Cell Biochemistry and Function

WILEY

CELL BIOCHEMISTRY & FUNCTION

RESEARCH ARTICLE OPEN ACCESS

Characterizing the Role of Endocannabinoid Receptor Cnr1 in Mouse Ovarian Granulosa Cells

Jasmine Randhawa[1] | Ejimedo Madogwe[1] | Aire McCall[1] | Jaswinder Singh[2] | Raj Duggavathi[1]

[1]Department of Animal Science, McGill University, Sainte-Anne-de-Bellevue, Quebec, Canada | [2]Department of Plant Science, McGill University, Sainte-Anne-de-Bellevue, Quebec, Canada

Correspondence: Raj Duggavathi (raj.duggavathi@mcgill.ca)

Received: 10 October 2024 | **Revised:** 5 March 2025 | **Accepted:** 13 March 2025

Funding: This work was supported by the Natural Sciences and Engineering Research Council of Canada.

Keywords: Cnr1 | endocannabinoid system | granulosa cells | mouse | ovulation

ABSTRACT

The endocannabinoid receptors Cnr1 and Cnr2 have been found in reproductive organs such as the oviduct and uterus. These receptors bind to endocannabinoids, the arachinodoylethanolamine (AEA) and arachinodoylglycerol (2-AG), respectively. Both cannbinoid receptors have been investigated for their role in implantation and fertilization. However, not much is explored in terms of their role in ovarian granulosa cells. As these two receptors (especially Cnr1) have affinity towards the major component of Cannabis, tetrahydrocannabinol (THC), its usage raises concerns about the potential effects of THC on ovarian functions. Hence, it is important to characterize the role of endocannabinoid system in the ovarian granulosa cells. The objectives of this study were to use the mouse model to: (1) profile the expression pattern of the *Cnr1* and *Cnr2* and the endocannabinoid metabolizing enzymes (*Faah* and *Mgll*) in granulosa cells and (2) to determine the effect of the Cnr1 antagonist, AM251 on ovarian functions. We found that *Cnr1* transcript abundance was higher ($p < 0.05$) at 4 h hCG than 24 h and 48 h eCG timepoints, whereas *Cnr2* transcript decreased ($p < 0.05$) with follicular development. Conversely, *Faah* and *Mgll* transcripts were higher at 14 h hCG ($p < 0.05$) suggesting their upregulation after ovulation. The ovulation rate was lower in AM251 than vehicle-treated mice ($p < 0.05$), indicating that *Cnr1* signaling may regulate ovulation. Further investigating the effect of AM251, we found that it significantly downregulated *Ptgs2* and *Pappa* ($p < 0.05$). Overall, these data suggest that Cnr1, an important player in the endocannabinoid system, is important for ovulation.

1 | Introduction

The cannabinoid receptors, which are members of G protein-coupled receptors (GPCR) namely, Cnr1 which was first cloned from rat cerebral cortex cDNA library [1] and CNR2 was first cloned from human promyelocytic leukemia cell HL60 cDNA [2], led to the discovery of the endocannabinoid system. The endogenous ligand that binds to CNR1 with high affinity is N-arachidonoylethanolamine (AEA, a.k.a. Anandamide) investigated in procine brain and the one that binds to CNR2 is 2-arachidonoylglycerol according to a study in canine intestine (2-AG) [3, 4]. The endocannabinoids, AEA and 2-AG are degraded by enzymes fatty acid amide hydrolase (Faah) and monoglyceride lipase (Mgll), respectively [5, 6]. The receptors with endogenous ligands and their metabolizing enzymes constitute the endocannabinoid system, whose roles in physiology are being uncovered in different organ systems. For example, Cnr1 has been extensively studied investigating brain where it is known to have an inhibitory effect on adenylyl cyclase and the cAMP/PKA pathway (Reviewed in [7]). This receptor is also known to activate the MAPK/PI3K pathway leading to cell survival shown in human astrocytoma cells [8]. Another study in mice using a Cnr1 agonist (WIN 55, 212-22) in GT1-7, immortalized GnRH neurons showed that Cnr1 has an

Summary

- The Cnr1 and Cnr2 receptors are well-studied cannabinoid receptors in the brain.
- Their presence in ovaries hints at a regulatory role in ovarian functions.
- This study aimed to examine how the expression of these receptors and cannabinoid metabolizing enzymes changes during follicular development and the role of Cnr1 in ovulation using the mouse model.
- We found that Cnr1 is upregulated by the preovulatory hCG treatment in immature superovulated mice.
- Inhibition of Cnr1 reduced ovulation rate and downregulated key ovulatory genes. These data demonstrate that the cannabinoid receptor, Cnr1 plays a key role in ovulation in mice.

inhibitory effect on Gonadotropin-releasing hormone (GnRH) release in dose dose-dependent manner and this negative relationship was confirmed using an antagonist (AM251) [9]. This study observed the elongation of proestrous stage, which is one of the four stage of estrous cycle in mice, by 2 days in Cnr1 agonist (CP 55,940) treated group [9].

Several studies, based on the ubiquitous distribution of the cannabinoid receptors proposed that they have a spectrum of physiological roles in multiple organ systems including pain, energy metabolism, cardiovascular and reproductive functions [10]. While endocannabinoids and by extension cannabis have positive effects on physiology, cannabis use may not necessarily lead to positive health outcomes. For example, though the antiepileptic effects of cannabis are long known, there are case reports of increased seizure frequency after marijuana smoking [11]. Mouse studies have also shown that while AEA modulates the seizure threshold and severity [12], it increases seizures in mice lacking the *Faah* gene [13]. These observations together suggest dose-dependent effects of the endocannabinoids. Similarly, the use of cannabis may have positive or negative health outcomes in humans. Therefore, it is necessary to understand the tissue-specific roles of the endocannabinoid receptors.

The endocannabinoid receptors are expressed in female reproductive organs such as the fallopian tube and uterus, suggestive of their potential role in female fertility [14]. A *Cnr1* and *Cnr2* knockout mouse study reported embryo retention in the oviduct indicating their importance in embryo transfer to the uterus for implantation [15]. A human study revealed that plasma AEA levels increase during the follicular phase of the menstrual cycle peaking around ovulation, signifying its potential role in follicular development and ovulation [16]. As the major cannabis component, tetrahydrocannabinol (THC), is an exogenous ligand for Cnr1, multiple studies have posited the negative effects of THC on reproductive processes. One study demonstrated that THC treatment in vitro resulted in lower follicle-stimulating hormone (FSH)-induced steroidogenesis and luteinizing hormone/choriogonadotropin receptor (LHCGR) expression in rat granulosa cells [17]. A mouse study showed that THC administered orally resulted in reduced litter size [18]. These observations indicate that Cnr1/2 may play regulatory roles in the ovary, which have not been thoroughly explored.

