The endocannabinoid pathway and the female reproductive organs

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Abstract

Endocannabinoids are endogenous ligands of cannabinoid, vanilloid and peroxisome proliferator-activated receptors that activate multiple signal transduction pathways. Together with their receptor and the enzymes responsible for their synthesis and degradation, these compounds constitute the endocannabinoid system that has been recently shown to play, in humans, an important role in modulating several central and peripheral functions including reproduction. Given the relevance of the system, drugs that are able to interfere with the activity of endocannabinoids are currently considered as candidates for the treatment of various diseases. In this review, we will summarise the current knowledge regarding the effects of endocannabinoids in female reproductive organs. In particular, we will focus on some newly reported mechanisms that can affect endometrial plasticity both in physiological and in pathological conditions.

Key Words
- Uterus/endometrium
- Female reproduction
- Ovary

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Introduction

Endocannabinoids are endogenous ligands that bind to the same receptors as the principal biologically active component of *Cannabis sativa*, Δ^9-tetrahydrocannabinol (Δ^9-THC). Δ^9-THC and its related molecule Δ^8-THC exert their central psychoactive effects acting through the cannabinoid receptor CB1, while their peripheral effects are mostly mediated by the cannabinoid receptor CB2 (Matsusa *et al*. 1990, Munro *et al*. 1993). However, some peripheral tissues express both receptor isotypes (Zygmunt *et al*. 2000). The effects of exogenous cannabinoids on human fertility have been extensively studied in marijuana smokers, and it has been clearly demonstrated that, at high doses, they can alter trophoblast development and invasiveness, oviductal transport, prostaglandin production by human gestational tissues and endometrial decidualisation (Kessler *et al*. 2005, Wang *et al*. 2006, Mitchell *et al*. 2008). Recent studies have underlined that endocannabinoids, under the control of sex hormones and cytokines, mimic similar effects in female reproduction (Karasu *et al*. 2011). In this review, we will summarise the importance of the endocannabinoid system in female reproductive organs and will point out new mechanisms affecting endometrial plasticity.

The endocannabinoid pathway

N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) are the principal endocannabinoids and act primarily at cannabinoid receptors CB1 and CB2. Additional endocannabinoids have also been discovered. They either activate the same receptors or potentiate the effects of AEA and 2-AG by inhibiting their degradation (Ho *et al*. 2008). In addition, AEA also binds to type 1 vanilloid receptor (TRPV1; Starowicz *et al*. 2007) and to peroxisome proliferator-activated receptor γ (PPARγ; Gasperi *et al*. 2007). AEA and 2-AG together

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with other cannabinoid-like compounds, their metabolic enzymes, the target receptors and the putative membrane transporter form the endocannabinoid system.

Synthesis, transport and degradation

Endocannabinoids are synthesized on demand from phospholipid precursors and are not stored (Habayeb *et al*. 2002). AEA is formed by cleavage of its precursor *N*-arachidonoyl phosphatidylethanolamine (NAPE) into AEA and phosphatidic acid by NAPE-hydrolysing phospholipase D (NAPE-PLD), which is a calcium-sensitive enzyme (Okamoto *et al*. 2004, Wang *et al*. 2006). Alternative pathways for AEA synthesis also exist. They include hydrolysis of NAPE by phospholipase C to form phospho-AEA, which is then dephosphorylated by a protein tyrosine phosphatase (Liu *et al*. 2006) or acyl hydrolysis of NAPE to form glycerophospho-NAPE subsequently hydrolysed to AEA by a phosphodiesterase (Simon & Cravatt 2006). 2-AG is also released on demand after conversion of diacylglycerol by a diacylglycerol lipase (Prescott & Majerus 1983).

The biological activity of endocannabinoids is regulated by fine mechanisms of intracellular uptake and degradation to free arachidonic acid. The principal enzymes involved are serine hydrolase's fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase for AEA and 2-AG respectively (Ligresti *et al*. 2005, McKinney & Cravatt 2005). Additional degradation mechanisms catalysed by lipoxygenase (van der Stelt *et al*. 2002), cyclooxygenase-2 (Rouzer & Marnett 2008) or cytochrome P450 (Snider *et al*. 2008) have also been demonstrated. By contrast, AEA transport through the plasma membrane has not been fully elucidated yet. Most likely, it is catalysed by a carrier protein that also takes up 2-AG (Battista *et al*. 2005, Hermann *et al*. 2006). However, diffusion driven by FAAH (Day *et al*. 2001), diffusion driven by compartmentalisation of intracellular AEA (Hillard & Jarrajan 2003) and endocytosis (McFarland *et al*. 2004) have been hypothesised.

