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1 **The integration of multiple signaling pathways provides for bidirectional control**

2 **of *CRHR1* gene transcription in rat pituitary cell during hypoxia**

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28 **Abstract**

29 Hypoxia upregulates hypothalamic corticotrophin releasing hormone (CRH) and its receptor type-1 (CRHR1)
30 expression and activates the HPA axis and induces hypoxic sickness and behavioral change. The transcriptional
31 mechanism by which hypoxia differently regulates CRHR1 expression remains unclear. Here we report hypoxia
32 time-dependently induced biphasic expression of CRHR1mRNA in rat pituitary during different physiological status.
33 Short exposure of gestational dams to hypoxia reduced CRHR1mRNA in the pituitary of P1-P14 male rat offspring.
34 A short- and prolonged-hypoxia evoked biphasic response of CRHR1mRNA characterized initially by decreases and
35 subsequently by persistent increases, mediated by a rapid negative feedback via CRHR1 signaling and positive
36 transcriptional control via NF-κB, respectively. Further analysis of CRHR1 promoter in cultured primary anterior
37 pituitary and AtT20 cells showed that c-Jun/AP-1 delivered negative while HIF-1α and NF-κB delivered positive
38 control of transcription at CRHR1 promoter. The negative and positive inputs are integrated by hypoxic initiation
39 and duration in CRHR1 transcription.

40

41 **Keywords:** AP-1; CRH; Corticotropin-releasing hormone receptor 1;Hypoxia;NF-κB;Transcription;

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44 **1. Introduction**

45 CRH and CRHR1 are well known to play a crucial role in homeostasis, endocrine and behavior modulation (Ramot
46 et al., 2017; Hillhouse and Grammatopoulos, 2006; Carlin et al.,2006; Refojo et al.,2011; Nikodemova et al., 2002;
47 Westphal etal., 2009; Klenerova et al., 2008; Potter et al., 1994; Kolasa et al., 2014) by coordinating the response of
48 the brain and the HPA axis during stresses, including hypoxia (Chen et al., 2012; Fan et al., 2009; Wang et al., 2013;
49 Wang et al., 2004; Xu et al., 2006). Hypoxia is a common pathophysiological event with a potential influence on
50 gene transcription. Hypoxia stimulation of the HPA axis may be experienced by embryos in utero, neonates, adults,
51 and elders, showing a distinct spatio-temporal change in CRHR1 mRNA expression. We have previously reported
52 that exposure of neonatal rats to hypoxia activates CRH and CRHR1 mRNA expression and the HPA axis, and
53 gestational hypoxia induces an anxiety-like behavior and down-regulates the methylation of CRHR1 promoter but
54 upregulates CRHR1 mRNA in the hypothalamus paraventricular nucleus (PVN) of male offspring (Fan et al., 2009;
55 Fan et al., 2013; Wang et al., 2013). In regard to the HPA axis, hypoxia can downregulate or upregulate CRHR1
56 mRNA expression in rat anterior pituitary (Wang et al., 2004; Xu et al.,2006), but the precise mechanisms
57 underlying the transcriptional modulation are not as yet assessed. Increasing numbers of publications suggest that
58 hypoxia-activated or depressed gene expressions are implicated in many physiological and pathological processes.
59 Hypoxia exerts profound effects on the transcription of a large number of genes across a wide range of oxygen
60 tensions (Chen et al., 2012; Chen et al., 2014; Semenza, 2009; Rocha, 2007; Cummins and Taylor, 2005; Seta and
61 Millhorn, 2004; Bruning et al., 2012), including hypoxia-inducible factor (HIF-1), a major transcription factor in
62 controlling the ubiquitous transcriptional response to hypoxia, CREB, a c-AMP response element bind protein,
63 nuclear factor-κB (NF-κB), and activator protein-1 (AP-1) (Semenza, 2009; Cummins and Taylor, 2005; Bruning et
64 al., 2012). NF-κB is a family of five proteins including RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2
65 (p100/p52) that are ubiquitously expressed, form homo- or heterodimers and act as a transcriptional mediators of

66 gene in response to numerous stimuli (O'Dea and Hoffmann, 2010; Hoffmann et al., 2006). Significantly, the
67 modulation of hypoxia-sensitive genes by NF-κB is commonly complemented by AP-1, a dimeric immediate-early
68 transcription factor that is an important pleiotropic facilitator of transcriptional cascades (Cummins and Taylor,
69 2005).

