Absent, but not glucocorticoid-modulated, corticotropin-releasing hormone (Crh) regulates anxiety-like behaviors in mice

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Abstract (248 words)

The hypothalamic-pituitary-adrenal (HPA) axis is a well characterized endocrine response system. Hypothalamic Crh in the paraventricular nucleus of the hypothalamus (PVH) initiates HPA axis signaling to cause the release of cortisol (or corticosterone in rodents) from the adrenal gland. PVH-specific deletion of *Crh* reduces anxiety-like behaviors in mice. Here we report that manipulation of PVH *Crh* expression in primary adrenal insufficiency or by dexamethasone (DEX) treatment do not alter mouse anxiety behaviors. In Experiment 1, we compared wildtype (WT) mice to those with primary adrenal insufficiency (*Mrap*KO) or global deletion of Crh (*Crh*KO). We analyzed behaviors using open field (OF) and elevated plus maze (EPM), PVH Crh mRNA expression by spatial transcriptomics, and plasma ACTH and corticosterone after a 15-minute restraint test with ELISAs. EPM analysis showed *Crh*KO mice were less anxious than WT and *Mrap*KO mice, and *Mrap*KO mice had no distinguishing behavioral phenotype. In Experiment 2, we evaluated HPA axis habituation to chronically elevated *Crh* expression by comparing mice treated with 5-8 weeks of DEX with those similarly treated followed by DEX withdrawal for 1 week. All mice regardless of genotype and treatment showed no significant behavioral differences. Our findings suggest that reduced anxiety associated with low *Crh* expression requires extreme deficiency, perhaps outside of those PVH Crh neurons negatively regulated by glucocorticoids. If these findings extend to humans, they suggest that increases in Crh expression with primary adrenal insufficiency, or decreases with exogenous glucocorticoid therapy, may not alter anxiety behaviors via modulation of Crh expression.

Introduction

The World Health Organization estimates that approximately 300 million people have some form of an anxiety disorder, with this number increasing by 26% during the COVID-19 pandemic.[1] In general, patients living with anxiety disorders have excessive worry and/or fear. Typically, patients with anxiety disorders have significant disturbances in their thinking which often result in dysregulation of their emotions and/or behavior that may consequently impair daily functions.[1] There is a wide spectrum in the severity of symptoms patients may experience, and many factors may contribute to the source of their anxiety.

Stressors are defined as conditions in which there is a threat to homeostasis. This includes perceived or anticipated threats established from prior experience and perceived threats within a current situation.[2] When the body is subjected to these stressors, the stress response incudes autonomic, endocrine, and behavioral components. In the presence of an acute stressor, the autonomic nervous system utilizes both the sympatho-adrenomedullary limb, which secretes epinephrine from the adrenal medulla into the systemic circulation, and the sympatho-neuronal limb, which secretes norepinephrine from sympathetic neurons distributed throughout the body's organs, to elicit a rapid-onset 'fight-or-flight' response.[2-4] These catecholamines work to restore homeostasis by increasing heart rate, blood pressure, plasma glucose, and oxygen consumption within seconds.[2,5]

61 Endocrine responses to stressors have a longer time course and are mediated by the
62 hypothalamus, anterior pituitary gland, and adrenal gland, which together constitute the
63 hypothalamic-pituitary-adrenal (HPA) axis.[2-7] In response to a stressor, corticotropin-
64 releasing hormone (CRH) is released from parvocellular neurons within the medial
65 paraventricular nucleus of the hypothalamus (PVH). Acting through its G-protein
66 coupled CRH receptor-1 (CRHR1), located in corticotrophs of the anterior pituitary
67 gland, CRH stimulates these cells to increase the synthesis and secretion of
68 adrenocorticotropin hormone (ACTH). ACTH stimulates zona fasciculata cells of the
69 adrenal cortex through its G protein-coupled melanocortin-2 receptor (MC2R) to
70 stimulate the synthesis and release of cortisol (or corticosterone in rodents). In addition
71 to their effects to maintain vascular, energy and metabolic homeostasis by promoting
72 vasoconstriction, hepatic gluconeogenesis, proteolysis in muscle, and lipolysis in
73 adipocytes, glucocorticoids cause negative feedback inhibition on CRH in the PVH and
74 ACTH in anterior pituitary corticotrophs via glucocorticoid receptors (GR) to restore the
75 axis to its baseline state.[2,4,6]
76
77 The behavioral stress-response has yet to be fully characterized. While CRH plays a
78 role in mouse anxiogenesis, the biological conditions and mechanisms remain unclear.[8
79 3,9-14] This study aims to expand upon our and others' prior work by characterizing the
80 relationship between the body's endocrine and behavioral stress responses. We have
81 previously shown that deletion of hypothalamic Crh using a Cre recombinase[15] driven
82 by the expression of transgenic *Sim1* (a transcription factor that regulates PVH
83 development[16,17]), results in both suppression of the HPA stress response and an

84 anxiolytic behavioral phenotype in mice.[18] This phenotype persisted in mice with *Sim1*
85 deletion of Crh even when they were administered corticosterone-supplemented
86 drinking water, suggesting that the reduction in anxiety was due to loss of hypothalamic
87 Crh rather than the reduction in blood corticosterone concentration.[18] Another group
88 demonstrated that PVH Crh neurons coordinate rodent behavior in response to stress,
89 as their metaplastic[19] capability to anticipate[20] and interpret environmental cues[21]
90 promotes adaptable behavioral decision making.[20,22] Through
91 photoactivation/inactivation of PVH Crh neurons in mice, this group found a relationship
92 between PVH Crh activity and the onset of anxiety-like behaviors.[23] They found that
93 optogenetic stimulation of PVH Crh neurons caused a rapid onset of intense grooming
94 behavior that rapidly ceased with termination of photostimulation[21]. These studies
95 demonstrate that modulation of Crh in PVH neurons controls not only the endocrine
96 response but also aspects of the behavioral response to stressors. This could provide
97 an explanation for the tight coupling between these two aspects of the stress response,
98 which are invariably linked following exposure to a stressor. [21]
99
100 Our present study examines the impact of more subtle, physiological changes in PVH
101 Crh on behavioral responses to stressors by modulating PVH Crh via the negative
102 feedback of glucocorticoids upon PVH Crh expression and comparing these responses
103 to those in *Crh*KO mice. We have used *Mrap*KO mice as a model of high endogenous
104 Crh. These animals have a genetic form of severe, primary adrenal insufficiency due to
105 deletion of *Mrap*, a single-pass transmembrane accessory protein required for signaling
106 of ACTH via Mc2r, causing markedly elevated PVH *Crh* expression.[24] We have used

107 mice treated with dexamethasone (DEX) as a model of low endogenous Crh. DEX is a
108 potent, synthetic glucocorticoid that suppresses HPA axis activity, as it inhibits both PVH
109 Crh and anterior pituitary ACTH secretion through GR mediation.[25 26] We administered a
110 supraphysiological amount of DEX in mouse drinking water to WT, *Crh*KO and *Mrap*KO
111 mice, and examined the endocrine and behavioral stress responses in these mice
112 before, during, and after withdrawal from DEX treatment. Surprisingly, we found that
113 while *Crh*KO mice with global deletion of *Crh* have markedly decreased anxiety-like
114 behaviors, modulation of endogenous Crh in mice via alteration in glucocorticoid
115 negative feedback has no effect on behavioral stress responses.

