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Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation

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Running title: Cannabinoid ligands and receptors in implantation

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Summary

The mechanisms by which synchronized embryonic development to the blastocyst stage, preparation of the uterus to the receptive state and reciprocal embryo-uterine interactions for implantation are coordinated are still unclear. We show here that preimplantation embryo development becomes asynchronous in mice deficient in cannabinoid receptor genes, brain-type (*CB1*) and/or spleen-type (*CB2*). Furthermore, while the levels of uterine anandamide (endocannabinoid) and blastocyst CB1 are coordinately downregulated with the onset of uterine receptivity and blastocyst activation prior to implantation, these levels remain high in the non-receptive uterus and dormant blastocysts during delayed implantation, and in pregnant leukemia inhibitory factor (*LIF*) deficient mice with implantation failure. The results suggest that a tight regulation of endocannabinoid signaling is important for synchronizing embryo development with uterine receptivity for implantation. Indeed, this is consistent with our finding that while experimentally induced sustained level of an exogenously administered natural cannabinoid inhibits implantation in wild-type mice, it fails to do so in *CB1*$^{-/-}$/*CB2*$^{-/-}$ double mutant mice. The present study is clinically important because of the widely debated medicinal use of cannabinoids and their reported adverse effects on pregnancy.

Introduction

Previous investigation suggested adverse effects of cannabinoid exposure to pregnancy outcome (reviewed in 1-5). Cannabinomimetic drugs interact with two types of cannabinoid receptors, brain-type (CB1) and spleen-type (CB2) (6-9). These receptor subtypes are negatively coupled to adenylate cyclase, N-type and P/Q-type calcium channels, and positively coupled to

mitogen activated protein (MAP) kinase and A-type potassium channels through $G_{i/o}$ proteins (7,10). CB1 is expressed in brain and other peripheral tissues (7-11). CB2 is expressed primarily in immune tissues including spleen, leukocytes and tonsils (12). We have previously shown that CB1 is expressed in the preimplantation mouse embryo at much higher levels than in the brain (1,2). The discovery of cannabinoid receptors led to the identification of endogenous cannabinoid ligands, *N*-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) (13-17). We observed that anandamide is synthesized in the pregnant mouse oviduct and uterus (18) and that its levels are far higher in the uterus than in any other mammalian tissue examined (3). However, anandamide levels are significantly lower at implantation sites than at interimplantation sites, suggesting endocannabinoid ligand-receptor signaling during implantation (3). Indeed, anandamide at higher levels adversely affects embryo development and implantation, while at lower levels it stimulates embryo growth and differentiation via CB1 (1-5). Interestingly, 2-AG is present in mouse uterus at amounts similar to or lower than the lowest anandamide levels (1-5 nmol/g tissue) and its level does not vary significantly between implantation and interimplantation sites (unpublished results). Using cannabinoid receptor mutant mice (19) and physiological approaches, we further defined here the role of ligand-receptor signaling with cannabinoids in preimplantation embryo development and implantation.

EXPERIMENTAL PROCEDURES

Animals - Adult CD-1 (Charles River), *CB1* and *CB2* mutant mice on C57BL/6J background or *LIF* mutant mice on a mixed background were housed in the Animal Care Facility at the University of Kansas Medical Center. Story Landis (NINDS/NIH) kindly provided leukemia inhibitory factor (*LIF*) mutant mice that were originally generated by Philippe Brulet (Pasteur Institute, France). We

have characterized these mice previously (20). Heterozygous breeding was performed to generate littermate (+/+), (+/-) and (-/-) mice of specific mutation. Homozygous males and females with specific null mutations were crossed to generate homozygous embryos with respective mutations or double mutation. Females were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy (day 1=vaginal plug), respectively. To induce conditions of delayed implantation, mice were ovariectomized on the morning (0800-0900) of day 4 of pregnancy and maintained with daily injection (sc) of progesterone (P_4, 2 mg/mouse) from days 5-7. To activate dormant blastocysts and initiate implantation, P_4-primed delayed implanting pregnant mice were injected with estradiol-17β (E_2, 25 ng/mouse) (21).

Embryo culture - Two-cell embryos recovered on day 2 were cultured in 25 μl of Whitten's medium in groups of 5-10 under silicon oil in an atmosphere of 5% CO_2 in air for 72 h with or without anandamide (7.5 nM) (1,2). The control cultures contained the same concentration of the vehicle. The embryos were observed every 24 h to monitor their development. At termination of culture, the number of embryos forming blastocysts was recorded.

Anandamide binding - Autoradiographic ligand binding in blastocysts was performed as previously described (1,2). Blastocysts were incubated with 4.5 nM [^{3}H]anandamide (Sp. Act. 221 Ci/mmol, DuPont/NEN) in the presence or absence of 500-fold molar excess of unlabelled anandamide. After incubation, blastocysts were washed, fixed in 2% paraformaldehyde, cytospun onto slides, air dried and subjected to autoradiography using a liquid emulsion. Blastocysts were post-stained in hematoxylin. The autoradiographic signals were quantitated under darkfield using OPTIMA II

program with an image analysis system. Statistical analysis was performed using one-way ANOVA followed by Newman-Keuls test.

