Article

17β-oestradiol and progesterone regulate anandamide synthesis in the rat uterus

Ana Franchi received her initial training in chemistry at the School of Sciences, University of Buenos Aires and became interested in prostaglandin metabolism in the sex cycle. As a post-doctoral researcher she began to work in pregnancy and developed two rodent models of diseases that take place during pregnancy: pre-term labor and septic abortion. She is currently a researcher of the National Research Council and director of CEFYBO (School of Medicine).

Dr Ana Franchi

María L Ribeiro[1,2], Claudia A Vercelli[1], Micaela S Sordelli, Mariana G Farina, Marcos Cervini, Silvia Billi, Ana M Franchi
Laboratory of Physiopathology of Pregnancy and Labor, Center for Pharmacological and Botanical Studies, School of Medicine (National Research Council, University of Buenos Aires), Paraguay 2155 16th floor, CP (C1121ABG), Buenos Aires, Argentina
[1]Both authors contributed equally to this manuscript.
[2]Correspondence: e-mail: marialribeiro@yahoo.com.ar

Abstract

Anandamide is an endocannabinoid known to participate in reproductive processes. This study observed that 17β-oestradiol and progesterone modulated the production of anandamide and its metabolizing enzymes in the rat uterus. Anandamide production was highest at the oestrous stage and 17β-oestradiol and progesterone stimulated its synthesis in ovariectomized rats. During early pregnancy, anandamide production remained constant on days 1–5 of gestation and diminished towards day 6. On day 6, implantation sites showed lower synthesis compared with interimplantation sites. In the delayed implantation model, 17β-oestradiol inhibited anandamide synthesis compared with progesterone. During pseudopregnancy, anandamide production did not decrease towards day 6 as occurred during normal gestation. The administration of 17β-oestradiol augmented anandamide production in rats on day 5 of pseudopregnancy; the treatment with mifepristone did not produce any change in anandamide synthesis. Anandamide-metabolizing enzymes were regulated by progesterone and 17β-oestradiol. The effect of ovarian hormones on the synthesis of anandamide depends on different physiological conditions, oestrous cycle and early pregnancy, and on the presence of the activated blastocyst. Thus, ovarian hormones, as signals that emanate from the mother, operate in conjunction with the blastocyst intrinsic programme, regulating the synthesis of anandamide in a specific manner during crucial reproductive events that may compromise pregnancy outcome.

Keywords: *blastocyst, implantation, oestradiol, pregnancy, progesterone, uterus*

Introduction

Anandamide (*N*-arachidonoylethanolamine, AEA) belongs to a family of lipid mediators serving as endogenous ligands for the brain, type CB1, and the spleen, type CB2 cannabinoid receptors (Devane *et al*., 1992; Vogel *et al*., 1993). AEA seems to play an important role during early pregnancy but increased AEA concentrations are detrimental to the survival of the embryo. The murine uterus contains the highest concentrations of AEA yet discovered in mammalian tissues and changes in its concentrations are associated with uterine receptivity for embryo implantation (Schmid *et al*., 1997). Uterine anandamide synthase capacity and degradation have been described at early gestation in the mouse (Paria *et al*., 1996). Later, *N*-arachidonylphosphatidyl ethanolamine phospholipase-D (NAPE-PLD), one of the enzymes involved in AEA synthesis, was discovered (Guo *et al*., 2005). The effectiveness of AEA depends on its concentration in the extracellular space, which is regulated through its metabolism and turnover by the target organ. The best-characterized pathway is the breakdown of AEA by the enzyme fatty acid amide hydrolase (FAAH) (Cravatt *et al*., 1996). It is suggested that embryonic and/or uterine FAAH could play an important role in regulating uterine AEA concentrations (Paria *et al*., 1999; Xiao *et al*., 2002; Maccarrone *et al*., 2004). FAAH activity is higher in the implantation sites compared with the non-implantation sites.

However, low AEA concentrations at the implantation sites also correlate with high cyclo oxygenase-2 expression (Lim *et al.*, 1997), suggesting that more than one metabolizing enzyme may control the concentration of AEA.

Klinger *et al.* (2006) reported that both FAAH activity and protein content in the mouse uterus vary as a function of the natural oestrous cycle stages. Evidence has been provided that a critical balance between AEA synthesis and its degradation in mouse embryos and oviducts creates locally appropriate AEA tone for normal development of embryos and implantation (Wang *et al.*, 2006a).

This evidence suggests that AEA signalling is operative very early in pregnancy and that the systemic endocrine milieu in early pregnancy might be involved in the regulation of AEA concentration and the enzymatic changes described. Indeed, oestrogen and progesterone derived from the maternal ovary suppress the activity of FAAH in the mouse uterus (Maccarrone *et al.*, 2000a). Also, it is well known that rat uterine endometrium undergoes cyclical changes which are regulated by 17β-oestradiol and progesterone. Therefore, the synthesis of AEA in the rat uterus might be regulated by ovarian hormones. Thus, the aim of the present work was to study the regulation of AEA production by 17β-oestradiol and progesterone in virgin and pregnant rats. These findings may contribute to a better understanding of the significance of ligand–receptor signalling with AEA in coordinating the series of events that leads to a successful pregnancy.

Materials and methods

Animals

The experimental procedures reported here were approved by the Animal Care Committee of the Centre for Pharmacological and Botanical Studies (CEFYBO, School of Medicine-CONICET, University of Buenos Aires) and carried out in accordance with the Guide for Care and Use of Laboratory Animals (DHEW Publication, NIH, 80–23). Female rats of the Wistar strain were housed in group cages under controlled conditions of light (14 h light, 10 h dark) and temperature (23–25°C). Animals received food and water *ad libitum*. The stages of the oestrous cycle were identified by vaginal smears. Uterine horns were removed at proestrus, oestrus, dioestrus and metoestrus, cleaned of fat and frozen at –70°C until tested. To study the effect of ovarian steroid hormones on the synthesis of AEA in the non-pregnant uterus, virgin rats were ovariectomized without regard to the stage of the oestrous cycle and rested for 20 days before receiving any treatment. The ovariectomized rats were divided into four groups and respectively injected with corn oil (0.4 ml/rat, control rats), 17β-oestradiol (4 µg/kg) (Franchi *et al.*, 1989a) or progesterone (16 mg/kg) (Franchi *et al.*, 1989b). Steroids were dissolved in corn oil and injected s.c. with the same volume. Rats were killed 18 h after the injection. Uterine horns were collected and frozen at –70°C until tested.

Virgin female rats were mated with fertile males of the same strain. The morning the spermatozoa were observed in the vaginal fluid was defined as day 1 of pregnancy. Under the conditions of the study centre's animal facilities, spontaneous term labour occurs on day 22 of gestation. The blastocysts arrive at the uterus lumen between days 4 and 5 of gestation and implantation occurs during the afternoon of day 5. Rats on days 1–6 of pregnancy were sacrificed at 09.00–10.00 in the morning. Embryos were recovered on days 1–5 from the reproductive tract to confirm pregnancy. On day 6 of pregnancy distinct macroscopically visible uterine swellings indicated the implantation sites. Uterine horns of the rats on days 1–6 of pregnancy were excised and uterine horns on day 6 were separated into implantation and interimplantation sites.