Hence, in this study, using the mouse model, we aimed to (1) profile the expression pattern of the *Cnr1* and *Cnr2* and the endocannabinoid metabolizing enzymes (*Faah* and *Mgll*) in granulosa cells and (2) to determine the effect of the Cnr1 antagonist, AM251 on ovarian functions.

2 | Materials and Methods

2.1 | Animals

We used 3-weeks old C57BL/6 immature mice (10–12 g) bought from Charles River laboratories. They were kept in standard cages under 12 h light and dark cycles from 7:00 a.m. to 7:00 p.m. and were given feed ad libitum. The experiments were conducted following the Canadian Council of Animal Care guidelines and were approved by the Facility Animal Care Committee, McGill University (protocol number 2019-7550). The number of mice per treatment and timeline groups were based on previous studies involving immature superovulated mice [19–22].

2.2 | Superovulation

Immature mice were superovulated with equine chorionic gonadotropin (eCG; Folligon, Cat. 00806285, Intervet Canada; 5 IU/200 μL, ip) and human chorionic gonadotropic (hCG; Chorulon, CH-475-1, Intervet Canada; 5 IU/200 μL, ip) administered 48 h apart. First, ovaries were collected, for granulosa cell isolation, at different timepoints ($N = 3$–4 mice per timepoint) corresponding to different stages of follicle development, ovulation, and corpus luteum formation (Table 1). The ovulation process is completed by 12–14 h after hCG administration [21].

A second group of immature mice were superovulated as mentioned above but were treated with a specific Cnr1 antagonist, AM251 (5 μg/g, ip; Cat. S2819, Selleckchem), 30 min before hCG treatment. This drug has been shown to be efficient in inhibiting the Cnr1 receptor injected 30 min before other

TABLE 1 | Timepoints and their representative stages of folliculogenesis for granulosa cell collection in mice treated with the standard super-ovulation protocol (details in Methods).

Timepoints	Follicular development stages
0 h eCG	Small antral follicle
24 h eCG	Growing antral follicle
48 h eCG	Preovulatory follicle
4 h hCG	Ovulating follicle
14 h hCG	Corpus luteum

treatments [23] and effective for up to 24 h in zebrafish [24]. In the preliminary experiment, two different doses of AM251 drug (5 μg/g and 10 μg/g, ip) were tested. Since both these doses did not have significant difference in ovulation rate (data not shown), further experiments were performed using the lower dose. The control group mice were treated with DMSO (vehicle) 30 min before hCG treatment. Oviducts were collected at 18 h post-hCG to count the number of oocytes ovulated and observe cumulus-oocyte complex expansion between groups. The COC expansion was observed between the AM251 treated and vehicle group using the Nikon Eclipse TE2000-S microscope, and the images were captured with NIS software at 10× magnification and phase contrast setting. The image quantification was done using ImageJ 1.53e software.

A third group of mice were treated with AM251 as above, but ovaries were harvested at 4 h hCG ($N = 3$ per group) to collect granulosa cells from ovulating follicles. Ovaries were also harvested from three mice at 48 h post-eCG (0 h hCG) to collect granulosa cells from preovulatory follicles before hCG stimulation.

2.3 | Granulosa Cell Collection

Ovaries were collected in Eppendorf tubes containing 400 μL of 1× PBS on ice. These were then transferred to 35 × 10 mm petri dishes having 200 μL of 1× PBS and all the fat surrounding them was removed. The ovaries were then punctured with 27-gauge needles in new petri dishes with 200 μL 1× PBS. The exudates from follicles of both ovaries of each mouse were pooled and filtered through 40 μm sterile cell strainers (Cat. 352340, Fisher Scientific, Canada) separating cumulus oocyte complexes from granulosa cells [19]. The filtrate was then centrifuged at 7000 rpm for 10 min and the supernatant was discarded followed by RNA extraction from granulosa cells.

2.4 | RNA Purification and cDNA Synthesis

RNA from granulosa cells was purified using Direct-zol RNA Miniprep Kit (Cat. R2053, Zymo Research, VWR, Canada) as per manufacture's protocol. The quality and quantity of RNA was measured using Nanodrop 2000 (Thermoscientific). The cDNA was synthesized using iScript Advance cDNA Kit (Cat. 1725038, Bio-Rad, Canada) from 250 ng of RNA according to the manufacturer's protocol.

2.5 | Real Time qPCR

All the primers used (Table 2) were ordered from Integrated DNA Technologies (Iowa, USA) and they were validated for the efficiency between 90% and 110%, and R^2 from 0.98 to 1. The experiment was performed using manufacturer's protocol. The primer, *Pappa*, and *Ptgs2* were validated through amplicon sequencing because of low melt curve and was only included in data after verifying the presence of amplicon in the sample. The qPCR assays were performed according to MIQE guidelines [22]. For each qPCR reaction, Advanced qPCR Mastermix was used (Cat. 800-435-UL, Wisent, Canada) and the assay was performed according to the Wisent protocol. The following conditions were applied for qPCR assay: initial denaturation at 95°C for 2 min followed by 39 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s and final step at 95°C for 10 s. The readings were displayed on 384 CFX manager TM software (BioRad) and for the analysis of relative transcript

TABLE 2 | Primers used for real-time qPCR to quantify mRNA abundance in mouse granulosa cells.

Gene	Forward	Reverse
Areg	AGGGGACTACGACTACTCAG	GAAACTTGGCAGTGCATGGA
B2m	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCGGCTTCCCATTC
Cnr1	GTACATTCTCTGGAAGGCTCAC	CTGCACCTTGCCATCTTCT
Cnr2	GTTACCCGCCTACCTACAAAG	GAGCGGCAGGTAAGAAATCA
Cyp19A1	TGGAGAACAATTCGCCCTTTC	CCGAGGTGTCGGTGACTTC
Egr1	TCGGCTCCTTTCCTCACTCA	CTCATAGGGTTGTTCGCTCGG
Faah	TGAACGAGGGTGTGACATCG	TTCCACGGGTTCATGGTCTG
Mgll	AGGCGAACTCCACAGAATGTT	ACAAAAGAGGTACTGTCCGTCT
Pappa	CACAGGCAGAGCATCAGGAAG	TGCTTGCCATGAGGTAACCAG
Pgr	GGTGGAGGTCGTACAAGCAT	CTCATGGGTCACCTGGAGTT
Ptgs2	TGAGCAACTATTCCAAACCAGC	GCACGTAGTCTTCGATCACTATC
Ptx3	CCTGCGATCCTGCTTTGTG	GGTGGGATGAAGTCCATTGTC
Rp119	ATGAGTATGCTCAGGCTACAGA	GCATTGGCGATTTCATTGGTC
Scarb1	TTTGGAGTGGTAGTAAAAAGGGC	TGACATCAGGGACTCAGAGTAG
Sdha	GGAACACTCCAAAAACAGACCT	CCACCACTGGGTATTGAGTAGAA
Star	CAGAGGATTGGAAAAGACACGG	GGCATCTCCCCAAAATGTGTG
Tnfaip6	GGGATTCAAGAACGGGATCTTT	TCAAATTCACATACGGCCTTGG

abundance Starting Quantity values were taken and normalized against the three reference genes (succinate dehydrogenase complex, subunit A (*Sdha)*, ribosomal protein L19 *(Rpl19)* and beta-2-microglobulin *(B2m*) [22].

