

1 **Antagonizing the corticotropin releasing hormone receptor 1 with**  
2 **antalarmin reduces the progression of endometriosis.**

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24

## 25 **Abstract**

26           Endometriosis is a disorder in which endometrial tissue is found outside the uterus  
27 causing pain, infertility and stress. Finding an effective and long-term treatment for  
28 endometriosis still remains one of the most significant challenges in the field. Corticotropin  
29 releasing hormone (CRH) is one of the main signaling peptides within the hypothalamic pituitary  
30 adrenal (HPA) axis released in response to stress. CRH can affect nervous and visceral tissues  
31 such as the uterus and gut via activation of two types of CRH receptors: CRHR1 and CRHR2.  
32 Our aim was to determine if blocking CRHR1 with antalarmin will reduce endometriosis  
33 progression. First, we induced endometriosis in female rats by suturing uterine horn tissue next  
34 to the intestinal mesentery and allowed to progress for 7 days. We determined that after 7 days,  
35 there was a significant increase in CRHR1 within endometriotic vesicles as compared to normal  
36 uterus. A second group of rats received endometriosis but also antalarmin (20 mg/kg, i.p.)  
37 during the first 7 days after surgery. As separate group of sham surgery rats served as controls.  
38 Endometriosis was allowed to progress until 60 days after surgery. At time of sacrifice, rats  
39 were tested for anxiety behaviors and endometriotic vesicles, and uterus were collected. Rats  
40 with endometriosis that received antalarmin significantly reduced the size (67% decrease) and  
41 number (30% decrease) of endometriotic vesicles. Antalarmin also prevented the increase in  
42 CRH and CRHR1 within endometriotic vesicles but not of glucocorticoid receptor.  
43 Behaviorally, endometriosis increased anxiety in the zero-maze test but antalarmin did not  
44 modify it. Our data provides the first demonstration for the effective use on CRHR1 antagonist  
45 for the treatment of endometriosis with promising effects for long-term therapy of this  
46 debilitating disease.

47

## 48 **Introduction**

49 Corticotropin releasing hormone (CRH) is one of the main signaling molecules of the  
50 hypothalamic pituitary adrenal (HPA) axis. CRH has a myriad of physiological effects that  
51 include behavioral, endocrine, autonomic and immune responses (1,2). CRH acts mainly by  
52 binding to CRH receptors type 1 (CRHR1) and type 2 (CRHR2) with a 10-fold affinity for the  
53 CRHR1 versus CRHR2 (3). CRH receptors belong to the superfamily of G-protein coupled  
54 receptors and typically effect cellular activity via coupling to adenylate cyclase (3). CRHR1 is  
55 abundant in the brain (4) as well as in adrenal glands, uterine and colonic tissues, and  
56 lymphocytes, among others (5–7). Eleven splice variants of the CRHR1 receptor have been  
57 identified (8), with a tissue specific expression pattern (9,10). In addition, the CRH paralog,  
58 urocortin 1 (UCN1) can bind and activate both the CRHR1 and R2 (11).

59 Due to the variety of physiological activities that the CRH system exerts, CRHR1  
60 antagonists have been clinically used for more than three decades for a variety of conditions. For  
61 example, CRHR1 antagonists have been tested for the treatment of disorders including  
62 depression (12), irritable bowel syndrome(13) (IBS), and proposed as a possible treatment for  
63 anxiety disorders (14). In fact, phase II/III clinical trials are undergoing or have been completed  
64 for depression, IBS and anxiety (15). Antalarmin is a CRHR1 antagonist that has been widely  
65 used in animal research to investigate CRH effects on reproduction, inflammation, addictive  
66 disorders, sleep disorders, among others (16). Antalarmin is a non-peptide molecule that readily  
67 crosses the blood-brain-barrier. Both, anti-stress and anti-inflammatory activities of antalarmin  
68 have been documented in animal studies (17).

69 Endometriosis is a chronic inflammatory disorder defined as the presence of endometrial-  
70 like tissue (e.g., glands and stroma) outside the endometrial cavity. This condition is

71 characterized by peritoneal inflammation resulting in severe and chronic pelvic pain, and often  
72 infertility (18). Endometriosis can be commonly misdiagnosed as irritable bowel syndrome  
73 (IBS)(19) due to overlap in common symptoms and perhaps mechanisms of disease progression  
74 involving aberrant activation of inflammatory cascades. The causes of endometriosis onset are  
75 unknown; however, a relationship between stress, hypothalamic pituitary adrenal axis (HPA)  
76 dysregulation, and endometriosis severity has been documented by others and our own work in  
77 the rat model of endometriosis (20–23). Strong evidence (from both human and animal studies)  
78 suggest that abnormal functioning of the HPA axis, release of CRH and/or the inflammatory  
79 response system disrupts feedback of both neuroendocrine and immune systems contributing to  
80 the development of the disease (24,25). CRH and CRH receptors are abundant in female  
81 reproductive tissues and this axis has been shown to regulate several reproductive functions  
82 (26,27), mostly mediating pro-inflammatory activities such as ovulation, luteolysis and  
83 blastocyst implantation (2). Despite the well-documented role of CRH receptor in stress related  
84 disorders, reproductive function and inflammation, no previous study has addressed the potential  
85 role of CRHR1 blockade in the treatment of endometriosis.

86 In the current study, we took advantage of the well-established auto transplantation rat  
87 model of endometriosis to investigate the effects of the CRHR1 receptor antagonist antalarmin in  
88 endometriosis. Given the role of CRHR1 in inflammation, we first tested whether this receptor  
89 was up regulated in ectopically implanted endometrium shortly after disease induction.  
90 Following this, we administered antalarmin, early during endometriosis establishment to test  
91 whether it could block vesicle formation in this model. We hypothesized that blockade of  
92 CRHR1 during the first week after endometriosis induction will reduce the initiation of  
93 inflammatory processes that lead to endometriosis vesicle establishment and subsequent

94 development. In addition, we hypothesized that CRHR1 blockade at central levels may reduce  
95 stress associated behaviors previously linked to endometriosis such as anxiety and depression.  
96 Data presented herein suggest that the CRHR1 antagonist antalarmin might function as a  
97 completely new line of treatment for women suffering from endometriosis, highly needed in the  
98 management of this debilitating and still incurable disease.

99

## 100 **Materials and Methods**

### 101 **Animals and experimental groups**

102 Female Sprague Dawley rats of 60 days old were used in the experiments (weighing  
103 between 190 – 220 grams). Rats were housed two per cage and kept in a 12-hour light/dark  
104 cycle with food and water ad libitum. All experimental procedures were approved by the Ponce  
105 Health Sciences University and the University of Texas at Rio Grande Valley Institutional  
106 Animal Care and Use Committees and adhere to the NIH Guide for the Care and Use of  
107 Laboratory Animals. Rats were weighed twice per week to monitor their adequate development  
108 and once a week during drug administration period. Group 1 consisted of 32 female rats that  
109 underwent endometriosis induction or sham surgery (described below) and were sacrificed 7  
110 days after surgery (surgery - Day 0; Figure 1). This experiment was carried out to quantify the  
111 levels of CRHR1 receptors at 7 days after surgery and thus assess the feasibility of using a  
112 CRHR1 antagonist during this period. Group 2 consisted of 40 female rats that underwent  
113 endometriosis induction or sham surgery and received the CRHR1 antagonist antalarmin or  
114 vehicle control from days 1-7 after surgery. Parallel to this group, a separate group of 11 rats  
115 underwent sham surgery and were left untreated until day 60 after surgery. Only rats with

116 regular estrous cycles were used in the experiments as assessed by vaginal smear lavage during  
117 the 7 days prior to surgery and 7 days before sacrifice.

118

119 **Figure 1: Diagram of experimental protocols.** Rats in Group 1 received  
120 endometriosis or sham surgery and were allowed to progress for 7 days. Rats in Group 2  
121 received sham surgery or endometriosis. Rats from the endometriosis group were injected with  
122 either vehicle or antalarmin for seven consecutive days after surgery and allowed to progress  
123 for 53 additional days. During the endometriosis progression period, animals were undisturbed  
124 except for weekly weighing done at the same time of cage changing.

125

## 126 **Drug administration**

127 Animals in Group 2 received 1 daily injection (between 09:00 – 10:00 hours) for 7  
128 consecutive days of antalarmin (N-butyl-N-ethyl-[2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7H-  
129 pyrrolo[2,3-d]pyrimidin-4-yl]-amine; Tocris Bioscience, Bristol, UK) suspended in a vehicle  
130 composed of 10% Tween 80 and distilled water and given intraperitoneally at 20 mg/kg in a  
131 volume of 1 ml/kg. This dose of antalarmin was chosen based on previous published work from  
132 Cippitelli, et al., (2012) (28) dose-response study showing that the 20 mg/kg i.p. administration  
133 was the most effective dose to block withdrawal behaviors, thus it readily entered the blood-  
134 brain barrier in addition to the peripheral tissues. Antalarmin injections started the morning  
135 following surgery. After day 7, rats were left undisturbed except for cage change and weighing  
136 twice a week.