70 Whilst these data are suggestive of the possible molecular mechanisms involved in controlling hypoxia-induced
71 CRHR1 gene expression in the pituitary, details of transcription and the molecular pathways have not been
72 elucidated. Since the extent to which hypobaric hypoxia influences gene expression may depend on the time period
73 of exposure and also on the stage of an animal's development we studied CRHR1 transcription in four hypoxia
74 models with different physiological status: a) a short exposure of gestational dams to hypoxia (SGH) where dams
75 were exposed to simulated altitude of 5000 m for 4 h per day; b) a short period hypoxia (SH) where the adult rats
76 were exposed to simulated altitude of 7000 m for 1, 8 and 24 h; c) a prolonged hypoxia (PH) where adult rats were
77 exposed to simulated altitude of 5000 m for 2 and 5 days. Finally d) we established an *in vitro* cell culture model to
78 study the hypoxia induced cellular mechanisms of transcriptional control involved in the promoter of CRHR1. We
79 found that hypoxia distinctly induced a bidirectional (biphasic), initially down, followed by up expression of
80 CRHR1 mRNA in rat pituitary cells that was transcriptionally controlled negatively by corticosterone (Corts) and
81 Jun/AP-1, and positively by NF-κB, and HIF-1α signaling input.

82

83 **2. Materials and methods**

84 2.1. *Animals*

85 Virgin female, Sprague-Dawley rats weighing 220 ± 20 g were purchased from the Experimental Animal Center
86 of Zhejiang Province (Hangzhou, Zhejiang, China; License No. SCXK2008-0033; SCXK (Shanghai) 2012-0002).
87 Groups of three female rats were housed overnight with one eugamic male weighing 350 ± 20 g. The day on which

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88 sperm was microscopically observed in vaginal smears was designated as embryonic day 0 (E0). The pregnant rats
89 were randomly allocated to gestational dams to hypoxia (SGH) and control groups. Rats were housed individually
90 under a 12-h light/dark cycle (lights on at 06:00) in a temperature-controlled room at 22 ± 2°C. Food and water were
91 provided *ad libitum* and the cages were cleaned twice weekly. All experiments were conducted accordance with the
92 NIH laboratory animal care guidelines. All protocols concerning animal use were approved by the Institutional
93 Animal Care and Use Committee of School of Medicine, Zhejiang University (ZJU201304-1-01-025).
94

95 2.2. *Prenatal hypoxia stress*

96 A short exposure of SGH. Dams in the SGH group were placed into a hypobaric chamber (Avic Guizhou Fenglei
97 Aviation Armament Co., Ltd, China, FLYDWC-50-IIC) simulating hypoxia at 5000 m altitude (equivalent to
98 ~10.8% O_2 at sea level) for 4 h/day throughout pregnancy period (E1-E21). The treatment was imposed once daily
99 from 08:00 to 12:00 (Fig.1) (Fan et al., 2009; Fan et al., 2013; Wang et al., 2013). The dams in the control group
100 were kept in the same chamber at sea level (equivalent to ~ 21 % O_2) under the same conditions as the SGH group.
101 At the end of SGH experiment, the neonatal babies (litter) were kept with own mother until test. The day of the
102 litter's birth was considered as postnatal day 0 (P0). After birth, the pups were left undisturbed with their biological
103 mothers until weaning at P21. They were randomly distributed according to birth day (P1, P7, P14, P21 and P90)
104 and housed in groups of five or six per cage (Wang et al., 2013). The brains and pituitary of 8 of these post natal
105 offspring were studied without further exposure to hypoxia tests and to minimize intra-specific differences, no more
106 than two male rats from each litter were used for a further test of CRHR1 methylation and mRNA expression in
107 brain and pituitary.
108

