This is the peer reviewed version of the following article: Totorikaguena L, Olabarrieta E, Lolicato F, et al. *The endocannabinoid system modulates the ovarian physiology and its activation can improve in vitro oocyte maturation.* **J Cell Physiol**. 2020; 235: 7580–7591, which has been published in final form at https://doi.org/10.1002/jcp.29663. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions. This article may not be enhanced, enriched or otherwise transformed into a derivative work, without express permission from Wiley or by statutory rights under applicable legislation. Copyright notices must not be removed, obscured or modified. The article must be linked to Wiley's version of record on Wiley Online Library and any embedding, framing or otherwise making available the article or pages thereof by third parties from platforms, services and websites other than Wiley Online Library must be prohibited.

1 # The endocannabinoid system modulates the ovarian physiology and its activation
2 # can improve in vitro oocyte maturation

3 **Running title**: Cannabinoid system on folliculogenesis

4 Lide Totorikaguena[1], Estibaliz Olabarrieta[1], Francesca Lolicato[2], Jon Romero-
5 Aguirregomezcorta[1], Johan Smitz[2], Naiara Agirregoitia[1] and Ekaitz Agirregoitia[1]

6

7 [1]Department of Physiology, Faculty of Medicine and Nursing, UPV/EHU, Leioa, Bizkaia

8 [2]Laboratory of Follicular Biology (FOBI), UZ Brussel, Laarbeeklaan 101, 1090
9 Brussels, Belgium.

10

11 **Data availability statement**

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

14

15 **Correspondence**:

16 Ekaitz Agirregoitia, Dept. of Physiology, Faculty of Medicine and Nursing, UPV/EHU,
17 Leioa, Bizkaia. Email: e.agirregoitia@ehu.eus

18

19

20

Acknowledgments

The authors thank SGiker (UPV/EHU) for personal and technical support, particularly Ricardo Andrade and Alejandro Díez for his assistance with the confocal microscopy analysis. This work received financial support from the University of the Basque Country (Grant PPGA18/01). L.T. and J.R acknowledge the financial support given by University of the Basque Country (PIF15/149 and ESPDOC17/33, respectively).

Conflict Of Interest

The authors have no conflicts of interest to declare.

45 **ABSTRACT**

46 The cannabinoid system has been involved in many aspects of reproduction and it is
47 known that the system chronic use of exogenous cannabinoids are deleterious to
48 reproductive processes. Even so, it is not known what happens in relation to the
49 physiology of the ovary when cannabinoid receptors are absent. The present study
50 investigated the effect of the lack of CB1 and CB2 cannabinoid receptors in mice ovarian
51 morphology, folliculogenesis, oocyte retrieval and oocyte maturation and evaluated the
52 use of THC on oocyte in vitro maturation (IVM) by comparing classical IVM and two-
53 step IVM by analyzing the meiotic competence of the oocytes and their evolution towards
54 embryos. Thus, when CB1 and CB2 cannabinoid receptors were missed, the ovary area
55 and volume was significantly less and the action of the eCG hormone was diminished. In
56 addition, the mutant genotypes had fewer ovarian follicles and they were less competent
57 after eCG administration compared to wild type mice, and this lack of cannabinoid
58 receptors showed a mismatch of oocyte maturation. However, the in vitro use of THC
59 showed improvements in oocytes IVM after a Pre-IVM step for 48 h, since those oocytes
60 reached a significantly higher polar body rate, a larger diameter and the best result on
61 blastocysts rate was achieved when THC was used during the IVM step.

62

63 **Key words**: Cannabinoid receptors, ovary, oocyte, maturation, THC

64

65

66

67

68

69

70

71 **INTRODUCTION**

72

73 Phytocannabinoids are lipophilic compounds derived from the *Cannabis sativa L.*
74 Research on cannabinoids began in the 1960s, when cannabis Δ9-tetrahydrocannabinol
75 (THC) was purified and described (Mechoulam R & Gaoni Y, 1967). THC is the most
76 abundant cannabinoid in the plant and the primary responsible for bioactive effects
77 (ElSohly & Slade, 2005). Interestingly, an endogenous cannabinoid system (ECS) was
78 also described in animals (Correa, Wolfson, Valchi, Aisemberg, & Franchi, 2016). This
79 system consists of cannabinoid receptors (CB1 and CB2), their internal ligands
80 (endocannabinoids) and the synthesis and degradation enzymes (Correa et al., 2016).
81 The endocannabinoid system has been identified in the regulation of both female and
82 male reproductive events such as, gametogenesis, fertilization, preimplantation embryo
83 development, implantation and postimplantation embryonic growth (Walker, Holloway,
84 & Raha, 2019). Regarding the female, the ECS has been described in hypothalamus-
85 pituitary-ovary (HPO) axis (Brents, 2016), as well as, follicular fluid, oocytes and
86 granulosa cells from various species like mice, rats, cows and humans (Agirregoitia et al.,
87 2015; Agirregoitia et al., 2016; Bagavandoss & Grimshaw, 2010; El-Talatini, et al., 2009;
88 López-Cardona et al., 2016; López-Cardona et al., 2017; Peralta et al., 2011; Schuel et
89 al., 2002).
90 Generally, when the ECS is exogenously modulated, the cannabinoids alter HPO axis
91 regulation, potentially leading to disruption of the reproductive system and generating,
92 among others, anovulatory menstrual cycles; in the same way, altered ECS expression is
93 also associated with reduced fertility (Brents, 2016). It has been described that the lack
94 of cannabinoid receptors inhibits the hormone release in HPO axis (Cacciola et al., 2013;
95 Oláh, Milloh, & Wenger, 2008; Wenger, Ledent, Csernus, & Gerendai, 2001), although
96 it is not known what happens in the ovary of knock out mice for cannabinoid receptors.

97 Preliminary studies suggest that the absence of CB1 in oocytes causes, after fertilization,
98 embryo development failure in mice (López-Cardona et al., 2017).
99 Therefore, if both a cannabinoid overexposure and a cannabinoid under-exposure could
100 be harmful to the oocyte physiology, it is not surprising that there are evidences where
101 endocannabinoid signaling could regulate human follicle maturation and development
102 (El-Talatini, Taylor, & Konje, 2009; Schuel et al., 2002). Previous studies in mice and
103 cows, showed that cannabinoids are able to improve in vitro maturation (IVM) of oocytes
104 derived from the largest cohort of follicles, induced with exogenous equine chorionic
105 gonadotropin (eCG), (López-Cardona et al., 2016; López-Cardona et al., 2017;
106 Totorikaguena et al., 2019). Recent publications showed that a significant improvement
107 of maturation rate and developmental competence of mouse oocytes was achieved
108 introducing a "pre-maturation" (Pre-IVM) step (Romero, Sanchez, Lolicato, Van Ranst,
109 & Smitz, 2016; Zhang, Su, Sugiura, Xia, & Eppig, 2010). During Pre-IVM, meiotic arrest
110 is imposed via modulation of the cAMP signaling pathway using the C-type Natriuretic
111 Peptide-22 (CNP), the natural oocyte maturation inhibitor (Romero et al., 2016; Zhang et
112 al., 2010). We hypothesized that the modulation of the ECS may further enhance the two
113 step IVM system. To this purpose, we chose to culture in presence of cannabinoids
114 juvenile unprimed oocytes instead of oocytes retrieved from larger follicles at a more
115 advanced stage of development (Romero et al., 2016).
116 In summary, as oocyte meiotic maturation is an important process whereby immature
117 oocytes acquire the characteristics required for successful fertilization and embryogenesis
118 (Lonergan & Fair, 2016), the aim of this work is double. Firstly, in order to further
119 understanding the role of ECS in oocyte physiology, we studied the effect of the lack of
120 CB1 and CB2 cannabinoid receptors in ovarian morphology, folliculogenesis, oocyte
121 retrieval and oocyte maturation by generating knockout mice for CB1 and/or CB2

122 receptors. Secondly, to improve the effectiveness of the IVM technique, we evaluated the
123 use of THC on oocyte maturation and developmental competence by making use of a
124 double step IVM system.

125 **MATERIALS AND METHODS**

126 **Experimental animals**

127 Wild-type (WT), *Cnr1*$^{-/-}$ (Marsicano et al., 2002), *Cnr2*$^{-/-}$ (Buckley et al., 2000), and
128 *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ mice (C57BL/6J) used in this study were kept in an animal house under
129 controlled conditions of temperature (22 ± 1°C) and photoperiod (light/dark cycle 14 h:10
130 h). Animals were given free access to water and food. All experimental procedures
131 involving the use of mice were approved by the University of the Basque Country
132 (UPV/EHU, CEEA reference number: M20-2015-016-027-028-173) and were performed
133 according to the *Guide for Care and Use of Laboratory Animals*, endorsed by the Society
134 for the Study of Reproduction and European legislation.