## 137 **Endometriosis induction**

138 Endometriosis was surgically induced as previously described elsewhere (29,30). Briefly,  
139 rats were anesthetized with isoflurane and four pieces of the right uterine horn were auto  
140 transplanted to 4 different blood vessels in the intestinal mesentery. The control group were sham  
141 operated animals for which the right uterine horn was massaged for 2 minutes and sutures were  
142 placed in the intestinal mesenteric area with no uterine implants. For group 1, sham or  
143 endometriosis operated rats were allowed to progress for 7 days after surgery. For group 2,  
144 endometriosis was allowed to progress for 60 days before sacrificing, similar to our previous report  
145 (31;30).

## 146 **Behavioral assessment**

147 One day before behavioral assessment and sacrifice a subset of rats (6 vehicle and 6  
148 antalarmin) were subjected to an acute episode of swim stress of 10 min and compared to no  
149 stress controls to assess how rats respond to acute activation of the HPA axis. For this, animals  
150 were placed in a Plexiglas tank for 10 min in water at 25°C (modified from (32)). Rats were  
151 towel dried and kept in warm cage after swim until fur dried. The next day, we used two  
152 behavioral tasks to assess anxiety behaviors. The open field test is used to quantify exploratory  
153 and locomotor activity of a rodent in an open arena. The apparatus used was a square wood arena  
154 (91 x 91 x 38 cm) with overhead light illumination and video monitoring to record animal  
155 activity using Any-Maze software (Stoelting, Wood Dale, Illinois). We quantified the following  
156 behaviors during 20 minutes: 1) total distance moved, 2) time spent moving, 3) time spent in the  
157 center of the arena, 4) time spent near the walls of the arena (defined by the 15 cm of floor arena  
158 closest to the walls) and 5) total fecal pellets. The more time the animal spends in the center of  
159 the arena compared to the space adjacent to the wall is considered as having less anxiety. At the

160 end of the testing period, animals were returned to the home cage and after a 5-min break were  
161 tested in the elevated zero-maze.

162           The elevated zero-maze is very similar to the more traditional elevated plus maze test,  
163 with the advantage of not having a neutral (undefined) zone in the middle. The apparatus  
164 consisted of a circle with an arm width of 10cm and elevated 40cm from floor. Two sections of  
165 the circle were open without walls and two enclosed by 40 cm high walls. Rats were placed in  
166 the intersection of an open arm, facing the closed arm and opposite to the experimenter. Rats  
167 were allowed to run the maze for 5 consecutive minutes and recorded using the Any-Maze  
168 software (Stoelting, Wood Dale, Illinois). The following parameters were analyzed by the Any-  
169 Maze program: 1) total distance travelled in the maze, 2) time spent in the open/closed arms and  
170 2) number of entries made by the rodent onto the open/closed arms. When 60% of the animal  
171 body entered the arm, the program counted it as an entry. In addition, we quantified total fecal  
172 pellets in the maze. The more time the animal spends in the open arms is considered as having  
173 less anxiety. After the 5-min testing period, the rat was returned to the home cage and  
174 immediately anesthetized with an overdose of 65% sodium pentobarbital to proceed with  
175 laparotomy. The maze was thoroughly cleaned with 70% alcohol solution and allowed to dry  
176 before testing the next rat.

## 177 **Sample Collection and Processing**

178           We verified that the animals were deeply anesthetized. Rats were weighed, and a  
179 cytological smear taken to verify stage of the estrous cycle. The peritoneal and thoracic cavities  
180 were opened, and a blood sample was collected directly from the heart. Following this, we  
181 collected peritoneal fluid using a sterile plastic pipette. Then, we examined for the presence of  
182 endometriosis vesicles. The implants that developed into vesicles were excised from the



183 mesentery, weighed and measured using a digital caliper. Classification of vesicles was carried  
184 out as previously described (23,33) and assigned the following grades: grade 1= disappeared; grade  
185 2= 0.01- 1.99 mm; grade 3= 2 - 4.49 mm; grade 4= 4.5 – 5.99 mm; grade 5= 6.0 mm or larger. In  
186 sham animals, we counted and collected the empty suture sites. In addition to the endometriosis  
187 vesicles, we collected the adrenal glands, removed all surrounding fatty tissue and weighed them.  
188 We also collected tissues from colon, and the left uterine horn. All tissues were flash frozen and  
189 stored at -80° until further processing.

## 190 **Enzyme linked immunosorbent assays (ELISA)**

191 Serum and peritoneal fluid samples from animals were tested for levels of corticosterone,  
192 adrenocorticotrophic hormone (ACTH) and the pro-inflammatory cytokine IL-6 following  
193 instructions in the commercial kits. The following kits were used: Corticosterone rat/mouse kit  
194 (Cat. # 79175; IBL America, Minneapolis, MN); Rat IL-6 pre-coated ELISA kit (Cat. #437107;  
195 BioLegend, San Diego, CA); Mouse/rat ACTH ELISA kit (Cat. #AC018T-100, Calbiotech, El  
196 Cajon, CA).

## 197 **RNA isolation and cDNA synthesis**

198 Endometriosis vesicles and normal uterine tissue, from endometriosis or sham rats were  
199 lysed in an RLT buffer (Qiagen, Germantown, MD) using the Bullet Blender Tissue  
200 Homogenizer (Next Advance, Averill Park, NY). The total RNA from the lysates were extracted  
201 according to RNAeasy Mini Kit manufacturer's protocol (Qiagen, Germantown, MD). RNA  
202 concentration and purity were measured on a NanoDrop 2000 UV spectrophotometer (Thermo  
203 Scientific, Wilmington, USA). Concentration and quality of RNA samples were acquired based  
204 on the ratio of absorbance at 260/280 nm in the spectrophotometer. To carry out the synthesis of

205 cDNA from RNA samples a total reaction volume of 20  $\mu$ l including 0.1 $\mu$ g of total RNA  
206 concentration and synthesis reagents was used. We used the iScript cDNA Synthesis Kit  
207 according to manufacturer's protocol (Bio-Rad, Hercules, CA). Reactions were carried out in T-  
208 100 thermal cycler (Bio Rad, Hercules, CA). RT-PCR running method was as follows: 25°C for  
209 5 min, 42°C for 30 min, 85°C for 5 min. Samples were stored at -80° C for later experimentation  
210 or qRT-PCR.

## 211 **Quantitative real time PCR protocol**

212 We used real time quantitative PCR (qPCR) to evaluate changes in mRNA expression.  
213 For this, we used 25  $\mu$ l of a total volume of reaction assay with 1:10 dilution of cDNA with IQ  
214 SyBR Green Supermix (Bio Rad Hercules, CA) in a 96 well plate according to the manufacturer  
215 protocol and amplified in a Quant Studio 12K Flex Real time PCR System (Applied Biosystems,  
216 Carlsbad, CA). Commercial primers for CRH, UCN1 CRHR1, CRHR2 and GR were purchased  
217 from Qiagen (Germantown, MD). Real time PCR cycles protocol was as follows: 95°C for  
218 10 min. for enzyme activation followed by 40 cycles of denaturing at 95 °C for 15 sec. and  
219 annealing at 60°C for 1 min. All changes in gene expression were normalized against GAPDH  
220 of each sample. CT values and changes per gene expression level were automatically analyzed  
221 by the Quantstudio 12K Flex Software (Applied Biosystems, Carlsbad, CA). All samples were  
222 run in duplicate. For comparison purposes, mRNA from sham rats were always run within the  
223 same plate as experimental samples from vehicle treated and antalarmin treated groups.

## 224 **Statistical analyses**

225 GraphPad Prism 6.0 (Graph-Pad Software, San Diego, California) was used to prepare  
226 graphs and run statistical analyses. Data is presented as mean difference  $\pm$  SEM and a p value

227 <0.05 was considered statistically significant. The variability between groups was first assessed  
228 followed by a test for outlier values. A Student t-test was used for comparisons between two  
229 groups and when group variability was significantly different, a Welch corrected t-test was used.  
230 A one-way analysis of variance (ANOVA) was used to compare behaviors. A one-sample t-test  
231 against the sham rats value normalized to 1.0 was used to assess qRT-PCR results. A repeated  
232 measures one-way ANOVA was used to compare changes in weight gain between treatment  
233 groups across time.

234

## 235 **Results**

### 236 **Shortly after endometriosis induction, CRHR1 is elevated**

237 To evaluate the early endometriotic vesicle development, we induced the disease and  
238 sacrificed the animals after 7 days. It is during this early development period that we  
239 hypothesized that the major changes in CRHR1 will be observed. At 7 days, we observed that  
240 93.7 % of the implants have created a large vesicle, which in most cases was very large and filled  
241 with fluid. In the sham group, only sutures were observed as no uterus was transplanted. Table  
242 1 shows the morphological characteristics of the observed vesicles at 7 days post-induction  
243 surgery. We quantified the CRHR1 mRNA in the vesicles as compared to the normal uteri of the  
244 same rats and that of sham surgery controls. CRHR1 mRNA in endometriosis vesicles showed a  
245 two-fold increase as compared to normal uterus of sham rats ( $t=2.934$ ,  $d.f.=6$ ,  $p<0.05$ ; Figure 2).