Different methods of manipulating pregnancy-related uterine changes (pseudopregnancy and ovariectomy-induced delayed implantation) were used as tools to understand the relative roles played by the embryo and ovarian hormones in modulating the changes in AEA synthesis in the pregnant rat. A delayed implantation rat model was established as previously described with minor modifications (Xiao *et al.*, 2002). Briefly, rats were ovariectomized at 09.00–10.00 on day 4 of pregnancy. To maintain the implantation delay, rats were injected with progesterone (8 mg/kg) on days 5 and 6 of pregnancy. On day 7 of gestation, females were divided randomly into three groups and treated respectively with corn oil (0.4 ml/rat, s.c., control group), progesterone (8 mg/kg) or 17β-oestradiol (0.4 mg/kg). Steroids were dissolved in corn oil and injected s.c. with the same volume. These rats were sacrificed at 09.00–10.00 on day 8 and their uteri were collected and stored at –70°C until used. Uteri of rats with implantation sites were collected. If implantation sites were absent, uterine horns were flushed with saline to recover unimplanted blastocysts. Rats without implantation sites or blastocysts were excluded from the experiments. Pseudopregnancy could be induced in female rats treated with equine chorionic gonadotrophin (PMSG): the uterus undergoes all the normal changes that prepare it for implantation, but no embryos are present in the uterine lumen. Prepuber rats (25–28 days of age) received 50 IU PMSG dissolved in saline i.p. Day 1 of pseudopregnancy was considered as 24 h after the injection. Females were sacrificed at days 4, 5 and 6 of pseudopregnancy (Wang and Menon, 2005). Where indicated, a group of rats on day 4 of pseudopregnancy was injected with 17β-oestradiol (8 mg/kg, s.c.) or mifepristone (RU-486) (10 mg/kg, i.p.) (Farina *et al.*, 2004) and sacrificed on day 5 of pseudopregnancy. Control animals were injected with corn oil (0.4 ml, s.c.) or with ethanol 30% (0.3 ml, i.p.), respectively.

Determination of anandamide-synthesizing activity

The synthesis of AEA was assayed as previously described (Fernández-Solari *et al.*, 2004; Scorticati *et al.*, 2004) and others (Devane and Axelrod, 1994; Paria *et al.*, 1996) with minor modifications. Uterine tissue was homogenized in Tris buffer pH 7.6 (20 mmol/l Tris/HCl, 1 mmol/l EDTA) and incubated for 30 min with 0.1% v/v Triton X-100 at 0°C. One hundred and fifty µg of protein were incubated for 60 min at 37°C in Tris buffer pH 9.0 (50 µmol/l Tris/HCl) containing 20 mmol/l ethanolamine and 40 µmol/l [^{14}C]-arachidonic acid (0.1 µCi, specific activity 51 mCi/mmol). The reaction was stopped by the addition of chloroform:methanol (1:1 v/v). The aqueous phase was washed two times with chloroform. Samples were applied on thin layer chromatography (TLC) aluminium Silica Gel 60 sheets with concentration zone. Plates were run in the organic phase of a solvent system of ethyl acetate:hexane:acetic

acid:distilled water (100:50:20:100 v/v). Lipids were visualized with iodine, identified by co-migration with an AEA standard, scraped from the plates and quantified by liquid scintillation counting. The Rf values of AEA and arachidonic acid were 0.33 and 0.78 respectively. Each radioactive peak corresponding to AEA was counted and expressed as a percentage of the total radioactivity of the plates. Protein concentration was determined by the method of Bradford (1976). Anandamide-synthesizing activity is reported as nmol AEA/mg protein/h. The optimal reaction conditions were determined.

Polymerase chain reaction analysis

Total RNA from rat uterus was isolated using Trizol reagent according to the manufacturer's recommendations. Following extraction, RNA was quantified and further treated with RNase-free DNase I to digest contaminating genomic DNA. First strand cDNA was synthesized from total RNA (1 µg) using Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) and random primers according to the manufacturer's recommendations in the presence of ribonuclease inhibitor. After first-strand synthesis, polymerase chain reaction (PCR) was performed with specific intron-spanning primers. Oligonucleotide primers for FAAH were 5′-TTGGTTTGTGCGAGCCGAGT-3′ (sense) and 5′-CCTTTGTTCACTTCCCAGGC-3′ (antisense) and yielded a product of approximately 380 base pairs (bp). PCR cycle parameters were as follows: an initial denaturating step at 94°C for 5 min followed by 30 cycles of 94°C for 40 s, 57°C for 30 s and 72°C for 1 min followed by 72°C for 5 min. Oligonucleotide primers for NAPE-PLD were 5′-ATGAGAACAGCCAGTCTCCA-3′ (sense) and 5′-CCATTTCCACCATCAGCGTC-3′ (antisense) and yielded a product of approximately 407 bp. PCR cycle parameters were as follows: an initial denaturating step at 94°C for 5 min followed by 30 cycles of 94°C for 45 s, 59°C for 1 min and 72°C for 1 min followed by 72°C for 5 min. The β-actin sense and antisense primers used were 5′-TGTTACCAACTGGGACGACA-3′ and 5′-TCTCAGCTGTGGTGGTGAAG-3′ respectively yielding a product of approximately 392 bp. PCR cycle parameters were as follows: an initial denaturating step at 94°C for 5 min followed by 30 cycles of 94°C for 40 s, 57°C for 30 s and 72°C for 1 min followed by 72°C for 5 min. PCR products were resolved in 12% acrylamide gels and subjected to silver staining. Photographs were taken using a digital camera Olympus C-5060 and analysed using the Image J (open source) software package. Data were expressed as the relative amount of FAAH or NAPE-PLD versus β-actin mRNA.

Western blot analysis

Uterine slices were incubated in triple-detergent buffer (phosphate-buffered saline pH 7.4 with 0.02% w/v sodium azide, 0.1% w/v sodium dodecyl sulphate (SDS), 1% v/v Nonidet P-40, 0.5% v/v sodium deoxycholate) containing 10 µg/ml leupeptin, 2 µg/ml aprotinin, 100 µg/ml soybean-trypsin inhibitor, 1 mmol/l EDTA, 1 mg/ml benzamidine, 10 µg/ml dithiothreitol and 1 mg/ml caproid acid. Tissues were homogenized (Ultra Turrax, T25 basic, IKA Labortechnik, Germany) and sonicated for 30 s (Ultrasonic Cell Disrupter, Microson, Heat systems Inc.). After centrifugation at 1000 g for 15min, the supernatants were collected and stored at –70°C until western blotting was performed. Each lane was loaded with 100 µg protein. Samples were separated on 10% (w/v) SDS-polyacrylamide gels for FAAH and 12% SDS-polyacrylamide gel for NAPE-PLD by electrophoresis and transferred to a nitrocellulose membrane. Blots were incubated overnight with anti-FAAH (1:100) or anti-NAPE-PLD (1:200) and 30 min with anti-actin (1:4000). Blots were washed with buffer (10 mmol/l Tris, 100 mmol/l NaCl and 0.1% (v/v) Tween 20, pH 7.5) followed by 1 h incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody and developed using the enhanced chemiluminescence western blot system. Photographs of the membranes were taken using a digital camera and analysed using the Image J software package.