CB1 immunostaining - Blastocysts were cytospun onto slides, fixed in 2% formalin for 15 min, washed in PBS and incubated in blocking solution for 10 min. They were then incubated with an affinity-purified rabbit antipeptide antibody specific to CB1 (1 μg/ml) overnight at 4°C (2). Immunostaining was performed using a Zymed Histostain-SP kit (Zymed Laboratories, San Francisco, CA) (2). Blastocysts were counter-stained with hematoxylin. Red deposits indicate the sites of immunoreactive CB1 protein. Control blastocysts were incubated in parallel with the antibody preneutralized with excess antigenic peptide.

Analysis of anandamide and other N-acylethanolamines (NAEs) - Extraction, isolation and analysis of NAE were done essentially as previously described (3). Uteri were homogenized in chloroform/methanol (2:1) and the homogenate was partitioned against 2.5% aqueous NaCl. Internal standards (*N*-acyl-1,1,2,2-$^{2}H_4$-ethanolamines with chain lengths of 16:0, 17:0, 18:0, 18:1n-9, 18:2n-6, and 20:4n-6; d4 NAEs, 0.1 μg each) were added to the extract. The NAEs were isolated using solid phase silica extraction columns (Alltech). Neutral lipids were eluted with 4 ml of chloroform followed by elution of the NAEs with 4 ml of chloroform:methanol (98:2). The NAE fractions were taken to dryness and reacted with 50 μl of tert-butyldimethylchlorosilyl/imidazole reagent (Alltech) at 80°C for 1 h. The *tert*-butyldimethylchlorosilyl/imidazole (tBDMS) derivatives were extracted into 50 μl of hexane for analysis by gas chromatography-mass spectrophotometry (GC-MS) in the selected ion monitoring mode. Endogenous NAEs were quantitated by comparing the peak size of their (m-57) ions to those of the corresponding stable isotope-labeled internal standards (22). Anandamide accounted for over 90% of total NAEs.

Administration of drugs – (-)-Δ^9-tetrahydrocannabinol [(-)THC] or its inactive stereoisomer (+)THC was delivered at a constant rate via miniosmotic pumps (Alzet Corporation, Palo Alto, CA) to study their effects on implantation in wild-type and *CB1*$^{-/-}$/*CB2*$^{-/-}$ pregnant mice. Miniosmotic pumps (mean pumping rate of 0.52 µl/hour and fill volume 96 µl) containing either (-)THC or (+)THC were placed under the back skin of mice on day 2 (1000 h) of pregnancy and continued through day 5. These pumps released THC at a rate of 20 µg/h. Cytochrome P_{450} inhibitors, metyrapone and clotrimazole (50 mg/kg bd wt) were first injected intraperitoneally 2 h before the installation of pumps and injected twice daily till day 4 of pregnancy (4). On day 5 (1000 h), implantation sites were determined by the blue dye method (21). If implantation sites were absent, uterine horns were flushed with saline to recover unimplanted blastocysts. Mice without implantation sites or blastocysts were excluded from the experiments.

RESULTS

Embryo development is asynchronous in cannabinoid receptor deficient mice - Since synchronized embryo development and uterine preparation are important to successful implantation, we first compared *in vivo* developmental potential of cannabinoid receptor mutant embryos with wild-type embryos. We observed that the development of *CB1*$^{-/-}$, *CB2*$^{-/-}$ or *CB1*$^{-/-}$/*CB2*$^{-/-}$ double mutant embryos recovered from the oviduct on day 3 (Table 1) and from the uterus on day 4 (Table 2) of pregnancy was asynchronous. On the morning of day 3, about 84% of the wild-type embryos recovered were at the 8-cell stage. In contrast, only about 24% of *CB1*$^{-/-}$, 42% of *CB2*$^{-/-}$ and 50% of the *CB1*$^{-/-}$/*CB2*$^{-/-}$ double mutant embryos were at the 8-cell stage, resulting in an increased population of 2-cell, 4-cell and 6-cell embryos (Table 1). On day 4 morning, about 98% of the wild-

type embryos were blastocysts. However, only about 62% of *CB1*$^{-/-}$, 71% of *CB2*$^{-/-}$ or 61% of *CB1*$^{-/-}$/*CB2*$^{-/-}$ embryos were at the blastocyst stage; a considerable number of embryos was at the morula stage. However, these morulae were apparently viable and normal, since they rapidly developed to blastocysts in culture. We speculated that developmentally retarded embryos eventually form blastocysts and implant. Thus, we next compared the status of implantation in the wild-type and mutant mice on day 5 of pregnancy. Increased localized endometrial vascular permeability at the site of the blastocyst is one of the early markers of implantation (23). This can be monitored by an intravenous injection of a blue dye (Chicago blue B) solution which specifies implantation sites as distinct blue bands along the uterus (4,21,23). We observed that while all of the vaginal plug-positive wild-type or *CB2*$^{-/-}$ mice (n=7) showed an average of 9 or 7 implantation sites, respectively, 6 of 10 (60%) *CB1*$^{-/-}$ and 14/18 (78%) *CB1*$^{-/-}$/*CB2*$^{-/-}$ plug-positive mice showed an average of 7 and 9 implantation sites per mouse, respectively. These results suggest that the loss of CB1 had modest, if any, adverse effects on implantation. This is perhaps due to implantation of blastocysts eventually formed from the slowly growing embryos. Our next objective was to examine whether the mutant embryos are responsive to an endocannabinoid exposure *in vitro*.