109 2.3. *Adult hypoxia stress*

110 A short period hypoxia (SH). Healthy adult male Sprague-Dawley rats (Experimental Animal Center, Zhejiang,

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111 China) weighing 180 ± 20 g were group-housed in the behavior lab 7 days for environmental adaptation before
112 experiments. Rats in the hypoxia group were placed in a hypobaric chamber and exposed to hypobaric hypoxia of
113 7000 m altitude (~8.2% O_2)for 1, 8, 24 h (Fig. 2A, B,C,F,G,H,J) or a prolonged hypoxia (PH), which mimicked at
114 altitude of 5000 m (~10.8% O_2) for 2 or 5 days (4 h/per day, Fig.2D,E,I)(Hao et al., 2015). The normoxia group
115 (Control, Con) was placed in the same chamber set at sea level (~ 21 % O_2). Rats were randomized into different
116 groups. 1. The Control group was injected (ip) with 0.9% saline. 2. The Hypoxia group was injected with vehicle
117 (0.9% saline) before hypoxia stress. 3. Dex group was injected with Dexamethasone (Dex, 500 μg/kg, ip) for 2 days
118 (4 h hypoxia /day, Fig.2I). 4. PDTC group was injected with PDTC (The pyrrolidine dithiocarbamate, an inhibitor of
119 NF-κB, 150 mg/kg, Chen, et al., 2013) for 5 days (4 h hypoxia (10.8% O_2) /day, Fig.2E) or for 8 h hypoxia (8.2%
120 O_2, Fig.2B,G). 5. An antagonist group (Fig.2C,H) was treated with CP154,526 (an antagonist of CRHR1, 30 mg/kg,
121 kindly donated by Pfizer Inc.USA). After exposure, rats were rapidly decapitated within half an hour at 14:00 -14:30
122 to minimize circadian rhythm effects.

123

124 2.4. *Sample collection*

125 At E12 and E19, dams were anesthetized with intraperitoneal injection of sodium pentobarbital (30 mg/kg) after
126 SGH and sacrificed by decapitation. In the fetuses, the anogenital distance was measured, the sex was determined,
127 and the brain (E12) or hypothalamus (E19) was snap-frozen in liquid nitrogen and stored -80 ^{o}C until DNA and
128 RNA isolation.

129 The offspring (P1, P7, P14, P21, and P90) were sacrificed by decapitation, and the pituitary was removed, snap-
130 frozen in liquid nitrogen and stored -80 ^{o}C until DNA and RNA isolation. The adult rats were also sacrificed by
131 decapitation after exposure to hypoxia stress, and the pituitary was snap-frozen in liquid nitrogen and stored -80 ^{o}C,
132 the trunk blood were collected (in EDTA tube), plasma was obtained by centrifugation and stored at -80°C.

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133 Plasma corticosterone (Cayman Chemical) was estimated with commercial ELISA kits for rats. The sensitivity
134 of the assay was 0.40 ng/mL, and interassay and intraassay coefficients of variation were 6.5% and 4.5%,
135 respectively. The antibody cross-reacted 100% with corticosterone and <0.5% with other steroids.
136
137 2.5. *Real-time qPCR and DNA methylation analysis of the CRHR1 promoter*
138 Total RNA was reverse-transcribed to cDNA using TransScript™ First-Strand cDNA Synthesis SuperMix
139 (TransGen Biotech, Beijing, China). Changes in human CRHR1 (NM_001145146.1) and rat CRH (NM_031019.1),
140 CRHR1 (NM_030999.3), were assessed using SYBR Premix Ex Taq™ (TaKaRa Biotechnology Co., Ltd., Dalian,
141 China). In addition, rat 18S ribosomal RNA was amplified for each sample as an endogenous control, and the cycle
142 threshold was subtracted from the target threshold value. All samples and negative controls were prepared in
143 duplicate wells of a 384-well plate and analyzed using the PRISM7900HT real-time PCR system (Applied
144 Biosystems, Foster City, CA, USA). The cycle number at threshold (CT value) was used to calculate the relative
145 amount of mRNA. The CT value of each target was normalized by subtraction of the CT value of 18 s. Primers were
146 used in qPCR are shown in the supplementary material (Table S1).
147 Transcription factor binding sites were predicted using the MATCH software (http://www.gene-
148 regulation.com/cgi-bin/pub/programs/match/bin/match.cgi) and http://jaspar.genereg.net/, with cut-off selection for
149 matrix to minimize the false negatives (Supplementary Fig. 1 and Fig. 2 for transcription factor binding sites of
150 mouse and rat in the region of the *CRHR1* promoter). CpG island status within the promoter region of *CRHR1*
151 (NC_005109.2) and bisulfite DNA sequencing PCR (BSP) primer were both analyzed using MethPrimer-Design
152 Primers for Methylation PCRs (http://www.urogene.org/methprimer/index1. html) (Wang et al., 2013; Li and Dahiya,
153 2002). DNA was isolated from the E12 brain and hypothalamus of E19 embryos and pituitary in offspring. Genomic
154 DNA (500 ng) was bisulfite-converted using the EZ-DNA Methylation-Gold Kit™ (Zymo Research Corp., CA,
155 USA) according to the manufacturer's instructions. Bisulfite treatment of genomic DNA converts cytosine to uracil,