116 **Methods**

117 Animal Husbandry

118 Adult male mice ages 12-16 weeks were used for all experiments. Adrenal function [27]
119 including the effect of Crh on adrenal activity [28] is sexually dimorphic in mice, such that
120 future studies are warranted in female mice. All mice were bred on a C57BL/6J
121 background and housed in Boston Children's Hospital's animal facility following the
122 regulations of the Institutional Animal Care and Use Committee, which approved all
123 experiments. Mice were kept on a 12-hour light/dark cycle (lights on 7 am) in a
124 temperature and humidity-controlled room (21°C with relative humidity 35-64% set point
125 +/- 5%). Animals were kept on a standard chow diet *ad libitium* (Prolab IsoPro
126 RMH3000). *Mrap*KO mice have global deletion of *Mrap* [24] and were bred from an
127 *Mrap*KO father paired with a heterozygous (glucocorticoid-sufficient) *Mrap*KO mother.
128 *Crh*KO mice were generated as previously described from *Crh*KO x *Crh*KO pairings. [18]

129 To assure viability of postnatal mice, when either *Crh*KO or heterozygous *Mrap*KO mice
130 were visibly pregnant (around E10.5), mothers were switched from standard drinking
131 water to corticosterone-supplemented drinking water (see below). Mothers and pups
132 were kept on corticosterone-supplemented water until pups were weaned (P21). Mice
133 treated with DEX (see below) were immediately weaned onto DEX-supplemented water
134 (3.6 mcg/mL) and maintained on this treatment for 5-8 weeks.
135

136 Corticosterone Treatment

137 A 15 mg/mL stock solution was prepared by adding 500mg >92% pure corticosterone
138 (Sigma-Aldrich, C2505-500 mg) to 33 mL pure, non-denatured 200 proof ethanol
139 (Sigma-Aldrich, 493546) and vortexing until the material was completely dissolved. The
140 final stock solution was aliquoted and stored at -20°C until ready for use. Before use,
141 the aliquots were thawed and vortexed until all corticosterone was solubilized and then
142 added into mouse drinking water at a final concentration of 30 mcg/mL, as previously
143 described.[29]
144

145 Dexamethasone Treatment

146 A 10 mg/mL DEX stock solution was prepared by mixing 100 mg water-soluble DEX
147 (Sigma-Aldrich, D2915-100mg) with 10 mL of tap water. The material was vortexed until
148 completely dissolved. The stock solution was aliquoted and stored at -20°C until ready
149 for use. Before use, the aliquots were thawed and mixed by pipetting up and down and
150 then added to mouse drinking water at a final concentration of 3.6 mcg/mL.

Behavioral Experiments

Open Field

Mice were transferred to Boston Children's Hospital's Neurobehavioral Core's testing facilities (Day 0) and single-housed one week prior to the first testing date to allow the mice to acclimate to their new environment. All experiments were performed starting at 9 am. Open field (OF) experiments were performed (Day 7) in a 42 cm x 42 cm square box and scored using Kinder Scientific Motor Monitor II tracking software. Mice were placed at a 6 o'clock position facing towards the wall at the start time. Each run lasted for 15 minutes. Between each run, each OF box was wiped down with disinfecting/deodorizing wipes. Each mouse was returned to their home cage after testing.

Elevated Plus Maze

Elevated plus maze (EPM) experiments were performed (Day 8) on a platform raised 74 cm from the floor. Each arm of the platform was 77 cm in total length. Mice were placed at the center opening so that they were facing the open arms at the start time. Each trial ran for 6 minutes, and the data were automatically scored using Ethovision XT software (version 17.0). EPM heatmaps represent the average amount of time spent by location of each mouse. The average time and location data were generated by the same Ethovision XT software. In Fig. 2, the horizontal arms, denoted with "O", represent the open arms of the platform, and the vertical arms, denoted by "C", represent the closed arms on the platform. The center area of the EPM is not enclosed by walls, so it is

173 considered an open area of the maze. Between each run, the maze was wiped down
174 with disinfecting/deodorizing wipes. Each mouse was returned to its home cage after
175 testing. For both OF and EPM experiments, a lamp with an 8.5 W (800 Lumens) light
176 bulb was pointed up at the ceiling for low level ambient lighting.

177 Restraint Test and retro-orbital phlebotomy

178 On Day 9 of Experiments 1 and 2, one day after completing the EPM experiment, mice
179 were restrained in 50cc tubes that previously had the end of the tube cut off for
180 ventilation. After 15 minutes of restraint, mice were subjected to retro-orbital phlebotomy
181 using EDTA-coated capillary tubes and returned to their home cage. Blood samples
182 were immediately placed on ice and then centrifuged at 4°C for 10 minutes at 1500 x g.
183 The plasma was immediately aspirated and stored at -80°C until hormone analysis. A
184 separate group of WT, *Crh*KO heterozygotes, and *Crh*KO homozygotes was restrained
185 for 2 h to verify *Crh* mRNA expression in the *Crh*KO model used in this study that was
186 created by recombineering[18] and different from our initial *Crh*KO model.[28]
187
188 In Experiment 2, due to unavoidable experiment interference, 1 WT untreated animal
189 was restrained for 28 min instead of 15 min. Therefore, data from this animal were
190 removed from both plasma ACTH and corticosterone concentration evaluations. No
191 interference occurred during the prior behavioral experiments, so those data from this
192 animal were retained in OF and EPM analyses.
193

194 Hormone Assays

195 Plasma was slowly thawed on ice. Corticosterone concentration was measured using a
196 mouse/rat corticosterone ELISA kit (ALPCO; 55-CORMS-E01) as directed by the
197 manufacturer. The lower limit of detection is 0.061 mcg/dL. Animals which had
198 undetectable plasma corticosterone levels were recorded as having a value halfway
199 between 0 mcg/dL and the assay's lower limit of detection. ACTH levels were measured
200 using ACTH ELISA (ALPCO; 21-ACTH-E01) following the manufacturer's protocol. The
201 lower limit of detection is 0.22 pg/mL. Additional ACTH 0 calibrator (ALPCO; 11-C-270-
202 10-G25) was used to dilute plasma as necessary. Animals that had undetectable
203 plasma ACTH levels were recorded as having a value halfway between 0 pg/mL and the
204 assay's lower limit of detection.
205

206 *In situ* Hybridization

207 Experimental animals were euthanized (Day 10) by rapid decapitation without
208 anesthesia. Brains were removed and fixed in 10% formalin overnight and then
209 transferred to 70% ethanol for histology. Paraffin embedding was performed by either
210 the Histology Core Facility at Beth Israel Deaconess Medical Center or at Boston
211 Children's Hospital's Histology Department. Paraffin blocks were sectioned and H&E
212 stained by Boston Children's Hospital's Pathology Department. Three 5μM-thick coronal
213 brain sections were sectioned onto each slide, and every other slide was H&E stained.
214

215 We surveyed H&E slides under a Nikon E800 microscope to identify adjacent unstained
216 slides that contained PVH. Four unstained slides that were flanked by H&E-identified,

217 PVH-containing brain sections were selected for *Crh* mRNA hybridization using
218 RNAscope (ACDBio). The middle brain section on each of the 4 slides was stained for
219 *Crh* mRNA using a mouse *Crh* probe (ACD #316091) and 2.5 HD RED detection kit
220 (ACD, #316091) following manufacturer's instructions. Similar brain sections from
221 *Crh*KO mice of all treatment conditions for were analyzed for *Crh* mRNA as a negative
222 control.
223
224 For an individual biological sample, the brain section with the highest *Crh* mRNA
225 expression among the 4 stained sections was analyzed densitometrically (see below).
226 These selected slides were then compared across their respective biological replicates.
227 If there was variation in *Crh* expression among biological replicates, the selected slides
228 were ranked from least to greatest *Crh* expression, and the median image was chosen
229 as the final representative image. Semi-quantification of RNAscope images was
230 performed following the RNAscope manufacturer's protocol for FIJI (Fig. S1,
231 Supplemental Materials[30]). The area of the PVH was determined by first drawing two
232 triangles that were large enough to encompass the *Crh* signal on all slides (Fig. S1A,
233 Supplemental Materials[30]). These same triangles were then transposed onto each
234 image. The areas of the two transposed triangles were measured, and the average area
235 of these triangles was calculated. *Crh* expression was quantified by dividing the area of
236 the *Crh* densitometric signal by the average area of the PVH triangles.

237 <u>Genotyping</u> (see Supplemental Materials[30])

238

239 Statistical Analyses

240 Statistical analyses of behavioral experiments were performed using Prism GraphPad
241 10. Means were evaluated using One-way ANOVA multiple comparison testing with the
242 Bonferroni method and compared to untreated WT values. In Experiment 2, Figure 3G
243 used the same WT untreated values from Experiment 1 that were shown in Figure 1D.
244 Asterisks on graphs denote significance (*≤0.05, **≤0.001, and ***≤0.0001). Error bars
245 on graphs represent mean + SEM. Due to multiple undetectable plasma ACTH
246 concentrations in DEX treated animals, Gaussian distribution cannot be assumed.
247 Therefore, one-way ANOVA analysis is unable to test for significance compared to WT
248 untreated animals.