2.6 | Group Size

The size of the samples (*N*) varies among different objectives to fulfil the required number. The variation within an experiment occurs because of replicate being an outlier which could be due to varied mouse response to hormonal treatment. A number of 3 mice were decided for Figures 3 and 4 experiment because of minimum statistical requirement.

2.7 | Statistical Analysis

All data were analyzed using R package (4.0.3 version) and the level of significance was set to $p < 0.05$. Normality was tested using the Shapiro–Wilk test. The transcript abundance data and ovulation rates were analyzed by one-way ANOVA followed by the multiple comparison Tukey's test and Student's *t*-test respectively. Cumulus oocyte expansion quantification was analysed using Student's *t*-test.

3 | Results

3.1 | Transcript Abundance of Endocannabinoid Receptors and Degrading Enzymes in Granulosa Cells During Follicular Development Ovulation and CL Formation

Follicular development is a result of a tightly concerted gene expression program. Expression patterns of gene groups reveal their potential role in folliculogenesis and ovarian functions. Therefore, first, we sought to profile the transcript levels of the endocannabinoid system genes in granulosa cells collected at carefully selected timepoints representative of specific stages of follicular and corpous luteum (CL) development (Table 1). The expression patterns of the endocannabinoid receptors, *Cnr1* and *Cnr2*, and the endocannabinoid degrading enzymes, *Faah* and *Mgll*, were analyzed using qPCR analyses. There was a significant difference in mRNA abundance of *Cnr1*, *Cnr2*, *Faah* and *Mgll* transcripts at different timepoints analyzed. The mRNA level of the primary AEA and THC receptor, *Cnr1* was higher at 4 h post-hCG than 24 h and 48 h after eCG treatment ($p < 0.05$, $N = 3$, Figure 1). In contrast, mRNA levels of the 2-AG receptor, *Cnr2* were higher at 0 h eCG than all other timepoints ($p < 0.05$, $N = 4$, Figure 1). The transcript abundance of *Faah*, and *Mgll* were higher at 14 h hCG compared to all other timepoints ($p < 0.05$, $N = 3$ *(Faah)*, $N = 4$ *(Mgll)*, Figure 1).

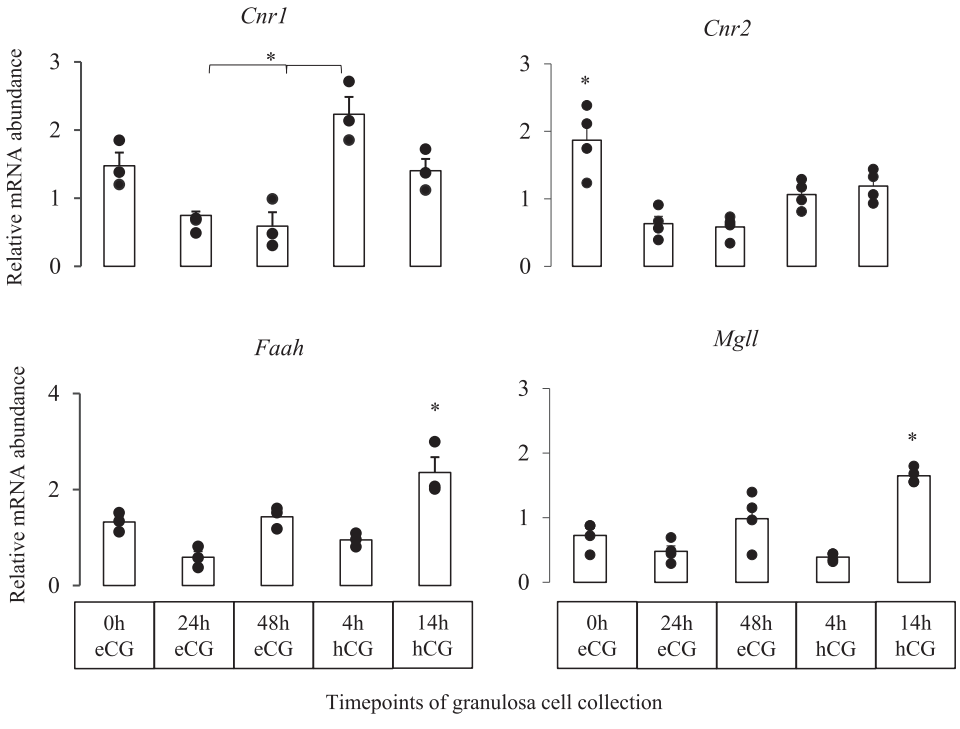

FIGURE 1 | Graphs of mRNA abundance of endocannabinoid receptors and degrading enzymes in granulosa cells collected at different timepoints. Quantitative-PCR was performed to profile the expression pattern of *Cnr1, Cnr2, Faah*, and *Mgll*. Data was normalized to reference genes *B2m, Rpl19* and *Sdha*. Data are represented as Mean ± SEM ($N = 3$–4 per time point). The bars are means and the points are individual data points (•). *Denotes $p < 0.05$.