Mutant embryos are resistant to anandamide - Two-cell wild-type or mutant embryos were cultured in the presence or absence of anandamide as previously described (1,2). As observed previously (1,2), most (>79%) of the 2-cell wild-type embryos failed to develop to the blastocyst stage in the presence of anandamide. In contrast, more than 80% of the *CB1*$^{-/-}$ or *CB1*$^{-/-}$/*CB2*$^{-/-}$ double mutant embryos developed to blastocysts in the presence of anandamide (Fig. 1). However, *in vitro* development of *CB2*$^{-/-}$ embryos, like wild-type embryos, was severely compromised in the presence of anandamide. These results provide genetic confirmation to our previous findings that CB1, but

not CB2, in wild-type blastocysts responds to cannabinoid agonists in a stage and dose-dependent manner *in vitro* (1-5), although these embryos express *CB2* mRNA (1). While the developmental response of *CB2*$^{-/-}$ embryos to anandamide is similar to wild-type embryos *in vitro* (1,2), early asynchronous development of *CB2*$^{-/-}$ embryos *in vivo*, like *CB1*$^{-/-}$ or *CB1*$^{-/-}$/*CB2*$^{-/-}$ embryos, is surprising. It is possible that the reproductive tract environment differentially modulates the development of mutant embryos in response to endocannabinoids *in vivo*, but not during their development *in vitro*. In this respect, the mouse uterus expresses the *CB1*, but not the *CB2* gene (24) and thus, uterine CB1 may influence embryonic development *in vivo* in the absence of embryonic CB2. Another possibility could be that uterine anandamide levels in mutant mice are different from those of the wild-type mice. However, uterine anandamide levels in *CB1*$^{-/-}$/*CB2*$^{-/-}$ double mutant mice are not significantly different from the wild-type uterine levels (unpublished results). Nonetheless, the present genetic evidence suggests that cannabinoid receptors have some role in synchronizing embryo development in the reproductive tract *in vivo*.

Anandamide levels are higher in the nonreceptive uterus - In the mouse, progesterone (P_4) and estrogen sequentially program the uterus into pre-receptive, receptive and non-receptive phases during pregnancy or pseudopregnancy (23,25). Blastocysts implant only in the receptive uterus. The P_4-primed uterus becomes receptive on day 4 when it is superimposed with preimplantation ovarian estrogen. Subsequently, the receptive uterus proceeds to the non-receptive phase and blastocysts can no longer implant (21,25). Similar uterine changes are experimentally produced during delayed implantation induced by ovariectomy before preimplantation ovarian estrogen secretion on day 4. Under this condition, blastocysts undergo dormancy and fail to implant. This condition is terminated by an injection of estrogen with activation of dormant blastocysts, attainment of the receptive uterus

and implantation (21,23,25). The mechanism by which estrogen initiates these events in the P_4-primed uterus is not clearly understood.

The mouse uterus is receptive on day 4, while it was considered to be nonreceptive on day 5 (21). We previously observed that not only are uterine anandamide levels higher on day 5 of pseudopregnancy, the levels are lower at implantation sites than at interimplantation sites in day 5 pregnant mice (3). Thus, the regulated levels of anandamide correlate with uterine receptivity and implantation. However, we have recently discovered that the mouse uterus on day 5 of pseudopregnancy is still receptive to implantation. For example, most (86%) of the day 5 pseudopregnant recipients receiving transfer of day 4 blastocysts showed implantation. In contrast, transferred blastocysts completely failed to implant in day 6 pseudopregnant recipient uteri (unpublished results). To examine whether uterine anandamide levels correlate with gradual progression of a receptive uterus to a nonreceptive state, we measured uterine anandamide levels on days 4-6 of pseudopregnancy. The results show that anandamide levels are lower on day 4, but increase gradually reaching maximum levels on day 6 (Fig. 2A). These results show that uterine non-receptivity results in higher anandamide levels that could be a cause of implantation failure. In fact, experimentally induced sustained and higher levels of exogenous cannabinoids postpone implantation via activation of CB1 (4). Higher levels of anandamide during the non-receptive phase could be due to lower levels of anandamide hydrolase activity in the uterus. Indeed, lower anandamide level at the implantation site is accompanied by higher anandamide hydrolase activity (18). A correlation between decreased anandamide hydrolase activity and increased pregnancy loss in women has recently been reported, suggesting an adverse effect of higher anandamide levels during pregnancy (26).

During delayed implantation, blastocysts undergo dormancy and implantation does not occur (21,23,25). We surmised that if the downregulation of uterine anandamide levels is important for the receptive state, then its levels should be higher in the P_4-treated delayed implanting uterus and lower in the estrogen-induced receptive uterus. Indeed, uterine anandamide levels were considerably higher in delayed implanting uterus than the estrogen induced receptive uterus (Fig. 2B). These results again suggest that uterine anandamide levels are critical to implantation.