249

250

251 **Results**

252 *Crh*KO mice lack hypothalamic *Crh* mRNA and have low basal and stress-induced
253 CORT responses

254 The *Crh*KO model used in this study was created by recombineering[18], whereas our
255 previously described *CrhKO* model was developed with restriction fragment ligation and
256 retained a *Pgk*neo cassete in the deleted *Crh* locus[28]. We thus first sought to phenotype
257 this novel, recombineered model by assaying hypothalamic *Crh* mRNA expression as
258 well as plasma corticosterone and ACTH levels both in the basal and stressed state in
259 *Crh* WT, *Crh*KO heterozygous, and *Crh*KO homozygous animals. Stress was induced
260 with a 2h physical restraint. Overall, *Crh* mRNA expression significantly differed by both
261 genotype and treatment condition using 2-way ANOVA (Fig S1; p=0.007). *Crh* mRNA

262 increased following 2h restraint in both WT and *Crh*Het animals, and expression was
263 higher in WT vs. *Crh*HET animals under both conditions. As expected, *Crh* mRNA was
264 absent in both basal and stressed *Crh*KO mice (Fig S1, Supplemental Materials[30]).
265 Corticosterone levels were similarly higher after 2h restraint compared to the basal state
266 in WT and *Crh*HET but not *CrhKO* mice (P<0.0001), though there was no significant
267 effect of genotype nor treatment condition on plasma ACTH levels (P=0.44, Fig S2,
268 Supplemental Materials[30]).
269
270 <u>Influence of endogenous *Crh* and glucocorticoids on behavioral and endocrine</u>
271 <u>responses to stress in WT, *Mrap*KO and *Crh*KO mice. (Experiment 1)</u>
272 In Experiment 1, we tested whether *Crh* expression links the endocrine and behavioral
273 stress responses. First, we exposed mice to a 15-minute open field (OF) trial followed
274 by a 6-minute elevated plus maze (EPM) trial 24 hours after the OF test. OF total
275 locomotor activity was not different among WT, *Mrap*KO and *Crh*KO mice (WT vs.
276 *Mrap*KO P>0.999; WT vs. *Crh*KO P>0.999), indicating there were no baseline locomotor
277 abnormalities in the mutant genotypes (Fig. S3A, Supplemental Materials[30]). Further,
278 *Mrap*KO and *Crh*KO mice were not different from WT mice in total distance traveled in
279 the OF center (WT vs. *Mrap*KO P=0.286; WT vs. *Crh*KO P>0.999), time spent in the OF
280 center (WT vs. *Mrap*KO P=0.0719; WT vs. *Crh*KO P=0.836), and number of entries into
281 the OF center (WT vs. *Mrap*KO P=0.462; WT vs. *Crh*KO P>0.999) (Figs. S3B-D,
282 Supplemental Materials[30]). These three measures are indicators of anxiety, suggesting
283 that neither absent (*Crh*KO) nor elevated (*Mrap*KO) *Crh* expression affects these
284 aspects of mouse anxiety-like behavior.
285

286 The morning following the OF trial, we exposed mice to a 6-minute EPM trial. Increased
287 time spent and number of entries into the EPM open areas indicate decreased anxiety-
288 like behavior in mice. An arial view heatmap, displaying genotype-averaged position x
289 time during 6-minute trials, showed that *Crh*KO mice (Fig. 1C, D), compared to WT mice
290 (Fig. 1A, D), spent more time in the open areas of the maze (*Crh*KO 145.2±10.8 s vs.
291 WT 69.5±7.6 s; P<0.0001). *Crh*KO mice also entered the open areas more frequently
292 than WT mice during the 6-minute test (*Crh*KO 49.4±4.7 entries vs. WT 29.7±2.5
293 entries; P=0.0015) (Fig. 1E). Interestingly, *Mrap*KO mice, with elevated PVH *Crh*
294 expression, scored comparably to WT mice in time spent in open areas (*Mrap*KO
295 99.4±9.6 s vs. WT 69.5±7.6 s; P=0.0595), with a nonsignificant trend towards more time
296 in these areas (Fig. 1B, D). Further, the number of entries into the open areas was not
297 different between *Mrap*KO and WT mice (*Mrap*KO 35.1±3.2 entries vs. WT 29.7±2.5
298 entries; P=0.541) (Fig. 1E). An increase in latency to enter the open areas of the EPM is
299 associated with increased anxiety-like behavior. Both *Mrap*KO mice (23.6±10.0 s;
300 P=0.911) and *Crh*KO mice (11.9±4.7 s; P>0.999) scored comparably to WT mice
301 (15.0±6.1 s) (Fig. S3E, Supplemental Materials[30] in this metric.
302
303 24 h after completing the EPM trial in Experiment 1, we characterized the HPA axis
304 status in these same WT, *Mrap*KO and *Crh*KO mice after 15 minutes of restraint stress
305 by measuring PVH *Crh* mRNA expression by RNAscope and plasma corticosterone
306 concentrations. Following this stressor, *Mrap*KO mice tended towards increased
307 expression of PVH *Crh* mRNA compared to WT (WT 5.8±0.2 vs. *Mrap*KO 16.4±2.6
308 arbitrary units; P=0.052) while no *Crh* transcript was detected in *Crh*KO mice (Figs. 2A-

309 D). After 15 minutes of restraint, WT mice plasma corticosterone was 11.6±1.5 mcg/dL,
310 whereas it was undetectable in *Mrap*KO mice (P<0.0001) (Fig. 2E). As we have
311 previously found[18], *Crh*KO had low plasma corticosterone concentrations following
312 restraint (0.59±0.24 mcg/dL compared to WT, P<0.0001) (Fig. 2E).
313

314 <u>Dexamethasone reduces PVH *Crh* expression and the HPA axis response to stress,</u>
315 <u>and its subsequent withdrawal restores PVH Crh expression and HPA axis</u>
316 <u>responsiveness, but neither treatment affects anxiety-like behaviors. (Experiment 2)</u>

317 In Experiment 2, we asked if the lack of increased anxiety-like behaviors in *Mrap*KO
318 mice in Experiment 1 was due to habituation to chronically elevated *Crh* expression. To
319 test this, we disrupted HPA axis signaling by administration of dexamethasone (DEX)-
320 supplemented drinking water. We compared two treatment groups to the WT-untreated
321 animals studied in Experiment 1, (Fig. 2A, D). The first group consisted of WT, *Mrap*KO
322 and *Crh*KO mice treated with DEX for 5-8 weeks. The second treatment group
323 consisted of WT, *Mrap*KO and *Crh*KO mice treated with DEX for 5-8 weeks and then
324 withdrawn from treatment for 1 week.
325
326 We measured mouse behavior using the same 15-minute OF and 6-minute EPM tests
327 as in Experiment 1. Unlike in Experiment 1, where untreated *Crh*KO mice compared to
328 WT mice had reduced anxiety-like behaviors in OF and EPM tests, following either DEX
329 treatment or its subsequent withdrawal, *Crh*KO mice behaved no differently than
330 untreated WT mice in time spent in EPM open areas (*Crh*KO-DEX 73.8± 16.9 s vs, WT-
331 untreated 100.8± 9.1 s, P=0.616; *Crh*KO-DEX withdrawn 95.8± 13.0 s vs. WT-untreated

332 100.8± 9.1 s, P>0.999) (Fig. 3A) or entry frequency in EPM open areas (*Crh*KO-DEX
333 39.7±8.4 entries vs. WT-untreated 40.9±2.4 entries, P>0.999; *Crh*KO DEX-withdrawn
334 41.8±6.8 entries vs, WT-untreated 40.9±2.4 entries, P>0.999) (Fig. 3B). Similarly,
335 behavior of *Mrap*KO mice in both treatment groups (DEX-treated, and DEX-withdrawn)
336 was not different from that of untreated WT (Fig. 3). All genotypes in both treatment
337 groups (DEX-treated or DEX-withdrawn) had no difference in OF total locomotor activity
338 compared to WT untreated mice, indicating DEX treatment does not interfere with
339 overall mouse locomotion (Fig. S4A, Supplemental Materials[30]). Distance traveled, time
340 spent, and number of entries in the OF center were also not different among all
341 genotypes and treatment groups compared to WT untreated animals (Figs. S4B-D,
342 Supplemental Materials[30]). EPM analysis of latency to open areas also found no
343 significant differences in animals on DEX treatment, nor in animals who were DEX-
344 withdrawn compared to WT-untreated animals (Fig. S4E, Supplemental Materials[30]).
345
346 Twenty-four hours after completion of behavioral testing, we analyzed the impact of
347 DEX treatment and its withdrawal on PVH *Crh* mRNA expression and HPA axis
348 hormones after a 15-minute restraint. Compared with *Crh* mRNA expression in
349 untreated WT mice (5.8±0.2 arbitrary units, Figs. 2A, D), DEX-treated WT (Fig. 4A, G,
350 1.6±0.1 arbitrary units) and *Mrap*KO (Figs. 4B, G, 1.6±1.4 arbitrary units) were lower.
351 Following 1 week of DEX withdrawal PVH *Crh* was higher in both WT (Fig. 4D, G,
352 6.6±0.4 arbitrary units) and *Mrap*KO (Figs. 4E, G, 7.7±4.3 arbitrary units) mice. As
353 expected, *Crh*KO mice had undetectable *Crh* mRNA irrespective of DEX treatment
354 status (Figs. 4C, F, G). None of the differences in PVH *Crh* were significant between WT

