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REVIEW

CB_2 receptors in reproduction

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Cannabinoids have been always identified as harmful drugs because of their negative effects on male and female reproduction. The discovery of the 'endocannabinoid system (ECS)', composed of bioactive lipids (endocannabinoids), their receptors and their metabolic enzymes, and the generation of mouse models missing cannabinoid receptors or other elements of the ECS, has enabled a wealth of information on the significance of endocannabinoid signalling in multiple reproductive events: Sertoli cell survival, spermatogenesis, placentation, fertilization, preimplantation embryo development, implantation and postimplantation embryonic growth. These studies have also opened new perspectives in clinical applications, pointing to the ECS as a new target for correcting infertility and for improving reproductive health in humans. This review will focus on the involvement of type-2 cannabinoid (CB_2) receptors in reproductive biology, covering both the male and female sides. It will also discuss the potential relevance of the immunological activity of CB_2 at the maternal/foetal interface, as well as the distinctiveness of CB_2 versus type-1 cannabinoid (CB_1) receptors that might be exploited for a receptor subtype-specific regulation of fertility. In this context, the different signalling pathways triggered by CB_1 and CB_2 (especially those controlling the intracellular tone of nitric oxide), the different activation of CB_1 and CB_2 by endogenous agonists (like anandamide and 2-arachidonoylglycerol) and the different localization of CB_1 and CB_2 within membrane subdomains, termed 'lipid rafts', will be discussed. It is hoped that CB_2-dependent endocannabinoid signalling might become a useful target for correcting infertility, in both men and women.
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Abbreviations: AEA, *N*-arachidonoylethanolamine (anandamide); 2-AG, 2-arachidonoylglycerol; AR, acrosome reaction; $CB_{1/2}$, type-1/2 cannabinoid receptor; ECS, endocannabinoid system; ERK, extracellular signal-regulated kinase; ICM, inner cell mass; IL, interleukin; IVF, *in vitro* fertilization; LH, luteinizing hormone; LR, lipid raft; MCP-1, monocyte chemoattractant protein-1; Th1/2, type 1/2 T-helper; THC, Δ^9-tetrahydrocannabinol; Tr, trophoblast

(Endo)cannabinoids and mammalian reproduction

Mammalian reproduction starts with the interaction between a male gamete (sperm) and a female gamete (egg), leading to fertilization. The fertilized egg (embryo) produces through several mitotic cell divisions the blastocyst, where an inner cell mass (ICM) is surrounded by a trophoblast (Tr) (Dey *et al*., 2004). The proper embryo is derived exclusively from the ICM, whereas the placenta and extraembryonic membranes are generated from cells contributed by the Tr. It is extremely difficult to define the identity and timeline of the molecular pathways engaged during human pregnancy, because of experimental difficulties generated in part by ethical restrictions. Thus, experiments on animal models like mice or boars, which share with humans a number of reproductive events, and the availability of genetically engineered mice have provided evidence that a number of lipid mediators serve as important signalling molecules during early pregnancy. Among these lipid messengers, prostaglandins, eicosanoids generated from arachidonic acid by cyclooxygenases and lysophosphatidic acid, a small lipid molecule belonging to the lysophospholipid group, are well-recognized signals in reproductive events (Dey, 2005).

During the last decade, increasing evidence has pointed towards the relevance of endocannabinoids, another group of bioactive lipids including amides and esters of arachidonic acid, in both female and male fertility (Park *et al*., 2004; Wang *et al*., 2006a). The endocannabinoids that have been best characterized to date are *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). Both act as endogenous ligands of cannabinoid (CB) receptors, thus mimicking several actions of the natural *Cannabis sativa*

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component Δ^9-tetrahydrocannabinol (THC), which accounts for the majority of the reproductive hazards in marijuana users (Hall and Solowij, 1998; Piomelli, 2004). With the characterization of the 'endocannabinoid system (ECS)', which comprises endocannabinoids (AEA, 2-AG and other congeners), their receptors, their metabolic enzymes and yet-putative transporters (Kogan and Mechoulam, 2006; Bari *et al*., 2006a), and with the generation of knockout mouse models where ECS elements were genetically deleted, a large body of molecular and physiological evidence has been accumulated to demonstrate that (endo)cannabinoid signalling is critical in Sertoli cell survival, spermatogenesis, placentation, fertilization, preimplantation embryo development, implantation and postimplantation embryo growth. Table 1 summarizes some of the effects of THC in human reproduction. For an extensive review on the effects of THC and ECS in human and animal reproduction, see Wang *et al*. (2006a). Here, the main focus will be on the role of type-2 cannabinoid (CB_2) receptors in endocannabinoid signalling implicated in reproductive biology, in both males and females. Also, the distinctiveness of CB_2 receptors versus type-1 cannabinoid (CB_1) receptors will be discussed, to speculate on the potential relevance of these two different receptor subtypes in regulating fertility. It is hoped that a deeper insight would lead to potential clinical applications of the CB_2-dependent endocannabinoid signalling as a target for correcting infertility and improving reproductive health in humans.