Statistics

Statistical analysis was performed using the GraphPad Prism Software (San Diego, CA, USA). Comparisons between values of different groups were performed using one-way ANOVA (analysis of variance). Significance was determined using Tukey's multiple comparison tests for unequal replicates. Time course for AEA production was analysed by a linear regression test. All values presented in this study represent means ± SEM Differences between means were considered significant when P was 0.05 or less.

Drugs and chemicals

[^{14}C]-Arachidonic acid (specific activity 51 mCi/mmol, 1.9 GBq/mmol) was provided by PerkinElmer (Boston, MA, USA). Anandamide, ethanolamine, 17β-oestradiol, progesterone, Triton X-100, secondary horseradish peroxidase-conjugated antibody and anti-β-actin antibody were purchased to Sigma Chemical Company (St Louis, MI, USA). TLC plates were from Merck KGaA (Darmstadt, Germany). PMSG (equine chorionic gonadotrophin, Novormon®) was kindly provided by Syntex SA, Buenos Aires, Argentina (www.syntexar.com, accessed 24 July 2008). Mifepristone (RU-486) was obtained from Biomol (Plymouth Meeting, PA, USA). Trizol reagent, RNase-free DNase I, Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) and random primers were purchased from Invitrogen (Life Technologies, Argentina). Silver staining detection kit was purchased from Winkler (Santiago, Chile). The anti-NAPE-PLD antibody was from Cayman Chemical (Ann Harbor, MI, USA). The anti-FAAH antibody was a gift from Dr Benjamin Cravatt. All other chemicals were analytical grade.

Results

Before studying the role of steroid hormones, anandamide-synthesizing capacity was characterized in the rat uterus. Optimal reaction conditions were determined using dioestrous uterine tissue. Because it has been previously observed that anandamide-synthesizing capacity is linear up to 200 µg of protein (data not shown), 150 µg of protein was incubated. Initial experiments showed that the production of AEA was linear for a period of 60 min after a lag of 5 min ($r^2 = 0.96$, $P < 0.05$, **Figure 1**). Similarly to previously reported results (Devane and Axelrod, 1994), it was observed that the synthesis of AEA was measurable at pH = 9.0 and was undetectable in the absence of ethanolamine (**Table 1**).

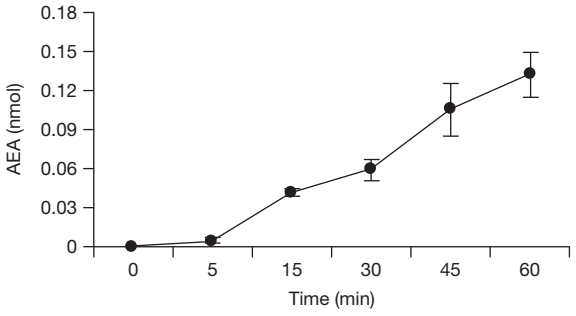

Figure 1. Anandamide (AEA) synthesis in the rat uterus as a function of time. Homogenate protein (150 µg/point) was incubated with 40 µmol/l arachidonic acid and 20 mmol/l ethanolamine for different time periods. Results are expressed as nmol AEA; $n = 5$ for each point.

Table 1. Anandamide synthesis in the rat uterus under different experimental conditions.

Ethanolamine (20 mmol/l)	+	–
Triton X-100 (0.01% v/v)	+	+
AEA synthesis (nmol AEA/mg protein/h)	0.8 ± 0.1	ND

Homogenate protein (150 µg/point) from dioestrus rat uterus was incubated at pH = 9 with 40 µmol/l arachidonic acid with or without 20 mmol/l ethanolamine. $n = 4$ for each point. ND = not detectable.

When the production of AEA in virgin females was analysed, it was shown that AEA production was regulated during the oestrous cycle, being significantly higher at oestrus compared with proestrus, metoestrus or dioestrus ($P < 0.01$, **Figure 2a**). Thus, it was decided to study the effect of ovarian hormones, 17β-oestradiol and progesterone, on uterine AEA production in ovariectomized rats. The ovariectomized rat uterus showed detectable AEA production (control, **Figure 2b**). The administration of 17β-oestradiol or progesterone significantly augmented the synthesis of AEA compared with the control group ($P < 0.01$, **Figure 2b**).

Then the production of AEA was observed during early pregnancy in the rat uterus. AEA synthesis remained unchanged between days 1 and 5 of gestation (**Figure 3a**). A significant decrease occurred on day 6 compared with days 1–5 of pregnancy ($P < 0.01$, **Figure 3a**). When the production of AEA was analysed in separated implantation versus interimplantation sites on day 6 of gestation, AEA production was lower at the sites of embryo implantation ($P < 0.01$, **Figure 3a**).

As the synthesis of AEA was differentially regulated between implantation and interimplantation sites, it was decided to investigate if this modulation was supported by a crosstalk between the blastocyst and the uterus and/or by ovarian hormones. Thus, AEA production was studied in the delayed implantation model and during pseudopregnancy. In the group of delayed implantation rats, the treatment with 17β-oestradiol inhibited the production of AEA compared with progesterone ($P < 0.001$, **Figure 3b**). Then the synthesis of AEA was measured in the pseudopregnant rat uterus, an experimental model in which the embryos are not present. The results showed that in the absence of a blastocyst, the production of AEA did not diminish from day 5 to day 6 of pseudopregnancy, as was observed during normal pregnancy (**Figure 3c**).

Based on these results, it was postulated that although AEA production was modulated by ovarian hormones, implantation of the blastocyst was also necessary. In order to confirm this hypothesis, pseudopregnant rats on day 4 were treated with 17β-oestradiol or with RU-486, an antagonist of the progesterone receptor. 17β-oestradiol administration increased the production of AEA on day 5 of pseudopregnancy (**Figure 4a**). On the other hand, the administration of RU-486 did not modify AEA synthesis compared with the control animals (**Figure 4b**).