135 For the pre-IVM experiments, immature oocytes were obtained from C57BL/6J x CBA/ca
136 unprimed juvenile mice. These animals were housed and bred according to Belgian
137 legislation and with the consent of the ethical committee of the Vrije Universiteit Brussel
138 (Project numbers: 09-216-1 and 14-216-1).

139 **Ovary collection and histology**

140 Ovaries from female mice 8 to 10-week-old WT (C57BL/6) or KO on a C57BL/6
141 background (*Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$) were collected. In some cases, mice
142 were superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin
143 (Folligon, Intervet, Castle Hill, NSW, Australia). Twenty-four ovaries (n= 24) were
144 transferred to M2 medium at 37ºC and cleaned of any connective tissue. Part of the

145 ovaries were used to measure the total volume and the area by FIJI software. To this
146 purpose, digital images of the whole ovary were captured using an Olympus BX50 optical
147 microscope (Olympus Optical Co.) connected to a digital colour camera (Olympus
148 XC50).

149 The ovaries were mixed with Bouin's solution for 2-4 h and then processed for histology
150 protocol. Briefly, ovaries were dehydrated in alcohol, clarified using xylol, embedded in
151 histological paraffin, and the blocks were sectioned at 8 µm with a retraction microtome
152 (Shandon AS 325). Finally, the slides were stained with hematoxylin-eosin every fifth
153 section and analyzed (48 µm between analysed sections).

154 **Follicle counting**

155 As it has been previously described, the number of follicles was estimated by determining
156 the mean number of follicles per section after sampling every fifth section selecting a
157 section thickness of 8 µm (Tilly, 2003). The slides were examined and captured using and
158 Olympus BX50 optical microscope with 40x enlargement, by which the follicles and
159 other structures of the ovary were observed. After that, all areas of the fragment were
160 photographed using a digital colour camera (Olympus XC50) coupled to the objective of
161 the light microscope to assess the structures of interest.
162 Follicles were classified as primordial (a single layer of flattened granulosa cells
163 surrounding the oocyte); primary follicles (a single layer of cubic-shaped granulosa cells
164 surrounding the oocyte); secondary follicles (a single layer of cuboidal-shaped granulosa
165 cells surrounding the oocyte), and antral or preovulatory (a fluid-filled cavity inside the
166 oocyte) (Silva et al., 2004).

167

168 The total volume of each ovary was measured (section area x section thickness x number
169 of sections), and the follicle count was stated as follicles per millimetre cubed of ovarian
170 tissue (Aiken, Tarry-Adkins, & Ozanne, 2013).

171 **Isolation and *in vitro* maturation of Cumulus–Oocyte Complexes to determine**
172 **meiotic progression**

173 Female mice 8 to 10 week old WT, *Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ were
174 superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin, and
175 ovaries were collected 46 to 48 h later. The ovaries were cleaned of any connective tissue
176 and placed in M2 medium supplemented with 4 mg/ml bovine serum albumin fraction V.
177 Antral follicles were punctured with 30-gauge needles, and immature cumulus–oocyte
178 complexes (COCs) were collected in M2 medium. COCs were matured for 17 h in TCM-
179 199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/ml epidermal growth
180 factor at 37°C under an atmosphere of 5% CO_2 in air with maximum humidity (López-
181 Cardona et al., 2017).

182 In order to determine the role of cannabinoids receptors in mice oocyte maturation, COCs
183 at 0, 1, 2, 4, 8,12 and 17 h of IVM (n = 30 per time point and group in three independent
184 replicates) were used as previously described (Khatir, Lonergan, & Mermillod, 1998).
185 Briefly, COCs were partially denuded by vortexing during 3 min in 0.1% of hyaluronidase
186 (Sigma, MI, USA) and fixed in 4% paraformaldehyde (Panreac, Barcelona, Spain) for 20
187 min. Then were washed twice in PBS and incubated in PBS containing 10 μg/mL Hoechst
188 33342 (Thermo Fisher, MA, USA) for 15 min. Oocytes were then placed in glass slides
189 and squashed with coverslip for observation with an immunofluorescence microscope
190 (Zeiss Axioskop, NY, USA) under UV light.

191 **Incubation with Tetrahydrocannabinol (THC)**

192 The THC stock solutions were prepared in DMSO. During maturation (17 h), COCs were
193 incubated with 100 nM THC to evaluate the effects of activation of cannabinoid receptor
194 by this agonist. The control group was performed using only the same amount of DMSO
195 and the experiment control without any additives.

196 **Collection and culture of Cumulus-Oocyte Complexes from Small Antral Follicles**
197 **of Unprimed Mice and Pre-Ovulatory Cumulus-Oocyte Complexes from Large**
198 **Antral Follicles**

199 Compact COCs of the first wave of follicular development were collected from small
200 antral follicles of prepubertal mice (19–21 days old) without prior gonadotropin
201 administration. Pre-ovulatory COCs (controls), were collected by puncturing large antral
202 follicles of prepubertal female mice (25–27 days old) following 48 h of priming with 2.5
203 IU eCG. Cumulus-oocyte-complexes from small antral follicles were collected in
204 Leibovitz L-15 containing 10% heat-inactivated FCS, (all from Life Technologies),
205 supplemented with 200 μM 3-isobutyl-1-methylxanthine (Sigma, MI, USA) to prevent
206 meiosis resumption during the period of collection and preculture handling (Romero et
207 al., 2016).

208

209 For Pre-IVM experiments, COCs from small antral follicles of unprimed female mice
210 aged 19–20 days old were cultured for 48 h in presence of 25 nM CNP-22 (CNP; Phoenix
211 Europe, London, UK) and 10 μM 17-β-estradiol (E2; Sigma, MI, USA). For IVM
212 experiments, recombinant epidermal growth factor was used as ovulatory stimuli,
213 recombinant follicle stimulating hormone (FSH; Merck-Serono, Darmstadt, Germany)
214 was added and COCs were directly incubated in the medium of IVM in the presence or

215 absence of THC 10^{-7} M for 18 h. Basal culture medium for the culture of COCs (Pre-IVM and
216 IVM phases) consisted of a-MEM, 2.5% fetal bovine serum (both from Life Technologies, CA,
217 USA), 5 µg/ml insulin, 5 µg/ml apo-transferrin, and 5 µg/ml sodium selenite (all from
218 Sigma, MI, USA).
219
220 Additionally, two conditions were evaluated for oocyte developmental capacity: 1) an
221 IVM control, in which COCs from small antral follicles of unprimed mice aged 20 days
222 old were in vitro matured for 18 h without pre-maturation step and, 2) an in vivo control,
223 where in vivo grown and matured oocytes were obtained from female aged 25–27 days
224 old primed for 48 h with 2.5 IU eCG, followed by 14 h with 2.5 IU hCG (Chorulon;
225 Intervet).

226 **Evaluation of Meiotic progression**

227 Maturation rates of the COCs, exposed to THC phytocannabinoid were evaluated by
228 scoring the presence of the first polar body and measuring MII oocyte diameter. Meiotic
229 progression was analyzed by assessing the nuclear maturation stage under an inverted
230 microscope equipped with a Hoffman modulation contrast system (Nikon). Nuclear
231 maturation was scored as GV (germinal vesicle stage), GVBD (when GV is not visible),
232 PB (first polar body observed in the perivitelline space), or DEG (when the oocyte was
233 degenerated). Oocyte diameter was measured under an inverted microscope by using a
234 calibrated ocular micrometer and excluding the zona pellucida.

235 ***In vitro* fertilization**

236 To assess the developmental competence after pre-IVM and IVM culture, COCs derived
237 from each experimental condition [control, vehicle (DMSO), THC] were in vitro

238 fertilized with the same sperm sample as previously described in Romero et al., (19).
239 Briefly, COCs were transferred to 200 µl equilibrated IVF medium (M16, NEAA, BSA)
240 and overlaid with mineral oil and a 2×10^6 spermatozoa/ml concentration of spermatozoa
241 obtained from CBAB6F1 male donor aged 6 to 12 weeks. After 1 h of co-incubation at
242 37ºC, 5% CO_2, 5% O_2, and 100% humidity, presumptive zygotes were denuded, washed
243 twice, and cultured in groups of 10–15 zygotes in 30 µl of embryo culture medium (M16,
244 NEAA, NAA) overlaid with oil for embryo culture (Sigma, MI, USA) at 37ºC in 5%CO_2,
245 5%O_2, and 100% humidity. Cleavage (2- cell) rate was scored 24 h after IVF. On Day 5,
246 blastocyst development and hatching were recorded. In total, 60–70 COCs (from four
247 independent replicates) were assessed per condition.

