3:207-212
168. Yang ZZ, Tschopp O, Hemmings-Mieszczak M, Feng J, Brodbeck D, Perentes E, Hemmings BA 2003 Protein kinase B alpha/Akt1 regulates placental development and fetal growth. J Biol Chem 278:32124-32131
169. Gomez del Pulgar T, Velasco G, Guzman M 2000 The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. Biochem J 347:369-373
170. Molina-Holgado E, Vela JM, Arevalo-Martin A, Almazan G, Molina-Holgado F, Borrell J, Guaza C 2002 Cannabinoids promote oligodendrocyte progenitor survival: involvement of cannabinoid receptors and phosphatidylinositol-3 kinase/Akt signaling. J Neurosci 22:9742-9753
171. Parast MM, Aeder S, Sutherland AE 2001 Trophoblast giant-cell differentiation involves changes in cytoskeleton and cell motility. Dev Biol 230:43-60
172. Cross JC, Hemberger M, Lu Y, Nozaki T, Whiteley K, Masutani M, Adamson SL 2002 Trophoblast functions, angiogenesis and remodeling of the maternal vasculature in the placenta. Mol Cell Endocrinol 187:207-212
173. Hemberger M, Hughes M, Cross JC 2004 Trophoblast stem cells differentiate in vitro into invasive trophoblast giant cells. Dev Biol 271:362-371
174. Tanaka M, Gertsenstein M, Rossant J, Nagy A 1997 Mash2 acts cell autonomously in mouse spongiotrophoblast development. Dev Biol 190:55-65
175. Simmons DG, Fortier AL, Cross JC 2007 Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. Dev Biol 304:567-578
176. Ozaita A, Puighermanal E, Maldonado R 2007 Regulation of PI3K/Akt/GSK-3 pathway by cannabinoids in the brain. J Neurochem 102:1105-1114
177. Cella M, Leguizamon GF, Sordelli MS, Cervini M, Guadagnoli T, Ribeiro ML, Franchi AM, Farina MG 2008 Dual effect of anandamide on rat placenta nitric oxide synthesis. Placenta 29:699-707