355 and MrapKO genotypes or DEX treatment status, possibly because of the small number
356 of animals studied (Fig 4G; P=0.4948).
357
358 After 15 minutes of restraint stress in WT and *Mrap*KO DEX-treated animals, plasma
359 ACTH was undetectable compared to WT mice not given DEX (410.1±33.9 pg/mL) (Fig.
360 5A). Plasma ACTH was also very low in DEX-treated *Crh*KO mice (31.8±26.9 pg/mL).
361 One week after DEX withdrawal, WT mice had plasma ACTH concentrations not
362 different from those of WT-untreated mice (WT-withdrawn 833.6±230.6 pg/mL vs. WT-
363 untreated 410.1±33.9 pg/mL; P>0.999) (Fig. 5A), whereas *Mrap*KO mice had
364 significantly elevated plasma ACTH (2399±841.3 pg/mL; P<0.0001) (Fig. 5A). Notably,
365 two DEX-withdrawn *Mrap*KO animals had low (357.9 pcg/mL) or undetectable plasma
366 ACTH after the restraint test. When analyzing the behavior of these two animals, they
367 were not identified as outliers when using the Grubbs' outlier test (alpha=0.05). Plasma
368 ACTH remained low in DEX-withdrawn *Crh*KO mice (14.6±10.6 pg/mL) (Fig. 5A).
369
370 After 15 minutes of restraint stress, plasma corticosterone in WT-untreated mice was
371 10.9±0.7 mcg/dL (Fig. 5B), similar to that observed in Experiment 1 (Fig. 2E) and was
372 markedly lower in DEX-treated WT mice (0.2±0.1 mcg/dL; P<0.0001) (Fig. 5B). Plasma
373 corticosterone was also very low in DEX-treated *Mrap*KO mice (0.8±0.3 mcg/dL;
374 P<0.0001) and *Crh*KO mice (1.0±0.7 mcg/dL; P<0.0001) (Fig. 4B), with levels similar to
375 those found following restraint without DEX treatment (Fig.1E). Although WT mice
376 withdrawn from DEX treatment had increased plasma ACTH compared to restrained WT
377 mice that had not received DEX (Fig. 4A), corticosterone concentrations after restraint

378 in DEX-withdrawn compared to DEX-untreated restrained WT mice were markedly
379 lower (WT-withdrawn 2.3±0.6 mcg/dL vs. WT-untreated 10.9±0.7 mcg/dL; P<0.0001)
380 (Fig. 4B).
381

382 **Discussion**

383 Many studies have explored the role of Crh and its receptor, Crhr1, in anxiety behavior
384 regulation in rodents, [9,31-35], including some that have found administration of Crhr1
385 antagonists results in a reduction of anxiety-like behavioral phenotypes,[10,36], and
386 another which demonstrated that a Crhr1 antagonist reversed an anxiogenic phenotype
387 in mice with Crh-neuron specific deletion of GABA.[37] Based on these preclinical data,
388 several pharmaceutical companies developed small molecule CRH receptor 1
389 antagonists, which unfortunately all failed to reduce anxiety in multiple human clinical
390 trials. [38] We previously found that selective deletion of murine *Crh* in the brain regions of
391 *Sim1* expression, including in the PVH, reduces both stressor-induced anxiety-like
392 behaviors and endocrine responses of the HPA axis.[18] Our findings are consistent with
393 the fact that behavioral and endocrine responses to stressors are usually temporally
394 associated[21], and suggest that CRH, at least in part, may mediate this linkage. [23]
395

396 In our prior studies of selective deletion of PVH *Crh, Sim1Crh*KO mice were deficient in
397 both hypothalamic Crh as well as in systemic corticosterone, the major mouse
398 glucocorticoid. Thus, their reduced anxiety could have been due to the reduction in
399 either hormone. To evaluate this further we supplemented those mice with short-term
400 systemic glucocorticoid via their drinking water and found that they still exhibited

401 anxiolytic behavior.[18] We therefore concluded that this behavior was due to
402 hypothalamic Crh, rather than glucocorticoid, deficiency. We undertook the current
403 studies to further examine the impact of manipulation of endogenous Crh via changes in
404 negative feedback by glucocorticoids in mediating stressor-related behaviors. To that
405 end, we have compared wildtype mice to those with global deletion of either *Crh*
406 (*Crh*KO mice) or *Mrap* (*Mrap*KO mice). Mrap is necessary for ACTH function via
407 signaling through the adrenal ACTH/Mc2r receptor. [24] Humans with mutations in *MRAP*
408 have a severe form of primary adrenal insufficiency, Familial Glucocorticoid Deficiency
409 Type 2 (FGD2).[39] Mice with global deletion of *Mrap* have a similar phenotype, with
410 severe glucocorticoid deficiency causing secondary elevation in hypothalamic Crh and
411 pituitary ACTH.[24] We reasoned that since *Mrap*KO mice have both very low systemic
412 glucocorticoid concentrations and very high hypothalamic PVH *Crh* expression (Fig. 2),
413 they would be useful in understanding whether modulation of one or both of these
414 hormones is involved in stressor-related anxiety behaviors.

415

416 In this study, *Crh*KO mice in Experiment 1 exhibited a reduction in anxiety-like
417 behaviors, as they spent more time in the EPM open areas (Fig. 2) and entered the
418 open areas more frequently compared to WT mice (Fig. 2). Their anxiolytic behavioral
419 phenotype was not due to differences in locomotor activity between null mutants and
420 WT mice as determined by both groups' comparable total distance traveled during the
421 15-minute OF test (Fig. S3, Supplemental Materials[30]). Thus, the total absence of Crh
422 expression reduces anxiety-like behaviors in mice. We had previously found that a
423 different *Crh*KO mouse line had no anxiolytic phenotype[9], which was confirmed by

424 another research group using this same mouse. [40] However, the mouse model used for
425 those studies[28] was different from the *Crh*KO mice we used in the present study. The
426 cloned genomic DNA in the original mouse model was obtained from a Balb/c DNA
427 library and resulted in a *Crh*KO mouse on a mixed 129SV/C57BL6 background. The
428 gene-targeting vector replaced the coding region of *Crh* with a neomycin resistance
429 gene that was continuously expressed from a *Pgk* promoter. The *Crh*KO mouse used in
430 the current study was created by breeding a mouse with loxP sites flanking *Crh* exon 2
431 with an *EIIA*Cre mouse, resulting in germline deletion of the entire *Crh* exon 2.[18]
432 Further, we have studied this mouse on a pure C57BL6 background instead of on a
433 mixed 129SV/C57BL6 background.
434
435 To reverify our new *Crh*KO mouse strain, we used *in situ* transcriptomics to
436 demonstrate our ability to manipulate *Crh* expression in WT and Crh-deficient mice, and
437 affirm our ability to accurately locate and measure PVH *Crh* mRNA. We compared PVH
438 *Crh* expression in WT, *Crh*HET, and *Crh*KO animals in their basal state (Fig. S1,
439 Supplemental Materials[30]) and after a 2 h restraint test (Fig. S2, Supplemental
440 Materials[30]). WT animals had the highest basal PVH *Crh* expression which increased
441 after restraint. *Crh*HET animals had decreased PVH *Crh* expression compared to their
442 respective WT treatment controls and as expected, *Crh*KO animals had no PVH *Crh*
443 expression detected in either. Though WT mice trended towards higher plasma ACTH
444 than *Crh*HET, there was no significant difference among genotypes and treatment
445 groups after 2 h of restraint, possibly because the rise in corticosterone during the 2 h

446 restraint period (Fig. S2, Supplemental Materials[30]) inhibited ACTH production and/or
447 secretion.
448
449 The hypothalamus is the central regulator of the body's endocrine response to an
450 external stressor, as it initiates the HPA axis signaling cascade with Crh. Within the
451 hypothalamus, the highest density of Crh-expressing neurons is located in the PVH,[10 41]
452 and PVH Crh is activated in the presence of multiple external stressors. In one study,
453 mice were exposed to physical (ether), physiological (high salt), psychological
454 (restraint), immune (lipopolysaccharide), and innate (predator odor) stressors. Out of
455 the 95 brain regions that were examined, the PVH was 1 of 12 areas of Crh expression
456 that had significant increase in Fos expression compared to controls when exposed to
457 all 5 stressors.[41] The crucial endocrine role of PVH Crh, the dense population of Crh-
458 expressing cells within the PVH, the selective activation of PVH in response to a variety
459 of stressors neurons, and numerous studies that demonstrate the behavioral
460 consequences when PVH Crh is activated or inactivated[18 22], indicate that PVH Crh has
461 significant importance in rodent stress-response regulation.
462
463 Since *Crh*KO mice, with global deletion of Crh, have reduced anxiety-like behaviors, we
464 hypothesized that mice with increased Crh would display an increase in anxiety-like
465 behaviors. However, *Mrap*KO mice, with high PVH Crh expression due to primary
466 adrenal insufficiency (Fig. 2), have normal behavioral responses to stressors (Fig. 1). In
467 fact, analysis of time spent in EPM open areas showed *Mrap*KO mice had a non-
468 significant decrease in anxiety-like behaviors compared to WT mice, spending more