**Table 1: Characteristics of endometriosis vesicles at seven days after auto-transplantation surgery**

Endo vesicles at 7 days	Percent developed (%)	Total weight (g)	Total area (mm <sup>2</sup> )	Total volume (mm <sup>3</sup> )
Average per rat ± S.E.M.	93.75 ± 2.80	1.19 ± 0.25	160.92 ± 15.64	2190.25 ± 1110.58

246 In contrast, the mRNA levels in uteri of rats that received endometriosis were not different from  
247 the uteri of sham rats ( $t=0.829$ , d.f.=6,  $p>0.05$ ; Figure 2).

248

249 **Figure 2: qRT-PCR of CRHR1 within endometriosis vesicles and uterus of the**  
250 **endometriosis rats and sham rats.** At 7 days after the autotransplantation surgery to induce  
251 endometriosis, we observed a significant two-fold increase in CRHR1 mRNA within  
252 endometriosis vesicles only.

253

## 254 **Antalarmin did not affect stress reactivity or anxiety behaviors**

255 To block the significant increase in CRHR1 receptor within the endometriotic vesicles,  
256 we administered antalarmin during the first 7 days after endometriosis induction surgery. After  
257 that, we allowed the endometriosis to progress for 53 additional days. At Day 59 after  
258 endometriosis surgery, a subset of the animals (6 vehicle and 6 antalarmin) were subjected to a  
259 5-min swim stress challenge. Antalarmin treated animals were not different from the vehicle  
260 control group in any of the behavioral parameters measured such as immobility, swimming,  
261 struggling behaviors and diving episodes (data not shown). Therefore, rats tested in the stress  
262 challenge were collapsed within the not-tested ones within treatment groups (vehicle or  
263 antalarmin). The next day, all animals were tested using the open field and the zero maze to  
264 evaluate trait and state anxiety, respectively. The total distance traveled (Figure 3A) was  
265 significantly lower in rats that received antalarmin as compared to the sham group ( $F_{(2,45)}= 3.507$ ,  
266  $p< 0.05$ ), but the amount of time rats spend in the center of the open field arena was similar  
267 between groups ( $F_{(2,45)}= 0.12$ ,  $p> 0.05$ ; Fig. 3B). On the zero maze, a higher locomotor activity  
268 was observed for both groups of rats with endometriosis that received vehicle or antalarmin

269 compared to the sham group ( $F_{(2,49)} = 6.01$ ,  $p < 0.01$ ; post-hoc,  $p \leq 0.01$  both comparisons; Fig.  
270 3C). Despite a higher locomotor activity, there was a strong trend for both groups of rats with  
271 endometriosis to spend less time in the open arms of the zero maze ( $F_{(2,49)} = 2.82$ ,  $p = 0.06$ ; Fig.  
272 3D) suggesting increased anxiety. In summary, antalarmin administered shortly after  
273 endometriosis induction does not have long-term effects on anxiety behaviors. However,  
274 endometriosis tended to increased anxiety in the zero-maze compared to sham controls,  
275 regardless of treatment.

276

277 **Figure 3: Behavioral assessment for anxiety.** Rats that received sham surgery or  
278 endometriosis and antalarmin or vehicle treatment were tested in the open field (A and B) or the  
279 elevated zero maze (C and D). In comparison to sham, we observed a significant decrease in  
280 locomotor activity of the group that received antalarmin. However, time spent in the center of  
281 the open field was not different between groups. In the elevated zero maze, a significant  
282 increase in locomotion was observed for rats that had endometriosis as compared to sham,  
283 regardless of the drug treatment (C). Rats with endometriosis, regardless of drug treatment,  
284 showed a trend towards spending less time in the open segment of the zero maze as compared  
285 to sham group. \* represents  $p < 0.05$ .

286

287

## 288 **Antagonizing CRHR1 early in endometriosis produced a significant** 289 **decrease in vesicle development**

290 Antalarmin administration during the 7 days after endometriosis induction resulted in a  
291 30% significant decrease in the number of developed endometriosis vesicles at 60 days (Welch

292 corrected t-test,  $t = 3.38$ ,  $d.f. = 22.86$ ,  $p < 0.01$ ; Fig. 4A). The total weight of endometriosis  
293 vesicles (sum per rat) in the antalarmin treated group was 67% less than the vehicle control  
294 group ( $t = 3.175$ ,  $d.f. = 38$ ,  $p < 0.01$ ; Fig. 4B). The reduced weight was a direct result of the  
295 smaller size of the vesicles in average volume (68% difference, Welch corrected,  $t = 2.515$ ,  $d.f. =$   
296  $25.39$ ,  $p < 0.05$ ; Fig. 4C) and area (55% difference,  $t = 3.067$ ,  $d.f. = 38$ ,  $p < 0.01$ ; Fig. 4D) per rat.  
297 Similar to our previous reports, (21,30) we classified the vesicles in grades (1 – 5) based on a  
298 length scale for each vesicle where 1 denotes an implant that disappeared and 5 an implant that  
299 developed into a vesicle equal or larger than 6mm (Fig. 4E). In the antalarmin treated group  
300 compared to the vehicle treated control, there was a larger percentage of endometriosis vesicles  
301 that disappeared, as well as a reduced percentage of vesicles of grade 3 and 5. In summary,  
302 seven days of antalarmin treatment resulted in a smaller percentage of endometriosis implants  
303 developed and those that did develop were significantly smaller in size compared to vehicle  
304 treated control group.

305

306 **Figure 4: Morphological characteristics of endometriosis vesicles.** (A) The percent  
307 of implants that developed into vesicles was significantly lower in the antalarmin treated group  
308 compared to the vehicle control group. (B) The total weight of all vesicles per rat was smaller  
309 for the antalarmin treated rats. (C) The average vesicle volume per rat was significantly smaller  
310 for the antalarmin treated group compared to the vehicle control group. (D) The average vesicle  
311 area per rat was significantly smaller in the antalarmin group compared to the vehicle group.  
312 (E) Vesicles that developed were classified by grade based on a scale by size. \*  $p < 0.05$ , \*\*  $p <$   
313  $0.01$ .

314

315 **Antalarmin produced a long-lasting increase in serum ACTH**

316 At the time of sacrifice, we collected peritoneal fluid and blood serum from rats to later  
317 examine how the treatment with antalarmin might have altered HPA axis markers and also the  
318 pro-inflammatory cytokine IL-6. Corticosterone was slightly elevated in rats with endometriosis  
319 treated with vehicle or with antalarmin, however this apparent difference, did not reach statistical  
320 significance ( $F_{(2,43)} = 1.99, p > 0.05$ ; Fig. 5A). On the other hand, we observed significantly  
321 elevated levels of serum adrenocorticotrophic hormone (ACTH) in rats that received antalarmin.  
322 ANOVA statistical test revealed a significant main effect of drug treatment ( $F_{(2,39)} = 518, p < 0.05$ ;  
323 Fig. 5B). Post hoc tests showed that the groups of rats that received antalarmin was significantly  
324 higher than sham and vehicle treated groups ( $p < 0.05$  both comparisons). While anti-  
325 inflammatory effects of antalarmin have been reported, the short treatment of antalarmin 53 days  
326 before sacrifice did not produce any change in IL-6 in peritoneal fluid, which is in direct contact  
327 with the endometriotic vesicles (Fig. 5C).

328

329 **Figure 5: Serum and peritoneal markers of stress and inflammation.** We used  
330 ELISA to measure (A) corticosterone and (B) adrenocorticotrophic hormone (ACTH) in the serum  
331 of rats and (C) IL-6 in peritoneal fluid at the time of sacrifice. There was no significant difference  
332 between groups in serum corticosterone levels at the time of sacrifice. However, a significantly  
333 higher level of ACTH was observed for rats that received antalarmin compared to the two other  
334 groups. No differences in IL-6 were observed between groups. \* represents  $p < 0.05$ .

335

336 **Antalarmin blocked mRNA increase in CRH and CRHR1 of uterus**  
337 **and vesicles.**

338 We quantified the mRNA for urocortin and CRH, which are the main agonists of the  
339 CRHR1 receptor, within developed endometriosis vesicles in rats from both treatment groups  
340 using qRT-PCR. As a comparative parameter, we also quantified the mRNA with the uteri of the  
341 same animals and used uteri of sham controls to normalize the data. We observed a significant  
342 two-fold increase in CRH for vehicle treated rats, both in uterus (one sample t-test:  $t= 2.66$ , d.f.=  
343 13,  $p< 0.05$ ) and vesicles ( $t= 2.29$ , d.f.= 13,  $p< 0.05$ ; Fig. 6A). However, this increase was not  
344 observed in the antalarmin treatment group (Fig. 6A). In contrast, UCN1 mRNA was not altered  
345 in any of the groups measured (Fig. 6B).