Table 1 Effect of *Cannabis sativa* component THC on human reproductive events

Effect	*Reference*
In male fertility	
Induction of gynecomastia	Harmon and Aliapoulios (1972)
Decrease of testosterone levels	Kolodny *et al*. (1974)
Decrease of spermatogenesis and motility (oligospermia)	Kolodny *et al*. (1974), Hembree *et al*. (1978) and Hong *et al*. (1982)
Decrease of serum LH	Kolodny *et al*. (1974), Cone *et al*. (1986) and Vescovi *et al*. (1992)
Induction of sperm anomalies	Issidorides (1978)
Blockade of acrosome reaction	Whan *et al*. (2006)
In female fertility	
Induction of foetal abnormalities and early pregnancy termination	Persaud and Ellington (1967)
Disruption of menstrual cycle	Bauman (1980)
Inhibition of prolactin secretion	Bauman (1980) and Mendelson *et al*. (1985)
Induction of greater difficulty at delivery	Greenland *et al*. (1982, 1983)
Suppression or increase of serum LH levels in a menstrual stage-specific manner	Mendelson and Mello (1984) and Mendelson *et al*. (1986)
Increase of the incidence of preterm birth	Fried *et al*. (1984), Hatch and Bracken (1986), Day *et al*. (1991) and Sherwood *et al*. (1999)
Induction of intrauterine foetal growth restriction	Qazi *et al*. (1985), Frank *et al*. (1990)
Induction of poor oocyte retrieval rate upon IVF treatment	Klonoff-Cohen *et al*. (2006)

Abbreviations: IVF, *in vitro* fertilization; LH, luteinizing hormone; THC, Δ^9-tetrahydrocannabinol.

Type-2 cannabinoid receptors in female fertility: animal studies

In mice, both CB_1 and CB_2 receptor subtypes are expressed in preimplantation embryos, whereas only CB_1 is expressed in the oviduct and uterus (Das *et al*., 1995; Paria *et al*., 1995, 2001; Wang *et al*., 2004). CB_2 mRNA is present already at the one-cell stage and is maintained through the blastocyst stage; instead, CB_1 mRNA is detected only from the four-cell through the blastocyst stage (Paria *et al*., 1995). The presence of CB_1 mRNA and protein in preimplantation embryos correlates well with high-affinity binding sites for [^{3}H]AEA (Yang *et al*., 1996; Paria *et al*., 2001), and consistently blastocyst CB_1 is biologically active (Das *et al*., 1995; Paria *et al*., 1995). Indeed, the development of two-cell embryos into blastocysts can be arrested by the addition of synthetic (CP55940, WIN55212-2), natural (THC) or endogenous (AEA and 2-AG) CBs in culture (Paria *et al*., 1995; Paria *et al*., 1998). Moreover, a reduction in trophectodermal cells was noted in those blastocysts that escaped the developmental arrest in the presence of CB agonists (Yang *et al*., 1996), whereas the addition of selective antagonists of CB_1 (SR141716), but not of CB_2 (SR144528), reversed these adverse effects (Paria *et al*., 1998). However, recent observations of CB_2 expression in early embryos and embryonic stem cells by microarray analysis (Sharov *et al*., 2003), and the absence of its expression in Tr stem cells derived from preimplantation blastocysts (Wang *et al*., 2006a), seem to suggest that CB_2 is restricted to the ICM cells. Taken together, these results suggest that (endo)cannabinoids mediate their actions on preimplantation embryos via CB_1, whereas the physiopathological relevance of CB_2 expression in the early embryo is yet to be defined.

With the availability of CB receptor knockout mice in the late 1990s (Ledent *et al*., 1999; Zimmer *et al*., 1999), the physiological relevance of CB signalling during early embryo development has been further examined. Incidentally, CB_1 knockouts developed by Zimmer's group and those developed by Ledent's group did not differ in litter size, yet the former mice showed an increased mortality rate between 8 and 24 weeks, although not neonatally (Zimmer *et al*., 1999). In studies comparing the *in vivo* developmental potential of CB receptor-mutant embryos with wild-type embryos, asynchronous development was observed in $CB_1(-/-)$, $CB_2(-/-)$ or $CB_1(-/-)/CB_2(-/-)$ double-mutant embryos (Paria *et al*., 2001; Wang *et al*., 2004). These studies provided genetic evidence that CB_2, as well as CB_1, is critical for preimplantation embryo development *in vivo*, and in fact $CB_2(-/-)$ embryos show the same asynchronous development as $CB_1(-/-)$ or $CB_1(-/-)/CB_2(-/-)$ double-mutant embryos (Paria *et al*., 2001). In this context, it should also be recalled that double-knockout mice did give birth to live pups, suggesting that the activity of uterine and embryonic CB receptors in controlling reproduction can be compensated by other yet-unknown mechanisms (Paria *et al*., 2001). To further ascertain whether embryos deficient in CB receptors respond to endocannabinoids *in vitro*, two-cell wild-type or mutant embryos were cultured in the presence or absence of AEA. While a comparable development of wild-type and mutant embryos was observed in the absence of