Finally, NAPE-PLD and FAAH gene expression was observed at mRNA and protein level in the animal models. NAPE-PLD mRNA (~407 bp) and protein (~43 kDa) did not change during the oestrous cycle (**Figure 5a**,**f**) or when virgin females were treated with 17β-oestradiol or progesterone (**Figure 5b**,**g**). However, its expression (mRNA and protein) was modulated during pregnancy and in the delayed implantation model, being highest at the interimplantation sites (**Figure 5c**,**h**) and when the animals were treated with progesterone compared with 17β-oestradiol (**Figures 5d**,**i**). In pseudopregnant animals, a model in which the embryo is absent, NAPE-PLD expression was not modified (**Figures 5e**,**j**), thus suggesting that during pregnancy the blastocyst regulates its expression in the uterus. FAAH mRNA (~380 bp) was highest in proestrous and oestrous stages (**Figure 6a**) but the protein (~63 KDa) did not seem to be regulated during the cycle (**Figure 6f**). Under 17β-oestradiol or progesterone treatment neither FAAH mRNA nor FAAH protein changed (**Figures 6b**,**g**). During pregnancy, FAAH mRNA and protein remained constant in the implantation sites compared with the interimplantation sites (**Figure 6c**,**h**) and the administration of 17β-oestradiol or progesterone modulated FAAH expression to similar levels (**Figure 6d**,**i**). While FAAH mRNA was increased in days 5 and 6 of pseudopregnancy (**Figure 6e**), its protein was not regulated in this model (**Figure 6j**).

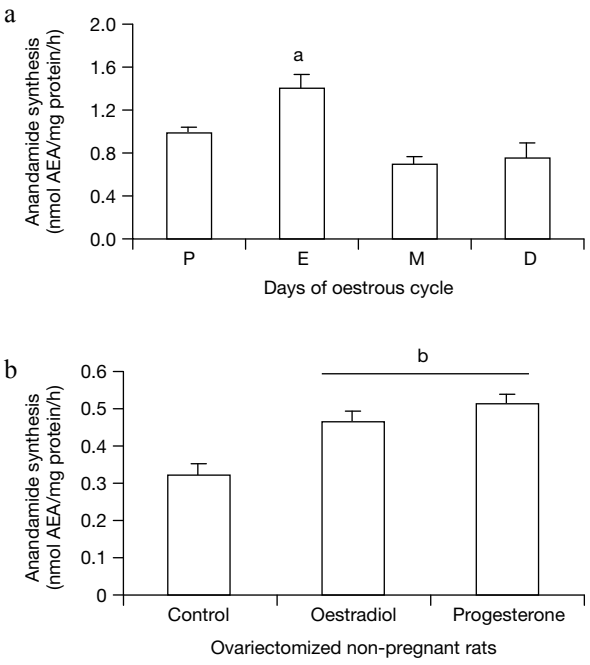

Figure 2. Anandamide (AEA) synthesis in the rat uterus during the oestrous cycle (**a**) and in ovariectomized rats treated with ovarian hormones (**b**). Homogenate proteins from proestrus (P), oestrus (E), metoestrus (M) and dioestrous (D), from ovariectomized rats (control) and from ovariectomized rats treated with 17β-oestradiol or progesterone were incubated. Results are expressed as nmol AEA/mg protein/h. a: $P < 0.01$ versus the rest; b: $P < 0.01$ versus control, $n = 5$ for each point.

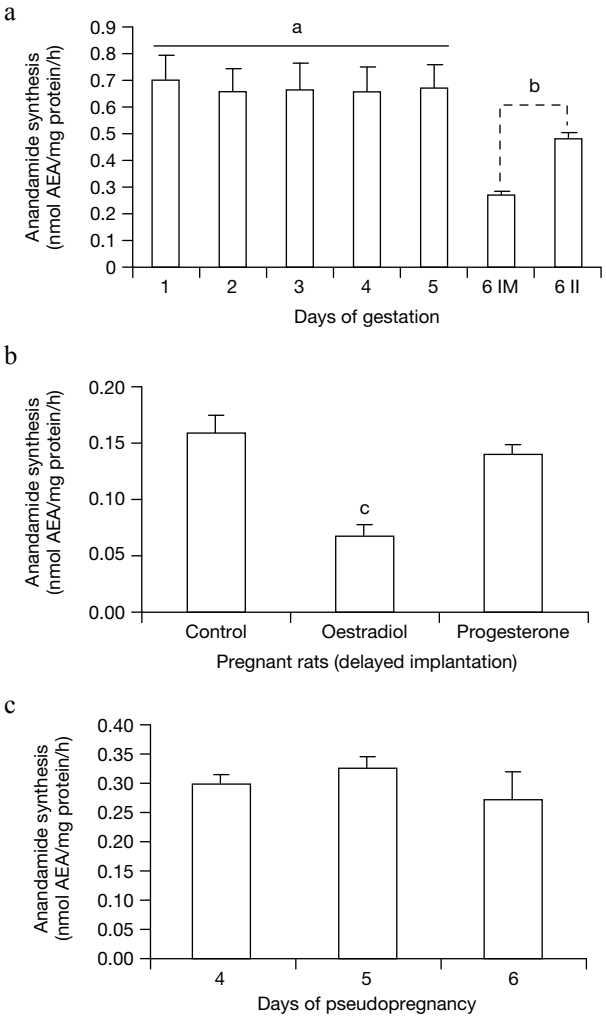

Figure 3. Uterine anandamide (AEA) synthesis during rat early pregnancy. Homogenate proteins from early pregnant (**a**), delayed implantation (**b**) and pseudopregnant (**c**) rat uteri were incubated. Results are expressed as nmol AEA/mg protein/h. a: $P < 0.01$, days 1–5 versus day-6 implantation sites (IM) and versus day-6 interimplantation sites (II); b: $P < 0.01$, day 6 IM versus day 6 II. c: $P < 0.001$, 17β-oestradiol versus control and progesterone. $n = 5$ for each point.

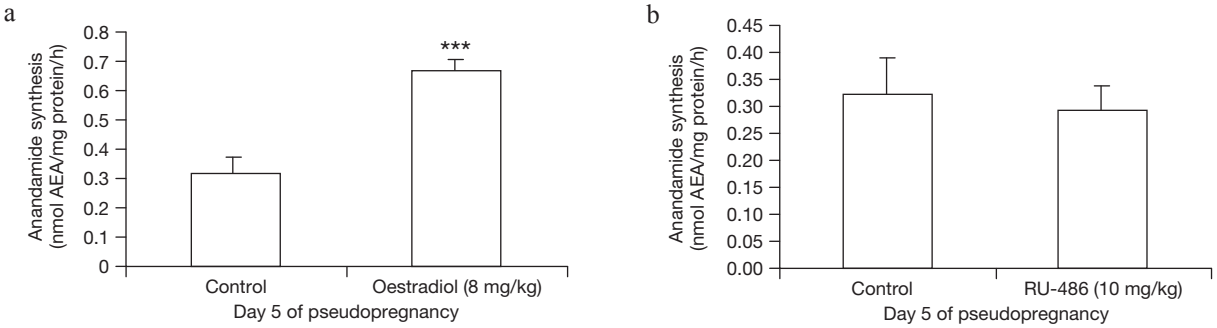

Figure 4. Uterine anandamide (AEA) synthesis in females on day 5 of pseudopregnancy treated with 17β-oestradiol (**a**) or RU-486 (**b**). Homogenate proteins from control rats and from rats treated with 17β-oestradiol or RU-486 were incubated with ethanolamine and [^{14}C]-arachidonic acid. Results are expressed as nmol AEA/mg protein/h. ***: $P < 0.001$, 17β-oestradiol versus control. $n = 4$ for each point.