Uterine anandamide levels are higher in LIF deficient mice - In the mouse, *leukemia inhibitory factor* (*LIF*) is expressed in a biphasic manner, first in endometrial glands on day 4 morning and then in stromal cells adjacent to blastocysts during the attachment reaction on day 4 night (20), suggesting its role in implantation. Indeed, mice deficient in LIF show implantation failure and blastocysts undergo dormancy (27). This is similar to delayed implantation produced by ovariectomy during normal pregnancy. However, *LIF*$^{-/-}$ blastocysts implant successfully after transfer into wild-type uteri (27), establishing LIF as an essential maternal factor for uterine preparation to implantation. We speculated that uterine anandamide levels should remain high in *LIF*$^{-/-}$ uteri as compared to wild-type uteri on day 4 of pregnancy. Indeed, uterine levels were significantly higher in *LIF*$^{-/-}$ mice than those in wild-type mice (Fig. 2C). Collectively, the above results suggest that regulated levels of anandamide are associated with various phases of uterine receptivity for implantation.

Cannabinoid receptors are higher in dormant blastocysts - Because an intimate interaction between the blastocyst and uterus is essential for implantation, we examined whether uterine anandamide levels at different phases of receptivity correlate with cannabinoid receptor expression in the

blastocyst. As previously reported (1,2), significant levels of [^{3}H]anandamide binding were observed in normal blastocysts collected at 0900 h on day 4 of pregnancy (Figs. 3Aa and 3B). This binding remarkably decreased in blastocysts recovered at 2000 h on day 4 just prior to the attachment reaction between the blastocyst and uterine luminal epithelium (Figs. 3Ac and 3B). These results suggest that downregulation of anandamide binding to blastocyst is important for the initiation of implantation. If this speculation is correct, then anandamide binding should remain high in dormant blastocysts during delayed implantation, but diminish with blastocyst activation. Indeed, dormant blastocysts exhibited an increased population of anandamide binding sites (Figs. 3Ae and 3B) which significantly diminished by 12 h after termination of the delay by an estrogen injection (Figs. 3Ag and 3B). CB1 immunoreactive protein paralleled anandamide binding in dormant (delayed) and activated blastocysts (Fig. 3C). As stated before, blastocysts fail to implant and undergo dormancy in *LIF*$^{-/-}$ mice (20,27). We observed that the levels of anandamide binding to dormant *LIF*$^{-/-}$ blastocysts were also higher as compared to active wild-type blastocysts (data not shown). Collectively, these results demonstrate that the decreased levels of cannabinoid receptors in active blastocysts just prior to implantation correlate with decreased levels of anandamide in the receptive uterus.

Cannabinoid receptor mutant mice are resistant to cannabinoid-induced implantation failure - We speculated that if a tight regulation of ligand-receptor signaling is important for implantation, maintaining a sustained level of exogenously administered cannabinoids should disrupt uterine receptivity for implantation in wild-type mice, but not in mutant mice. An active natural cannabinoid, (-)THC or its inactive stereoisomer (+)THC was delivered subcutaneously at a constant rate via miniosmotic pumps in wild-type or *CB1*$^{-/-}$/*CB2*$^{-/-}$ double mutant mice from days 2-

5 of pregnancy. To inhibit rapid systemic degradation of THC by cytochrome P_{450} enzymes, mice were injected twice daily with P_{450} inhibitors as previously described (4). The mouse uterus accumulates (-)THC when its infusion accompanies P_{450} inhibitors; the levels are below the limit of detection when (-)THC alone is infused (4). Mice were examined for implantation on day 5 by the blue dye method. As observed previously (4), (-)THC in the presence of P_{450} inhibitors inhibited implantation in wild-type mice and morphologically dormant-looking blastocysts were recovered from these mice. In contrast, similar treatments failed to inhibit implantation in *CB1*$^{-/-}$/*CB2*$^{-/-}$ double mutant mice (Table 3). Administration of (+)THC plus the P_{450} inhibitors or the inhibitors alone had no adverse effects on implantation in wild-type or double mutant mice. These results, indeed, demonstrate that sustained and higher levels of cannabinoids are inhibitory to implantation and this effect is mediated by cannabinoid receptors, since the double mutant, but not the wild-type, mice are resistant to this effect.

DISCUSSION

The mechanism(s) by which the development of preimplantation embryos to active blastocysts is synchronized with uterine receptivity for implantation is not clearly understood. Our present observations of asynchronous development of embryos deficient in cannabinoid receptors during the preimplantation period, and coordinated downregulation of both uterine anandamide levels and blastocyst cannabinoid receptors prior to implantation in wild-type mice suggest that ligand-receptor signaling with endocannabinoids locally helps in regulating the "window" of implantation. Although the Mendelian frequency of offspring resulting from heterozygous crossings of *CB1* null mice is skewed, resulting in a somewhat reduced number of homozygous offspring (19) and although the pregnancy rate in mutant mice resulting from

homozygous mating is somewhat lower (unpublished results), birth of viable *CB1*$^{-/-}$, *CB2*$^{-/-}$ or *CB1*$^{-/-}$/*CB2*$^{-/-}$ double mutant offspring suggests that the absence of embryonic and/or uterine cannabinoid receptors is not indispensable for embryonic development or implantation. However, the observed increased mortality of *CB1*$^{-/-}$ offspring perhaps could be due to inferior fetal development resulting from implantation of slowly developing embryos (19). The major phenotypes of *CB1*$^{-/-}$ or *CB1*$^{-/-}$/*CB2*$^{-/-}$ double mutant mice are their resistance to exogenous cannabinoid exposure with respect to embryo development and implantation *in vitro* and *in vivo*. This suggests that embryonic development and implantation are likely to be affected by aberrant levels of exogenous or endogenous cannabinoids in the uterus and/or aberrant embryonic expression of cannabinoid receptors during early pregnancy.