346

347 **Figure 6: mRNA levels measured by qRT-PCR from the uterus and endometriosis**  
348 **vesicles.** (A) corticotropin releasing hormone (CRH). (B) Urocortin 1 peptide. Data normalized  
349 to the uterus of sham rats. \* represents  $p< 0.05$  compared to sham rats' uterus.

350

351 The mRNA of the CRHR1 receptor measured in endometriosis vesicles of the vehicle  
352 group was significantly increased as compared to sham uterus ( $t= 3.45$ , d.f.= 8,  $p< 0.01$ ; Fig.  
353 7A), but this increase was not observed in the vesicle of antalarmin treated rats ( $p>0.05$ ). Due to  
354 the intricate balance of CRH receptor activity in uterine tissue, we also quantified the CRHR2  
355 receptor mRNA. For this receptor, we observed a small but significant fold increase in mRNA  
356 only in the vesicles of vehicle treated animals ( $t= 3.2$ , d.f.= 8,  $p< 0.05$ ; Fig. 7B). No other  
357 changes were observed for CRHR2. The glucocorticoid receptor showed an interesting pattern  
358 with a significant mRNA fold increase that was observed in vesicles from both, the vehicle  
359 treated ( $t= 2.88$ , d.f.= 8,  $p< 0.05$ ; Fig. 7C) and antalarmin treated ( $t= 4.65$ , d.f.= 8,  $p< 0.01$ ; Fig.  
360 7C) groups, but no changes in uterus.

361



362 **Figure 7: mRNA levels measured by qRT-PCR from the uterus and endometriosis**  
363 **vesicles.** (A) corticotropin releasing hormone receptor type 1 (CRHR1). (B) corticotropin  
364 releasing hormone receptor type 2 (CRHR2). (C) glucocorticoid receptor (GR). Data  
365 normalized to the uterus of sham rats. \* represents  $p < 0.05$  compared to sham rats' uterus.

366

367 **Antalarmin decreased body weight and the decrease persisted in**  
368 **treated animals**

369 Inconsistencies in the effect of antalarmin on male rodent body weight have been  
370 reported (34,35). We monitored the rat weight changes during the drug administration period  
371 and every week afterwards during the development of endometriosis. By day 7 and for 2 days  
372 after antalarmin has stopped, rats receiving the drug weighed significantly less than vehicle  
373 control groups (Repeated measures ANOVA of treatment:  $F_{(1,38)} = 7.615$ ,  $p < 0.01$ , post hoc,  
374  $p < 0.01$  on day 7 and  $p < 0.05$  on days 8 and 9; Fig. 8A). While the rats maintained a constant  
375 weight gain rate, by week 7 and 8 after endometriosis induction (Figure 8B), a significantly  
376 lower weight gain compared to vehicle group was also recorded ( $F_{(1,38)} = 33.89$ ,  $p < 0.001$ , post  
377 hoc,  $p < 0.05$  for weeks 6 and 7).

378

379 **Figure 8: Percent increase in weight for rats with endometriosis treated with**  
380 **antalarmin or vehicle** (A) After seven days of treatment, antalarmin treatment significantly  
381 decreased the weight of the rats and this difference persisted for two additional days after the  
382 drug treatment has stopped. (B) During the subsequent weeks, antalarmin group remained  
383 weighing less than control group and by weeks 6 and 7 this difference reached statistical  
384 significance. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

## 385 **Discussion**

386 Here we present evidence that a short treatment with the CRHR1 antagonist antalarmin  
387 had long-term efficacy in reducing endometriosis with minimal changes in behavior. More  
388 importantly, the vesicles that developed were significantly smaller, suggesting that antalarmin  
389 interfered with both the establishment and the development of vesicles. We also demonstrated  
390 that antalarmin prevented the increase in CRH and CRHR1 mRNA within endometriosis vesicles  
391 as compared to vehicle treated rats that lasted for almost 2 months after treatment stopped. This  
392 suggests that a short treatment might produce long-lasting effects for the treatment of this  
393 disease. To our knowledge, our work provides the first *in vivo* evidence of efficacy and use of  
394 CRHR1 antagonist antalarmin for the treatment of endometriosis.

395 In the present study, only one time point was tested corresponding to a major increase in  
396 CRHR1 mRNA, early in endometriosis development, as demonstrated herein. Sampson's theory  
397 postulates that retrograde menstruation leads to endometriosis. Based on this theory, during  
398 every menstruation, there is opportunity for new endometriosis implants to develop. Therefore,  
399 we suggest that in the clinical scenario, treatment with CRHR1 antagonist would need to be used  
400 right after laparoscopic surgery or for several years, similar to contraceptive pills, in order to be  
401 effective. In our animal model, we observed that about 60% of the implants sites developed into  
402 endometriosis vesicles. Perhaps a longer or continuous treatment with the CRHR1 antagonist  
403 might have produced a larger decrease in endometriosis vesicle development or even completely  
404 abolish it. In the clinical setting, there is a significant lag in the diagnosis of endometriosis,  
405 which is on average 7 years after symptoms appear. Our work opens the possibility for future  
406 testing of antalarmin or other CRHR1 antagonist at later time points in disease progression.

407 Not all CRHR1 antagonists are equal. The clinical use of CRHR1 antagonists has been  
408 limited by several factors that include lack of consistent efficacy (36,37), elevated tissue  
409 accumulation and prolonged half-life (38,39). Recently, a group of orally administered CRHR1  
410 antagonists have been shown to have high bioavailability and low lipophilicity in animal models  
411 of IBS (40). The availability of these new antagonists opens significant possibilities for the  
412 advancement of testing new CRHR1 compounds in endometriosis. However, certain challenges  
413 still remain. Eleven isoforms of the CRHR1 receptor have been identified in humans (8), and  
414 splicing of CRHR1 seems to be tissue specific. For example, CRHR1 $\beta$  is present in pituitary  
415 myometrium and endometrium but not in adrenal, placenta or synovium (41,42). It still needs to  
416 be determined whether ectopic endometrium will display a different profile of CRHR1 splice  
417 variants as compared to eutopic endometrium both in pre-clinical studies as well as in the clinical  
418 setting.

419 One of the most interesting findings of the current study was the elevated levels of ACTH  
420 in plasma that persisted even after antalarmin administration stopped. However, this elevated  
421 response was not followed by an increase in plasma corticosterone (see Fig.5). ACTH binds to  
422 the melanocortin receptor type 2 (MC2) to stimulate the release of glucocorticoids from the  
423 adrenal glands (43). In human endometrium, all five types of melanocortin receptors have been  
424 found (MC1-5) and when exposed to ACTH, decreased vascularity was observed in cultured  
425 decidual biopsies (44). Therefore, some of the long-term effects of antalarmin on decreasing  
426 endometriosis progression in our current study might be attributed to an increased level of  
427 circulating ACTH, resulting in decreased endometrial vesicle development. While further  
428 experiments are necessary to elucidate the increase in ACTH without a concomitant increase in  
429 corticosterone observed herein, the most plausible explanation is a de-sensitization of intra-

430 adrenal signaling system. In support of this, there is evidence that human adult adrenal tissues  
431 express, ACTH, CRH, CRHR1 and CRHR2 mRNA and that exposure of adrenal cells to  
432 antalarmin blocks the production of cortisol (45). Therefore, CRHR1 are involved in the control  
433 of glucocorticoid secretion, and antalarmin administration might have led to long-term  
434 dampening or desensitization within adrenal tissues.

435         Long-term changes in behavior due to the antalarmin administration were minimal. This  
436 suggest that compensatory activity most likely occurred in the amygdala and/or other regions  
437 involved in controlling anxiety-like behaviors. A recent study using intracerebroventricular  
438 administration of antalarmin showed that blocking CRHR1 provides neuroprotection and blunts  
439 neuroinflammation resulting from global cerebral ischemia (46). Blocking CRHR1 in the  
440 hippocampus results in a reduction of excitatory activity onto CA3 pyramidal cells in  
441 hippocampus (47). Clinically, CRHR1 signaling has been implicated in mediating abnormal  
442 brain responses to expected abdominal pain in patients with IBS (48). Based on the significant  
443 role of CRHR1 in homeostasis and behaviors, one of the challenges is to optimize CRHR1  
444 peripheral blockade, while producing minimal changes in the brain. This provides an  
445 opportunity for drug design that targets peripheral blockade of CRHR1 but prevents lasting  
446 effects in brain and behavior.