156 but leaves methylated 5' cytosine unchanged. The BSP primer pairs used for the assessment of the CRHR1 CpG
157 islands (106 bp) were shown in the supplementary material (Table S1).
158 The BSP products were sequenced using the forward primer by Genscript Biotechnology Co. (Nanjing, China),
159 two samples for each E12 brain and pituitary was sequenced. The first CpG island between -609 and -502bp
160 functions is the major regulatory domain of CRHR1 transcription activity (Wang et al., 2013), methylation of each
161 CpG site within the region were tested. The percentage methylation of each CpG site within the region amplified
162 was determined by the ratio between the peak values of C and T (C/[C+T]), and these levels were determined using
163 Chromas software 2.31.
164
165 2.6. *Plasmid construction and site-directed mutagenesis*
166 Genomic DNA was isolated from the anterior pituitary of male Sprague Dawley rats, and used as a template to
167 amplify the 5'-flanking region of the CRHR1 gene ranging from -2161 to +347 by using a primer set
168 (supplementary Table S1). Primers were designed based on Rattus norvegicus genome data resources
169 (NW_047340.1, Rn10_WGA1860_4:1983790-1984316). The amplified PCR product was subcloned into pMD18-T
170 vector (Takara) and sequenced, The PCR fragment was isolated again by digesting of XhoI and HindIII, and then
171 subcloned into a promoter less luciferase vector (pGL3-basic; Promega) in the sense orientation to generate
172 p2161Luc. A series of truncated pGL3-basic plasmids containing the 5'-flanking region of the rat CRHR1 gene (-
173 2161/+347, -1833/+347, -1795/+347, -1692/+347, -1289/+347, -1248/+347, -1218/+347, -1140/+347, -838/+347, -
174 687/+347 and -360/+347) were constructed in the similar manner. Two sets of mutants with the NF-κB site
175 positioned at -809~-800 (p838) mutation and c-Jun(AP-1) mutation were constructed by site-directed mutagenesis to
176 create Mutated Luc using primers (supplementary Table S1). The full open reading frame of rat c-Jun gene was
177 amplified by PCR from rat cDNA based on published sequence (1,005 bp; NM_021835.3) by using forward primer

178 (supplementary Table S1). pcDNA3.1-c-Jun was generated by inserting the PCR fragment into the pcDNA3.1 vector
179 (Invitrogen) and sequencing.
180
181 2.7. *Cell culture and transfection and Cell treatment*
182 We established an *in vitro* cell culture models. AtT20[mouse pituitary tumor cells AtT20, American Type
183 Culture Collection (ATCC) CCL-89™]cell lines were grown in RPMI medium 1640 (Gibco) or DMEM (Gibco),
184 respectively, containing 10% (vol/vol) FBS, and 100 U/ml penicillin at 37 °C in a humidified incubator with 5%
185 (vol/vol) CO_2. AtT20 cells were plated into 12-well plates with approximately 80% confluence. Two days after
186 plating, cells were transiently transfected with 1 μg reporter construct using the Lipofectamine 2000 reagent
187 (Invitrogen) according to the manufacturer's instructions. For cotransfection experiments, 1 μg reporter plasmid was
188 cotransfected with 1 μg pcDNA 3.1-c-Jun/AP-1 or the empty pcDNA 3.1 vector. All analyses were performed 24-48
189 h after transfection.
190 For primary pituitary cell (RPC) culture, the anterior pituitary glands from male Sprague Dawley rats (male, 180-
191 200 g) were quickly removed, and then chopped into little pieces (about 1×1 mm) with a small dissecting scissor,
192 and dispersed by incubation with 1 mg/ml trypsin (Sigma, Madrid, Spain) in Hank's balanced salt solution (Life
193 Technologies, Inc., Paisley, UK) at 37°C for 15 min. The primary anterior pituitary cells were cultured in DMEM
194 containing 100 U/ml penicillin G potassium, 1 mg/ml streptomycin sulfate and 10% FBS. The cells were maintained
195 in a humidified incubator at 37 °C in 5% CO_2 and 95% air for 7 days, and used for the following experiments. 5×10^6
196 primary anterior pituitary cells were nucleofected (Nucleofector, Amaxa Biosystems GmbH, Cologne, Germany) in
197 an electroporation cuvette along with nucleofector solution R and 2 μg plasmids using the programme A-023. Cells
198 were transferred into fresh pre-warmed media with 10% FBS and incubated for 24 h. Dual-luciferase reporter assays
199 were performed using a dual-luciferase reporter assay system (Promega Corp., WI, USA).