469 time in the open areas. That this might be due to the glucocorticoid deficiency in
470 *Mrap*KO mice is supported by a study that found that WT mice given corticosterone-
471 supplemented drinking water for 4 weeks developed an anxiogenic phenotype.[42] If an
472 increase in circulating glucocorticoids is an anxiogenic factor, it is reasonable to
473 question if the converse is true. *Mrap*KO mice have undetectable plasma corticosterone
474 which raises the possibility that despite high hypothalamic Crh, their concomitant low
475 glucocorticoid state could result in reduced anxiogenic behaviors, such that the two
476 opposing influences of high Crh and low glucocorticoid in the *Mrap*KO mouse model
477 counterbalance each other, leading to a normal behavioral phenotype.
478
479 The amygdala communicates with the hypothalamus to interpret perceived
480 threats.[13,14,37,43,44] Activation of Crh in central nucleus of the amygdala (CeA) neurons
481 using an AAV-DREADD system increased anxiety-like behavior in mice.[14] Rats with CeA
482 Crh over expression via stereotaxic viral injection have increased anxiety-like behaviors
483 when evaluated by EPM but not when analyzed with OF. [43] Unlike PVH Crh, Crh in the
484 central nucleus of the amygdala (CeA) is stimulated by GC secretion.[44] The low GC
485 state in *Mrap*KO mice may cause reduced Crh expression in the CeA, leading to a
486 reduction in anxiety that may counterbalance the anxiogenic effect of elevated PVH Crh.
487
488 Consistent with our behavioral results in *Mrap*KO mice, another mouse model with high
489 PVH Crh but also high systemic corticosterone did not have a distinct behavioral
490 phenotype.[45] Those investigators deleted exon 3 of *Nr3c1*, which encodes the
491 glucocorticoid receptor (GR) within the hypothalamus using *Sim1*Cre mice.

492 Homozygous *Nr3c1*FLOX::*Sim1*Cre+ mice had elevated PVH and CeA Crh expression
493 and elevated systemic corticosterone as a consequence of the PVH failing to
494 appropriately respond to GR-mediated glucocorticoid negative feedback. CeA GR
495 remained intact, as the *Sim1* field does not extend to the CeA, resulting in high systemic
496 corticosterone stimulating CeA Crh.[45].The absence of increased anxiety-like behaviors
497 in *Mrap*KO (low corticosterone) and in *Nr3c1*FLOX::*Sim1*Cre+ (high corticosterone)
498 mouse models suggests that glucocorticoids may make minimal contributions to the
499 manifestation of anxiety-like behaviors in mice.
500
501 It is also possible that the HPA axis in *Mrap*KO mice has somehow undergone
502 physiologic and/or behavioral adaptations to chronically elevated PVH Crh and/or
503 undetectable corticosterone production. A properly functioning stress response system
504 should adapt to learned, recurring internal and external stimuli. [46] Some researchers
505 propose neural network habituation as a mechanism for this adaptation, where repeat
506 exposure to a stimulus results in a reduction of stimulus-response pathway
507 transmission.[47] Anxiety disorders may arise due to perceived threats or disruption in
508 these brain signaling pathways. If a recurring threat or chronic neuronal dysregulation,
509 such as elevated PVH Crh, is then perceived as non-threatening, it could lead to a
510 decrease or cessation of the stress response and therefore any subsequent changes in
511 behavior.[13] Here we have shown how differing levels of PVH Crh or circulating
512 glucocorticoids do not have a strong relation to anxiety-like behaviors in mice. Though
513 inconclusive, these results together may have broader implications on how investigators

514 should define ‘stress’ in rodents, as many studies use plasma corticosterone
515 concentrations as the sole indicator of the stress response.
516
517 In the current study, we tested the hypothesis that the lack of increased anxiety-like
518 behaviors in *Mrap*KO mice was the result of HPA axis adaptations. We studied *Mrap*KO
519 mice that had PVH Crh suppressed by several weeks of DEX treatment, as well as
520 *Mrap*KO mice that had DEX treatment discontinued 1 week prior to behavioral testing.
521 Under these conditions, the endocrine axes of all three genotypes (WT, *Crh*KO, and
522 *Mrap*KO) behaved as anticipated (Figs. 4, 5): *Crh* mRNA expression was suppressed
523 by DEX treatment in WT and *Mrap*KO mice, and recovered 1 week after discontinuation
524 of DEX. Although we did not see total supp ression of PVH *Crh* in mice on DEX
525 treatment, we had previously found that a 70-80% reduction in PVH *Crh* mRNA (by *in*
526 *situ* hybridization) was associated with an anxiolytic behavioral phenotype in mice.[18]
527 Restraint-induced plasma ACTH was suppressed in all three genotypes by DEX
528 treatment and recovered above baseline one week after DEX was stopped. Plasma
529 corticosterone was also suppressed during DEX treatment in WT and *Crh*KO and was
530 undetectable in *Mrap*KO, as expected. [24] One week after DEX withdrawal, WT
531 corticosterone had not recovered to normal, untreated levels, despite simultaneous
532 ACTH concentrations being above normal.
533
534 Both during DEX treatment and 1 week following its withdrawal, none of the three
535 genotypes exhibited any changes in anxiety-like behaviors compared to untreated WT
536 mice (Figs. 3, S4). By testing mice one week after termination of DEX treatment, we had

537 hoped to study them during the acute phase of the increase in PVH Crh. However, it is
538 possible that by the end of the 1-week withdrawal period, these mice had already
539 adapted to their prior, pre-treatment neuroendocrine status, and thus negated our
540 attempt to avoid habituation. It is also possible that global genetic deletion of *Crh* and
541 glucocorticoid-mediated suppression of PVH Crh (and stimulation of CeA Crh) that
542 results in different behavioral outcomes Thus, we have found a reduction in anxiety-like
543 behavior only in mice with genetic manipulation of *Crh* using *Sim1*-Cre in our prior
544 study[18] and global *Crh* deletion in the present study. In contrast, glucocorticoid-
545 dependent manipulation of Crh expression did not result in any observable behavioral
546 phenotype whether Crh expression was increased or decreased. Our finding that
547 glucocorticoid-induced suppression of PVH Crh is not anxiolytic makes physiological
548 sense—otherwise feedback inhibition of Crh by prior activation of glucocorticoid
549 secretion by a stressor might terminate anxiety behaviors prematurely in response to
550 that stressor. The lack of anxiogenesis we have found in *Mrap*KO mice with elevated
551 PVH Crh requires further exploration.
552
553 In our prior studies of anxiolytic behavior in *Sim1*CreCrhKO mice, *Crh* was deleted in
554 ~70% of neurons within the *Sim1* field. [18] Although the strongest activity of *Sim1*Cre is in
555 the PVH, other brain structures that express Crh also express *Sim1* including the
556 anterior/lateral hypothalamic areas and the bed nucleus of the stria terminalis (BNST)
557 [15], but not the CeA.[18,45] Glucocorticoid modulates only a subset of brain neurons that
558 express Crh[48], so it is possible that glucocorticoid insufficiency in *Mrap*KO mice or

559 excess in DEX-treated mice does not affect all Crh-expressing neurons involved in
560 behavior.
561
562 In conclusion, we have found that complete deficiency of Crh is associated with
563 decreased anxiety-like behaviors in mice. These results are consistent with other
564 groups' work[22], which establishes Crh as a link between the endocrine and behavioral
565 responses to stress in rodents. The studies which document significant behavioral
566 impact of endogenous Crh, including our own, utilize profound technical manipulations
567 of *Crh* expression (e.g. global deletion, PVH-specific deletion [18], optogenetics) that may
568 have forced extreme behavioral outcomes that would otherwise not occur in nature. In
569 our present study, we manipulated *Crh* expression in a way that mimics what could
570 occur in disease (elevation of PVH Crh in primary adrenal insufficiency) or during clinical
571 treatment (PVH Crh suppression during and after glucocorticoid treatment). Although we
572 observed no changes in anxiety-like behaviors following modulation of *Crh* expression
573 by glucocorticoids, it may still be possible that PVH Crh links the endocrine and
574 behavioral responses to stress but in a more complex pathway than we initially
575 hypothesized. PVH Crh along with other limbic regions of the brain that express Crh,
576 may work together to interpret a perceived threat and then produce a behavioral
577 response—explaining why we see a reduction in anxiety when Crh is absent from all
578 brain regions in *Crh*KO mice. Humans with primary adrenal insufficiency[49] or secondary
579 adrenal insufficiency following exposure to long-term glucocorticoid [50] experience
580 increased anxiety. Further characterization of this system could aid in the development