Signal transduction and biological activity

AEA and 2-AG exert most of their biological effects by binding to the cannabinoid receptors CB1 and CB2. These are seven-transmembrane G-protein-coupled receptors that share 44% homology (Howlett *et al*. 2002). Initially, CB1R was isolated in the rat brain (Devane *et al*. 1988) and CB2R in the rat spleen and human myeloid cells (Munro *et al*. 1993), but it is now known that both of them are present in the CNS and several peripheral tissues (Karasu *et al*. 2011). A new putative cannabinoid receptor, CB3 or GPR55, has been recently identified (Lauckner *et al*. 2008), but its biological significance has not been completely elucidated (Ross 2009). As illustrated in Fig. 1, ligand binding of CB receptors activates several signalling

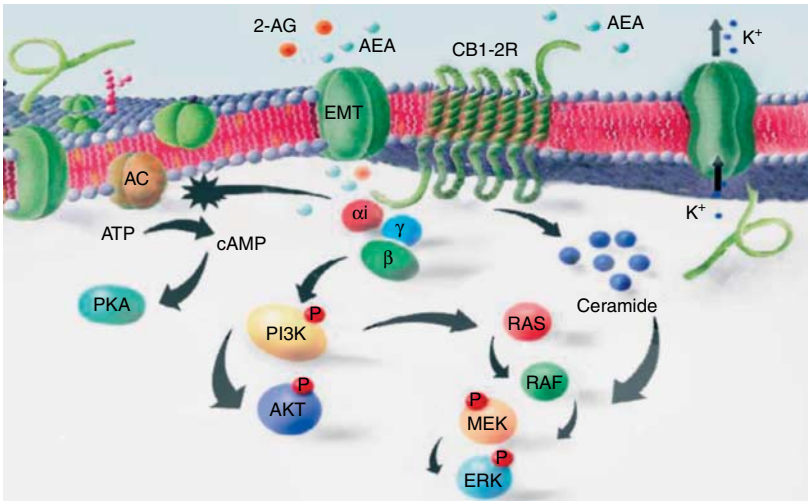

Figure 1

Overview of the most important biochemical pathways for cellular uptake and biological actions of the endogenous cannabinoids. Cannabinoids produce their effects by binding to specific G-protein-coupled plasma membrane receptors that can inhibit adenylate cyclase (AC); activate PKA, PI3K, AKT and MAPK; increase K^+ currents and stimulate ceramide production. Intracellular degradation is achieved following their transport through the plasma membrane mediated by the endocannabinoid membrane transporters (EMT).

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pathways leading to reduced intracellular cAMP concentrations, activation of MAP kinases, regulation of ionic current and activation or inhibition of inducible nitric oxide synthase (Diaz-Laviada & Ruiz-Llorente 2005).

The multiple signal transduction pathways activated by endocannabinoids underlie the different biological activities exerted within the CNS and the peripheral tissues. At the central level, endocannabinoids modulate pain initiation, psychomotor behaviour, memory, thermogenesis and appetite (Katona & Freund 2008), while in the periphery, they act on the immune (Klein 2005), cardiovascular (Pacher *et al*. 2008) and reproductive system (Maccarrone 2009) as well as on energy balance (Pagotto *et al*. 2006). Additional effects on pain transmission are mediated by binding to TRPV1 (Huang *et al*. 2002), while modulation of lipid and glucose metabolism is achieved by activation of PPARγ (O'Sullivan 2007).

The endocannabinoid system and the ovary

In the ovary, the endocannabinoid system has been poorly investigated, but the observations that, in humans, plasma AEA concentration increases in the natural menstrual cycle at the time of ovulation have stimulated researchers on this peculiar topic (Nir *et al*. 1973). Recently, it has been reported that the entire endocannabinoid system is active at the ovarian level and CB1R and CB2R, NAPE-PLD and AEA, have been identified in ovarian tissue (El-Talatini *et al*. 2009). Immunostaining shows expression of CB1R and CB2R in the medulla and cortex of the ovary. In the cortex, the receptors are expressed in the granulosa cells of primordial, primary, secondary and tertiary follicles and in the theca cells of secondary and tertiary follicles. Both receptors have also been observed in the corpus luteum and corpus albicans. NAPE-PLD, on the other hand, is expressed in the granulosa and theca cells of secondary and tertiary follicles, in the corpus luteum and in the corpus albicans, suggesting that AEA is mainly produced from the granulosa of growing follicles but not from oocytes (El-Talatini *et al*. 2009).