AEA, *$CB_1(-/-)$* and *$CB_1(-/-)/CB_2(-/-)$* mutant embryos, but not *$CB_2(-/-)$* or wild-type embryos, were resistant to the inhibitory action of AEA (Paria *et al*., 2001). This observation reinforces the tenet that CB_1 is the functional receptor for ensuring normal embryo growth and differentiation to blastocysts, and suggests that a role for CB_2 in these processes can be anticipated, but still awaits clarification. For instance, since CB_2 is expressed in the embryonic stem cells but not in Tr stem cells (Sharov *et al*., 2003), it is conceivable that CB_2 plays a role in specifying pluripotent ICM cell lineage during blastocyst formation. Moreover, a recent report has shown that the inhibitory effect of THC on Tr cell proliferation and gene transcription is mediated via the CB_2 receptor (Taylor *et al*., 2007), shedding some light on the possible functional meaning of this receptor in reproductive events.

During early pregnancy, another critical event occurring in parallel with embryonic preimplantation development is the embryos' timely transport from the oviduct into the uterus. In mice, embryos at the late morula or early blastocyst stage enter the uterus, where they develop and differentiate to achieve implantation competence, escape from the zona pellucida and implant into the receptive uterus. Thus, normal oviductal embryo transport is one of the prerequisites for on-time initiation of implantation in uterus, whereas a dysfunctional regulation of this process resulting from oviductal embryo retention would increase the incidence of pregnancy failure or cause tubal (ectopic) pregnancy in humans. Elegant studies on oviductal embryo transport in *$CB_1(-/-)$*, *$CB_2(-/-)$* or *$CB_1(-/-)/CB_2(-/-)$* double-mutant females have conclusively demonstrated that the maternal expression of CB_1 in the reproductive tracts plays a fundamental role in ensuring normal oviduct-to-uterus transport of embryos, and its deficiency results in embryo retention in the oviduct for an extended period, and hence in reduced fertility of *$CB_1(-/-)$* mice. On the other hand, CB_2 does not seem to play any role in the control of embryo transport through the oviduct (Wang *et al*., 2004), whereas a different regional expression of AEA-hydrolase fatty acid amide hydrolase (Maccarrone and Finazzi-Agrò, 2004) and of AEA-synthase *N*-acylphosphatidylethanolamine–phospholipase D (Guo *et al*., 2005) does contribute to generate an appropriate level of AEA conducive to preimplantation embryo growth and transportation (Wang *et al*., 2006b).

Collectively, these observations in mouse models provide evidence that while embryonic CB_1 primarily contributes to normal embryo development, oviductal CB_1 directs the timely oviductal transport of embryos. They also suggest that a role for CB_2 *in vivo* can be expected in embryo development, and possibly in ICM cell lineage determination.

Type-2 cannabinoid receptor in female fertility: human studies

The expression of CB receptors has been investigated also in human uterus during pregnancy, to ascertain the effects of (endo)cannabinoids on myometrial contractility (Dennedy *et al*., 2004). Using human biopsy specimens obtained at elective caesarean delivery, AEA and THC were found to exert a direct and equally potent relaxant effect on myometrial contractility *in vitro*. This relaxant effect was prevented by CB_1 antagonist SR141716 but not by CB_2 antagonist SR144528, although human endometrium expresses both receptor subtypes (Dennedy *et al*., 2004). These data highlight a possible role for exogenous and endogenous CBs during human parturition, suggesting that the relaxation component is under control of CB_1 only. On the other hand, the implications of CB_2 expression in human uterus during pregnancy remain to be elucidated.