On day 5 of pseudopregnancy when the uterus is still receptive, *LIF* expression persists in uterine glands. In contrast, *LIF* expression is undetectable or extremely low in the non-receptive day 6 uterus (unpublished results). Moreover, estrogen is essential for the induction of uterine LIF in mice (20,28). This is consistent with the absence of *LIF* expression in the P_4-primed delayed implanting mouse uterus, but its rapid induction after an estrogen injection (20). Thus, the virtual absence of uterine LIF on day 6 and during delayed implantation correlates with higher uterine anandamide levels and implantation failure. However, we do not know whether the absence of LIF is the cause of higher uterine anandamide levels or whether the higher levels are the consequence of implantation failure in the absence of LIF.

The physiological significance of anandamide in the uterus and cannabinoid receptors in the blastocyst is still not fully understood. Although it is clear that higher uterine anandamide and

blastocyst cannabinoid receptor levels are detrimental to the implantation process, uterine anandamide and blastocyst cannabinoid receptors still persist, albeit at lower levels, at the time of implantation. This suggests that lower levels of anandamide and cannabinoid receptors are beneficial to implantation. This suggestion is consistent with our recent observation that while higher anandamide levels are detrimental to blastocyst outgrowth in culture, lower levels stimulate this event (5). Similar biphasic (inhibitory and stimulatory) effects of anandamide at high and low concentrations are evident for other neural and behavioral functions (29), although the definitive cause of these biphasic effects of anandamide is not yet clearly understood (29). More recently, a bi-directional regulation of airway responsiveness by endogenous cannabinoids has been documented (30). Nonetheless, it is envisioned that a biphasic paracrine signaling via anandamide and cannabinoid receptors influences the fate of the embryo-uterine interactions during implantation, and aberrant levels of uterine anandamide and/or embryonic cannabinoid receptors are likely to adversely affect embryonic development and implantation. This could be a "quality control" mechanism to prevent implantation of abnormal embryos resulting from exposure to aberrant levels of cannabinoids. In conclusion, the present study highlights the importance of the ligand-receptor signaling with cannabinoids in female fertility and places the embryo and/or the uterus as targets for this signaling.

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FIGURE LEGENDS

Fig. 1 Effects of anandamide on *in vitro* development of preimplantation embryos. Two-cell embryos were recovered from oviducts of wild-type, *CB1*$^{-/-}$, *CB2*$^{-/-}$ *or CB1*$^{-/-}$/*CB2*$^{-/-}$ double mutant mice mated with males of the same genotypes and were cultured in the presence or absence of anandamide for a period of 72 hours. Embryonic development was assessed every 24 h under a dissecting microscope. The numbers at the top of each bar indicate the number of blastocysts developed per total number of 2-cell embryos cultured.

Fig. 2 Levels of endogenous anandamide in the mouse uterus. **(A)** Uterine levels on days 4-6 of pseudopregnancy. Levels in day 4 uteri were significantly ($P<0.05$; n=4 or 5) lower than those on days 5 and 6 of pseudopregnancy**. (B)** Anandamide levels in delayed implanting and receptive uterus. Levels in P_4-primed delayed uterus were significantly ($P<0.05$) higher than in E_2 treated P_4-primed receptive uterus (n=6 or 7). **(C)** Levels in day 4 pregnant uteri of *LIF*$^{-/-}$ and *LIF*$^{+/+}$ mice. Levels in *LIF*$^{+/+}$ uteri were significantly ($P<0.05$) lower than in *LIF*$^{-/-}$ uteri (n=5 or 6). Statistical analysis was performed by ANOVA followed by Student's t test. Uterine anandamide assay for each experiment within the same group was run simultaneously. Variations in uterine anandamide levels among three groups are likely due to different experimental conditions and the use of mice of different genetic backgrounds. CD-1 mice were used in experiments A and B, while mice on a mixed background were used for experiments in C.