447         One of the challenges in the clinical setting is to decrease endometriosis sites, while still  
448 preserving reproductive abilities. Antalarmin has been shown in rodents to reduce the number of  
449 implantation sites by 70% (49) by a Fas-ligand immune tolerance dependent mechanism (50). It  
450 is possible that antalarmin treatment will compromise reproductive abilities. However, the long-  
451 lasting effect of antalarmin in the current study opens the possibility of developing short-term

452 treatments that will provide long-lasting protection and allow for reproductive abilities to return  
453 to normal. This still remains to be tested.

## 454 **Conclusion**

455 A single week of antalarmin treatment was effective in reducing endometriosis in the rat  
456 model by reducing the number of developed vesicles by 30 % and the size of the vesicles that  
457 developed by 67%. CRHR1 Inhibitors are pharmacological agents that are advanced in the  
458 pipeline of clinical trials in safety and efficacy profiles for other inflammatory disorders such as  
459 IBS. Our study opens the possibility for a new application of CRHR1 inhibitors for the  
460 treatment of endometriosis. We predict that translation of our work into the clinical application  
461 can produce significant benefits for many women that suffer from endometriosis.

462

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472

473

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Fig. 1. Torres-Reveron, et al.

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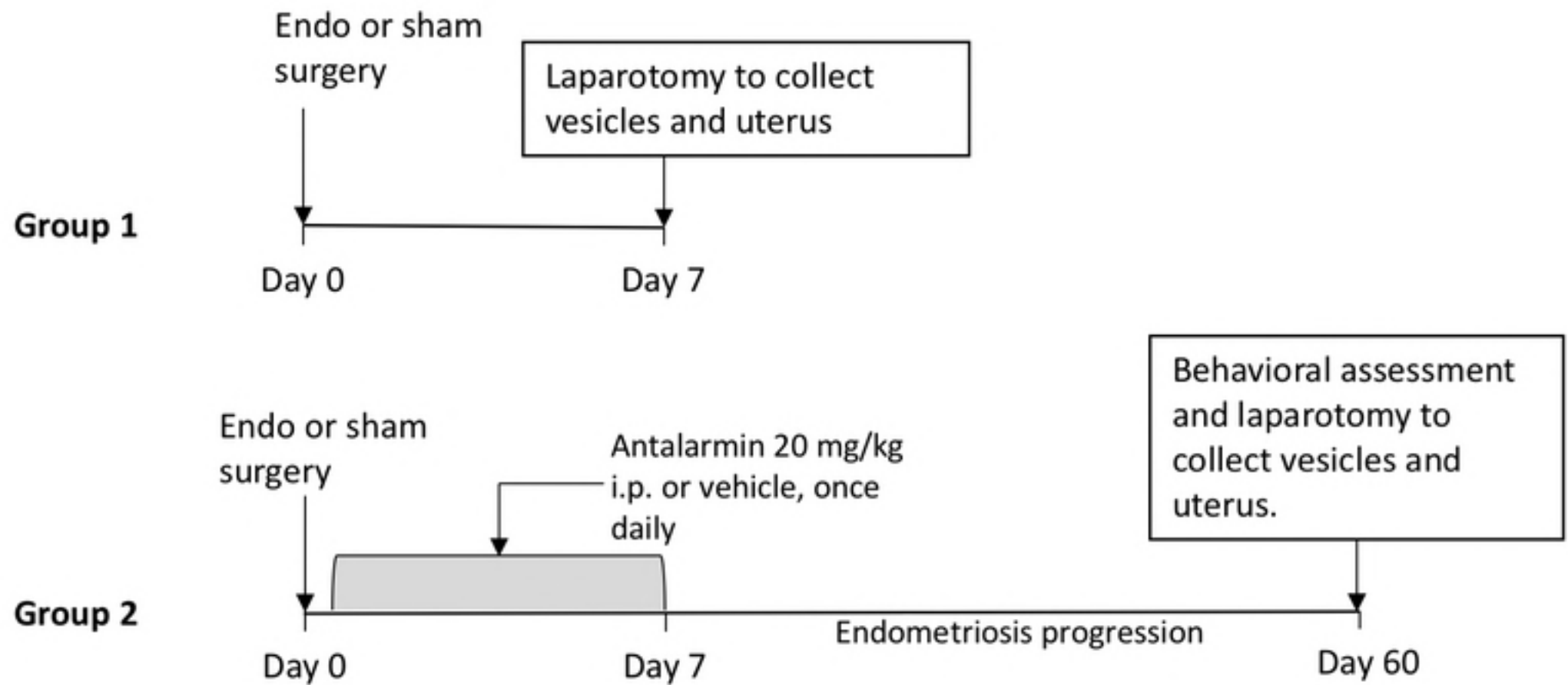


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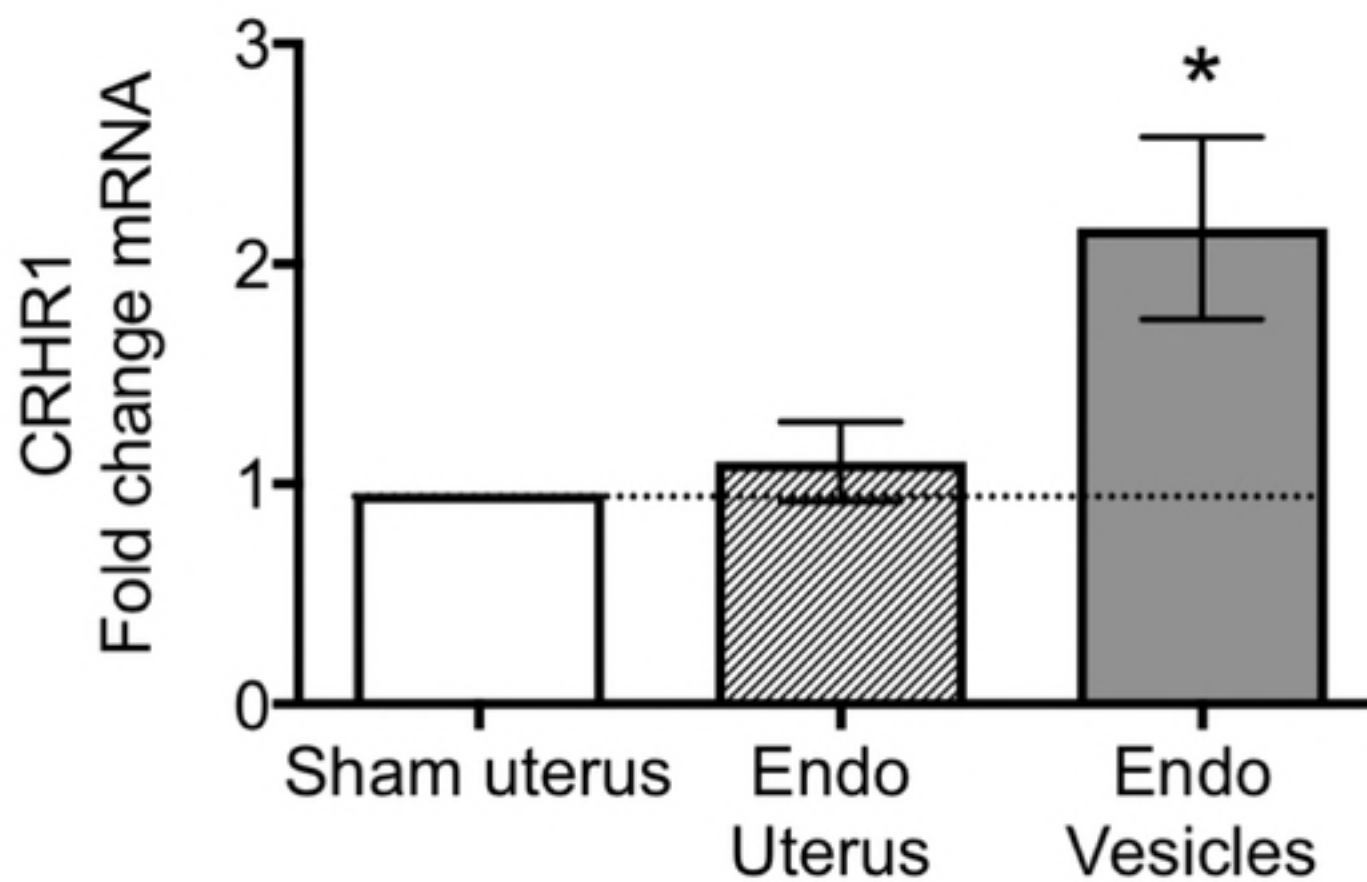