248 **Statistical analysis**

249 All statistical tests were performed by using Microsoft Excel software and Graphpad
250 software (GraphPad Software, Inc. La Jolla, CA, USA).
251 All the results were indicated as the mean ± S.E.M. Differences in ovary volume, ovary
252 area, oocyte meiotic resumption, oocyte diameter and rates of fertilization and blastocyst
253 formation between the 4 genotypes were compared by one-way ANOVA, followed by a
254 Bonferroni`s Multiple Comparison test. Values of $P < 0.05$ were considered significant.
255

256 **RESULTS**

257 **Effect of the absence of CB1 and CB2 receptors on the size of mouse ovary**
258 To study the role of CB1 and CB2 cannabinoid receptors in mouse folliculogenesis,
259 firstly, we observed the macroscopic morphology of mice ovary and we measured the
260 size of ovaries obtained from *Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ mutant genotypes,
261 as well as, the ovaries from wild type (WT) mice.

Ovaries from *Cnr1*$^{-/-}$ mice were smaller and more irregular than WT (Fig. 1A) and, although this difference was not significant. When both receptors, CB1 and CB2, were disrupted, the difference in ovary volume was significantly notable (Fig. 1A). After administration of 5 IU eCG, the volume of WT ovary was significantly higher than the rest of studied genotypes (Fig. 1B). Additionally, although the ovary volume increased regardless the mouse genotypes, the administration of eCG, induced a significant increase only in WT mice (Fig. 1C).

Similar outcomes were obtained when histology sections were used (Fig. 2A). The lack of cannabinoid receptors affected the size of the ovary, being smaller when CB1 receptor was missing but increasing this difference, until significant, when both receptors, CB1 and CB2, were missing in comparison with WT mice (Fig. 2A). Again, after the administration of eCG, the differences in the section of the ovary between genotypes grew, being significantly smaller the area when the CB1 receptor was missing and even smaller when CB1 and CB2 were missing (Fig. 2B).

Effect of the absence of CB1 and CB2 receptors on the number of mouse follicles

Ovarian morphology was assessed by histological examination of the different growing follicles using hematoxylin and eosin staining (Fig. 3). The number of total follicles varied between the different genotypes, being smaller in those mice ovaries where the CB1 receptor or both receptors, CB1 and CB2, were absent (Fig. 3A). Furthermore, total number of follicles 48 hours after eCG treatment maintained the same pattern (Fig. 3B).

In order to study in deep the development of those follicles, the initial (primordial and primary follicles) and advanced (secondary and antral follicles) growth follicles were counted. Although all genotypes showed a greater amount of primary follicles, the wild type mice showed a higher number of primary follicles (60.9 %) than the knock out genotypes (around 45-50%) (Fig. 3C). In fact, the KO mice, especially the Cnr1$^{-/-}$

287 (32.4%) and $Cnr1^{-/-}/Cnr2^{-/-}$ (24.4%), had a higher number of secondary follicles than
288 WT mice (10.8%) (Fig. 3C). The $Cnr1^{-/-}$ (13.5%) and $Cnr1^{-/-}/Cnr2^{-/-}$ (11.8%) genotypes
289 also showed a lower number of primordial follicles than the rest of genotypes (around
290 17%) (Fig. 3C). Finally, we did not find much difference in the number of antral follicles
291 between genotypes (Fig. 3C). After the ovarian stimulation (48h post-eCG injection),
292 wild type mice showed a higher number of antral follicles (33.7%) in comparison with
293 the other genotypes (Fig. 3D). In addition, the mutant mice for cannabinoid receptors
294 showed more follicles in early stages (primordial and primary) than WT mice (Fig. 3D).
295 To further elucidate the effect of the cannabinoid receptor absence in the quantity of
296 oocytes, we counted the number of oocytes after puncturing the ovary stimulated with
297 eCG derived from $Cnr1^{-/-}$, $Cnr2^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$ mutant genotypes, as well as,
298 the ovaries from WT mice. We classified the oocyte as compact, denuded or expanded
299 according to the appearance of their cumulus cells and the degree of expansion of the
300 mural cells (González-Fernández et al., 2018; Hinrichs, 2010). We retrieved less amount
301 of oocytes from $Cnr1^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$ ovaries, although the difference was only
302 significant when both receptors, CB1 and CB2, were absent (Fig. 3E). We obtained the
303 highest number of expanded COC from the WT mice, being significantly higher than
304 $Cnr1^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$ mice. The amount of expanded COC obtained from $Cnr2^{-/-}$
305 genotype was also significantly higher than the one obtained from double-KO mice (Fig.
306 3E).
307

308 **Effect of the absence of CB1 and CB2 receptors on the velocity of meiotic**
309 **progression of mice oocytes**

310 We next examined whether the absence of cannabinoid receptors would have any impact
311 on meiotic progression of mice oocytes (Fig. 4). We fixed oocytes after 0, 1, 2, 4, 8, 12

312 and 17h of IVM. Oocytes were classified as GV, GVBD, PMI, MI and MII, based on
313 nuclear maturation stage. As it can be seen in Fig. 4A, at 0h all the oocytes were in GV
314 stage and after 1 h of IVM, we observed oocytes on GVDB from mutant mice, while all
315 the WT oocytes remained in the GV phase (Fig. 4B). At 2h and 4h of IVM, the KO-
316 genotypes continued to be more advanced than WT oocytes (Fig. 4C and 4D) but, at 4h,
317 although the only oocytes that had not reached PM1 were those of the WT genotype, the
318 oocytes without the two types of receptors (CB1 and CB2) had the highest amount of
319 oocytes in GV (Fig. 4D). Thereafter, at 8h and 12h, the oocytes of WT and *Cnr2*$^{-/-}$
320 genotypes accelerated their maturation process in comparison with the genotypes lacking
321 the CB1 receptor (*Cnr1*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$), in fact, at 12h, almost all the oocytes of
322 WT and *Cnr2*$^{-/-}$ have reached the MII (Fig. 4E and 4F). Finally, at 17h of maturation,
323 most oocytes of *Cnr1*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$genotypes had also been able to reach MII
324 stage (Fig 4G).
325

326 **Developmental capacity of oocytes recovered from unprimed ovaries following**
327 **prolonged pre-IVM and IVM**

328 Experiments were set up to evaluate developmental competence of oocytes undergoing
329 the Pre-IVM called step, followed by an IVM culture period in absence or presence of 10^{-}
330 ^{7} M of THC (Fig. 5).
331

332 Following the Pre-IVM culture period, the oocytes from almost all culture conditions
333 showed a high rate of meiotic resumption compared to those who did not mature with the
334 previous stage (Fig. 5A). Even so, only the cumulus cell-oocyte complexes (COCs)
335 incubated in Pre-IVM medium for 48 h followed by maturation with THC 10^{-7} M had a
336 significant high occurrence of PB oocytes (69.2%) in comparison to control IVM media

337 ($P < 0.05$). In addition, the occurrence of PB of those COCs incubated directly in IVM
338 media with THC 10^{-7} M did not differ from the PB occurrence found in the Pre-IVM
339 conditions.

340 After measuring the diameter of the mature oocytes, the same pattern as the previous
341 results was maintained (Fig. 5B), although COCs obtained after Pre-IVM culture reached
342 a larger mean of diameter than COCs cultured directly in IVM medium, only those COCs
343 that were matured with THC 10^{-7} M had a significantly larger diameter than the COCs
344 cultured in IVM medium. Finally, is interesting to note that when COCs were incubated
345 with THC directly in IVM medium, the diameter of the COCs did not differ from the
346 diameter found in the Pre-IVM conditions.

347 **Evaluation of the developmental competence of prepuberal mice oocytes**
348
349 The last aim was to evaluate the developmental competence of oocytes undergoing Pre-
350 IVM followed by an IVM culture period in presence of THC 10^{-7} M, observing the
351 fertilization and the subsequent embryo development rate. Following IVM, the oocytes
352 were in vitro fertilized, and embryos were cultured up to Day 5.