581 of anxiety treatments and improve the quality of life for patients such as these who
582 experience anxiety as a consequence of disorders of their adrenal axis.
583

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595

596 **Supplemental Materials**

597 Supplemental materials (Figures S1-S4 and genotyping methods[30]) are available at:

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762 **Figure Legends**

763 **Figure 1.** Analysis of anxiety-like behaviors in WT, *Mrap*KO and *Crh*KO mice. (**A-C**)
764 Arial-view heat map of EPM displaying genotype-averaged position x time during 6 min
765 EPM test. In the pictogram, 'O' denotes the open arms of the maze while 'C' denotes
766 the closed arms. Color gradient indicates the relative time spent within a specified
767 region (Red=maximum, Black = minimum). (**D**) *Crh*KO mice, compared with WT mice,
768 spend the most time in the open areas of the EPM (P<0.0001), whereas *Mrap*KO show
769 no significant difference compared to WT mice (P=0.0595). (**E**) *Crh*KO mice, compared
770 with WT mice, enter the open areas of the EPM more frequently during the 6 min test
771 period (P=0.0015), while *Mrap*KO show no difference compared to WT mice
772 (P=0.5407).

773

774 **Figure 2.** HPA axis status in WT, *Mrap*KO and *Crh*KO mice following 15 minutes
775 exposure to restraint. Representative images of PVH *Crh* mRNA expression in (**A**) WT,
776 (**B**) *Mrap*KO, and (**C**) *Crh*KO mice. Compared to WT, *Mrap*KO have increased, and
777 *Crh*KO have absent *Crh* mRNA expression. (**D**) PVH *Crh* mRNA semi-quantification
778 analysis shows *Mrap*KO mice have a non-significant trend towards increased PVH *Crh*
779 mRNA compared to WT mice (P=0.052). (**E**) Plasma corticosterone concentrations in
780 WT, *Mrap*KO, and *Crh*KO mice immediately after 15 min of restraint stress. Asterisks
781 denote P≤ 0.0001 compared to WT using One-way ANOVA with Bonferroni correction.
782 Compared to WT mice, *Mrap*KO have undetectable, and *Crh*KO have minimal response
783 to restraint stress. Scale bars = 100 μM.

784

785 **Figure 3.** Analysis of anxiety-like behaviors in untreated WT mice compared to WT,
786 *Mrap*KO and *Crh*KO mice either treated with dexamethasone or withdrawn from
787 dexamethasone treatment for 1 week. (**A**) During a 6 min EPM trial, compared to
788 untreated WT mice, all 3 genotypes on dexamethasone treatment or withdrawn from
789 treatment spend comparable amounts of time in the open areas. (**B**) During a 6 min
790 EPM trial, compared to untreated WT mice, all 3 genotypes on dexamethasone
791 treatment or withdrawn from treatment enter the open areas of the maze at the same
792 frequency.
793
794 **Figure 4.** Comparison of paraventricular hypothalamic *Crh* mRNA expression in mice
795 treated with 5-8 weeks of dexamethasone. Representative images of (**A**) WT and (**B**)
796 *Mrap*KO show low *Crh* mRNA expression during dexamethasone treatment. When
797 dexamethasone treatment was discontinued for 1 week, (**D**) WT and (**E**) *Mrap*KO *Crh*
798 mRNA expression increased compared to dexamethasone-treated mice. (**C, F**) *Crh*KO
799 mice have no detectable *Crh* mRNA expression. (**G**) Semi-quantification of PVH *Crh*
800 mRNA shows no significant difference in *Crh* expression when compared to the WT
801 untreated animal values in Figure 2D (P=0.427). Scale bar = 100 μM.
802
803 **Figure 5.** Evaluation of the endocrine response in mice after a 15 min restraint stress
804 test. (**A**) Mice treated with dexamethasone have suppressed plasma ACTH
805 concentrations compared with untreated WT mice. ACTH secretion recovers after 1
806 week of withdrawing glucocorticoid treatment, with *Mrap*KO mice having significantly
807 elevated plasma ACTH concentrations (P<0.0001). (**B**) Plasma corticosterone

808 concentrations in mice either treated with dexamethasone or withdrawn from

809 dexamethasone. Compared with untreated WT mice, all dexamethasone-treated mice

810 have attenuated corticosterone responses to the restraint stress test, even after

811 withdrawal of dexamethasone 1 week previously. Asterisks denote $P<0.0001$.

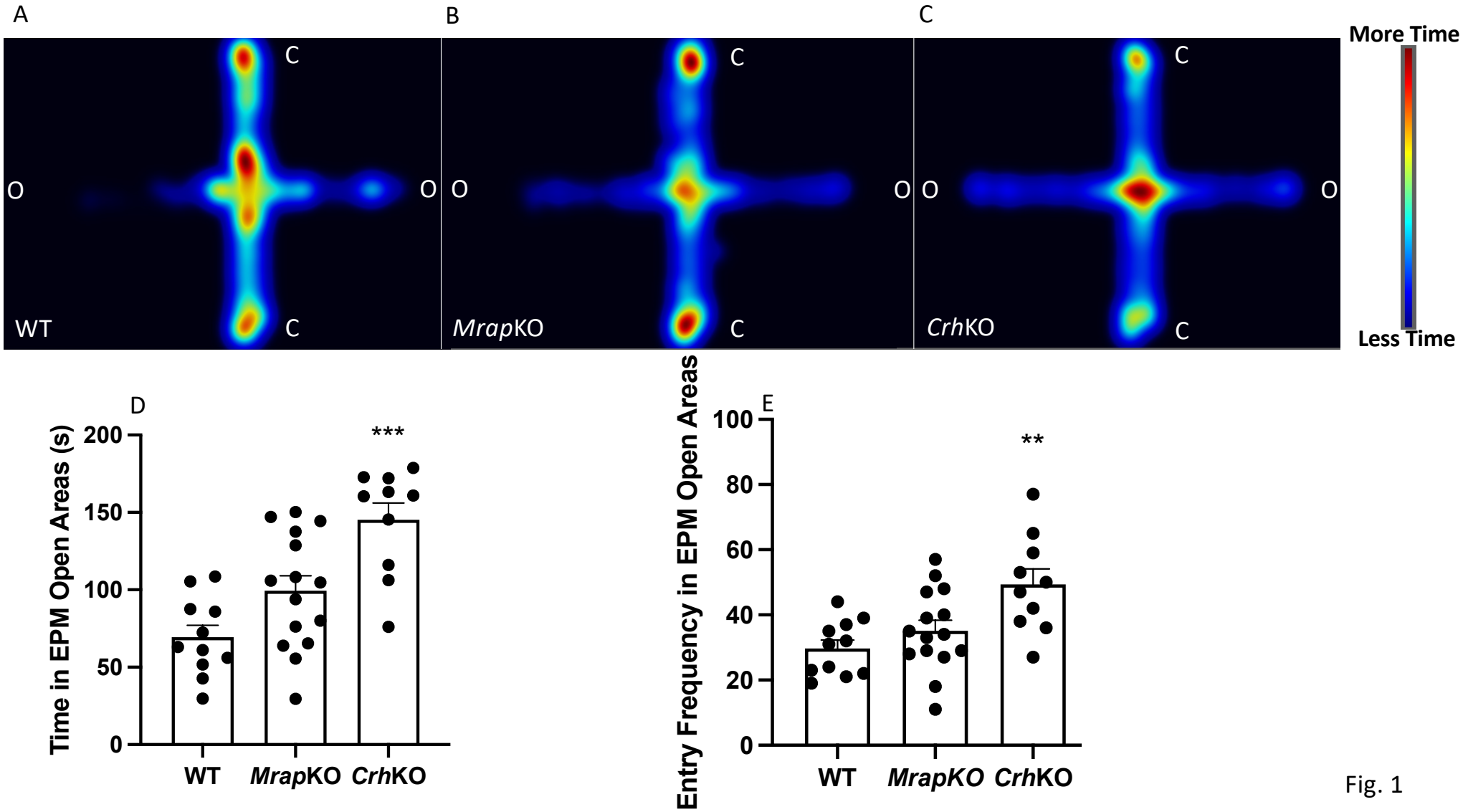

A
B
C
More Time
Less Time
C
O
O
C
WT
MrapKO
CrhKO
D
Time in EPM Open Areas (s)
200
150
100
50
0
***
WT
MrapKO
CrhKO
E
Entry Frequency in EPM Open Areas
100
80
60
40
20
0
**
WT
MrapKO
CrhKO