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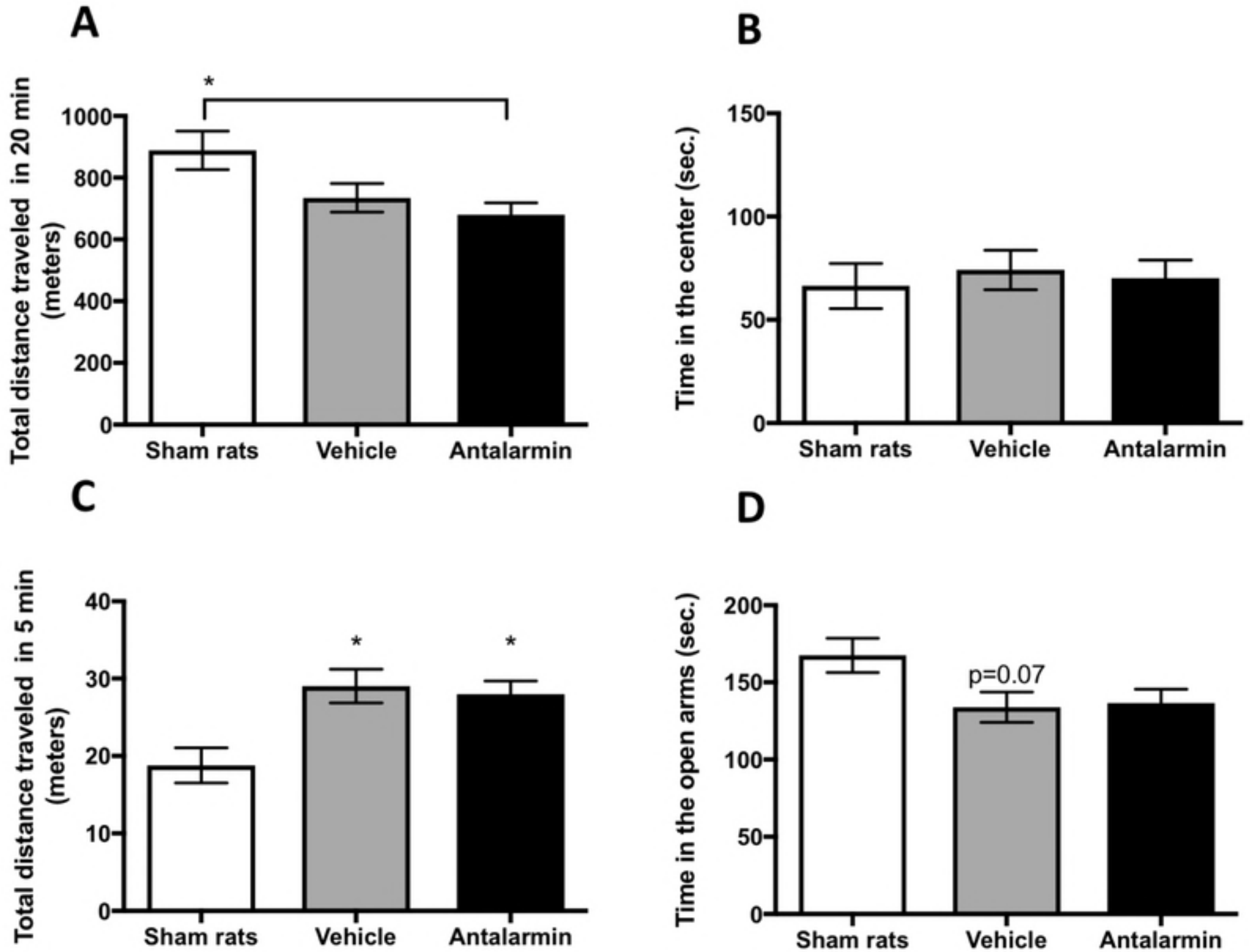


Fig. 4. Torres-Reveron, et al.

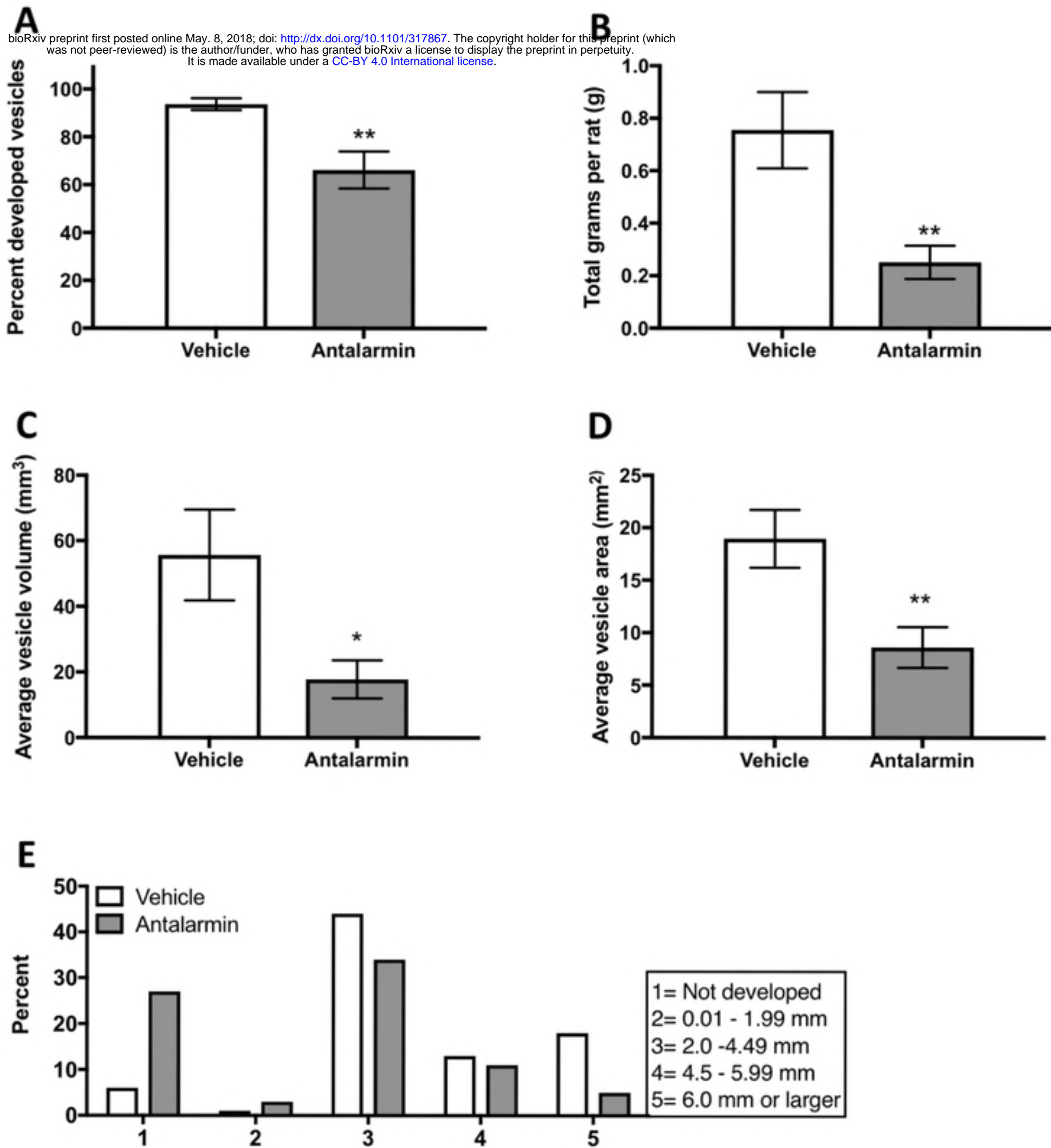
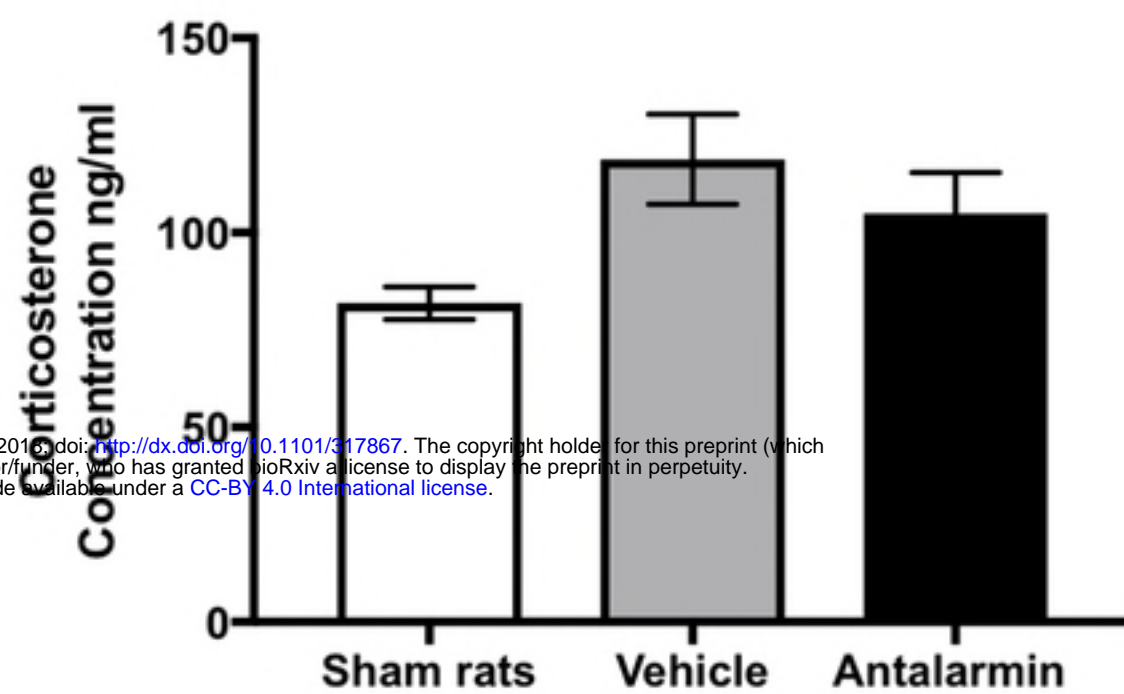