Another interesting human study has investigated the presence of ECS in human placenta (Helliwell *et al*., 2004). Remarkably, no CB_1 mRNA was detected by reverse transcriptase PCR in samples ($n = 14$) extracted from first trimester tissues, whereas CB_2 mRNA was evident in 10 of the 14 samples (Helliwell *et al*., 2004). In accordance with these data, no CB_1-positive immunoreactivity was identified in placental tissues, whereas CB_2-positive labelling was observed within the villous stroma (Helliwell *et al*., 2004). Interestingly, the CB_2-positive cells immunoreacted also to anti-CD14 antibodies, demonstrating that they were villous macrophages. Taken together, these data seem to suggest that human placenta expresses only CB_2 receptor, which might be engaged in the placentation process. In addition, it is likely that CB_2 receptors are involved in normal immune response during early pregnancy. In line with this, lipopolysaccharide-stimulated macrophages secrete AEA (Liu *et al*., 2003), suggesting that increased local levels of this endocannabinoid may be one mechanism contributing to poor pregnancy outcome after bacterial infection (Silver *et al*., 1995; Romero *et al*., 2002). In addition, AEA blocks the release of leukaemia inhibitory factor (Maccarrone and Finazzi-Agrò, 2004), which is essential for the successful reproduction of all mammals (Piccinni *et al*., 1998). More in general, it is well known that mammalian reproduction is subjected to tight immunoregulation. In fact, normal gestation is based on an early immunological adaptation that involves peripheral T lymphocytes of pregnant women (Piccinni *et al*., 1998; Sharkey, 1998; Maccarrone and Finazzi-Agrò, 2004). These cells produce type-1 T-helper (Th1) and type-2 T-helper (Th2) cytokines, which have opposite effects on Tr growth. Th2 cytokines (interleukin (IL)-3, IL-4 and IL-10) favour blastocyst implantation and successful pregnancy by promoting Tr growth either directly or indirectly through the inhibition of natural killer cell activity and the stimulation of natural suppressor cells. Conversely, type-1 T-helper cytokines (IL-2, IL-12 and interferon-γ) impair gestation by causing a direct damage to the Tr, by stimulating natural killer cells and by enhancing secretion of tumour necrosis factor-α by macrophages. The Tr stimulates the release of profertility Th2 cytokines from T lymphocytes (the so-called 'Th2 bias') through IL-4, whereas the antifertility type-1 T-helper cytokines bias is signalled by IL-2. Also, progesterone plays a role in this network, inducing the Th2 bias by binding to a specific receptor in T cells. The resulting hormone–cytokine network is a key element at the maternal/foetal interface, and a defect in its integrity may result in foetal loss (Chaouat *et al*., 1995; Szekeres-Bartho *et al*., 1996; Piccinni *et al*., 1998; Duval *et al*., 2000). On this background, it seems of major interest that in several experimental

Table 2 Immunological actions of CB_2 receptors

Evidence	*Reference*
Stimulation of peripheral CB_2 induces MCP-1 and IL-8 gene expression in the human promyelocytic cell line HL60	Jbilo *et al.* (1999)
Reduction of human monocytic cell (neuro) toxicity and cytokine secretion by ligands of CB_2	Klegeris *et al.* (2003)
Echinacea alkylamides modulate TNF-α gene expression via CB_2 and multiple signal-transduction pathways	Gertsch *et al.* (2004)
Activation of CB_2 negatively regulates IL-12p40 production in murine macrophages: role of IL-10 and ERK1/2 kinase signaling	Correa *et al.* (2005)
CB_2 agonists induce transcription of the μ-opioid receptor gene in Jurkat T cells	Borner *et al.* (2006)

Abbreviations: CB_2, type-2 cannabinoid receptors; ERK, extracellular signal-regulated kinase; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α.

paradigms, CB_2 has been shown to regulate the release of cytokines relevant for fertility, as summarized in Table 2. Therefore, it is conceivable that CB_2 in first trimester placenta might contribute to control the cytokine network responsible for the maternal/foetal cross talk.

Type-2 cannabinoid receptor in male fertility: human studies

(Endo)cannabinoid signalling has been proposed to regulate sperm functions required for fertilization in a variety of organisms, spanning from lower invertebrates to higher mammals (Schuel and Burkman, 2005). Evidence that *N*-acylethanolamines like AEA are present in human reproductive fluids in the low nanomolar range (up to ~70 nM) (Schuel *et al.*, 2002a) and that AEA may regulate human sperm functions (Schuel *et al.*, 2002b) has been recently presented. Additionally, *in vitro* studies have demonstrated that the AEA congener *N*-palmitoylethanolamine may affect the time course of capacitation of human sperm, by modulating the properties of their membranes (Ambrosini *et al.*, 2003). In this context, it should be recalled that ejaculated sperm from mammals must undergo a functional maturation to become capable of fertilizing an oocyte. Sperm acquire fertilization competence as they reside in the female genital tract where, after a series of physiological changes, they become 'capacitated', that is able to fertilize the egg (Yanagimachi, 1994). Capacitation consists of changes occurring at two sites: in the head of the sperm, which enable it to bind to the zona pellucida proteins and to undergo the acrosome reaction (AR); and in the flagellum, where hyperactivated sperm motility is facilitated.