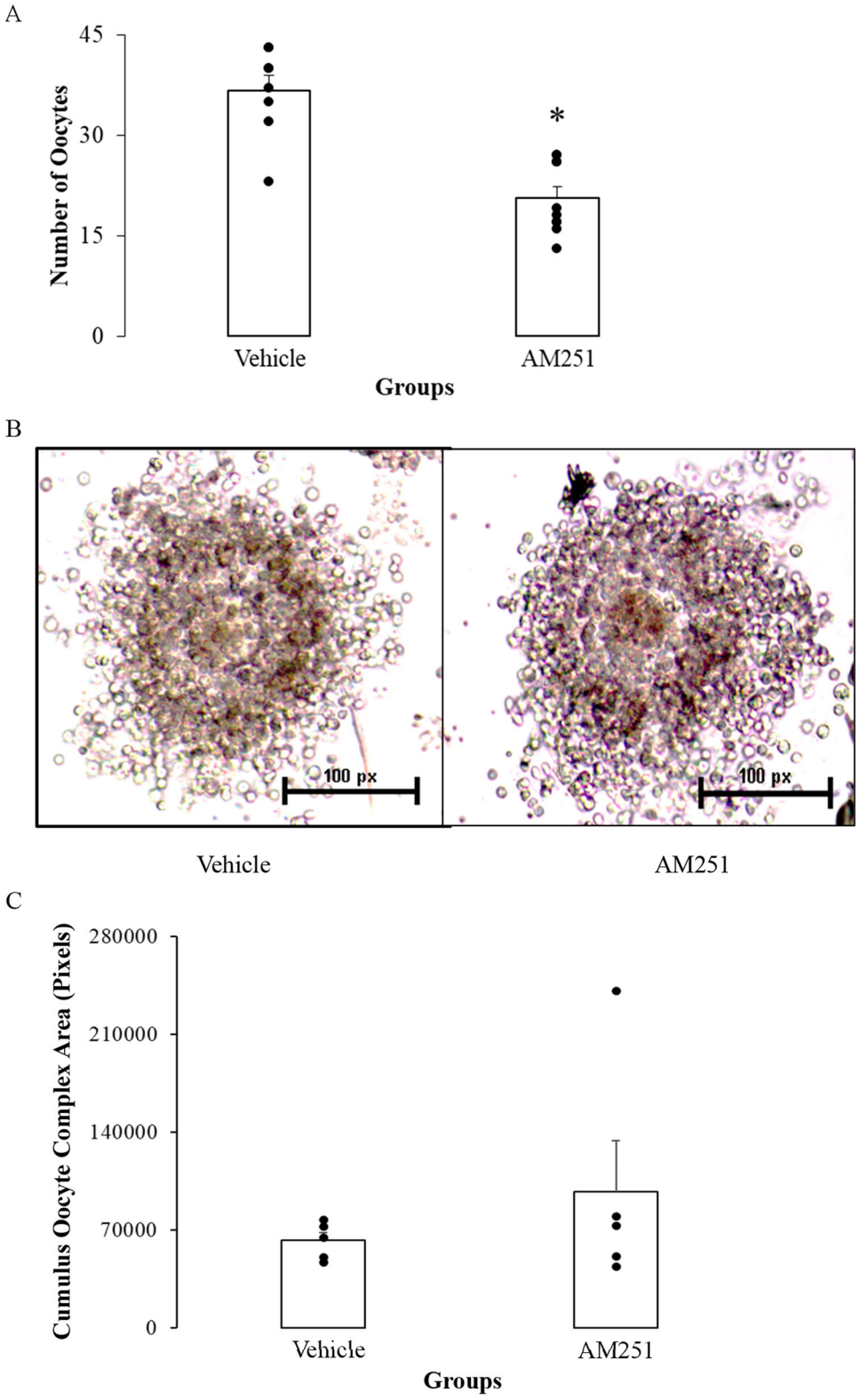

FIGURE 2 | (A) Graph showing the effect of Cnr1 antagonist (AM 251) on ovulation in mice post 18 h hCG. Data are represented as Mean ± SEM ($N = 8$-10). The bars are means and the points are individual data points (•). *Denotes $p < 0.0.5$. (B) Image showing the effect of Cnr1 antagonist (AM251) on cumulus oocyte complex (COC) in mice collected post 18 h hCG ($N = 5$). (C) Graph showing the effect of Cnr1 antagonist (AM251) on cumulus oocyte complex area post 18 h hCG. Data are represented as Mean ± SEM ($n = 5$). The bars are means and the points are individual data points (•).

3.2 | Effect of the Cnr1 Antagonist, AM251 on Ovulation

As there was a threefold increase in *Cnr1* expression in response to hCG stimulus, we hypothesized that Cnr1 signaling may be necessary for ovulation. We sought to test this hypothesis by inhibiting the Cnr1 signaling with a specific reverse agonist AM251 [25]. Intraperitoneal administration of AM251 (5 μg/g) had a significant effect on the hCG-stimulated ovulation in immature superovulated mice. The mean number of oocytes was 56.2% lower in AM251-treated than vehicle-treated mice ($p < 0.05$, $N = 8–10$, Figure 2A). However, among the ovulated oocytes, there was similar Cumulus oocyte expansion between the two groups of mice ($N = 5$, Figure 2B) hence the groups were not statistically significant ($N = 5$, Figure 2C).

3.3 | Inhibition of Cnr1 Decreases the mRNA Levels of *Ptgs2*, and *Pappa* Involved in Ovulation

With the observation that inhibition of Cnr1 signaling reduced ovulation rate, we pursued to uncover the disruption of the ovulatory gene expression program in ovulating follicles. We analyzed the expression of luteinizing hormone (LH) regulated genes involved in ovulation [22] in the vehicle and AM251-treated mice using qPCR analyses. The hCG-induced mRNA abundance of prostaglandin-endoperoxide synthase 2 (*Ptgs2)* and pappalysin 1 *(Pappa)* genes was lower at 4 h post-hCG in granulosa cells of AM251 compared to vehicle-treated mice ($p < 0.05$, $N = 3$, Figure 3). The transcript abundance of the luteinization marker genes, steroidogenic acute regulatory protein (*Star)*, pentraxin 3 *(Ptx3)*, and scavenger receptor class B *(Scarb)* were not different between vehicle and AM251-treated mice. The mRNA levels of the other hCG-regulated genes such as progesterone receptor *(Pgr)*, cytochrome P450 family 19 subfamily A member 1 *(Cyp19a1)*, early growth response 1 *(Egr1)*, TNF alpha-induced protein 6 *(Tnfaip6)* and amphiregulin *(Areg)* were similar between vehicle and AM251 treated mice groups ($N = 3$, Figure 4).