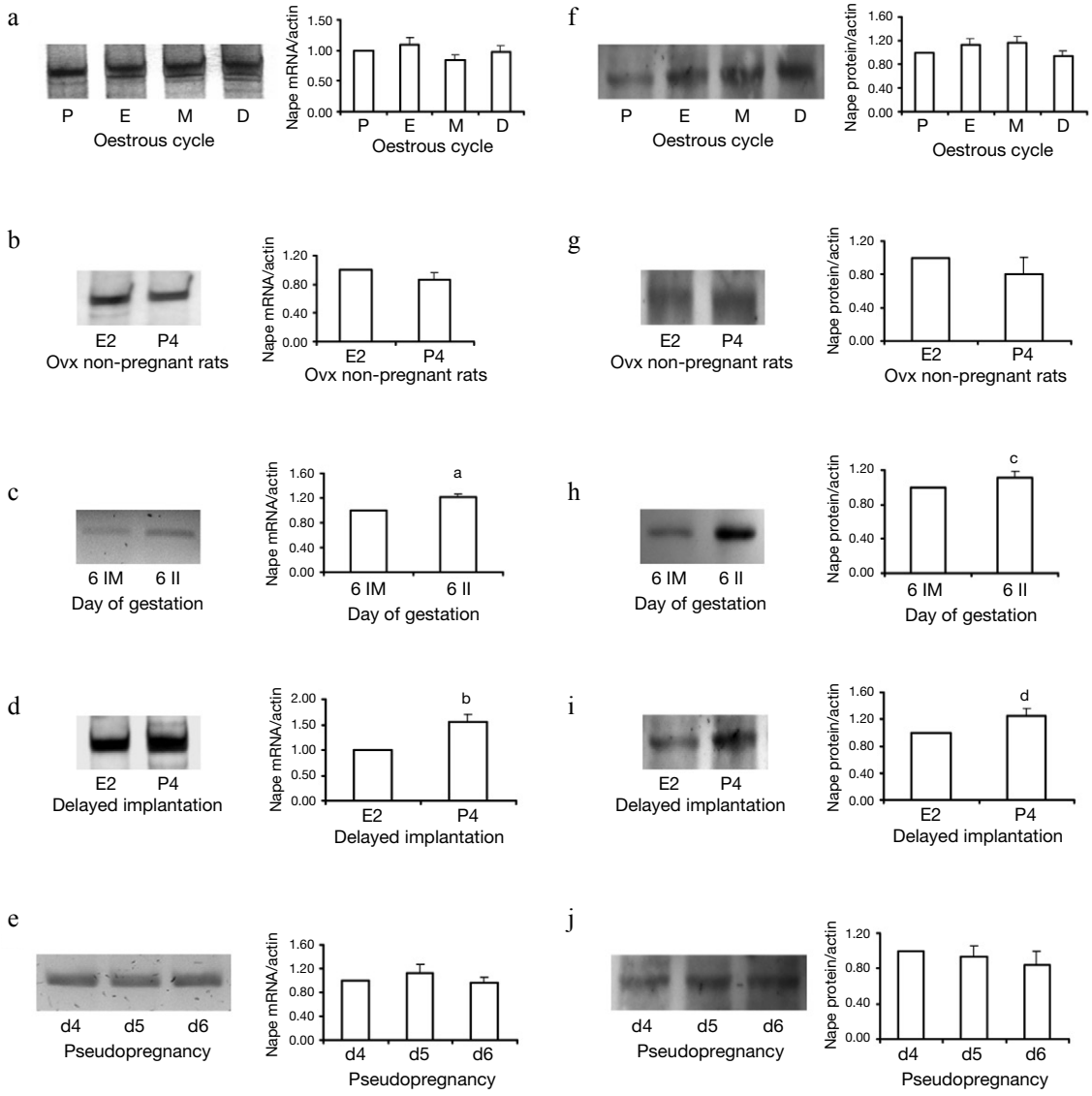

Figure 5. Densitometric analyses and protein and mRNA expression of *N*-arachidonylphosphatidyl ethanolamine phospholipase-D (NAPE-PLD) in uterine tissue obtained from different animal models: oestrous cycle (**a** and **f**), ovariectomized (ovx) rats treated with 17β-oestradiol or progesterone (**b** and **g**), day-6 implantation sites (IM) (**c** and **h**), delayed implantation (**d** and **i**) and pseudopregnancy (**e** and **j**). a: $P < 0.05$, day-6 IM versus day-6 interimplantation sites (II); b: $P < 0.05$, 17β-oestradiol versus progesterone; c: $P < 0.05$, day-6 IM versus day-6 II; d: $P < 0.05$, 17β-oestradiol versus progesterone. $n = 3$ for each point. D = dioestrus; d = day; E = oestrus; E2 = oestradiol; M = metoestrus; P = proestrus; P4 = progesterone.