Recently, AEA has been shown to reduce human sperm motility (Whan *et al.*, 2004). In addition, by means of reverse transcriptase PCR and immunochemistry, human sperm have been shown to express CB_1, whereas CB_2 protein could not be detected by western blot analysis (Rossato *et al.*, 2005). CB_1 was localized in the head and middle piece of the sperm, and its activation by AEA reduced sperm motility and inhibited capacitation-induced AR. Yet, CB_1 activation did not induce any variation in sperm intracellular calcium concentrations, but produced a rapid plasma membrane hyperpolarization that was reduced by the K^+ channel blocker tetraethylammonium. Notably, the effects of AEA on human sperm motility were dependent on the reduction of sperm mitochondrial activity as determined by rhodamine 123 fluorescence (Rossato *et al.*, 2005). These data lead to the suggestion that the activity of AEA on human sperm functions requires CB_1 activation, yet the presence of functional CB_1 receptors in human sperm was not ascertained. Neither the presence of the gene encoding for CB_2 receptors, nor the ability of human sperm to bind, synthesize, transport and degrade AEA was demonstrated. On this background, another study has investigated whether sperm cells of boar (*Sus scropha*) were able to bind and metabolize AEA, and whether this endocannabinoid might modulate sperm functions with a cause–effect relationship.

Type-2 cannabinoid receptor in male fertility: animal studies

It should be recalled that boar closely resembles the physiology of humans (Logan and Sharma, 1999), and thus its sperm are largely used as a model system for experiments on reproductive physiology (Barboni *et al.*, 1995; Mattioli *et al.* 1996; James *et al.*, 2004).

Boar sperm have been shown to possess the biochemical machinery to bind, synthesize and degrade AEA (Maccarrone *et al.*, 2005). Activation of CB_1 by the AEA stable analogue methanandamide inhibits capacitation and hence the ability of sperm cells to react to zona pellucida proteins with acrosome exocytosis, through a cAMP-dependent pathway. Unlike zona pellucida-induced AR, CB_1 was ineffective on spontaneous AR (Maccarrone *et al.*, 2005). Moreover, once sperm have completed capacitation, AEA stabilizes the acrosome membranes by activating vanilloid receptors, thus reducing spontaneous AR. Interestingly, boar sperm express also CB_2, which represents ~15% of total CB receptors according to western blot analysis and binding data (Maccarrone *et al.*, 2005). To date, it is not yet clear as to which role could be played by this receptor subtype, but chances are that it contributes, directly or indirectly, to sperm activation. In fact, the data suggest that sperm function is regulated by endocannabinoids through a dual stage-dependent effect. On the one hand, AEA, present in both seminal plasma and uterine fluids, prevents premature capacitation in freshly ejaculated sperm via a CB_1 (and potentially CB_2)-mediated mechanism; in this way, AEA contributes to maintain a suitable environment for the sperm to travel along the uterine tract without any fertilizing potential, but in a condition of membrane stability. On the other, a few hours later, when sperm have reached the oviduct (a condition that corresponds to the incubation *in vitro* under capacitating conditions), this inhibitory brake becomes looser. In fact, sperm are exposed to a progressively reduced concentration of AEA in the proximal female genital tract (Schuel *et al.*, 2002a), and sperm capacitation may occur as a consequence of CB_1 (and possibly CB_2) disinhibition. At this time, intracellular endocannabinoids may become a

major switch in the regulation of sperm function, and an increase in endogenous AEA may be necessary to activate vanilloid receptors and prevent spontaneous AR. As a consequence, AR will result only from sperm–egg interactions, thus maximizing the fertilizing potential of the sperm population. It should be stressed that these endocannabinoid-mediated cross talks between sperm and egg need further investigation. At any rate, the observation that sperm cells have a complete ECS adds a new player to hormone/cytokine networks regulating fertility in mammals. In the same line, the presence of a fully functional ECS in sperm supports the hypothesis that endocannabinoid signalling can contribute to block polyspermy, by controlling retrograde AEA activity in much the same way as it happens within the CNS (Schuel and Burkman, 2005). According to this model, AEA released from the egg following activation by their fertilizing sperm binds to CB_1 (and possibly CB_2) receptors on nearby sperm, to inhibit the AR, thereby preventing the sperm from penetrating the egg prior to completion of the cortical reaction (Schuel and Burkman, 2005).

Also, the function of mouse Sertoli cells has been shown to be altered by THC (Newton *et al*., 1993), although the molecular basis for this alteration has not been established. As Sertoli cells of the mammalian seminiferous epithelium are involved in the regulation of germ cell development by providing nutrients and hormonal signals needed for spermatogenesis, their ability to bind and degrade AEA may be important in controlling the spermatogenic output. Sertoli cells have the biochemical machinery to bind and degrade AEA, and this machinery was characterized in a cell age range 4–24 days, largely used as a model of immature mice in endocrinological studies (Maccarrone *et al*., 2003). Immature Sertoli cells express functional CB_2 receptors, but not CB_1 receptors, on their surface, and the level of these receptors is constant during ageing (Maccarrone *et al*., 2003). An interesting observation is that AEA can force Sertoli cells to apoptosis, and that this process is more evident upon ageing (Maccarrone *et al*., 2003). Additionally, CB_2 receptors expressed by Sertoli cells have a protective role against the toxic effects of AEA, and so has follicle-stimulating hormone. The latter substance dramatically impacts foetal and early neonatal Sertoli cell proliferation, and it is critical in determining the spermatogenic capacity in the adult mammals (Orth *et al*., 1998). It can be suggested that altered levels of follicle-stimulating hormone during testis development can control the proapoptotic potential of AEA, thus contributing to the differentiation process (Rossi *et al*., 2007). This observation, together with the well-established relationship of Sertoli cell number to the total spermatogenic output of the testis, can contribute to the negative effects exerted on testicular development by altered follicle-stimulating hormone concentrations, as well as by mutations of the follicle-stimulating hormone receptor gene (Tapanainen *et al*., 1997). Taken together, the finding that Sertoli cells partake in the peripheral ECS and that CB_2 regulates their survival (and hence the final spermatogenic output), seems to open new perspectives in the understanding and treatment of male infertility.