Figure 3 Cannabinoid receptors in the blastocyst. **(A)** Autoradiographic localization of [^{3}H] anandamide binding sites in the normal, dormant (delayed) and activated blastocysts. Autoradiographic signals appear as black grains in these bright-field photomicrographs of

blastocysts. (a) day 4 at 0900 h; (c) day 4 at 2000 h; (e) P_4-treated day 7 dormant (delayed); (g) P_4+E_2 treated day 7 activated. Corresponding non-specific binding is shown in the panels b, d, f and h. ICM, inner cell mass; Tr, trophectoderm. **(B)** Quantitation of anandamide binding to blastocysts. Non-specific bindings were subtracted from total bindings to determine specific bindings. Each experiment used 10-15 blastocysts and was repeated 3-4 times with similar results. Results are mean ± SEM ($^{*}P<0.05$). **(C)** Immunolocalization of CB1 protein in blastocysts. Reddish-brown deposits indicate the sites of immunoreactive CB1. Photomicrographs of three representative blastocysts for each experiment are shown. While intense immunostaining is observed in P_4-primed dormant blastocysts (upper panels), staining is remarkably lower in P_4 + E_2 treated activated blastocysts (lower panels). Blastocysts incubated with preneutralized antibodies showed no positive signals (data not shown). These experiments were repeated three times. ICM, inner cell mass; Tr, trophectoderm.

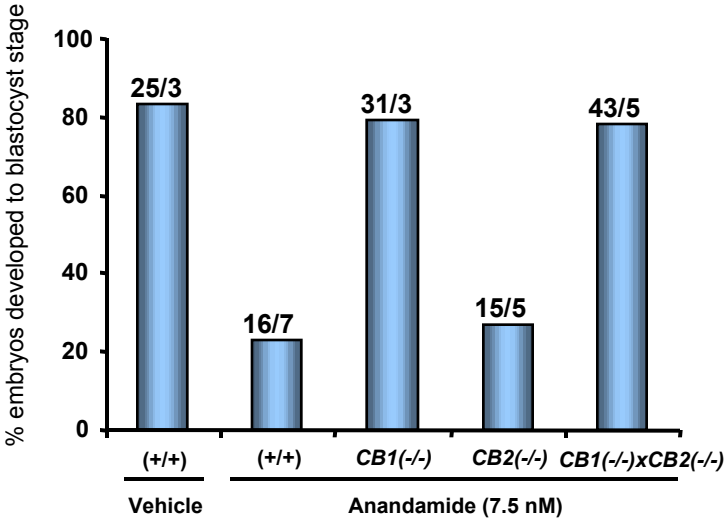
% embryos developed to blastocyst stage
100
80
60
40
20
0
25/3
16/7
31/3
15/5
43/5
(+/+)
(+/+)
CB1(-/-)
CB2(-/-)
CB1(-/-)xCB2(-/-)
Vehicle
Anandamide (7.5 nM)

Figure 1

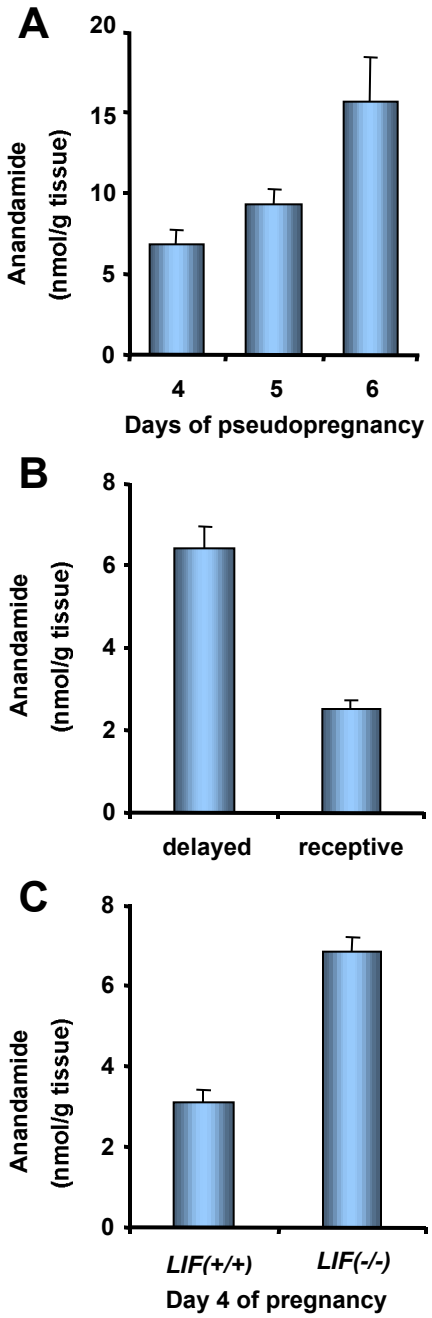
A
20
15
10
5
0
Anandamide
(nmol/g tissue)
4
5
6
Days of pseudopregnancy
B
8
6
4
2
0
Anandamide
(nmol/g tissue)
delayed
receptive
C
8
6
4
2
0
Anandamide
(nmol/g tissue)
LIF(+/+)
LIF(-/-)
Day 4 of pregnancy