Fig. 1

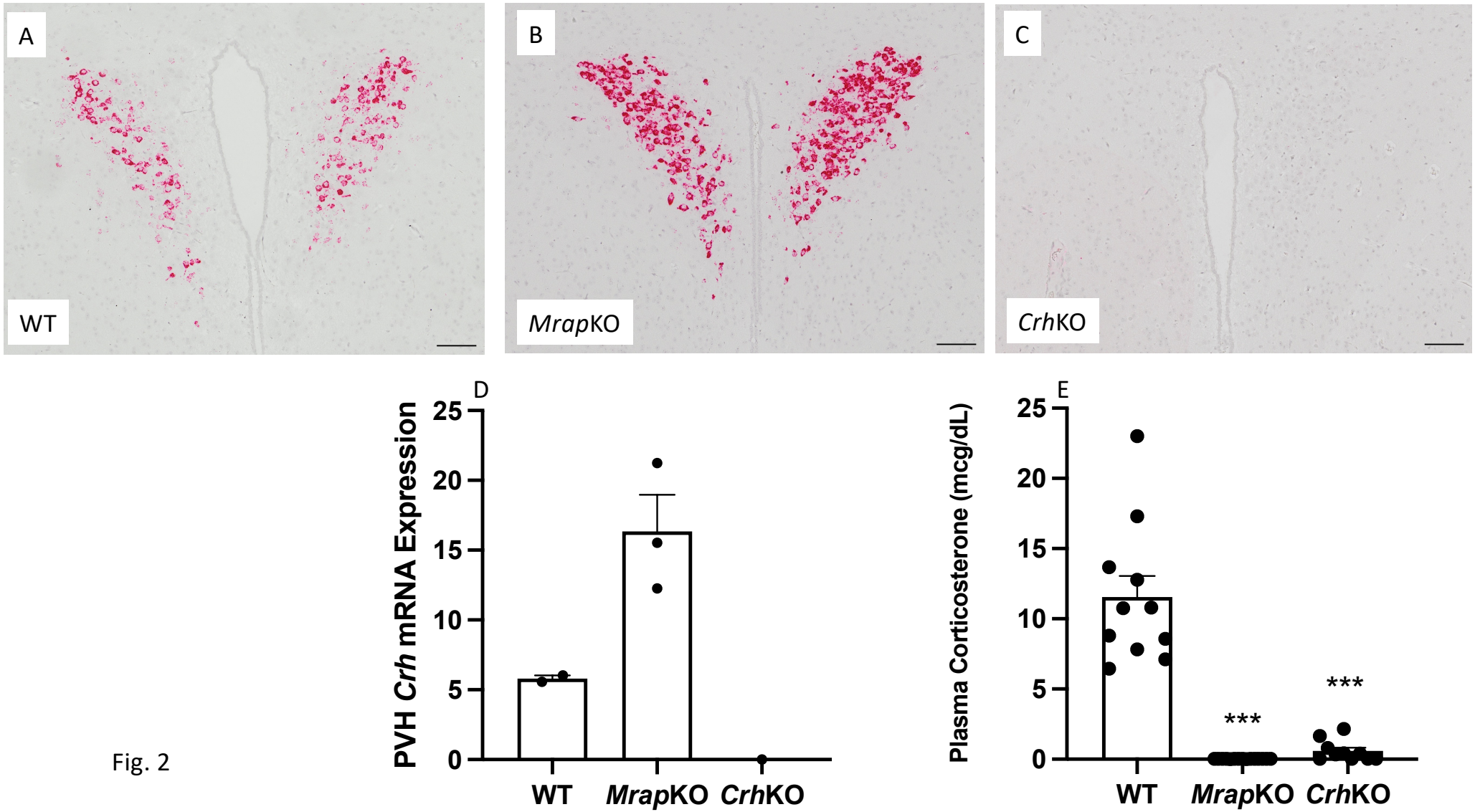
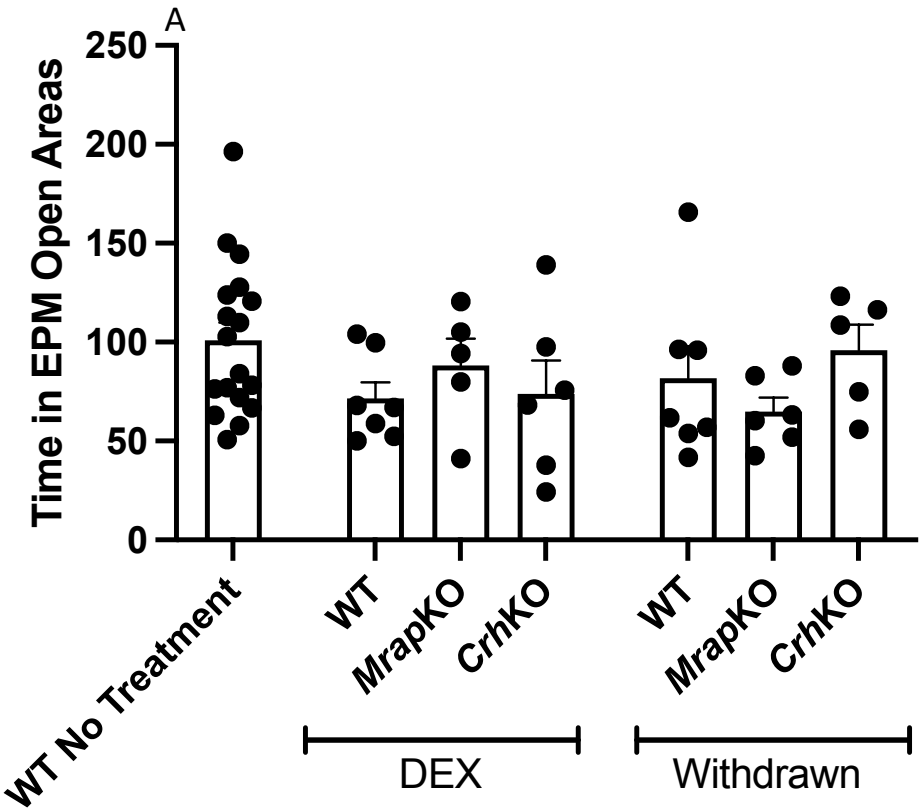

A
WT
B
MrapKO
C
CrhKO
D
PVH Crh mRNA Expression
25
20
15
10
5
0
WT
MrapKO
CrhKO
E
Plasma Corticosterone (mcg/dL)
25
20
15
10
5
0
***
***
WT
MrapKO
CrhKO

Fig. 2

Fig. 3


A
Time in EPM Open Areas
250
200
150
100
50
0
WT No Treatment
WT
MrapKO
CrhKO
WT
MrapKO
CrhKO
DEX
Withdrawn