The overall distribution of CB_2 in reproductive organs of both the male and female sides is summarized in Table 3. A major question that remains open is regarding the advantage that might be conferred by CB_2, in addition to or in place of CB_1, to reproductive performance. Some clues that might help to answer this question are discussed in the following section.

Table 3 Distribution and activity of CB_2 receptors in reproductive cells and tissues

Evidence	*Reference*	*Side of fertility impacted*
CB_2 mRNA is present atin the early stages of mouse embryo development and throughout the preimplantation period	Paria *et al*. (1995)	♀
$CB_2-/-$ mice show an early asynchronous embryo development *in vivo*	Paria *et al*. (2001)	♀
CB_2 mRNA is expressed in human uterus and myometrium	Dennedy *et al*. (2004)	♀
CB_2 mRNA and protein are expressed in first trimester human placenta	Helliwell *et al*. (2004)	♀
CB_2 mRNA is expressed in rat testis	Brown *et al*. (2002)	♂
CB_2 mRNA and protein are expressed in mouse Sertoli cells, and have a protective role against apoptosis	Maccarrone *et al*. (2003)	♂
CB_2 is weakly expressed in boar sperm cells	Maccarrone *et al*. (2005)	♂
CB_2 protein is expressed in normal and malignant human prostatic epithelium	Sarfaraz *et al*. (2005)	♂

Abbreviation: CB_2, type-2 cannabinoid receptors.

Distinctiveness of type-2 cannabinoid versus type-1 cannabinoid receptors

Both CB_1 and CB_2 receptors belong to the rhodopsin family of G-protein-coupled seven transmembrane spanning receptors. They show 44% overall identity with 68% identity within the transmembrane regions, and are coupled mainly to the $G_{i/o}$ family of G proteins (Howlett *et al*., 2002; Howlett *et al*., 2004). Signal-transduction pathways regulated by both CB receptor subtypes include the inhibition of AC and the activation of mitogen-activated protein kinase and of cytosolic phospholipase A2. However, only CB_1 regulates ionic currents (inhibition of voltage-gated L, N and P/Q-type Ca^{2+} channels, activation of K^+ channels), and CB_1 activates, whereas CB_2 inhibits nitric oxide synthase (Howlett *et al*., 2004; Demuth and Molleman, 2006).

The opposite effect of CB_1 versus CB_2 on nitric oxide (NO) release might be relevant for the *in vivo* control of reproduction, because NO plays several roles in male (Lewis *et al*., 1996; Donnelly *et al*., 1997; Herrero *et al*., 2003) and female fertility (Maul *et al*., 2003). For instance, NO regulates the contribution of Sertoli cells to fertility and inflammation-mediated infertility (O'Bryan *et al*., 2000; Fujisawa *et al*., 2001). In addition, it is produced by sperm and acts as an intracellular signalling molecule in the processes of capacitation and AR. It has been documented that during capacitation, NO interacts with the protein kinase A pathway and is also involved in tyrosine nitration of sperm proteins (Herrero

et al., 2001). On the other hand, during progesterone-induced AR, NO stimulates cyclooxygenase activity with a subsequent increase in prostaglandin E2. Furthermore, the ability of NO-releasing compounds to induce AR has been shown to occur via an increase in cGMP levels and subsequent protein kinase G activation. NO is also a major paracrine mediator and an important regulatory agent in various female reproductive processes, such as ovulation, implantation, pregnancy maintenance, labour and delivery (Maul *et al.*, 2003). In ovulation, circulating NO products are increased during follicle development and decreased right after ovulation. In implantation, NO also regulates endometrial functions such as endometrial receptivity, implantation and menstruation, so that NO donors may be useful for promoting fertility, while NO inhibitors might be used for contraception. Moreover, throughout gestation myometrial NO production is upregulated, thus contributing to achieve uterine quiescence; then, close to term NO production decreases, thus promoting effective contractions that result in labour (Maul *et al.*, 2003). Since human endometrium expresses both CB_1 and CB_2 (Dennedy *et al.*, 2004), it is conceivable that these two receptor subtypes are engaged at different time points to modulate in opposite ways NO content and thus NO-dependent effects. In contrast to the myometrium, NO production in the cervix is low during gestation and becomes upregulated once pregnancy advances to term. The findings from animal studies have been confirmed by several clinical trials, so that it is now believed that NO donors and nitric oxide synthase inhibitors may provide novel, effective, safe and inexpensive drugs to regulate and steer various functions in female reproductive life. Given this, it is tempting to speculate that a spatiotemporal regulation of CB_1 versus CB_2 expression might be instrumental in enhancing or reducing NO-dependent actions on both sides of mammalian fertility, thus opening a new avenue for CB-oriented therapeutic intervention. On a final note, it should be recalled that CB_2-specific signalling pathways may exist, which impact reproductive events in yet-unknown ways.