The changes in reproductive functions induced by cannabis derivatives strongly suggest that they exert potent negative effects on the ovulatory cycle. The primary negative effects are ascribed to a hypothalamic action, although some of these down-regulating influences may be mediated directly at the level of the pituitary and the ovary. At systemic level, cannabinoids are able to modulate the hypothalamic–pituitary–gonadal axis and they have been shown to down-regulate blood LH levels, by indirectly modifying GNRH secretion (Murphy *et al*. 1990). *In vitro* studies on rats have also demonstrated that Δ^9-THC exerts a direct inhibitory effect on both folliculogenesis (Adashi *et al*. 1983) and ovulation (El-Talatini *et al*. 2008). In support of these findings, in humans, a direct adverse effect on the ovary has been clearly documented, as cannabis users are at a higher risk of primary infertility due to anovulation (Mueller *et al*. 1990), and when they undergo IVF treatment, they produce poor-quality oocytes and have lower pregnancy rates compared with non-users (Klonoff-Cohen *et al*. 2006). Moreover, follicular fluid AEA concentrations are correlated with follicle size and are lower in follicles from which oocytes are not retrieved, indicating that AEA is probably involved in the maturation of the follicles and the oocytes (Schuel *et al*. 2002, El-Talatini *et al*. 2007, 2009).

It is well known that the endocannabinoid system regulates energy balance by modulating appetite, food intake and glucose metabolism (Bellocchio *et al*. 2007, Nogueiras *et al*. 2009). Some evidence also suggests that the endocannabinoid system could interact with ovary function through the modulation of pathways involved in energy balance and metabolism control. Indeed, obesity is commonly associated with menstrual irregularities, chronic oligo-anovulation and infertility (Pasquali *et al*. 2007), and regular ovulation is restored after simple management strategies aimed at weight reduction leading to improved natural conception (Zain & Norman 2008, Wilkes & Murdoch 2009).

Intriguingly, some authors suggested that there are several possibilities that specific, still undefined dysfunctions of the endocannabinoid system may be implicated in some pathological condition such as polycystic ovary syndrome (PCOS), which affects the ovary (Gambineri *et al*. 2004, Pasquali *et al*. 2006). PCOS is one of the most common causes of infertility due to anovulation in women. The disease is characterised by oligo- and/or anovulation, clinical and/or biochemical signs of hyperandrogenism and presence of polycystic ovaries. It is important to underlie that cardinal features of PCOS such as insulin resistance, androgen hypersecretion and obesity might be influenced by the endocannabinoid system. In rats, it has been shown that AEA activation of CB1R in pancreatic β cells can induce insulin hypersecretion and insulin resistance (Bermudez-Siva *et al*. 2006, Ahren 2009). These observations suggest that a local effect of endocannabinoid signalling in the pancreas might also play a role in PCOS-associated insulin resistance. Finally, PCOS anovulation may be, at least in part, the result of the complex interplay that exists between endocannabinoids, leptin production and obesity (Battista *et al*. 2008).

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However, further studies are mandatory to disentangle the relative contribution of endocannabinoids, local and systemic effects in the aetiopathogenesis of PCOS.

Endocannabinoid and the oviduct

The transport of the embryo from the oviduct to the uterus is aided by a wave of regulated contraction and relaxation of the oviduct muscularis. It is thought that the sympathetic neuronal circuitry, under the direction of ovarian hormones, coordinates the 'closing and opening' of the sphincter at the isthmus–uterine junction, thereby regulating the timely passage of embryos from the oviduct into the uterus (Howe & Black 1973, Heilman *et al*. 1976). It has been reported that CB1R and CB2R knockout mice are characterised by a high level of pregnancy loss (Wang *et al*. 2006). CB1R is colocalised with α1- and β2-adrenergic receptors (AdRs) in mouse oviductal muscularis at the isthmus region, and the β-AdR agonist isoproterenol restores normal embryo transport in CB1R knockout mice (Wang *et al*. 2004). These results indicate that maternal expression of CB1R in the reproductive tracts plays a fundamental role in ensuring normal embryo transfer from the oviduct to the uterus. Interestingly, wild-type females exposed to a stable AEA analogue (methanandamide) or natural THC also show pregnancy loss with embryo retention in the oviduct (Wang *et al*. 2006). These observations suggest that either a silenced or an enhanced cannabinoid signalling may influence embryo transport.