353 There were no significant differences in fertilization rate (two-cell) between the different
354 culture and treatments (Fig. 6A and 6B). On the other hand, although the blastocyst rates
355 of Pre-IVM and then matured in presence of THC 10^{-7} M was higher than controls and in
356 vivo matured oocytes, that difference was not significant (Fig. 6C). However, in the
357 unique medium where we obtained blastocysts from COCs cultured directly in IVM
358 medium (without Pre-IVM step) was where the COCs were matured in the presence of
359 THC (Fig. 6D). The microscopic visual quality of the blastocysts achieved in all
360 experimental groups was similar (data not shown).

361

362 **DISCUSSION**

363 The results of the present study indicate that the lack of cannabinoid receptors affects the
364 oocyte development, from the morphology of the ovary to the development of follicles
365 and the maturation of oocytes. Even so, the use of THC in the medium during the
366 maturation of wild type oocytes in vitro improves the maturation process, especially if
367 the oocytes have passed a "pre-maturation" stage prior to in vitro maturation (IVM).
368 Endocannabinoid system (ECS) is present in the hypothalamus, pituitary (Gammon CM1
369 et al., 2005) and ovary (El-Talatini et al., 2009; Galiègue et al., 1995) and, therefore, the
370 negative effects of cannabinoids (CBs) described in reproduction may come from their
371 action at different levels of the hypothalamic-pituitary-ovary (HPO) axis. The most
372 accepted hypothesis is that exogenous CBs exert untoward effects on reproduction by
373 reducing GnRH secretion (Gammon et al., 2005), preventing this hormone from
374 stimulating the release of gonadotropins [follicle stimulating hormone (FSH) and
375 luteinizing hormone (LH)] and suppressing gonadal function (Brents, 2016). This idea is
376 supported by the observation that the peripheral administration of exogenous GnRH
377 restores LH secretion in CB-treated animals (Ayalon D et al., 1977; Smith, Besch, Smith,
378 & Besch, 1979; Tyrey, 1978). It has been described that it would be the CB1 receptor
379 which regulates GnRH synthesis and release (Chianese, Ciaramella, Fasano, Pierantoni,
380 & Meccariello, 2011; Meccariello et al., 2008; Scorticati et al., 2004), although, it has
381 also been postulated that the activation of CB1 modulates the LH release at pituitary level
382 (Wenger et al., 2001). In addition, it has been demonstrated a direct inhibitory effect of
383 THC on folliculogenesis due to the interference with several FSH-dependent functions,
384 inhibiting the accumulation of estrogens and progesterone as well as inhibiting the
385 increase of LH receptors (Adashi, Jones, & Hsueh, 1983). In summary, it is known that
386 the regulation of the ECS and HPO axis are linked, although the mechanisms underlying
387 this link has not been fully described yet (Brents, 2016).

Another observation that reinforces the idea of the well-regulated ECS is required for the optimal function of HPO axis is that, not only cannabinoid treatment but also the lack of cannabinoid receptors causes a decrease in the levels of GnRH, FSH and 17-β-estradiol (Cacciola et al., 2013), as well as, LH (Oláh et al., 2008; Wenger et al., 2001). In addition, it is postulated that around the 40% of *Cnr1*$^{-/-}$ mice show pregnancy loss (Wang et al., 2004).

After the experiments carried out in this work, we have understood the effects on the ovary, folliculogenesis and maturation of the oocytes due to the lack of cannabinoid receptors. On the one hand, mice without CB1 or CB2 cannabinoid receptors generated smaller ovaries, which was accentuated when none of the receptors was present. This observation was reinforced by data that showed that CB-KO mice had fewer ovarian follicles compared to WT mice. Our observations indicate that the absence of cannabinoid receptors worsens the functional life span of the ovaries due to that life span is determined by the number of oocytes in the ovary, in fact, the infertility is characterised by a gradual decrease in follicle quantity and quality (Shi et al., 2016).

Ovarian stimulation is widely used to improve the efficiency of oocyte production (Takeo, Mukunoki, & Nakagata, 2019), but it is interesting to note how the ovarian stimulation with eCG did not generate an ovarian size growth as pronounced as in WT mice when cannabinoid receptors were absent. That fact probably was due to the less effect that eCG generated in CB-KO mice, since mutant mice for cannabinoid receptors showed less competent follicles than WT mice (the majority of the follicles in CB-KO mice are primordial and primary) after eCG administration. Following this observation, when we analysed the type of oocytes achieved from antral follicles punctured with needles, although the total amount of oocytes only was significantly less in *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ mice, both *Cnr1*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ mice achieved a smaller

413 number of expanded oocytes than WT mice. This fact is important because the meiotic
414 competence is different between the different types of oocytes, since it has been
415 demonstrated in equine species that only 21% of compact oocytes mature while 71% of
416 expanded ones reach the MII stage (Hinrichs, 2010).
417 Finally, although almost all the oocytes collected at GV stage reached the MII stage in all
418 studied genotypes, we observed an evident acceleration in the first steps of maturation of
419 the oocytes without cannabinoid receptors, more or less, until reaching the stage of MI,
420 because from MI the maturation was decelerated, especially in oocytes without CB1. It is
421 accepted that the failures in the acquisition of nuclear and cytoplasmic maturation (i.e.
422 condensed chromatin configuration, transcriptionally silencing) compromise the
423 obtention of oocyte development capacity and reduces the successful fertilization and
424 subsequent embryonic development (Coticchio et al., 2015; Sánchez et al., 2017). Even
425 so, it is difficult to know only by observing the velocity of maturation if an acceleration
426 or a deceleration will lead to some improvement or some failure in maturation. For
427 example, a systemic treatment with a CB2 agonist accelerates meiotic progression of fetal
428 oocytes, but decreasing the pool of primordial and primary follicles, negatively affecting
429 the ovarian reserve in the offspring (De Domenico et al., 2017). The acceleration in the
430 first steps of nuclear maturation also occurs when the bovine or mice WT oocytes are
431 incubated in vitro with CB agonists during the IVM (A. P. López-Cardona et al., 2016),
432 or deceleration when mice oocytes are incubated with CB antagonists (Cecconi, Rossi,
433 Oddi, Di Nisio, & Maccarrone, 2019). Even so, in the case of CB agonists, up to 50%
434 more blastocysts are achieved compared to the control (López-Cardona et al., 2016).
435 However, these oocytes matured in the presence of cannabinoids also arrive earlier at MII
436 stage (López-Cardona et al., 2016), unlike the KO oocytes used in the present work that
437 slowed maturation after reaching MI. The biggest difference is that, as we just said, while

438 incubating with cannabinoids during IVM improves the achievement of embryos, when
439 CB1-KO oocytes are used to perform IVF, 40-60% less blastocysts are achieved
440 compared to WT oocytes (López-Cardona et al., 2017). Therefore, it seems that the
441 maturation pattern observed in CB-KO oocytes cannot be said to be the most appropriate.
442 Considering all the data exposed so far, it seems that the alteration of the systemic ECS
443 affects the stages prior to the oocyte maturation, but, in the same way, the cannabinoids
444 are able to improve the oocytes IVM. Thus, as one of the biggest challenges is to develop
445 systems to improve the developmental competence of oocytes and to adapt culture
446 conditions to the stage-dependent oocyte needs (Sánchez et al., 2017), our last objective
447 was to test if the exposure to THC could improve oocyte competence acquisition from
448 small antral follicles from juvenile unprimed mice without eCG stimulation. The intention
449 was to try to improve the synchronization of meiotic and cytoplasmic maturation in antral
450 oocytes arrested at the immature GV-stage introducing a pre-maturation step during
451 which meiotic arrest was imposed via CNP, a “natural oocyte maturation inhibitor”
452 (Romero et al., 2016) and then, to perform the IVM in presence of THC. Thus, we
453 observed that those oocytes reached a significantly higher polar body rate and a larger
454 diameter when, after a Pre-IVM step for 48 h, were maturated with THC, in comparison
455 of oocytes without Pre-IVM step and without THC treatment. In addition, the highest
456 average on blastocysts rate was achieved when THC was used after a Pre-IVM step,
457 although that effect of the THC was not statistically significant compared to treatments
458 without THC. What did show statistical significance was that when the Pre-IVM step was
459 not used, blastocysts were only achieved in the medium with THC.
460 In conclusion, we have shown that the lack of cannabinoid signalling causes damages to
461 the ovarian function. In addition, since studies to date report a damage to reproduction
462 due to a systemic overexposure to cannabinoids, our study would raise the idea that, at

least the in vitro use of cannabinoids in oocyte maturation process could be positively assessed.