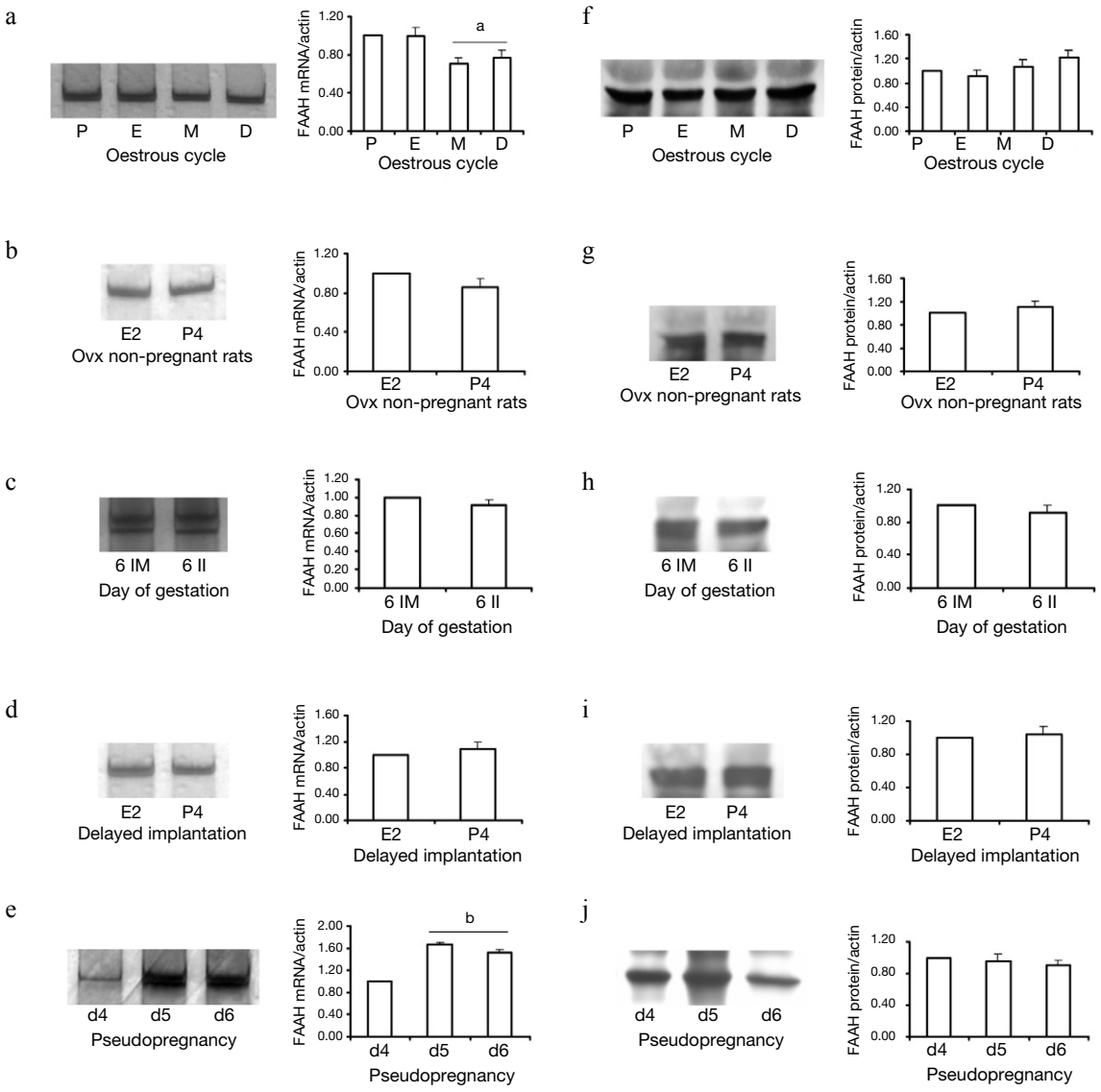

Figure 6. Densitometric analyses and protein and mRNA expression of fatty acid amide hydrolase (FAAH) in uterine tissue obtained from different animal models: oestrous cycle (**a** and **f**), ovariectomized (ovx) rats treated with 17β-oestradiol or progesterone (**b** and **g**), day-6 implantation sites (IM) (**c** and **h**), delayed implantation (**d** and **i**) and pseudopregnancy (**e** and **j**). a: $P < 0.05$, proestrus (P) and oestrus (E) versus metoestrus (M) and dioestrus (D); b: $P < 0.001$, day 4 versus day 5 and day 6. $n = 3$ for each point. II = interimplantation sites. See **Figure 5** for other abbreviations.

Discussion

The present work observed that 17β-oestradiol and progesterone regulated anandamide-synthesizing capacity depending on the animal's reproductive state. Also, the results shown here suggest that the implantation of the blastocyst was crucial in the modulation of AEA by ovarian hormones.

The discovery of AEA caused intense investigation regarding its biosynthetic pathway (Schmid, 2000). Enzymatic synthesis of AEA was first described in bovine brain as the conjugation of arachidonic acid with ethanolamine (Devane and Axelrod, 1994). This biosynthetic route has been previously described in mice uteri (Paria *et al.*, 1996; Schmid *et al.*, 1997). However, there are no reports in the rat so far. Guo *et al.* (2005) reported differences in the overall concentrations of uterine AEA between pregnant CD1 mice that were currently used at Vanderbilt University compared with the concentrations of uterine AEA in the same strain of mice at Kansas University Medical Centre (Schmid *et al.*, 1997). Thus, before studying the effect of ovarian hormones on AEA production, it was decided to characterize uterine AEA synthesis in Wistar rats housed under the conditions of the study centre's animal facilities. The results are consistent with previous findings in mice (Schmid *et al.*, 1997). It is now known that AEA could be also released by the cleavage of the phospholipid precursor, *N*-arachidonylphosphatidyl ethanolamine (NAPE), in a process catalysed by a selective phospholipase D, known as NAPE-PLD (Ueda *et al.*, 2005). Also, Liu *et al.* (2006) observed that in mice brain and RAW264.7 macrophages, the synthesis of AEA involves the phospholipase (PLC)-catalysed

cleavage of NAPE to generate phospho-AEA, which is subsequently dephosphorylated by phosphatases. Collectively, this evidence indicates the existence of multiple pathways for AEA synthesis that depend on the species and cell type. This work observed that AEA-synthesizing capacity is regulated by ovarian hormones in the rat uterus. The incorporation of arachidonic acid into AEA presented here has requirements for an alkaline pH and relatively high K_m for both arachidonic acid and ethanolamine. However, under pathological conditions such as lipopolysaccharide–induced infections, the cells are activated and generate numerous lipid-signalling molecules (Wang *et al*., 2006b). Thus, the biodisposability of arachidonic acid and ethanolamine could be augmented, promoting the generation of AEA by this route. Intrauterine infections due to Gram-negative bacteria and augmented AEA concentrations have been associated with early pregnancy loss (Maccarrone *et al*., 2000b; Burton and Jauniaux, 2004; Deb *et al*., 2004). The above evidence suggests that it is worthwhile to characterize the production of AEA by this pathway. In order to better understand which of these biosynthetic pathways is more relevant under this study's experimental conditions or if FAAH might function as anandamide synthase in the presence of higher concentrations of substrates, FAAH and NAPE-PLD expression was also determined.

The preparation of the endometrium during the oestrous cycle is essential for successful implantation. The uterus undergoes cellular remodelling during each sexual cycle, in order to be ready for a possible pregnancy. These changes are regulated by 17β-oestradiol and progesterone (Dey *et al*., 2004). It was observed that, while FAAH mRNA is highest at proestrus and oestrus, FAAH protein remained constant throughout the oestrous cycle. The discrepancy between the expression of FAAH mRNA and FAAH protein in the oestrous cycle and in the pseudopregnancy model could be explained by the sensitivity of the RT-PCR technique compared with the western blot. Others have shown that FAAH activity in the mouse uterus changes as a function of the oestrous stages (Klinger *et al*., 2006) and that the expression of FAAH mRNA in the rat uterine luminal epithelium is highest at oestrous (Xiao *et al*., 2002). However, there are no reports about the regulation of AEA production during the oestrous cycle in the rat. The highest synthesis occurred at the oestrous stage while NAPE-PLD mRNA was not regulated. To better understand the physiological relevance of differential AEA gradients during the oestrous cycle, this study investigated whether AEA production was regulated by 17β-oestradiol and/or progesterone. The synthesis of AEA was examined in a defined system, i.e. ovariectomized rats after ovarian hormone treatment. Both 17β-oestradiol and progesterone significantly increased the production of AEA in ovariectomized rat uterus but they did not alter FAAH or NAPE-PLD expression. With respect to FAAH, Xiao *et al*. (2002) reported that the highest FAAH mRNA expression in the rat uterine luminal epithelium is observed in the oestrous state. The discrepancy observed between this and the results from this study could be due to differences in the techniques used. These results suggest that in non-pregnant rats the local concentrations of AEA are tightly regulated and that ovarian steroid hormones could differentially influence AEA synthesis and degradation. When the production of AEA was studied in pregnant rats, the lowest production of AEA was detected at the sites of implantation. Similar results have been previously reported in mice uteri (Paria *et al*., 1996; Schmid *et al*., 1997). Indeed, consistent with mice NAPE-PLD enzymatic activity (Guo *et al*., 2005), the production of AEA, determined as the incorporation of arachidonic acid into AEA, exhibited a similar pattern showing higher production at interimplantation sites but lower synthesis at implantation sites. As previously described by Guo *et al*. (2005), this study also observed that NAPE-PLD mRNA was lower at the implantation sites compared with interimplantation zones. The results obtained by Wang *et al*. (2006a) suggest that FAAH is an important metabolic gatekeeper, regulating local AEA to direct preimplantation events that determine the fate of pregnancy. With respect to FAAH expression, neither the protein nor the mRNA were regulated between the implantation zones. Thus, based on previous evidence (Maccarrone *et al*., 2004; Hess *et al*., 2007; Sherwin *et al*., 2007), it is postulated that the blastocyst might be regulating FAAH activity at the sites of implantation.