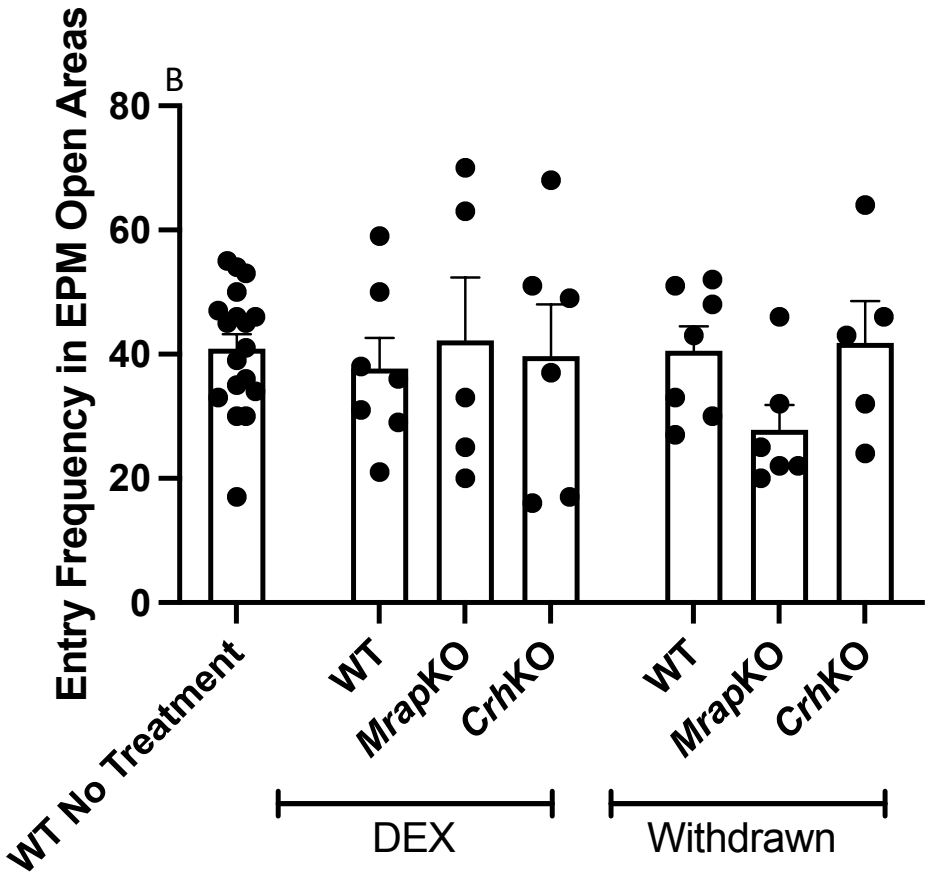

B
Entry Frequency in EPM Open Areas
80
60
40
20
0
WT No Treatment
WT
MrapKO
CrhKO
WT
MrapKO
CrhKO
DEX
Withdrawn

Fig. 4

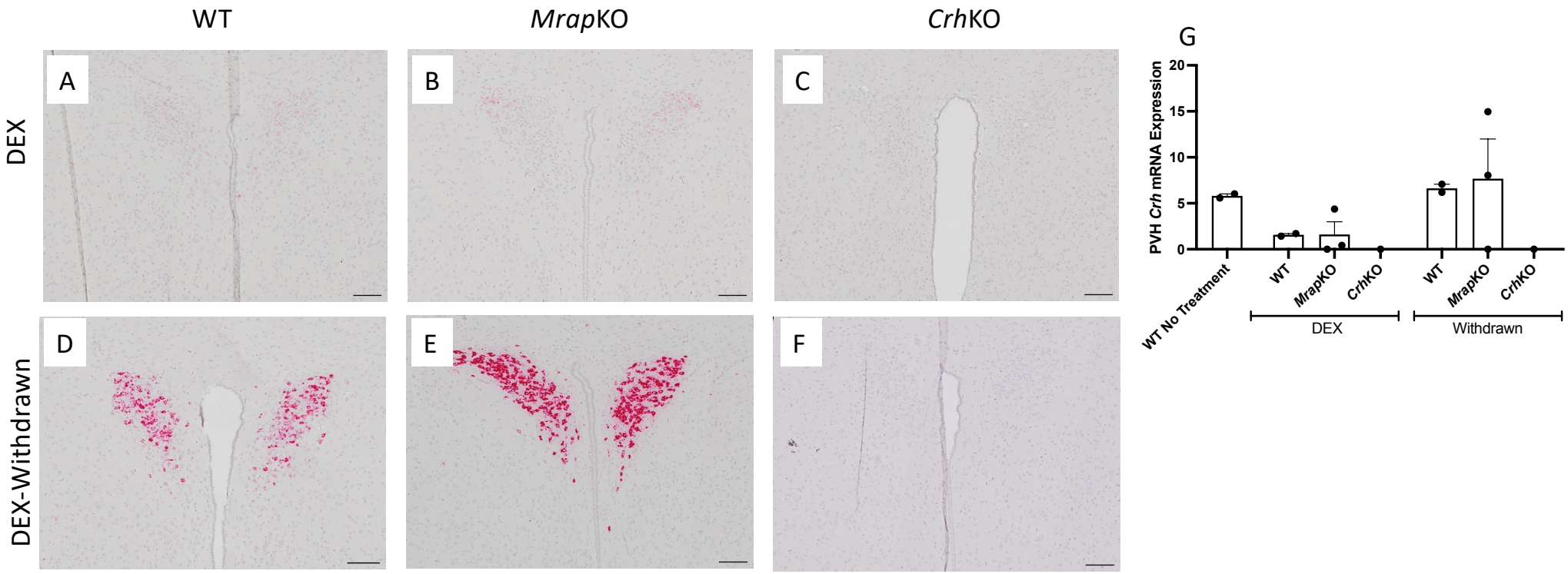
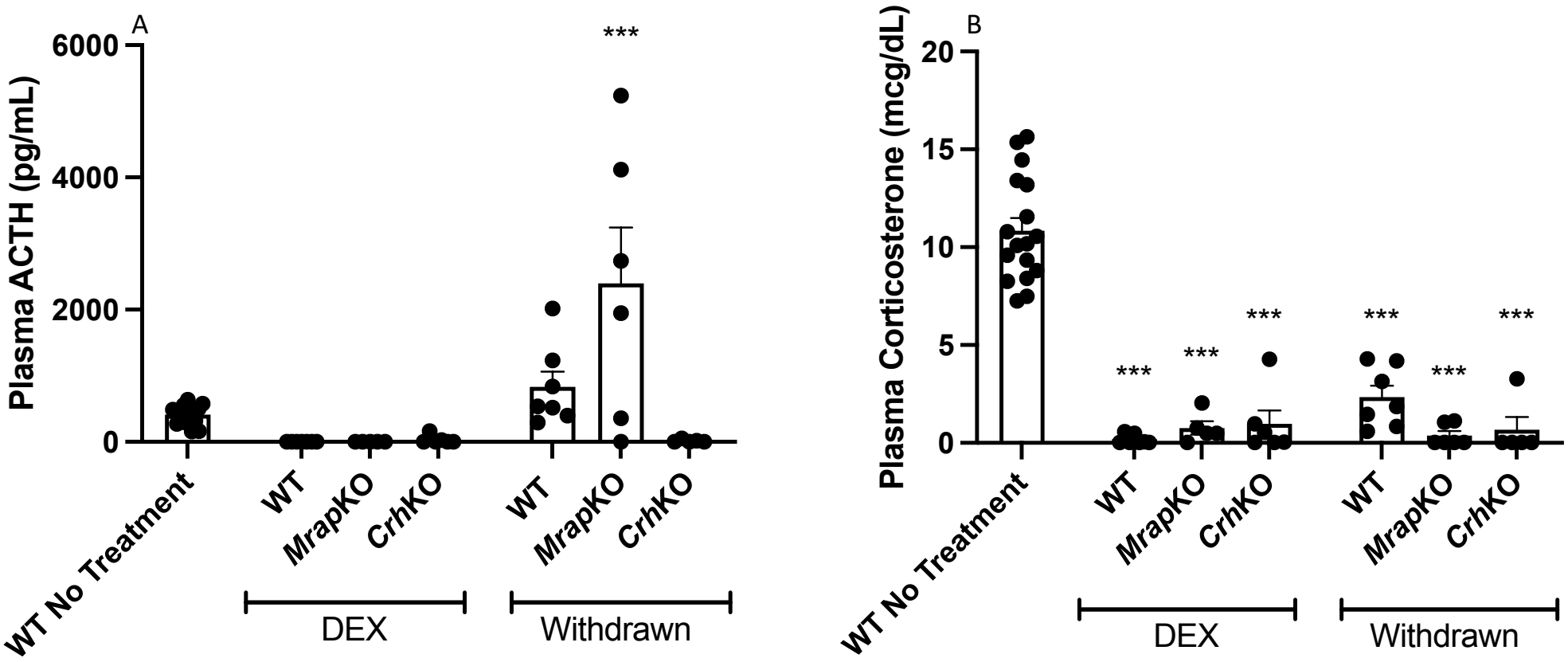

WT
*Mrap*KO
*Crh*KO
DEX
DEX-Withdrawn
A
B
C
D
E
F
G
PVH *Crh* mRNA Expression
20
15
10
5
0
WT No Treatment
WT
*Mrap*KO
*Crh*KO
WT
*Mrap*KO
*Crh*KO
DEX
Withdrawn

Fig. 5


A
Plasma ACTH (pg/mL)
6000
4000
2000
0
***
WT No Treatment
WT
MrapKO
CrhKO
WT
MrapKO
CrhKO
DEX
Withdrawn
B
Plasma Corticosterone (mcg/dL)
20
15
10
5
0
***
***
***
***
***
***
WT No Treatment
WT
MrapKO
CrhKO
WT
MrapKO
CrhKO
DEX
Withdrawn