REFERENCES

Adashi, E. Y., Jones, P. B. C., & Hsueh, A. J. W. (1983). Direct antigonadal activity of cannabinoids: Suppression of rat granulosa cell functions. *American Journal of Physiology - Endocrinology and Metabolism*, *7*(2).

Agirregoitia, E., Ibarra-Lecue, I., Totorikaguena, L., Mendoza, R., Expósito, A., Matorras, R., … Agirregoitia, N. (2015). Dynamics of expression and localization of the cannabinoid system in granulosa cells during oocyte nuclear maturation. *Fertility and Sterility*, *104*(3). https://doi.org/10.1016/j.fertnstert.2015.06.013

Agirregoitia, Ekaitz, Totorikaguena, L., Expósito, A., Mendoza, R., Matorras, R., & Agirregoitia, N. (2016). Dynamic of expression and localization of cannabinoid-degrading enzymes FAAH and MGLL in relation to CB1 during meiotic maturation of human oocytes. *Cell and Tissue Research*, 1–9. https://doi.org/10.1007/s00441-016-2381-2

Aiken, C. E., Tarry-Adkins, J. L., & Ozanne, S. E. (2013). Suboptimal nutrition in utero causes DNA damage and accelerated aging of the female reproductive tract. *FASEB Journal*, *27*(10), 3959–3965. https://doi.org/10.1096/fj.13-234484

Ayalon D, Nir I, Cordova T, Bauminger S, Puder M, Naor Z, … Lindner HR. (1977). Acute effect of delta1-tetrahydrocannabinol on the hypothalamo-pituitary-ovarian axis in the rat. In *Neuroendocrinology* (Vol. 23).

Bagavandoss, P., & Grimshaw, S. (2010). Temporal and spatial distribution of the cannabinoid receptors (CB 1, CB2) and fatty acid amide hydroxylase in the rat ovary. *Anatomical Record*, *293*(8), 1425–1432. https://doi.org/10.1002/ar.21181

Brents, L. K. (2016). Marijuana, the Endocannabinoid System and the Female

488 Reproductive System. *Yale Journal of Biology and Medicine*, *89*(2), 175–191.

489 Buckley, N. E., McCoy, K. L., Mezey, É., Bonner, T., Zimmer, A., Felder, C. C., …
490 Zimmer, A. (2000). Immunomodulation by cannabinoids is absent in mice
491 deficient for the cannabinoid CB2 receptor. *European Journal of Pharmacology*,
492 *396*(2–3), 141–149. https://doi.org/10.1016/s0014-2999(00)00211-9

493 Cacciola, G., Chioccarelli, T., Altucci, L., Ledent, C., Mason, J. I., Fasano, S., …
494 Cobellis, G. (2013). Low 17beta-Estradiol Levels in Cnr1 Knock-Out Mice Affect
495 Spermatid Chromatin Remodeling by Interfering with Chromatin Reorganization.
496 *Biology of Reproduction*, *88*(6), 152–152.
497 https://doi.org/10.1095/biolreprod.112.105726

498 Cecconi, S., Rossi, G., Oddi, S., Di Nisio, V., & Maccarrone, M. (2019). Role of Major
499 Endocannabinoid-Binding Receptors during Mouse Oocyte Maturation.
500 *International Journal of Molecular Sciences*, *20*(12).
501 https://doi.org/10.3390/ijms20122866

502 Chianese, R., Ciaramella, V., Fasano, S., Pierantoni, R., & Meccariello, R. (2011).
503 Anandamide modulates the expression of GnRH-II and GnRHRs in frog, Rana
504 esculenta, diencephalon. *General and Comparative Endocrinology*, *173*(3), 389–
505 395. https://doi.org/10.1016/j.ygcen.2011.07.001

506 Correa, F., Wolfson, M. L., Valchi, P., Aisemberg, J., & Franchi, A. M. (2016).
507 Endocannabinoid system and pregnancy. *Reproduction*, *152*(6), R191–R200.
508 https://doi.org/10.1530/REP-16-0167

509 Coticchio, G., Dal Canto, M., Mignini Renzini, M., Guglielmo, M. C., Brambillasca, F.,
510 Turchi, D., … Fadini, R. (2015). Oocyte maturation: gamete-somatic cells
511 interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic
512 reorganization. *Human Reproduction Update*, *21*(4), 427–454.

513 https://doi.org/10.1093/humupd/dmv011
514 De Domenico, E., Todaro, F., Rossi, G., Dolci, S., Geremia, R., Rossi, P., & Grimaldi,
515 P. (2017). Overactive type 2 cannabinoid receptor induces meiosis in fetal gonads
516 and impairs ovarian reserve. *Cell Death and Disease*, *8*(10).
517 https://doi.org/10.1038/cddis.2017.496
518 El-Talatini, M. R., Taylor, A. H., Elson, J. C., Brown, L., Davidson, A. C., & Konje, J.
519 C. (2009). Localisation and function of the endocannabinoid system in the human
520 ovary. *PLoS ONE*, *4*(2). https://doi.org/10.1371/journal.pone.0004579
521 El-Talatini, M. R., Taylor, A. H., & Konje, J. C. (2009). Fluctuation in anandamide
522 levels from ovulation to early pregnancy in in-vitro fertilization-embryo transfer
523 women, and its hormonal regulation. *Human Reproduction*, *24*(8), 1989–1998.
524 https://doi.org/10.1093/humrep/dep065
525 ElSohly, M. A., & Slade, D. (2005). Chemical constituents of marijuana: The complex
526 mixture of natural cannabinoids. *Life Sciences*, *78*(5), 539–548.
527 https://doi.org/10.1016/j.lfs.2005.09.011
528 Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., …
529 Casellas, P. (1995). Expression of Central and Peripheral Cannabinoid Receptors
530 in Human Immune Tissues and Leukocyte Subpopulations. *European Journal of*
531 *Biochemistry*, *232*(1), 54–61. https://doi.org/10.1111/j.1432-1033.1995.tb20780.x
532 Gammon CM1, Freeman GM Jr, Xie W, Petersen SL, Wetsel WC., Petersen, S. L., &
533 Wetsel, W. C. (2005). Regulation of Gonadotropin-Releasing Hormone Secretion
534 by Cannabinoids. In *Endocrinology* (Vol. 146). Retrieved from
535 http://www.endojournals.org/.
536 González-Fernández, L., Sánchez-Calabuig, M. J., Alves, M. G., Oliveira, P. F.,
537 Macedo, S., Gutiérrez-Adán, A., … Macías-García, B. (2018). Expanded equine

538 cumulus-oocyte complexes exhibit higher meiotic competence and lower glucose
539 consumption than compact cumulus-oocyte complexes. *Reproduction, Fertility and*
540 *Development*, *30*(2), 297–306. https://doi.org/10.1071/RD16441
541 Hinrichs, K. (2010). In Vitro Production of Equine Embryos: State of the Art.
542 *Reproduction in Domestic Animals*, *45*(SUPPL. 2), 3–8.
543 https://doi.org/10.1111/j.1439-0531.2010.01624.x
544 Hinrichs, Katrin. (2010). The equine oocyte: Factors affecting meiotic and
545 developmental competence. *Molecular Reproduction and Development*, *77*(8),
546 651–661. https://doi.org/10.1002/mrd.21186
547 Khatir, H., Lonergan, P., & Mermillod, P. (1998). Kinetics of nuclear maturation and
548 protein profiles of oocytes from prepubertal and adult cattle during in vitro
549 maturation. *Theriogenology*, *50*(6), 917–929. https://doi.org/10.1016/S0093-
550 691X(98)00196-4
551 Lonergan, P., & Fair, T. (2016). Maturation of Oocytes in Vitro. *Annual Review of*
552 *Animal Biosciences*, *4*(1), 255–268. https://doi.org/10.1146/annurev-animal-
553 022114-110822
554 López-Cardona, A. P., Sánchez-Calabuig, M. J., Beltran-Breña, P., Agirregoitia, N.,
555 Rizos, D., Agirregoitia, E., & Gutierrez-Adán, A. (2016). Exocannabinoids effect
556 on in vitro bovine oocyte maturation via activation of AKT and ERK1/2.
557 *Reproduction*, *152*(6), 603–612. https://doi.org/10.1530/REP-16-0199
558 López-Cardona, Angela Patricia, Pérez-Cerezales, S., Fernández-González, R., Laguna-
559 Barraza, R., Pericuesta, E., Agirregoitia, N., … Agirregoitia, E. (2017). CB1
560 cannabinoid receptor drives oocyte maturation and embryo development via
561 PI3K/Akt and MAPK pathways. *FASEB Journal*, *31*(8), 3372–3382.
562 https://doi.org/10.1096/fj.201601382RR

563 Marsicano, G., Wotjak, C. T., Azad, S. C., Bisogno, T., Rammes, G., Cascio, M. G., … Lutz, B. (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature*, *418*(6897), 530–534. https://doi.org/10.1038/nature00839

567 Meccariello, R., Franzoni, M. F., Chianese, R., Cottone, E., Scarpa, D., Donna, D., … Fasano, S. (2008). Interplay between the endocannabinoid system and GnRH-I in the forebrain of the anuran amphibian Rana esculenta. *Endocrinology*, *149*(5), 2149–2158. https://doi.org/10.1210/en.2007-1357

571 Mechoulam R, & Gaoni Y. (1967). The absolute configuration of delta-1-tetrahydrocannabinol, the major active constituent of hashish. *Tetrahedron Lettera*, (12), 1109–1111.