Taking these results together with those obtained by other authors, it can be speculated that AEA production described here and previously by Paria *et al*. (1996), NAPE-PLD (Guo *et al*., 2005) and FAAH (Wang *et al*., 2006a) might contribute to create an AEA gradient in the uterus during early pregnancy that allows blastocyst implantation. Also, it is important to consider that as the blastocyst expresses both NAPE-PLD (Guo *et al*., 2005) and FAAH proteins (Wang *et al*., 2006a), it could be modulating the concentration of AEA that impacts on the uterus before, during and after the implantation process. Two recent studies have come to similar conclusions on the complex embryonic factors operating during the invasive stages of implantation, i.e. the implantation window (for details see Edwards, 2007).

This study used ovariectomy-induced delayed implantation and pseudopregnancy models as tools to understand the relative roles played by the embryo and ovarian steroid hormones in modulating the synthesis of AEA in the pregnant rat uterus. In rodents, pre-receptive, receptive and non-receptive phases of the uterus during pregnancy or pseudopregnancy are sequentially programmed by ovarian progesterone and 17β-oestradiol. In the pseudopregnancy model, where the blastocyst is absent, uterine AEA production on day 6 did not decrease compared with days 4 and 5, as occurred during normal pregnancy. Mouse *Nape-pld* mRNA concentration and activity are higher in the nonreceptive uterus on day 6 compared with the concentrations observed in the receptive uterus on day 4 of pseudopregnancy (Guo *et al*., 2005). However, this study did not detect differences in NAPE-PLD expression under its experimental conditions. When FAAH expression was studied, its messenger increased on days 5 and 6 of pseudopregnancy, but this difference could not be detected at the protein level. These results suggest that the presence of the blastocyst is necessary to modulate NAPE-PLD and FAAH expression, thus being coincident with these findings on AEA-synthesizing capacity. The association between AEA production and receptive/nonreceptive uterine phases emphasizes the notion that it could be locally regulated in the uterus by implanting blastocysts. Thus, the synthesis of AEA was further examined in a physiologically relevant delayed implantation model. In the delayed implanting uterus, blastocysts undergo dormancy and fail to implant. However, this condition is terminated by an oestrogen injection with activation of dormant blastocysts, attainment of uterine receptivity and implantation (Parr and Parr, 1989; Xiao *et al*., 2002). Reactivation of blastocysts by 17β-oestradiol significantly down-regulated the synthesis of

AEA with the initiation of implantation. On the other hand, progesterone treatment maintained high AEA production compared with 17β-oestradiol treated animals. This finding is consistent with results obtained during normal implantation on day 6 of pregnancy and is in accordance with results in the rat and with the observation that NAPE-PLD expression and activity in mice are decreased in uterine regions close to the blastocyst (Guo *et al*., 2005). Xiao *et al*. (2002) reported that the expression of FAAH mRNA in the rat uterus was significantly stimulated in the delayed implantation model treated with 17β-oestradiol. Coincident with observations during pregnancy, FAAH expression was not modulated in this model, reinforcing the notion that the blastocyst might regulate FAAH activity in order to control AEA concentration at the implantation sites (Maccarrone *et al*., 2004; Hess *et al*., 2007; Sherwin *et al*., 2007). Based on these results, it was postulated that the blastocyst was mediating the effect of ovarian steroid hormones on uterine AEA production. The fact that the administration of 17β-oestradiol or RU-486 to pseudopregnant rats, a model in which embryos are absent, exerted different effects on AEA production compared with the model in which the blastocysts are present, support this notion. These results together with the ones obtained in the pseudopregnancy and in the delayed implantation models strongly support this idea, as it is thought that 17β-oestradiol-activated blastocysts down-regulate the production of AEA. As previously mentioned, Maccarrone *et al*. (2004) reported that mice blastocysts rapidly release a soluble compound that increases FAAH activity present in mice uteri. Overall, it could be suggested that activated blastocysts may protect them against the noxious effects of uterine AEA by locally inhibiting AEA produced by different synthetic pathways and increasing FAAH activity. Also, Sherwin *et al*. (2007) stressed the importance of paracrine signals from the preimplantation embryo in influencing the formation of a receptive endometrium. Embryo-derived signals clearly rescue the corpus luteum and regulate the endometrium during implantation. Also, Hess *et al*. (2007) observed a significant induction of pro-inflammatory cytokines and chemokines, and of angiogenic/static factors in decidualized endometrial stromal cells in response to products secreted by trophoblasts. These observations together with these results strongly suggest that steroids do not have sole control of endometrial receptivity.

In summary, the present work shows that the effect of maternal 17β-oestradiol and progesterone on uterine AEA production depends on the rat's reproductive state and on the activation of the blastocysts. In this sense, 17β-oestradiol and progesterone, as signals that emanate from the mother, might operate in conjunction with the blastocyst intrinsic programme regulating crucial events in early gestation and successful pregnancies. Embryonic and/or uterine FAAH play roles in creating optimal concentrations of AEA beneficial to implantation (Maccarrone *et al*., 2004; Hess *et al*., 2007; Sherwin *et al*., 2007). However, aberrant AEA synthesis may cause implantation failure or defective implantation. This is clinically relevant, since high peripheral AEA concentrations due to low FAAH activity are associated with spontaneous pregnancy loss in women (Maccarrone *et al*., 2000b). Understanding these mechanisms promises to help alleviate infertility, enhance fetal health and improve contraceptive design because that would open new perspectives in clinical applications.