4 | Discussion

The expression pattern of endocannabinoid receptors and degrading enzymes during follicle development provided the first hint of the potential mechanism by which endocannabinoid signaling regulates ovarian functions. We found that *Cnr1* expression was induced during ovulation indicating that hCG reverses the *Cnr1* expression that was downregulated by eCG in granulosa cells. This is in accordance with a study conducted in rat and human ovarian samples where *Cnr1* expression was observed in granulosa cells of antral follicles post 48 h PMSG injections and Metaphse I stage, respectively and both of which corresponds to preovulatory stage [26, 27]. This LH-driven expression pattern is similar to other genes such as *Star*, *Ptgs2,* and *Pgr*, which are all known to play critical roles during ovulation [28, 29]. The observation that *Cnr2* transcript abundance decreased through follicle development and ovulation indicated that this receptor may not mediate the endocannabinoid signaling at later stages of follicle development and ovulation. We observed that the expression of *Faah* and *Mgll* increased after ovulation suggesting that endocannabinoid signaling may be actively reduced during the formation of the corpus luteum. These results are in accordance with a human study using the immunohistochemistry analysis of the ovary, which showed that CNR2 was more prominently expressed in early follicles and CNR1 during the late follicle stage along with FAAH expression (MGLL expression was not analyzed) in luteal cells [30]. Another human study also suggests CNR1, FAAH, and MGLL expression in granulosa cells [26]. However, these results are in contrast to a rat study, which shows that *Cnr2* and *Faah* expression are absent in granulosa cells and are present in luteal cells [27]. Our results suggest that Cnr1 may be involved during the preovulatory stage of follicle development. This inference is supported by a study that showed that plasma AEA levels were high at ovulation and were positively correlated with LH, FSH, and estradiol but not with progesterone in women [16]. These observations provided justification for the experiment testing the role of Cnr1 signaling during ovulation.

We observed that treatment with two doses of the Cnr1-specific antagonist AM251 had a negative effect on the ovulation rate but not cumulus oocyte complex (COC) expansion. These findings are comparable to another study in mice where a change in ovulation rate was shown despite no difference in

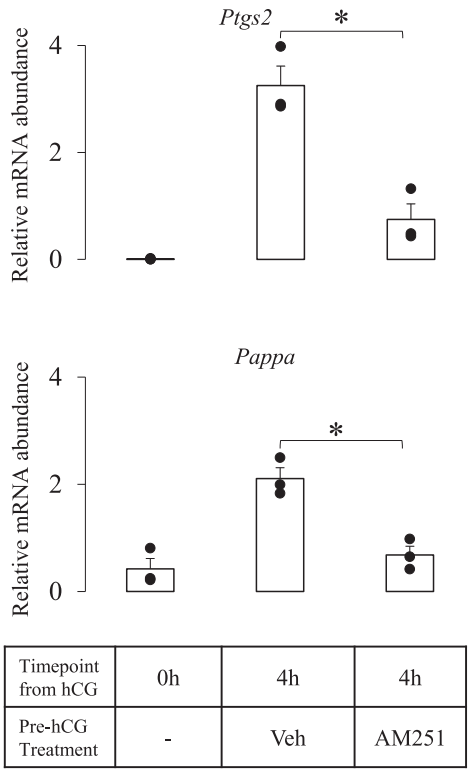

FIGURE 3 | Graphs of mRNA abundance of *Ptgs2* and *Pappa* in granulosa cells collected at 0 h (post 48 h eCG) and 4 h hCG timepoint from vehicle (veh) and AM251 treated mice groups. Quantitative-PCR was performed to profile the expression pattern of these genes. Data was normalized to reference genes *B2m, Rpl19,* and *Sdha*. Data are represented as Mean ± SEM ($N = 3$ per time point per group). The bars are means and the points are individual data points (●). *Denotes $p < 0.05$.

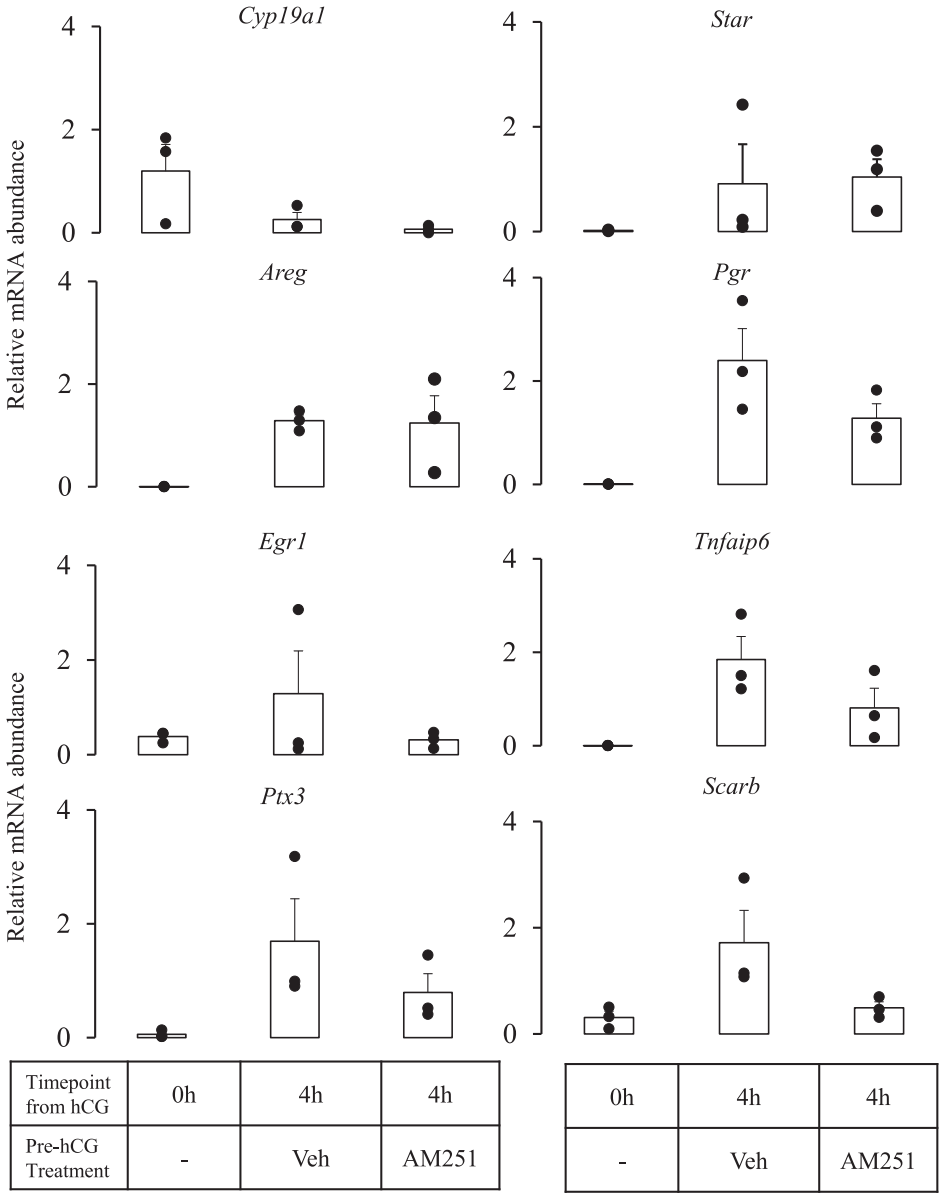

FIGURE 4 | Graphs of mRNA abundance of *Cyp19a1*, *Star, Areg, Pgr, Egr1, Tnfaip6, Ptx3*, and *Scarb* and in granulosa cells collected at 0 h (post 48 h eCG) and 4 h hCG timepoint from vehicle (veh) and AM251 treated mice groups. Quantitative-PCR was performed to profile the expression pattern of these genes. Data was normalized to reference genes *B2m, Rpl19*, and *Sdha*. Data are represented as Mean ± SEM ($N = 3$). The bars are means and the points are individual data points (•).