574 Oláh, M., Milloh, H., & Wenger, T. (2008). The role of endocannabinoids in the regulation of luteinizing hormone and prolactin release. Differences between the effects of AEA and 2AG. *Molecular and Cellular Endocrinology*, *286*(1-2 SUPPL. 1). https://doi.org/10.1016/j.mce.2008.01.005

578 Peralta, L., Agirregoitia, E., Mendoza, R., Expósito, A., Casis, L., Matorras, R., & Agirregoitia, N. (2011). Expression and localization of cannabinoid receptors in human immature oocytes and unfertilized metaphase-II oocytes. *Reproductive BioMedicine Online*, *23*(3), 372–379. https://doi.org/10.1016/j.rbmo.2011.05.011

582 Romero, S., Sanchez, F., Lolicato, F., Van Ranst, H., & Smitz, J. (2016). Immature Oocytes from Unprimed Juvenile Mice Become a Valuable Source for Embryo Production When Using C-Type Natriuretic Peptide as Essential Component of Culture Medium. *Biology of Reproduction*, *95*(3), 64–64. https://doi.org/10.1095/biolreprod.116.139808

587 Sánchez, F., Lolicato, F., Romero, S., De Vos, M., Van Ranst, H., Verheyen, G., …

588 Smitz, J. E. J. (2017). An improved IVM method for cumulus-oocyte complexes
589 from small follicles in polycystic ovary syndrome patients enhances oocyte
590 competence and embryo yield. *Human Reproduction*, *32*(10), 2056–2068.
591 https://doi.org/10.1093/humrep/dex262
592 Schuel, H., Burkman, L. J., Lippes, J., Crickard, K., Mahony, M. C., Giuffrida, A., …
593 Makriyannis, A. (2002). Evidence that anandamide-signaling regulates human
594 sperm functions required for fertilization. *Molecular Reproduction and*
595 *Development*, *63*(3), 376–387. https://doi.org/10.1002/mrd.90021
596 Scorticati, C., Ferná Ndez-Solari, J., De Laurentiis, A., Mohn, C., Prestifilippo, J. P.,
597 Lasaga, M., … Rettori, V. (2004). The inhibitory effect of anandamide on
598 luteinizing hormone-releasing hormone secretion is reversed by estrogen.
599 *Proceedings of the National Academy of Sciences*, *101*(32), 11891–11896.
600 Retrieved from www.pnas.orgcgidoi10.1073pnas.0404366101
601 Shi, L., Zhang, J., Lai, Z., Tian, Y., Fang, L., Wu, M., … Wang, S. (2016). Long-term
602 moderate oxidative stress decreased ovarian reproductive function by reducing
603 follicle quality and progesterone production. *PLoS ONE*, *11*(9), 1–18.
604 https://doi.org/10.1371/journal.pone.0162194
605 Silva, J. R. V., Van Den Hurk, R., De Matos, M. H. T., Dos Santos, R. R., Pessoa, C.,
606 De Moraes, M. O., & De Figueiredo, J. R. (2004). Influences of FSH and EGF on
607 primordial follicles during in vitro culture of caprine ovarian cortical tissue.
608 *Theriogenology*, *61*(9), 1691–1704.
609 https://doi.org/10.1016/j.theriogenology.2003.09.014
610 Smith, C. G., Besch, N. F., Smith, R. G., & Besch, P. K. (1979). Effect of
611 Tetrahydrocannabinol on the Hypothalamic-Pituitary Axis in the Ovariectomized
612 Rhesus Monkey**Supported by National Institute on Drug Abuse Grant 1-R01-

613 DA1346-02. *Fertility and Sterility*, *31*(3), 335–339. https://doi.org/10.1016/s0015-
614 0282(16)43885-9
615 Takeo, T., Mukunoki, A., & Nakagata, N. (2019). Ovulation of juvenile, mature, and
616 aged female C57BL/6 mice following coadministration of inhibin antiserum and
617 equine chorionic gonadotropin. *Theriogenology*, *135*, 1–6.
618 https://doi.org/10.1016/j.theriogenology.2019.05.028
619 Tilly, J. L. (2003). Ovarian follicle counts - Not as simple as 1, 2, 3. *Reproductive*
620 *Biology and Endocrinology*, *1*, 1–4. https://doi.org/10.1186/1477-7827-1-11
621 Totorikaguena, L., Olabarrieta, E., López-Cardona, A. P., Agirregoitia, N., &
622 Agirregoitia, E. (2019). Tetrahydrocannabinol Modulates in VitroMaturation of
623 Oocytes and Improves the Blastocyst Rates after in Vitro Fertilization. *Cellular*
624 *Physiology and Biochemistry*, *53*(3), 439–452. https://doi.org/10.33594/000000149
625 Tyrey, L. (1978). A-9-Tetrahydrocannabinol Suppression of Episodic Luteinizing
626 Hormone Secretion in the Ovariectomized Rat*. *Endocrinology*, *102*(6), 1808–
627 1814.
628 Walker, O., Holloway, A., & Raha, S. (2019). The role of the endocannabinoid system
629 in female reproductive tissues. *Journal of Ovarian Research*, *12*(SUPPL. 1), 2–10.
630 https://doi.org/10.1186/s13048-018-0478-9.
631 Wang, H., Guo, Y., Wang, D., Kingsley, P. J., Marnett, L. J., Das, S. K., … Dey, S. K.
632 (2004). Aberrant cannabinoid signaling impairs oviductal transport of embryos.
633 *Nature Medicine*, *10*(10), 1074–1080. https://doi.org/10.1038/nm1104
634 Wenger, T., Ledent, C., Csernus, V., & Gerendai, I. (2001). The central cannabinoid
635 receptor inactivation suppresses endocrine reproductive functions. *Biochemical*
636 *and Biophysical Research Communications*, *284*(2), 363–368.
637 https://doi.org/10.1006/bbrc.2001.4977

Zhang, M., Su, Y. Q., Sugiura, K., Xia, G., & Eppig, J. J. (2010). Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science*, *330*(6002), 366–369. https://doi.org/10.1126/science.1193573

LEGENDS TO FIGURES

Figure 1. Effects of cannabinoid receptors' lack in ovarian volume . Representative photographs and the measurement of the volume of ovaries of wild type (WT), Cnr1$^{-/-}$, Cnr2$^{-/-}$ and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice **(A)** before and **(B)** after eCG administration. **(C)** Ovary volume comparison between eCG treated and not treated mice. Results are the means ± S.E.M. of 6 independent experiments. The different combinations of letters or an asterisk (*) indicate significant differences between groups; $p<0.05$ in all cases.

Figure 2. Effects of cannabinoid receptors' lack in ovarian size. Representative photographs of histological cross-section and the measurement of the area of ovaries of wild type (WT), Cnr1$^{-/-}$, Cnr2$^{-/-}$ and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice before **(A)** and **(B)** after eCG administration. Results are the means ± S.E.M. of 6 independent experiments. The different combinations of letters indicate significant differences between groups; $p<0.05$ in all cases. Scale bar: 500 μm.