Endocannabinoid and the uterus

In the uterus, the endometrium represents a significant source of endogenous cannabinoids, and AEA levels are higher than in other reproductive tissues (Das *et al*. 1995, Schmid *et al*. 1997). The expression of the individual components of the endocannabinoid system was initially demonstrated in mice uteri (Das *et al*. 1995, Paria *et al*. 1999, 2001) and then also in humans, with both species expressing similar AEA levels (Taylor *et al*. 2010*a*). According to Taylor *et al*. (2010*a*) CB1R immunoreactivity is more intense in the glandular epithelium compared with the stroma and its expression is not regulated throughout the menstrual cycle. These findings were not confirmed by Resuehr *et al*. (2012) who recently reported a dramatic increase in CB1R mRNA and protein in normal endometrial samples in the secretory phase likely due to progesterone modulation. CB2R immunoreactivity is found in both glands and stroma; its expression is minimal at the beginning of the cycle and reaches a peak during the late proliferative phase (Taylor *et al*. 2010*a*). NAPE-PLD is intensively expressed in the menstrual, early-proliferative and late secretory glands with its lowest levels in the early-secretory phase. This enzyme is also found in the stroma (Taylor *et al*. 2010*a*). Glandular expression of FAAH increases during the menstrual cycle reaching a peak during menstruation. Similarly, FAAH is also expressed in the stroma (Taylor *et al*. 2010*a*). The expression of these two enzymes in the endometrium suggests that, during the menstrual cycle, they modulate AEA concentrations that are lower in the mid-luteal phase consistent with the idea that low levels are beneficial, and high levels detrimental, to blastocyst development (Maccarrone *et al*. 2002, Taylor *et al*. 2010*b*).

The human endometrium is a plastic tissue in which cells undergo a variety of adaptation reactions in response to the physiological changes that occur during the menstrual cycle and embryo implantation (Salamonsen 2003). Endometrial cell migratory behaviour can be considered as a central feature of endometrial plasticity and crucial for endometrial physiology. The stromal component begins to grow when the endometrial wound is completely re-epithelialised and endometrial cell movements are essential to repopulate the space created by tissue loss and to avoid excessive fibroplasia. These events are mainly regulated by steroid hormones, the timing and concentrations of which dictate the balance between endometrial growth and transformation (Guzeloglu-Kayisli *et al*. 2004). Interestingly, recent data support the idea that the endocannabinoid system plays an important role in the control of endometrial plasticity by regulating endometrial cell motility (Gentilini *et al*. 2010). We demonstrated for the first time that methanandamide stimulates endometrial stromal cell migration in a dose-dependent manner (Fig. 2) via CB1R and not via CB2R activation as indicated by the CB1R antagonist AM251-selective inhibition. This effect is mediated by activation of ERK1/2 and PI3K/Akt pathways as selective inhibitors of both pathways can prevent the stimulatory effect of methanandamide whereas inhibition of adenylate cyclase is probably not involved.

The protein–protein interactions and signalling events that regulate cell migration involve cytoskeleton rearrangement and formation of different actin structures indispensable for cell movements (Anand-Apte & Zetter 1997, Alessandro & Kohn 2002, Friedl & Wolf 2003). In agreement with these mechanisms, methanandamide activation of ERK1/2 and PI3K/Akt in endometrial stromal cells is able to induce rapid changes in the cytoskeleton architecture of the cells that thus acquire a migratory

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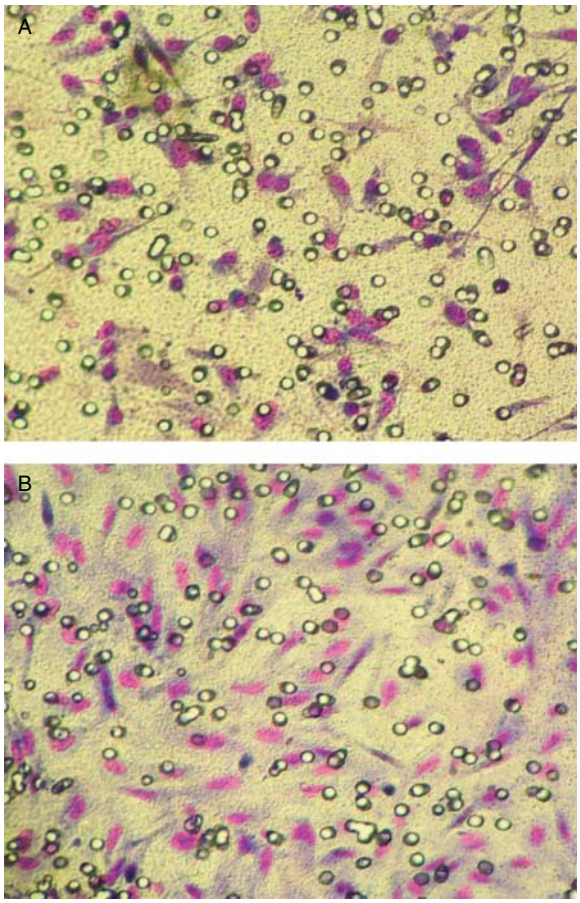