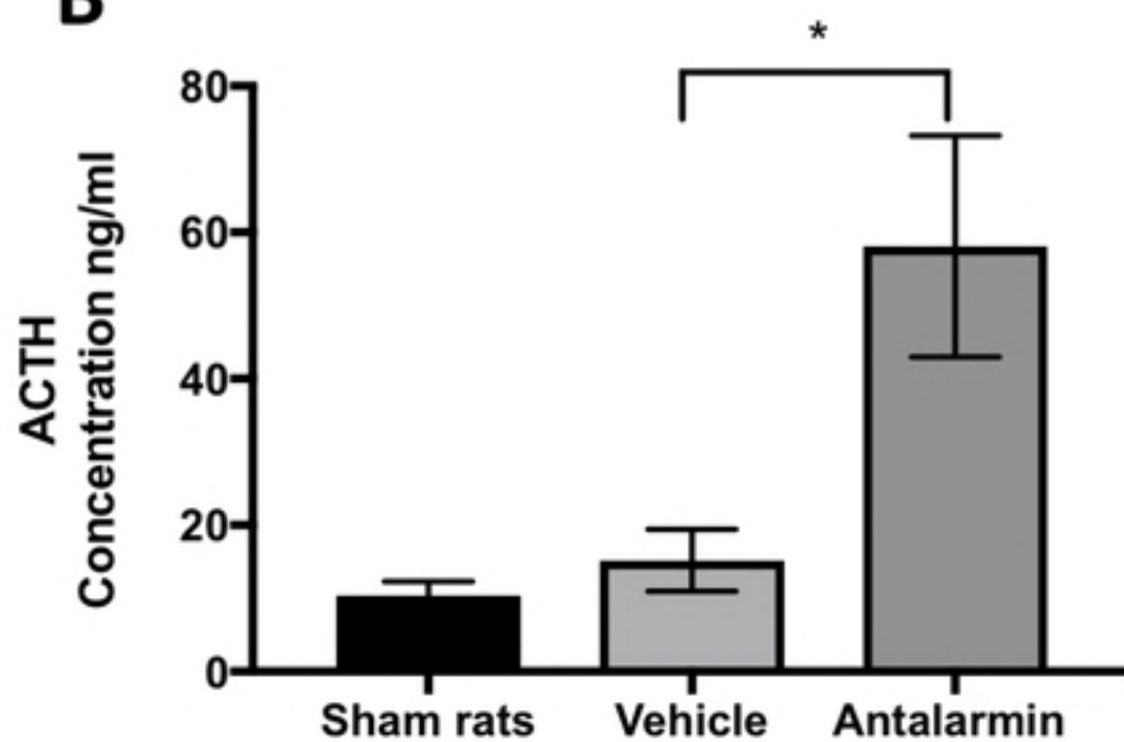
Fig. 5. Torres-Reveron, et al.

**A**



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**B**



**C**

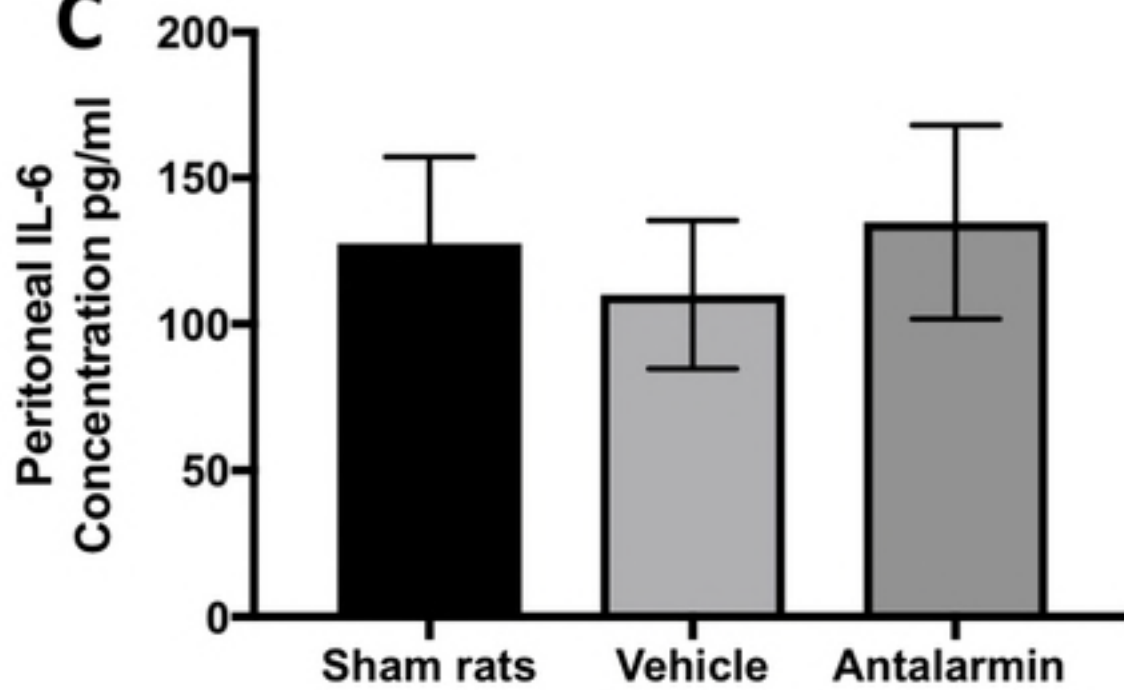




Fig. 6. Torres-Reveron, et al.