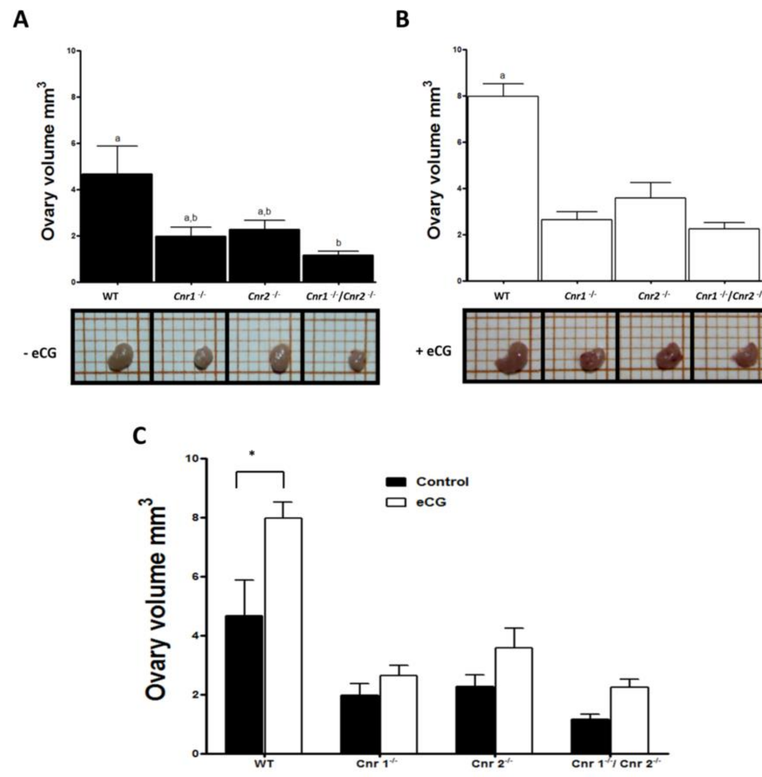
Figure 3. Effects of cannabinoid receptors' lack in ovarian folliculogenesis. (A and **B)** Total number of follicles per ovary of wild type (WT), Cnr1$^{-/-}$, Cnr2$^{-/-}$ and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice **(A)** before and **(B)** after eCG administration. Results are the means ± S.E.M. of 6 independent experiments. The different combinations of letters indicate significant differences between groups; $p<0.05$ in all cases. **(C** and **D)** Percentage of primordial, primary, secondary and antral ovarian follicles per ovary of wild type (WT), Cnr1$^{-/-}$, Cnr2$^{-/-}$ and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice **(C)** before and **(D)** after eCG administration.

662 **(E)** The number of oocytes achieved after the punctured antral follicles of eCG stimulated
663 wild type (WT), Cnr1$^{-/-}$, Cnr2$^{-/-}$ and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice. Results are the means ±
664 S.E.M. of 6 independent experiments. The different combinations of letters indicate
665 significant differences between groups; $p<0.05$ in all cases.

666 **Figure 4. Changes in nuclear status of oocytes throughout maturation.** Results are
667 expressed as percentage of oocytes at each stage of maturation at each time point: **(A)** 0h
668 **(B)** 1h **(C)** 2h **(D)** 4h **(E)** 8h **(F)** 12h and **(G)** 17h. Five stages are shown in different
669 colours: germinal vesicle (GV), germinal vesicle breakdown (GVBD), pro-metaphase I
670 (PMI), metaphase I (MI) and metaphase II (MII).
671
672 **Figure 5. Evaluation of THC in meiotic maturation :** meiotic completion up to **(A)** PB
673 extrusion and **(B)** diameter of MII oocytes. Oocytes were collected from unprimed mice
674 following Pre-IVM step in the presence of CNP conditions or without doing the Pre-IVM
675 step. Then, the IVM was performed in presence or in absence of THC 10^{-7} M. Results
676 are the mean ± S.E.M. of 4 independent experiments. At least 60 MII oocytes/treatment
677 were measured. Significant differences between treatments are indicated with different
678 letters; $p < 0.05$ in all cases.
679
680 **Figure 6: Evaluation of THC in developmental competence of oocytes collected from**
681 **unprimed prepuberal mice.** Oocytes were collected from unprimed mice (**A** and **C**)
682 following Pre-IVM step in the presence of CNP conditions or (**B** and **D**) without doing
683 the Pre-IVM step. Then, the IVM was performed in presence or in absence of THC 10^{-7}
684 M. Finally, the oocytes were in vitro fertilized and embryos were cultured for up to 5
685 days. Evaluation parameters were (**A** and **B**) 2-cell rate and (**C** and **D**) blastocyst
686 formation on Day 5 related to 2-cell rate (D5 Blast/2-cell). Results are the mean ± S.E.M.

687 of 4 independent experiments. Differences between treatments are indicated with
688 different letters; $p < 0.05$ in all cases.
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705