Acknowledgements

This work was supported by PICT 10901 and PIP 6127 to Dr Ana Franchi and PICT 34784 to Dr María L Ribeiro. The authors would like to thank Mrs Ramona Morales and Mrs Ana Inés Casella for their excellent technical support. They also thank Mr Ricardo Horacio Orzuza (Animal Facility Technician, School of Odontology, University of Buenos Aires) for providing us with the prepuberal rats, Syntex SA (Buenos Aires, Argentina) for kindly providing PMSG (Novormon®) and Dr Benjamin Cravatt for kindly providing the anti-FAAH antibody.

References

Bradford M 1976 A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Burton GJ, Jauniaux E 2004 Placental oxidative stress: from miscarriage to preeclampsia. *Journal of the Society for Gynecologic Investigation* **11**, 342–352.

Cravatt BF, Giang DK, Mayfield SP *et al*. 1996 Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* **384**, 83–87.

Deb K, Chaturvedi MM, Jaiswal YK 2004 A 'minimum dose' of lipopolysaccharide required for implantation failure: assessment of its effect on the maternal reproductive organs and interleukin-1alpha expression in the mouse. *Reproduction* **128**, 87–97.

Devane WA, Axelrod J 1994 Enzymatic synthesis of anandamide, the endogenous ligand for the cannabinoid receptor, by brain membranes. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 6698–6701.

Devane WA, Hanus L, Breuer A *et al*. 1992 Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* **258**, 1946–1949.

Dey SK, Lim H, Das SK *et al*. 2004 Molecular cues to implantation. *Endocrine Reviews* **25**, 341–373.

Edwards RG 2007 Chorionic gonadotrophin, genes and embryonic signals regulating the implantation window. *Reproductive BioMedicine Online* **14**, 538–539.

Farina M, Ribeiro ML, Weissmann C *et al*. 2004 Biosynthesis and catabolism of prostaglandin F2 (alpha) (PGF2α) are controlled by progesterone in the rat uterus during pregnancy. *Journal of Steroid Biochemistry and Molecular Biology* **91**, 211–218.

Fernández-Solari J, Scorticati C, Mohn C *et al*. 2004 Alcohol inhibits luteinizing hormone-releasing hormone release by activating the endocannabinoid system. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 3264–3268.

Franchi AM, Gimeno AL, Gimeno MA 1989a Influences of estradiol and of catechol and non-catechol estrogens on the output of prostaglandins in uteri from spayed rats. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **36**, 25–29.

Franchi AM, Gimeno MA, Gimeno al. 1989b Factors subserving the enhancing effect of progesterone on the output of prostaglandin F2 alpha in uteri from ovariectomized rats. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **35**, 113–118.

Guo Y, Wang H, Okamoto Y *et al*. 2005 *N*-acylphophatidylethanolamine-hydrolyzing phospholipase D is an important determinant of uterine anandamide levels during implantation. *Journal of Biological Chemistry* **280**, 23429–23432.

Hess AP, Hamilton AE, Talbi S *et al*. 2007 Decidual stromal cell response to paracrine signals from the trophoblast: amplification of immune and angiogenic modulators. *Biology of Reproduction* **76**, 102–117.

Klinger FG, Battista N, De Felici M *et al*. 2006 Stage-variations of anandamide hydrolase activity in the mouse uterus during the natural oestrous cycle. *Journal of Experimental and Clinical Assisted Reproduction* **3**, 3–6.

Lim H, Paria BC, Das SK *et al.* 1997 Multiple female reproductive failures in cyclooxygenase-2 deficient mice. *Cell* **91**, 197–208.

Liu J, Wang L, Harvey-White J *et al.* 2006 A biosynthetic pathway for anandamide. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 13345–13350.

Maccarrone M, DeFelici M, Klinger FG *et al.* 2004 Mouse blastocysts release a lipid which activates anandamide hydrolase in intact uterus. *Molecular Human Reproduction* **10**, 215–221.

Maccarrone M, De Felici M, Bari M *et al.* 2000a Downregulation of anandamide hydrolase in mouse uterus by sex hormones. *European Journal of Biochemistry* **267**, 2991–2997.

Maccarrone M, Valenise H, Bari M *et al.* 2000b Relation between decreased anandamide hydrolase concentrations in human lymphocytes and miscarriages. *Lancet* **355**, 1326–1329.

Paria BC, Zhao X, Wang J *et al.* 1999 Fatty-acid amide hydrolase is expressed in the mouse uterus and embryo during the periimplantation period. *Biology of Reproduction* **60**, 1151–1157.

Paria BC, Deutsch DD, Dey SK 1996 The uterus is a potential site for anandamide synthesis and hydrolysis: differential profiles of anandamide synthase and hydrolase activities in the mouse uterus during the preimplantation period. *Molecular Reproduction and Development* **45**, 183–192.

Parr MB, Parr E 1989 *The Implantation Reaction*. Plenum Medical Book Company, New York, pp. 233–277.

Schmid HH 2000 Pathways and mechanisms of *N*-acylethanolamine biosynthesis: can anandamide be generated selectively? *Chemistry and Physics of Lipids* **108**, 71–87.

Schmid PC, Paria BC, Krebsbach RJ *et al.* 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.

Scorticati C, Fernández-Solari J, De Laurentiis A *et al.* 2004 The inhibitory effect of anandamide on luteinizing hormone-releasing hormone secretion is reversed by estrogen. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 11891–11896.

Sherwin JR, Sharkey AM, Cameo P *et al.* 2007 Identification of novel genes regulated by chorionic gonadotrophin in baboon endometrium during the window of implantation. *Endocrinology* **148**, 618–626.

Ueda N, Okamoto Y, Morishita J 2005 *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D: a novel enzyme of the β-lactamase fold family releasing anandamide and other *N*-acylethanolamines. *Basic Life Sciences* **77**, 1750–1758.

Vogel Z, Barg J, Levy R *et al.* 1993 Anandamide, a brain endogenous compound, interacts specifically with cannabinoid receptors and inhibits adenylate cyclase. *Journal of Neurochemistry* **61**, 352–355.

Wang L, Menon KMJ 2005 Regulation of luteinizing hormone/chorionic gonadotropin receptor messenger ribonucleic acid expression in the rat ovary: relationship to cholesterol metabolism. *Endocrinology* **146**, 423–431.

Wang H, Xie H, Guo Y *et al.* 2006a Fatty acid amide hydrolase deficiency limits early pregnancy events. *Journal of Clinical Investigation* **116**, 2123–2131.

Wang H, Dey SK, Maccarrone M 2006b Jekyll and Hyde: two faces of cannabinoid signaling in male and female fertility. *Endocrine Reviews* **27**, 427–448.

Xiao AZ, Zhao YG, Duan EK 2002 Expression and regulation of the fatty acid amide hydrolase gene in the rat uterus during the estrous cycle and peri-implantation period. *Molecular Human Reproduction* **8**, 651–658.

Declaration: The authors report no financial or commercial conflicts of interest.

Received 19 September 2007; refereed 23 October 2007; revised received 30 May 2008; accepted 29 August 2008.