COC expansion between control and mitogen-activated protein kinase 1 (MAPK1/2 also known as ERK1/2) inhibitor [20]. This study showed that ERK1/2 levels are required post LH surge for ovulation whereas these maintained levels are not essential for COC expansion [20]. This implies that Cnr1 may be involved in oocyte release rather than COC expansion. It has been shown that Cnr1 regulate ERK1/2 pathway in the mouse hippocampus [31]. It indicates that ERK is a downstream molecule to Cnr1 signaling in the brain. Based on these studies we speculate that Cnr1 is involved in maintaining the ERK1/2 levels in ovarian granulosa cells post LH surge and regulates follicular rupture during ovulation. Taken together, this and the previous results demonstrate that Cnr1 signaling appears to be important for ovulation. These results also reinforce a previous study done in mice geminal vesicle staged oocytes surrounded by cumulus cells, which showed that oocyte resumption of meiosis was slower in response to the treatment with Cnr1 and Cnr2 antagonists, SR1 and SR2, respectively [32].

The process of ovulation involves changes in the structure of a follicle leading to follicle rupture and therefore release of oocyte. This consists of disruption in extracellular matrix within the cells. There are various mediators that act downstream to the LH signaling pathway which play a role in ovulation [33]. We showed here that inhibiting Cnr1 suppressed *Ptgs2* mRNA levels and decreased ovulation rate. One study investigated the administration of prostaglandin E2 (PGE2) in *Ptgs2*-knockout mice leading to the restoration of ovulation [34]. This implies that Ptgs2 is required for release of an oocyte as prostaglandin receptors are present in granulosa cells and are involved in

inflammatory processes during ovulation [35, 36]. Other studies on human macrophages showed that the use of AM251 had a negative effect on interleukin-1 β (IL-1β), which is a cytokine [37]. This study can be supported by another research investigation in inflammation-driven mice models where it was demonstrated that Cnr1 is required for leukocyte infiltration [38]. Taken together, Cnr1 could be involved in the inflammation process during follicle rupture at the time of ovulation. Our study also showed that the blockade of Cnr1 decreased the hCG-induced transcript abundance of *Pappa* in granulosa cells of ovulating follicles. A study in human ovarian follicular fluid revealed that PAPPA is involved in proteolysis of the insulin growth factor binding protein-4 (IGFBP4) [39, 40], increasing the bioavailability of the insulin-like growth factor 1 (IGF), which is known to potentiate the gonadotropin actions on follicles [41]. The observation of decreased ovulation rate and litter size in *Pappa*-knockout mice [42] strikes similarity with our findings of reduced *Pappa* expression and lower ovulation rate. Further, as many genes known to be LH-induced in granulosa cells of ovulating follicles such as *Star, Areg, Scarb* and others were not significantly affected by the AM251 treatment in our study. These observations indicate that Cnr1 signaling regulates some but not all of the gene expression programs necessary for ovulation in mice.

Although we did not further explore the signaling mechanisms by which Cnr1 pathway regulates ovulation, it is possible to speculate the potential mechanism based on what is currently known about the endocannabinoid signaling. The endocannabinoid receptors belong to Gi class of the G-protein coupled receptor (GPCR) family reducing the intracellular cAMP levels upon activation [1]. It is therefore plausible that Cnr1 plays a modulatory role in regulating cAMP concentration by exerting a negative effect on the adenylyl cyclase activity that is activated by the LH receptor, which is a Gs class of GPCR. This hypothesis is supported by the observation that treatment with forskolin, an adenylyl cyclase activator, results in lower number of ovulations in a dose-dependent manner compared to hCG treatment in mice [43]. But further studies would be required to thoroughly understand the role of the endocannabinoid signaling in LH-regulated pathways leading to ovulation. It would be interesting to see if granulosa-specific deletion of *Cnr1* results in reduced ovulation rate like our observation of lower ovulation rate in response to AM251 treatment.

Overall, the results of the present study show that *Cnr1* expression is upregulated by hCG. Pharmacological inhibition of this receptor reduced ovulation and downregulated the important ovulatory genes implying its role in ovulation. This study will also provide a basis to further explore the Cnr1 signaling pathway in ovaries and possible effects of cannabis and THC in the ovarian functions.

Author Contributions

J.R. contributed ideas, designed experiments, collected and processed samples, analyzed and interpreted data, prepared figures and wrote manuscript. E.M. took part in collecting samples and planning experiments. A.M. took part in AM251 ovulation experiment. J.S. contributed ideas, interpreted data, and reviewed the manuscript. R.D. conceived the study, designed experiments, analyzed data, and edited the manuscript.

Acknowledgments

The authors would like to thank Dr. Vilceu Bordignon for the use of their lab equipment. This study was supported by the National Science Research Council (NSERC) grant—R.D.; Graduate Excellence Award and GEFSES-CREATE Scholarship—J.R.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. L. A. Matsuda, S. J. Lolait, M. J. Brownstein, A. C. Young, and T. I. Bonner, "Structure of a Cannabinoid Receptor and Functional Expression of the Cloned cDNA," *Nature* 346, no. 6284 (1990): 561–564, https://doi.org/10.1038/346561a0.

2. S. Munro, K. L. Thomas, and M. Abu-Shaar, "Molecular Characterization of a Peripheral Receptor for Cannabinoids," *Nature* 365, no. 6441 (1993): 61–65, https://doi.org/10.1038/365061a0.

3. R. Mechoulam, S. Ben-Shabat, L. Hanus, et al., "Identification of an Endogenous 2-Monoglyceride, Present in Canine Gut, That Binds to Cannabinoid Receptors," *Biochemical Pharmacology* 50, no. 1 (1995): 83–90, https://doi.org/10.1016/0006-2952(95)00109-d.