Figure 2
Representative chemotaxis experiment. Migrated endometrial stromal cells have been stained: the image shows cell migration in basal condition (A) and after stimulation with methanandamide (10^{-5} M) (B).

phenotype. This phenotype is characterised by the rapid dissolution of F-actin stress fibres, progressive localisation of F-actin towards the edge of the cell membrane and by the presence of numerous stress fibre arcs (Fig. 3; Hotulainen & Lappalainen 2006, Gentilini *et al*. 2010). In addition, we also showed that methanandamide can regulate endometrial stromal cell motility by increasing electrical signal generated by K^+ channels that is an essential component of cellular migration. The ability of endocannabinoids to activate K^+ currents appears especially suggestive in this context because it is well known that external electric fields can also regulate embryo invasiveness and implantation (Schwab *et al*. 2008). The effects of endocannabinoids on endometrial cell migration have also been confirmed in a study from McHugh *et al*. (2011), although they suggest a CB1R-independent mechanism.

To date, there is only a single study that investigated the role of cannabinoids in the proliferation of endometrial cells. Leconte *et al*. (2010) showed that *in vitro* treatment with the agonist WIN 55212-2 has an antiproliferative effect mediated by a mechanism involving reduction of reactive oxygen species production and inactivation of the Akt pathway. Preliminary data from our laboratory confirm these findings. Indeed, high doses of methanandamide (10^{-5} M) influence endometrial stromal cell proliferation in a bimodal manner. The first 24-h stimulation increases cell proliferation, while prolonged treatments (48 h or more) lead to apoptosis (Fig. 4). The signalling pathways responsible for this bimodal effect have not been clarified yet, but it is tempting to speculate that prolonged treatment may lead to synthesis of apoptotic molecules such as ceramide (Ellert-Miklaszewska *et al*. 2013).

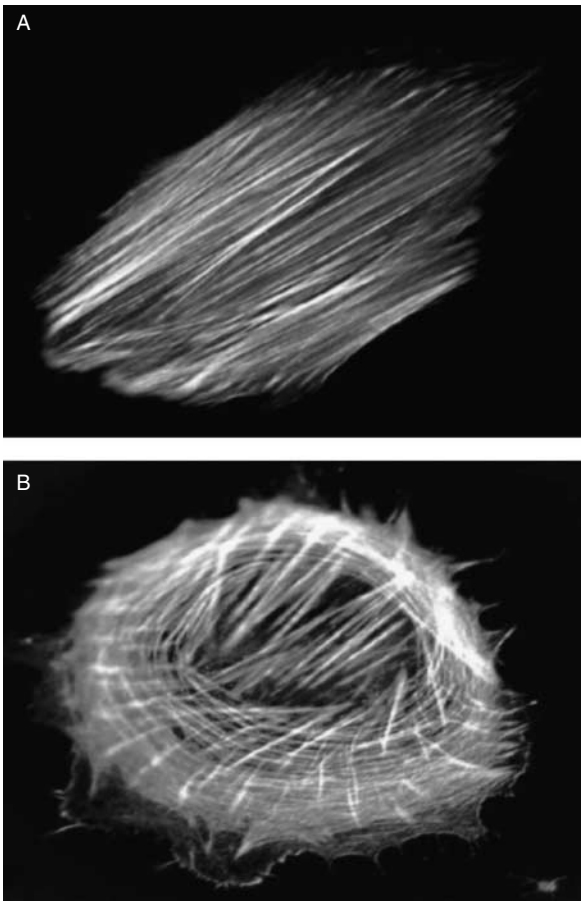

Figure 3
Effects of methanandamide (10^{-5} M) on actin cytoskeleton pattern of endometrial stromal cells. Untreated cells show a classic static phenotype (A). Treatment with methanandamide induces cytoskeleton rearrangements and a migratory phenotype (B).