A second point of interest is that AEA is only a partial agonist of CB_2, whereas 2-AG is a full agonist of both CB receptor subtypes (Howlett *et al.*, 2004; Demuth and Molleman, 2006). Therefore, it is conceivable that a differential regulation of 2-AG metabolism, through its synthetic enzyme diacylglycerol lipase (Bisogno *et al.*, 2003) or hydrolytic enzyme monoacylglycerol lipase (Dinh *et al.*, 2002), might serve to modulate CB_2 rather than CB_1. However, preliminary data on 2-AG metabolism in the uterus of leptin knockout (*ob/ob*) mice (Maccarrone *et al.*, 2004) that are infertile (Ahima and Flier, 2000), and more recent evidence in the mouse uterus during embryo implantation (Wang *et al.*, 2007), seem to suggest that changes in 2-AG are superimposable on those of AEA. Interestingly, in both studies, 2-AG levels were found to be $\sim$2 orders of magnitude higher than those of AEA, and this finding raises the question as to why 2-AG, which is detrimental to early embryo development (Wang *et al.*, 2007), is present in the pregnant uterus at such higher levels than AEA. It is possible that a specific, yet-to-be-disclosed activity of 2-AG, possibly mediated through CB_2, might be needed for regulating the 'window' of implantation by synchronizing embryo development and blastocyst activation with preparation of the uterus to the receptive state (Wang *et al.*, 2007).

A third point of interest stems from mounting evidence accumulated in the last few years, showing that lipid rafts (LRs) are involved in the trafficking and functioning of CB_1 receptors (McFarland and Barker, 2005). LRs are specialized membrane microdomains biochemically defined by the insolubility of their components in cold non-ionic detergents (Simons and Toomre, 2000). They are believed to mediate the regulation of signal transduction through several different mechanisms, for example, by favouring the internalization of specific components of the signalling chain, such as G-protein-coupled receptors and G proteins (Le *et al.*, 2002; Barnett-Norris *et al.*, 2005). LRs disruption by methyl-β-cyclodextrin, a membrane cholesterol depletor, has been shown to double CB_1-dependent signalling via AC and mitogen-activated protein kinase in neuronal cells (Bari *et al.*, 2005). In line with this, a recent paper has demonstrated that CB_1 is physically associated with LRs in breast cancer cells, suggesting that membrane subdomains might represent a cellular device for its intracellular trafficking, as well as a favourable platform to regulate CB_1 signalling (Sarnataro *et al.*, 2006; Oddi *et al.*, 2007). Unlike CB_1, CB_2 in leukaemic cells is insensitive to raft perturbation, suggesting that this receptor subtype does not localize within LRs (Bari *et al.*, 2006b). The molecular basis of the different sensitivity of CB receptor subtypes to raft integrity might be very complex, and to be clarified it needs a more detailed analysis of the three-dimensional structures of the two receptors and their interactions in the context of the membrane bilayers. However, it should be recalled that the most important differences between CB_1 and CB_2 are located in the N-terminal extracellular loop II, in the C terminus of transmembrane helix 7 and in the C terminus (Montero *et al.*, 2005). Moreover, a recent study using combined high-resolution nuclear magnetic resonance and computer modelling has shown that CB_1 and CB_2 have conformational properties and salt bridge differences in the so-called juxtamembrane segment (or helix 8), which is critical for their activity and regulation and, more notably, is under the influence of the surrounding chemical environment (Xie and Chen, 2005). On this basis, it is tempting to speculate that LRs might regulate CB_1 receptor by interacting with the specific regions of its three-dimensional structure, such as helix 8 (Xie and Chen, 2005), or helices 3 and 6 (Tian *et al.*, 2005). Interestingly, a role for LRs in reproduction has been recently documented (Noble *et al.*, 2007). For instance, LRs have been recognized as a critical factor in the pathways involved in Ca^{2+} signalling in the uterus, with an impact on the prevention of preterm and difficult labours (Noble *et al.*, 2007). In fact, the increases in cytosolic Ca^{2+} and contractility that occur with raft disruption are due, at least in part, to effects on large conductance Ca^{2+}-activated K^+ channels, localized within LRs. It is possible that CB_1-dependent control of ion conductances may play a role in this mechanism, whereas activation of CB_2 might be a means of triggering signal-transduction pathways common to CB_1 without alteration of ion currents.