4. T. Sugiura, S. Kondo, A. Sukagawa, et al., "2-Arachidonoylgylcerol: A Possible Endogenous Cannabinoid Receptor Ligand in Brain," *Biochemical and Biophysical Research Communications* 215, no. 1 (1995): 89–97, https://doi.org/10.1006/bbrc.1995.2437.

5. T. P. Dinh, D. Carpenter, F. M. Leslie, et al., "Brain Monoglyceride Lipase Participating in Endocannabinoid Inactivation," *Proceedings of the National Academy of Sciences* 99, no. 16 (2002): 10819–10824, https://doi.org/10.1073/pnas.152334899.

6. B. Basavarajappa, "Critical Enzymes Involved in Endocannabinoid Metabolism," *Protein & Peptide Letters* 14, no. 3 (2007): 237–246, https://doi.org/10.2174/092986607780090829.

7. B. D. Heifets and P. E. Castillo, "Endocannabinoid Signaling and Long-Term Synaptic Plasticity," *Annual Review of Physiology* 71, no. 1 (2009): 283–306, https://doi.org/10.1146/annurev.physiol.010908.163149.

8. I. Galve-Roperh, D. Rueda, T. Gómez del Pulgar, G. Velasco, and M. Guzmán, "Mechanism of Extracellular Signal-Regulated Kinase Activation by the CB1 Cannabinoid Receptor," *Molecular Pharmacology* 62, no. 6 (2002): 1385–1392, https://doi.org/10.1124/mol.62.6.1385.

9. C. M. Gammon, G. M. Freeman, W. Xie, S. L. Petersen, and W. C. Wetsel, "Regulation of Gonadotropin-Releasing Hormone Secretion by Cannabinoids," *Endocrinology* 146, no. 10 (2005): 4491–4499, https://doi.org/10.1210/en.2004-1672.

10. S. Zou and U. Kumar, "Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System," *International Journal of Molecular Sciences* 19, no. 3 (2018): 833, https://doi.org/10.3390/ijms19030833.

11. M. H. Keeler and C. B. Reifler, "Grand Mal Convulsions Subsequent to Marijuana Use. Case Report," *Diseases of the Nervous System* 28, no. 7 Pt 1 (1967): 474–475.

12. M. J. Wallace, B. R. Martin, and R. J. DeLorenzo, "Evidence for a Physiological Role of Endocannabinoids in the Modulation of Seizure

Threshold and Severity," *European Journal of Pharmacology* 452, no. 3 (2002): 295–301, https://doi.org/10.1016/s0014-2999(02)02331-2.

13. A. B. Clement, E. G. Hawkins, A. H. Lichtman, and B. F. Cravatt, "Increased Seizure Susceptibility and Proconvulsant Activity of Anandamide in Mice Lacking Fatty Acid Amide Hydrolase," *Journal of Neuroscience* 23, no. 9 (2003): 3916–3923, https://doi.org/10.1523/jneurosci.23-09-03916.2003.

14. M. G. Gervasi, M. Rapanelli, M. L. Ribeiro, et al., "The Endocannabinoid System in Bull Sperm and Bovine Oviductal Epithelium: Role of Anandamide in Sperm–Oviduct Interaction," *Reproduction* 137, no. 3 (2009): 403–414, https://doi.org/10.1530/rep-08-0204.

15. H. Wang, Y. Guo, D. Wang, et al., "Aberrant Cannabinoid Signaling Impairs Oviductal Transport of Embryos," *Nature Medicine* 10, no. 10 (2004): 1074–1080, https://doi.org/10.1038/nm1104.

16. N. Cui, L. Wang, W. Wang, et al., "The Correlation of Anandamide With Gonadotrophin and Sex Steroid Hormones During the Menstrual Cycle," *Iranian Journal of Basic Medical Sciences* 20, no. 11 (2017): 1268–1274, https://doi.org/10.22038/ijbms.2017.9488.

17. E. Y. Adashi, P. B. Jones, and A. J. Hsueh, "Direct Antigonadal Activity of Cannabinoids: Suppression of Rat Granulosa Cell Functions," *American Journal of Physiology-Endocrinology and Metabolism* 244, no. 2 (1983): E177–E185, https://doi.org/10.1152/ajpendo.1983.244.2.e177.

18. A. Kostellow, D. Ziegler, J. Kunar, G. Fujimoto, and G. Morrill, "Effect of Cannabinoids on Estrous Cycle, Ovulation and Reproductive Capacity of Female A/J Mice," *Pharmacology* 21, no. 1 (1980): 68–75, https://doi.org/10.1159/000137418.

19. H. Y. Fan, Z. Liu, P. F. Johnson, and J. S. Richards, "CCAAT/Enhancer-Binding Proteins (C/EBP)-α and -β Are Essential for Ovulation, Luteinization, and the Expression of Key Target Genes," *Molecular Endocrinology* 25, no. 2 (2011): 253–268, https://doi.org/10.1210/me.2010-0318.

20. E. Madogwe, Y. Schuermann, D. Siddappa, V. Bordignon, P. P. Roux, and R. Duggavathi, "Sustained ERK1/2 Signaling Is Necessary for Follicular Rupture During Ovulation in Mice," *Reproduction* 161, no. 2 (2021): 183–193, https://doi.org/10.1530/rep-20-0087.

21. J. S. Richards, D. L. Russell, S. Ochsner, and L. L. Espey, "Ovulation: New Dimensions and New Regulators of the Inflammatory-Like Response," *Annual Review of Physiology* 64, no. 1 (2002): 69–92, https://doi.org/10.1146/annurev.physiol.64.081501.131029.

22. D. Siddappa, É. Beaulieu, N. Gévry, P. P. Roux, V. Bordignon, and R. Duggavathi, "Effect of the Transient Pharmacological Inhibition of Mapk3/1 Pathway on Ovulation in Mice," *PLoS One* 10, no. 3 (2015): e0119387, https://doi.org/10.1371/journal.pone.0119387.

23. M. A. L. C. da Veiga, F. Fonseca Bloise, R. H. Costa-e-Sousa, et al., "Acute Effects of Endocannabinoid Anandamide and CB1 Receptor Antagonist, AM251 in the Regulation of Thyrotropin Secretion," *Journal of Endocrinology* 199, no. 2 (2008): 235–242, https://doi.org/10.1677/joe-08-0380.

24. L. S. Khara and D. W. Ali, "The Endocannabinoid System's Involvement in Motor Development Relies on Cannabinoid Receptors, TRP Channels, and Sonic Hedgehog Signaling," *Physiological Reports* 11, no. 1 (2023): e15565, https://doi.org/10.14814/phy2.15565.