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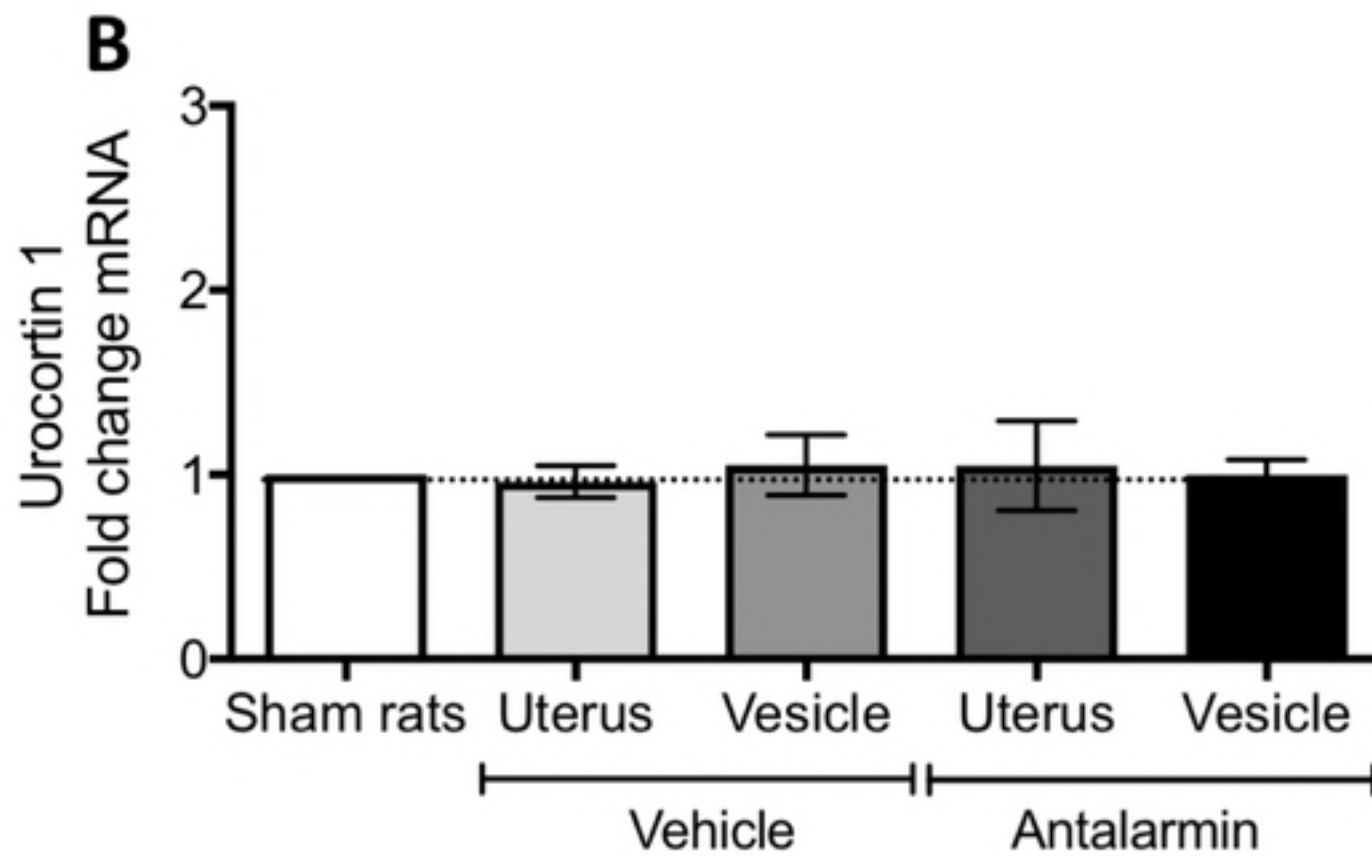
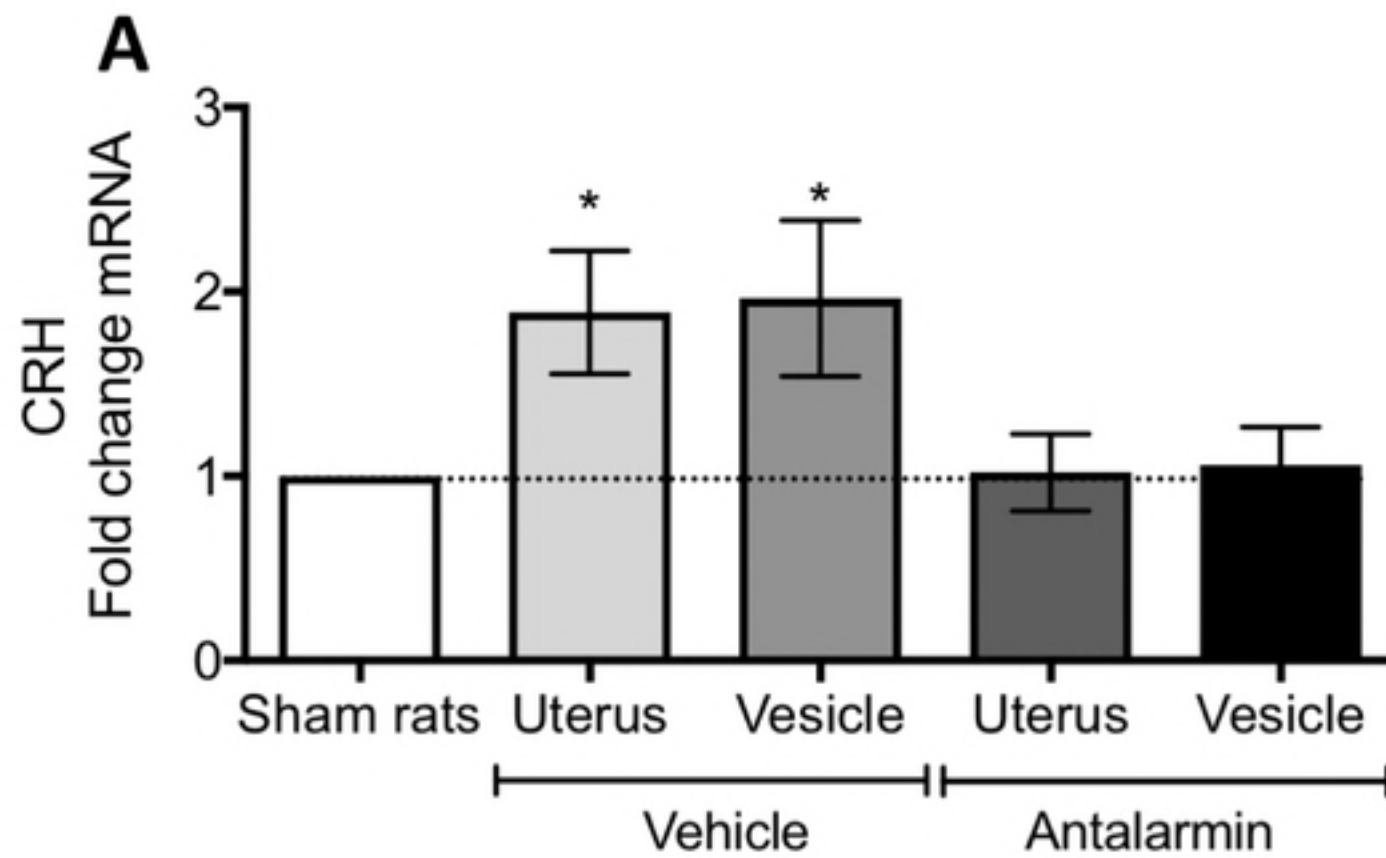
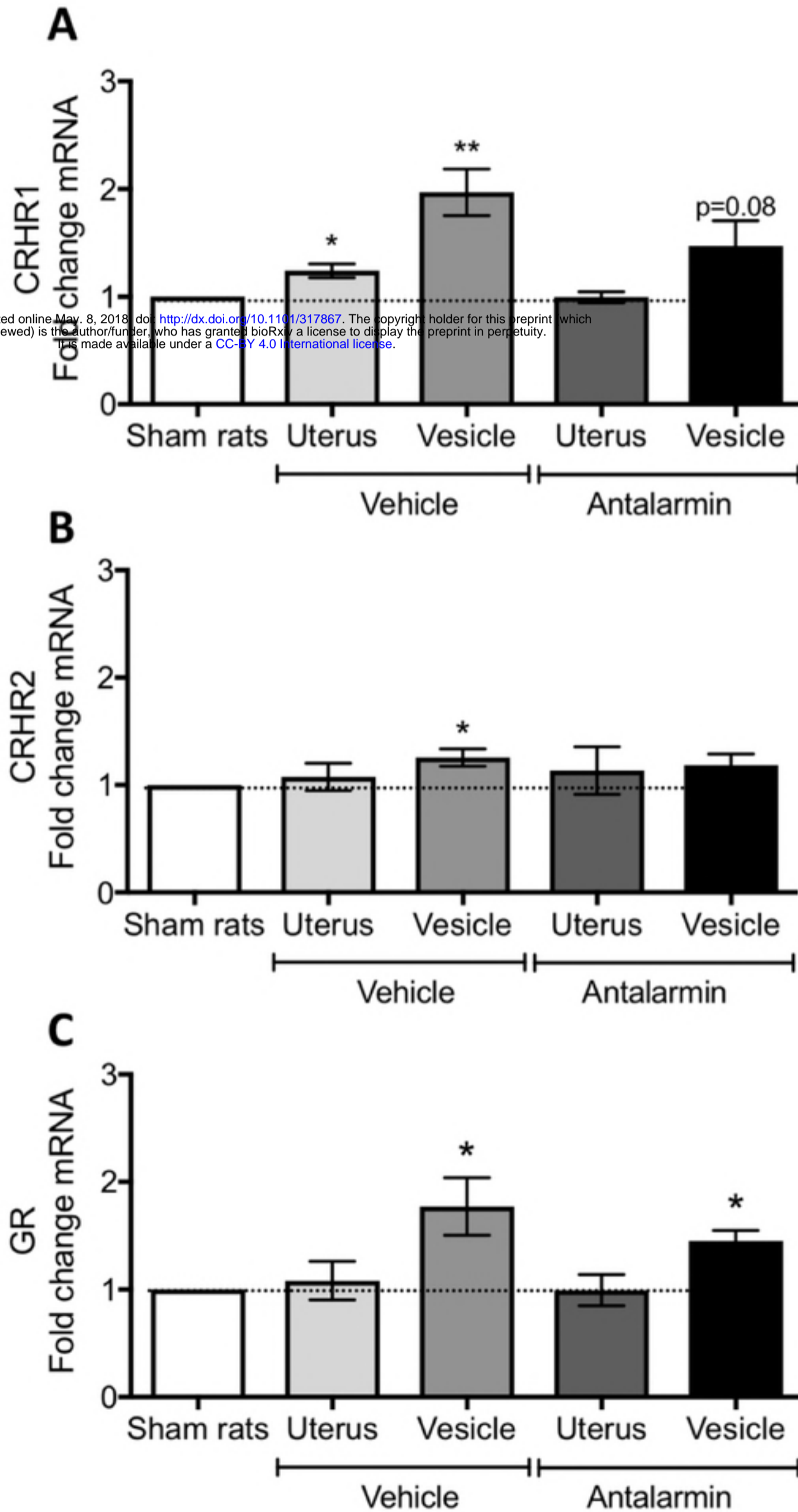


Fig. 7. Torres-Reveron, et al.



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Fig. 8. Torres-Reveron, et al.

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