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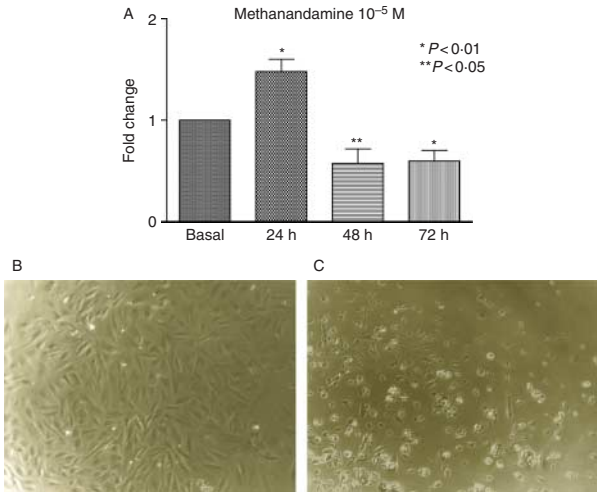

Figure 4
Effect of methanandamide (10^{-5} M) on endometrial stromal cell proliferation. Data are expressed as fold changes and are reported for 24, 48 and 72 h treatments ($n=12$) (A). (B) Endometrial stromal cells in basal condition. (C) Apoptotic endometrial stromal cells after 72-h stimulation with methanandamide.

Considering the possible role of the endocannabinoid system in regulating endometrial proliferation, Resuehr *et al*. investigated its involvement in endometriosis, a benign pathological condition characterised by deregulated endometrial cell proliferation and invasion. They demonstrated that expression of CB1R, at both the mRNA and protein level, is lower in eutopic endometrium of patients affected by endometriosis compared with that observed in samples obtained from control healthy women. This reduced CB1R expression has been attributed to the effects of persistent environmental toxicants and interleukin-1α that induce a progesterone resistance phenotype in patients affected by the disease (Resuehr *et al*. 2012). Thus, it is possible to speculate that reduced cannabinoid signalling might underlie the enhanced proliferative capacity of endometriotic lesions.

Conclusions

As summarised in Fig. 5, endocannabinoids exert important actions in female reproductive organs. Their biological activities include regulation of oocyte and follicle maturation, embryo transport through the oviduct and implantation of the blastocyst. Impairment of the endocannabinoid system has been associated with pathological conditions involving these organs. In addition, it has recently emerged that endocannabinoids also regulate endometrial plasticity modulating endometrial cell migration and proliferation. Although these last effects certainly need to be better elucidated, we feel that the endocannabinoid system may represent an important task for researchers dealing with diseases of the female reproductive system characterised by increased invasiveness and proliferation of the endometrium. Among these diseases, endometrial cancer does certainly have a primary role, but also benign pathologies such as endometriosis could benefit from the results of such researches. Indeed,

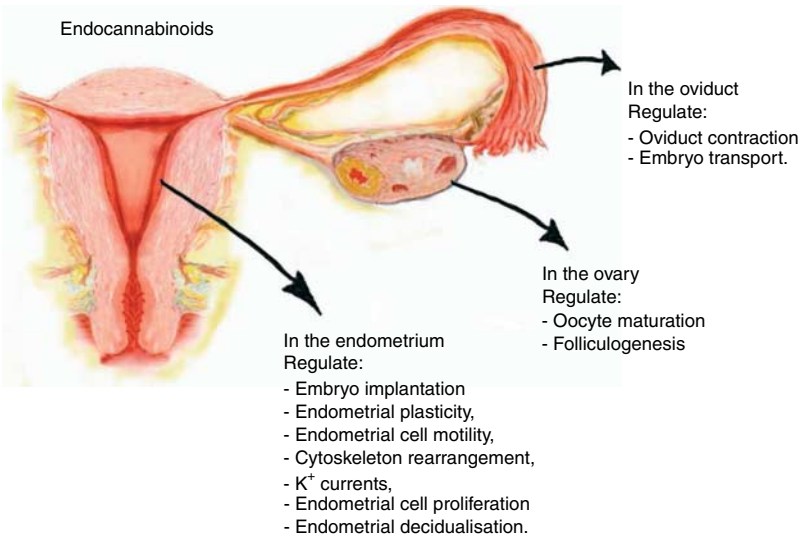

Figure 5
Overview of the most important biological activities of endocannabinoids in the female reproductive organs.

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the evaluation of chemical compounds acting on the endocannabinoid system will pave the way to develop alternative pharmacological strategies for a disease that, at present, heavily relies on surgical treatments.

Declaration of interest

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

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