Figure 2

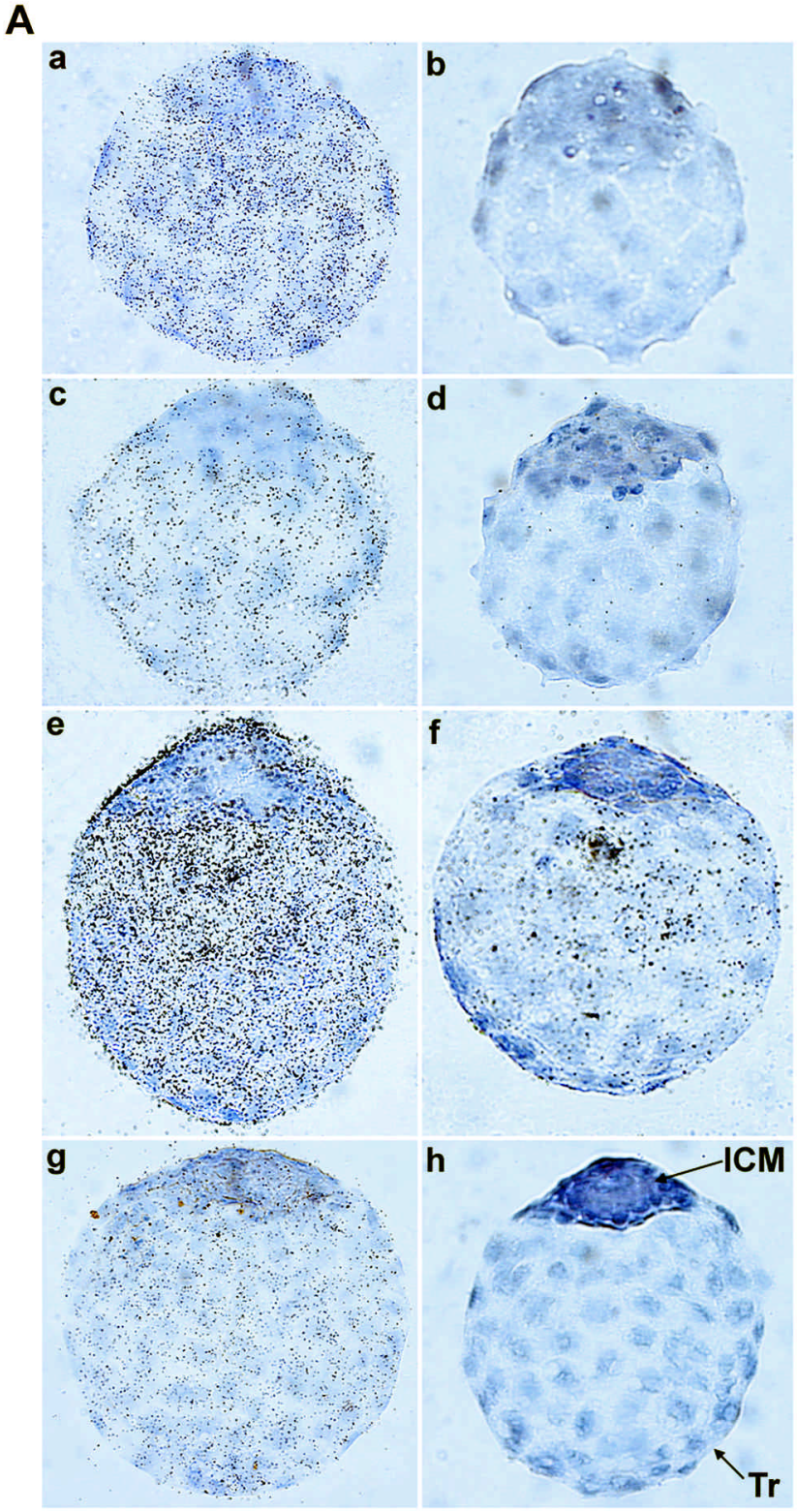

Figure 3A

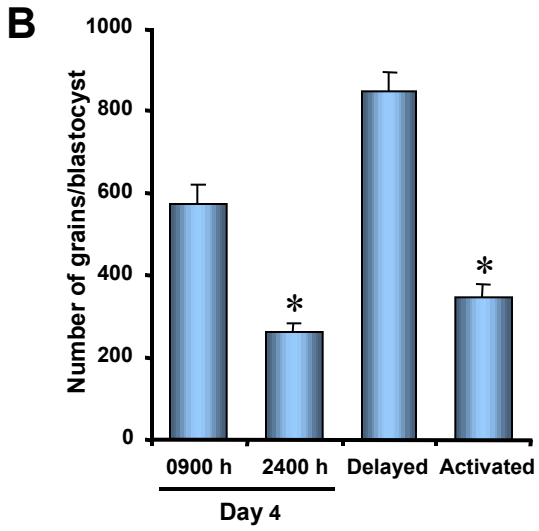

Figure 3B

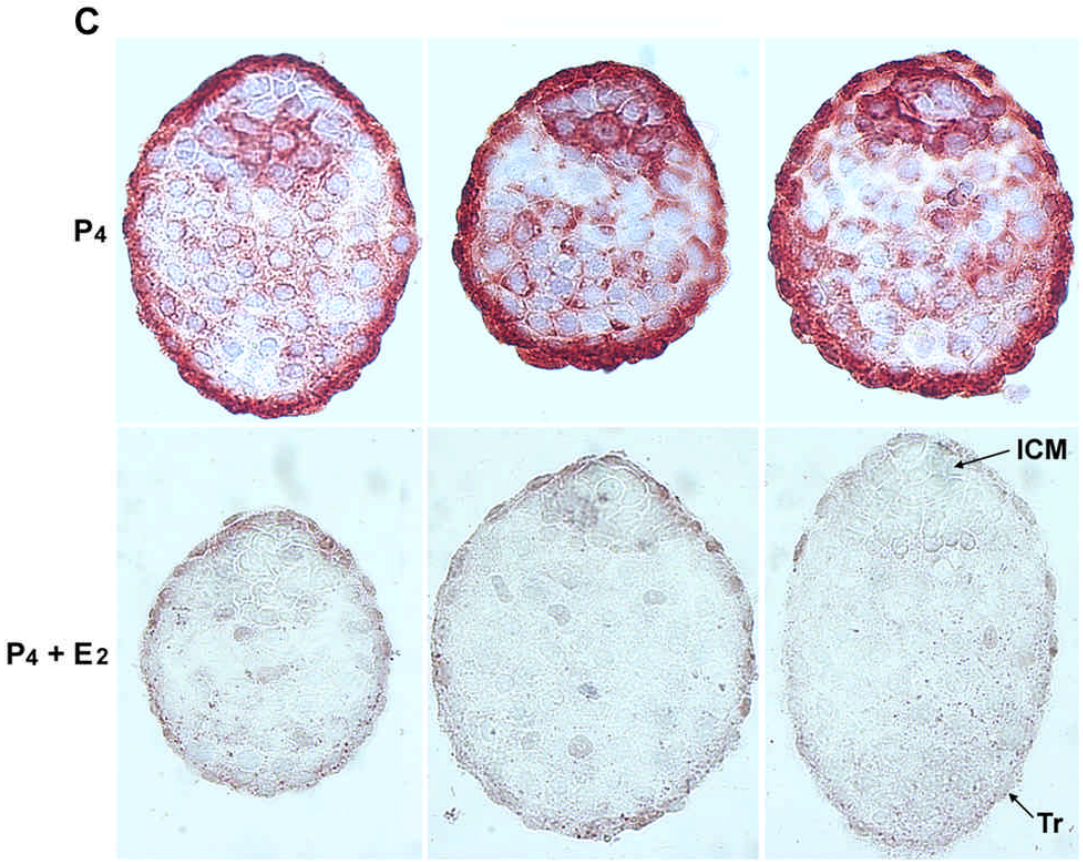

Figure 3C

Table 1. Developmental stages of embryos on day 3 of pregnancy

Genotypes	No. of mice with embryos	No. of total embryos	Stages of embryo development (%)			
			2-cell	4-cell	6-cell	8-cell
Wild-type	12	75	1 (1.3)	5 (6.7)	6 (8.0)	63 (84.0)
CB1(-/-)	9	62	1 (1.5)	34 (55.0)	12 (19.5)	15 (24.0)
CB2(-/-)	17	118	0 (0)	49 (41.5)	20 (17.0)	49 (41.5)
CB1(-/-)xCB2(-/-)	10	58	6 (10.5)	17 (29.0)	6 (10.5)	29 (50.0)

Embryos were recovered by flushing oviducts between 0900-1000 h on day 3 of pregnancy. Developmental stages were recorded under a stereo dissecting microscope. Mice without eggs (fertilized or unfertilized) were excluded from the experiments.

Table 2. Developmental stages of embryos on day 4 of pregnancy

Genotypes	No. of mice with embryos	No. of total embryos	Stages of embryo development (%)			
			4-cell	8-cell	M	B
Wild-type	7	42	0 (0)	0 (0)	1 (2.5)	41 (97.5)
CB1(-/-)	10	70	1 (1.5)	0 (0)	26 (37.0)	43 (61.5)
CB2(-/-)	11	70	1 (1.5)	1 (1.5)	18 (25.7)	50 (71.3)