25. J. Fiori, M. Sanghvi, M. O'Connell, S. Krzysik-Walker, R. Moaddel, and M. Bernier, "The Cannabinoid Receptor Inverse Agonist AM251 Regulates the Expression of the EGF Receptor and Its Ligands via Destabilization of Oestrogen-Related Receptor α Protein," *British Journal of Pharmacology* 164, no. 3 (2011): 1026–1040, https://doi.org/10.1111/j.1476-5381.2011.01384.x.

26. E. Agirregoitia, I. Ibarra-Lecue, L. Totorikaguena, et al., "Dynamics of Expression and Localization of the Cannabinoid System in Granulosa Cells During Oocyte Nuclear Maturation," *Fertility and Sterility* 104, no. 3 (2015): 753–760, https://doi.org/10.1016/j.fertnstert.2015.06.013.

27. P. Bagavandoss and S. Grimshaw, "Temporal and Spatial Distribution of the Cannabinoid Receptors (CB1, CB2) and Fatty Acid Amide Hydroxylase in the Rat Ovary," *Anatomical Record* 293, no. 8 (2010): 1425–1432, https://doi.org/10.1002/ar.21181.

28. J. P. Lydon, F. J. DeMayo, C. R. Funk, et al., "Mice Lacking Progesterone Receptor Exhibit Pleiotropic Reproductive Abnormalities," *Genes & Development* 9, no. 18 (1995): 2266–2278, https://doi.org/10.1101/gad.9.18.2266.

29. J. S. Richards, "Hormonal Control of Gene Expression in the Ovary," *Endocrine Reviews* 15, no. 6 (1994): 725–751, https://doi.org/10.1210/edrv-15-6-725.

30. M. R. El-Talatini, A. H. Taylor, J. C. Elson, L. Brown, A. C. Davidson, and J. C. Konje, "Localisation and Function of the Endocannabinoid System in the Human Ovary," *PLoS One* 4, no. 2 (2009): e4579, https://doi.org/10.1371/journal.pone.0004579.

31. P. Derkinderen, E. Valjent, M. Toutant, et al., "Regulation of Extracellular Signal-Regulated Kinase by Cannabinoids in Hippocampus," *Journal of Neuroscience* 23, no. 6 (2003): 2371–2382, https://doi.org/10.1523/jneurosci.23-06-02371.2003.

32. S. Cecconi, G. Rossi, S. Oddi, V. Di Nisio, and M. Maccarrone, "Role of Major Endocannabinoid-Binding Receptors During Mouse Oocyte Maturation," *International Journal of Molecular Sciences* 20, no. 12 (2019): 2866, https://doi.org/10.3390/ijms20122866.

33. D. M. Duffy, C. Ko, M. Jo, M. Brannstrom, and T. E. Curry, "Ovulation: Parallels With Inflammatory Processes," *Endocrine Reviews* 40, no. 2 (2018): 369–416, https://doi.org/10.1210/er.2018-00075.

34. B. J. Davis, D. E. Lennard, C. A. Lee, et al., "Anovulation in Cyclooxygenase-2-Deficient Mice Is Restored by Prostaglandin E2 and Interleukin-1β*," *Endocrinology* 140, no. 6 (1999): 2685–2695, https://doi.org/10.1210/endo.140.6.6715.

35. S. M. Harris, L. C. Aschenbach, S. M. Skinner, B. L. Dozier, and D. M. Duffy, "Prostaglandin E2 Receptors Are Differentially Expressed in Subpopulations of Granulosa Cells From Primate Periovulatory Follicles1," *Biology of Reproduction* 85, no. 5 (2011): 916–923, https://doi.org/10.1095/biolreprod.111.091306.

36. C. J. Park, P. C. Lin, S. Zhou, et al., "Progesterone Receptor Serves the Ovary as a Trigger of Ovulation and a Terminator of Inflammation," *Cell Reports* 31, no. 2 (2020): 107496, https://doi.org/10.1016/j.celrep.2020.03.060.

37. K. Sugamura, S. Sugiyama, T. Nozaki, et al., "Activated Endocannabinoid System in Coronary Artery Disease and Antiinflammatory Effects of Cannabinoid 1 Receptor Blockade on Macrophages," *Circulation* 119, no. 1 (2009): 28–36, https://doi.org/10.1161/circulationaha.108.811992.

38. S. Marquart, P. Zerr, A. Akhmetshina, et al., "Inactivation of the Cannabinoid Receptor CB1 Prevents Leukocyte Infiltration and Experimental Fibrosis," *Arthritis and Rheumatism* 62, no. 11 (2010): 3467–3476, https://doi.org/10.1002/art.27642.

39. E. Gaidamauskas, C. Gyrup, H. B. Boldt, et al., "IGF Dependent Modulation of IGF Binding Protein (IGFBP) Proteolysis by Pregnancy-Associated Plasma Protein-A (Papp-A): Multiple PAPP-A–IGFBP Interaction Sites," *Biochimica Et Biophysica Acta (BBA)—General Subjects* 1830, no. 3 (2013): 2701–2709, https://doi.org/10.1016/j.bbagen.2012.11.002.

40. C. A. Conover, C. Oxvig, M. T. Overgaard, M. Christiansen, and L. C. Giudice, "Evidence That the Insulin-Like Growth Factor Binding Protein-4 Protease in Human Ovarian Follicular Fluid Is Pregnancy Associated Plasma Protein-A," *Journal of Clinical Endocrinology and Metabolism* 84, no. 12 (1999): 4742–4745, https://doi.org/10.1210/jcem.84.12.6342.

41. P. Afradiasbagharani, E. Hosseini, A. Allahveisi, and M. Bazrafkan, "The Insulin-Like Growth Factor and Its Players: Their Functions, Significance, and Consequences In All Aspects of Ovarian Physiology,"

Middle East Fertility Society Journal 27, no. 1 (2022): 27, https://doi.org/10.1186/s43043-022-00119-1.

42. M. Nyegaard, M. T. Overgaard, Y. Q. Su, et al., "Lack of Functional Pregnancy-Associated Plasma Protein-A (PAPPA) Compromises Mouse Ovarian Steroidogenesis and Female Fertility1," *Biology of Reproduction* 82, no. 6 (2010): 1129–1138, https://doi.org/10.1095/biolreprod.109.079517.

43. K. F. Rodriguez, J. F. Couse, F. L. Jayes, et al., "Insufficient Luteinizing Hormone-Induced Intracellular Signaling Disrupts Ovulation in Preovulatory Follicles Lacking Estrogen Receptor-β," *Endocrinology* 151, no. 6 (2010): 2826–2834, https://doi.org/10.1210/en.2009-1446.