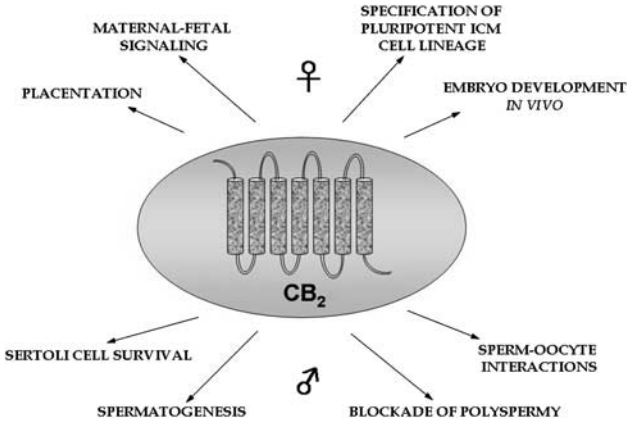

Figure 1 Potential roles of CB_2 receptors in male and female reproductive events. See text for details. CB_2, type-2 cannabinoid.

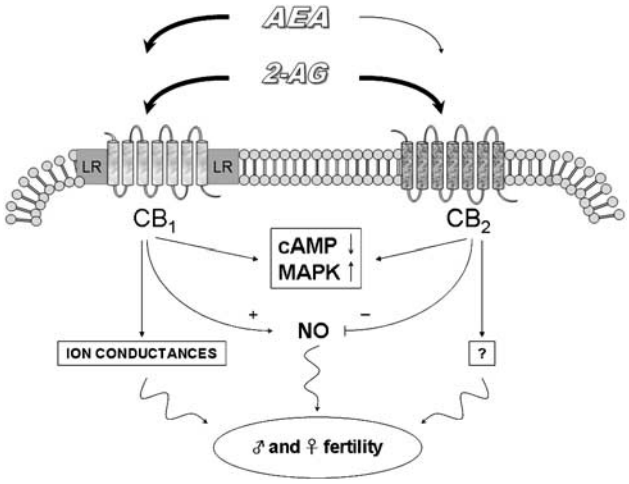

Figure 2 Distinctiveness of CB_2 versus CB_1 receptors that may be relevant for reproduction. See text for details. CB_1, type-1 cannabinoid; CB_2, type-2 cannabinoid.

Concluding remarks and future perspectives

A role for the endogenous CB system in several aspects of mammalian reproduction has been proposed, through the activation of CB and vanilloid receptors, and/or via non-receptor-mediated actions. In the case of AEA, the control of its activity on the reproductive system seems to be dependent on its endogenous tone, which in turn depends on fatty acid amide hydrolase. In fact, fatty acid amide hydrolase is under control of well-known regulators of fertility, such as type-1 T-helper/Th2 cytokines, progesterone and leptin, so that this enzyme can be considered a 'guardian angel' (Maccarrone and Finazzi-Agrò, 2004) or a 'gatekeeper' (Wang *et al*., 2006b) of mammalian reproduction. Fluctuations of endocannabinoid levels during the human ovulatory cycle (Lazzarin *et al*., 2004) reinforce the tenet that ECS contributes to the regulation of fertility. Also, CB_1 receptors play a major role in the effect of endocannabinoids on reproductive events, and it remains to be established which potential advantage could be conferred by the expression of CB_2 for improving reproductive performance. Here, we have summarized the distribution of CB_2 mRNA and/or protein in reproductive cells and tissues, and have shown that this receptor subtype may be involved in placentation, maternal/foetal signalling, specification of ICM cell lineage and embryo development *in vivo* (in females), as well as in Sertoli cell survival, spermatogenesis and sperm–oocyte interactions including blockade of polyspermy (in males). Figure 1 summarizes the reproductive events potentially under control of CB_2-dependent signalling. Awaiting for new experimental data that may further clarify the impact of CB_2 on reproduction, we have also speculated that certain distinctiveness of CB_2 versus CB_1 may represent an advantage for reproductive performance. In line with this, it is tempting to propose that (i) a differential signal transduction (especially for the pathway that regulates the endogenous tone of NO), (ii) a differential regulation of the metabolism of the CB_2 full agonist 2-AG and (iii) a differential sensitivity of CB_2 versus CB_1 to LRs integrity may represent useful aspects that might favour reproduction in mammals (Figure 2). Overall, it is hoped that a deeper understanding of endocannabinoid signalling, in particular the CB_2-dependent branch, might help to correct human infertility, in both men and women.

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Conflict of interest

The author states no conflict of interest.

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