CB1(-/-)xCB2(-/-)	13	97	1 (1)	5 (5)	32 (33.0)	59 (61.0)

Embryos were recovered by flushing uteri between 0900-1000 h on day 4 and their developmental stages were recorded. M, morula; B, blastocyst. Mice without eggs (fertilized or unfertilized) were excluded from the experiment

Table 3. Effects of infusion of (-)THC or (+) THC on implantation in wild-type and *CB1(-/-)*x*CB2(-/-)* double mutant mice

Genotype	Treatment	No. of mice	No. of mice with IS	No. of mice without IS	No. of IS	No. of blastocysts recovered*
Wild-type	(-)THC+Met+Clot	5	0	5	0	31
	(+)THC+Met+Clot	5	5	0	10.4±0.4	0
CB1(-/-)xCB2(-/-)	(-)THC+Met+Clot	6	5	1	7.4±0.7	4
	(+)THC+Met+Clot	5	5	0	9.8±0.5	0

(-)THC or (+)THC was administrated via miniosmotic pumps in the presence of cytochrome P_{450} inhibitors, metyrapone (Met) and clotrimazole (Clot) from days 2-5 of pregnancy. On day 5 (1000 h), implantation sites (IS) were determined by the blue dye method. Uteri without IS were flushed with saline to recover unimplanted blastocysts. Mice without IS or blastocysts were excluded from the experiments.
* Numbers indicate unimplanted blastocysts recovered from mice without implantation sites.