706 Figure 1



707

708

709

710

711

712

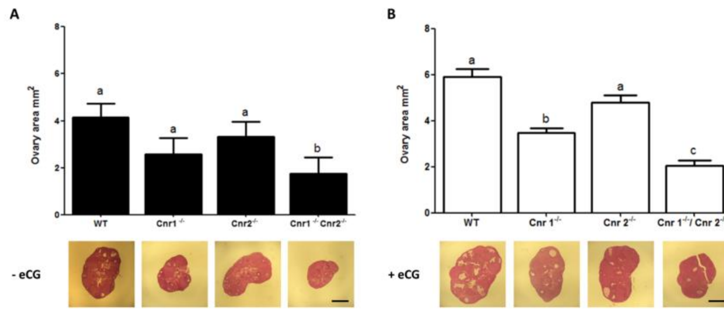
713

714

715

716

717 Figure 2



718

719

720

721

722

723

724

725

726

727

728

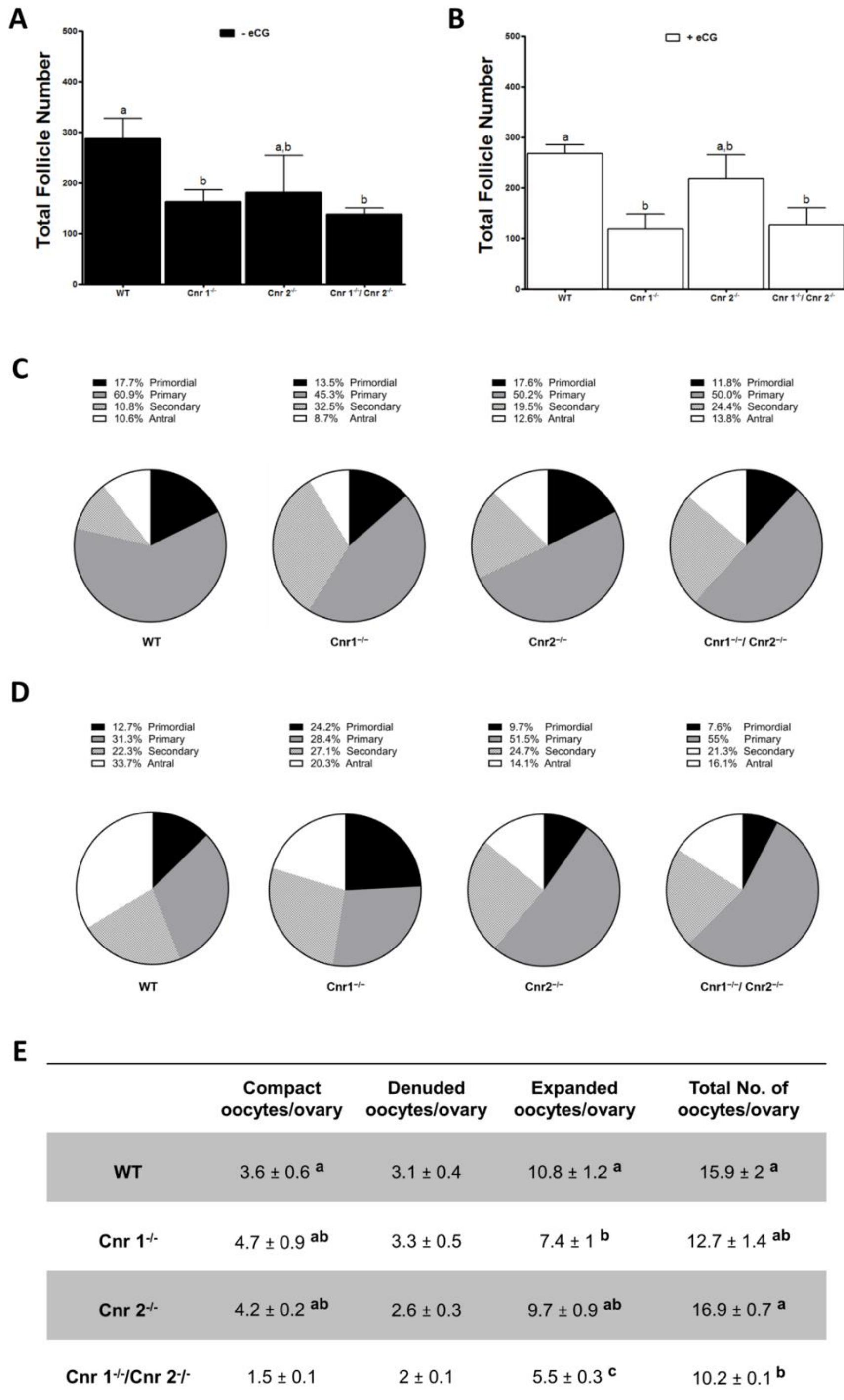
729

730

731

732

733 Figure 3



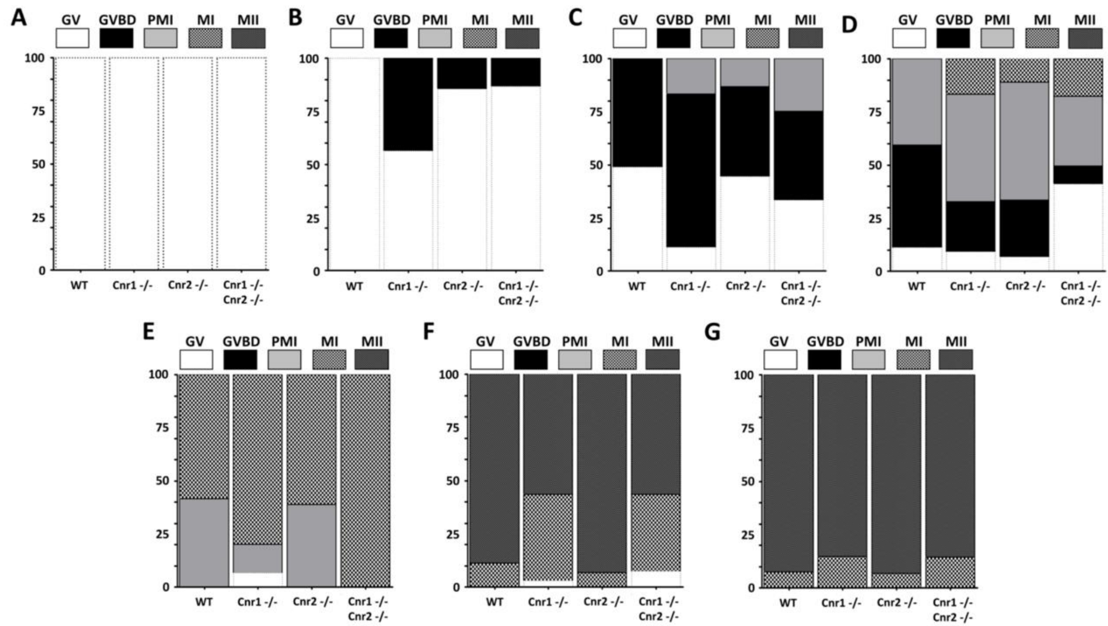
	Compact oocytes/ovary	Denuded oocytes/ovary	Expanded oocytes/ovary	Total No. of oocytes/ovary
WT	3.6 ± 0.6^{a}	3.1 ± 0.4	10.8 ± 1.2^{a}	15.9 ± 2^{a}
Cnr $1^{-/-}$	4.7 ± 0.9^{ab}	3.3 ± 0.5	7.4 ± 1^{b}	12.7 ± 1.4^{ab}
Cnr $2^{-/-}$	4.2 ± 0.2^{ab}	2.6 ± 0.3	9.7 ± 0.9^{ab}	16.9 ± 0.7^{a}
Cnr $1^{-/-}$/Cnr $2^{-/-}$	1.5 ± 0.1	2 ± 0.1	5.5 ± 0.3^{c}	10.2 ± 0.1^{b}

734

735

736

737 Figure 4



738

739

740

741

742

743

744

745

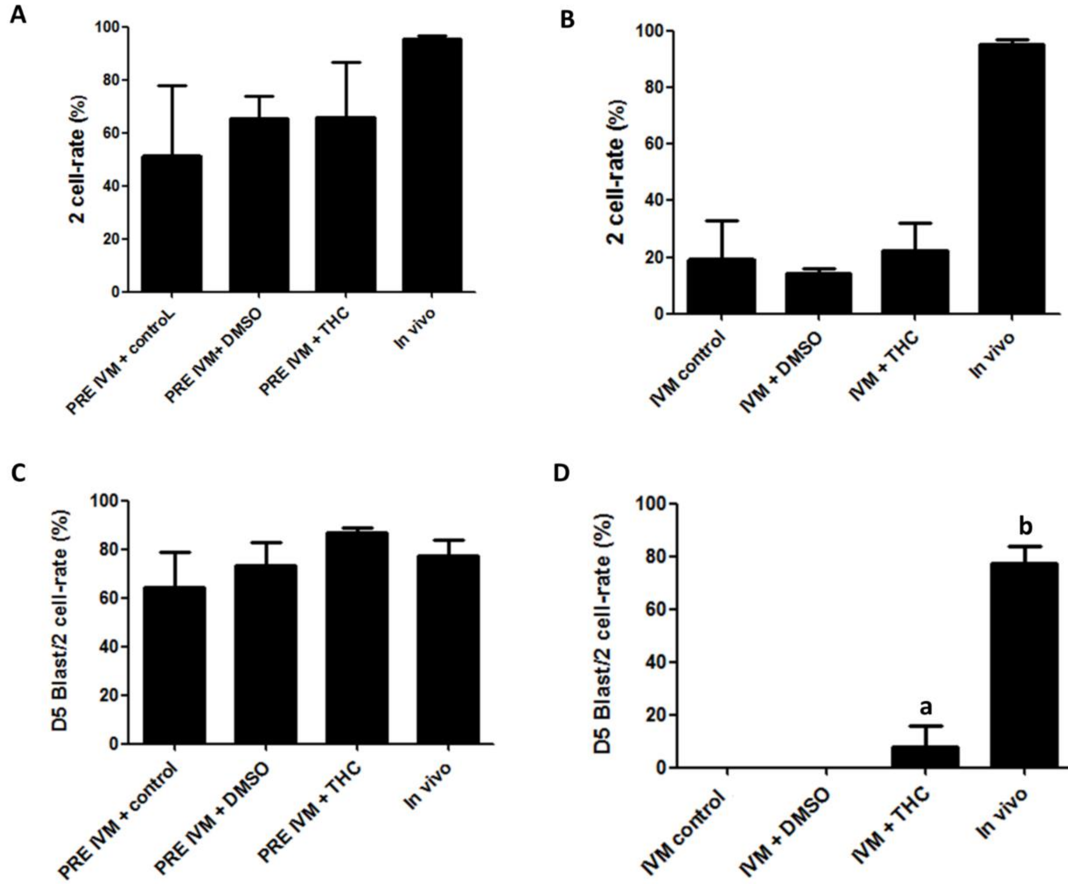
746

747

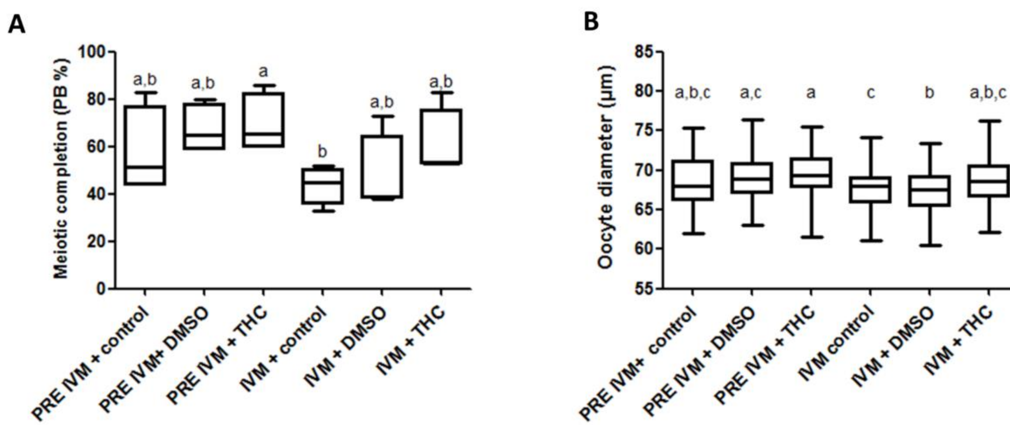
748

749 Figure 5

750



751



752