200 The hypoxia treatment was performed using the Proox Model P110 and ProCO2 Model P120 hypoxia systems
201 (BioSpherix, USA). AtT20 and RPC were moved to the hypoxia incubator in which the oxygen level was set as
202 indicated (1% O_2 hypoxia chamber with 1% O_2, 5 % CO_2, and 94% N_2) or normoxia condition (21%O_2) (Zhang et
203 al., 2016; Zhao et al., 2013; Zhang et al., 2013). AtT20 cells were treated with CRH(10 nM, Tocris Bioscience),
204 PDTC (10 or 100 μM), antibody (NF-κB or AP-1) , or AP-1 inhibitor (SR11302,1 or 10 μM, Tocris Bioscience)
205 for 24 h.

206

207 2.8 *Electrophoretic mobility shift assays (EMSA) and Western blot*

208 EMSAs were done to illustrate the activation of AP-1 or NF-κB in rat pituitary or cultured AtT20 cells under
209 hypoxia condition. The nuclear extracts from the rat pituitary or cultured AtT20 cells were prepared with NE-PER
210 Nuclear and Cytoplasmic Extraction reagents (Pierce Biotechnology, USA), according to the manufacturer's
211 protocol. The protein concentration of the nuclear extract was quantitated using the Bradford protein assay.
212 Oligonucleotides probe were 3'-end-biotinylated (50 fmol) encompassing the NF-κB binding sequence
213 (supplementary Table S1). The probe with sequences (CGGAGACTCC or TGAGTCA) specifically binds NF-κB or
214 AP-1 respectively. The probes were synthesized by company (Takara Biotechnology Co., Ltd., Dalian, China). Non
215 relative antibody (NA) and non relative competitor (NN) for experimental control, antibody of NF-κB (anti-p65/p50,
216 sc -372, dilution 1:1000, Santa Cruz, USA), c-Jun antibody p-c-Jun (Ser 63/73): sc-16312, dilution 1:1000, Santa
217 Cruz, USA) were used. Western blot was performed to determine the protein level of CRHR1 in tissue, monoclonal
218 antibody against CRHR1(48 KD, R&D systems), GAPDH(36 KD, 1:1000, Abcom) were used. Lysed samples were
219 centrifuged (14,000x g for 15 min) at 4 ^{o}C, and boiled with 6 x loading buffer at 95 ^{o}C for 5 min. After
220 electrophoresis, proteins were transferred to PVDF membrane and incubated antibodies.

221

222 2.9 *Chromatin Immunoprecipitation (ChIP) Assay*

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223 The chromatin immunoprecipitation (ChIP) assay was performed using ChIP kit according to the manufacturers'
224 instructions (EZ ChIP^{TM}-Catalog # 17-371, Millipore, USA). AtT-20 cells were seeded in 60-mm dishes, after
225 treatment for 24 h, AtT-20 cells were fixed with 1% formaldehyde at room temperature for 10 minutes and
226 terminated with glycine. The fixed cells were harvested with ice-cold PBS containing Protease Inhibitor Cocktail II,
227 and the pellet was resuspended in SDS lysis buffer. Then, the DNA was sheared to 200- to 1000-bp fragments by
228 sonication. Immunoprecipitation was performed using c-Jun antibody (1:50, Cell Signaling) or NF-κB p65 antibody
229 (1:100, Cell Signaling) with rotation overnight at 4°C. Protein/DNA complexes were captured in elution buffer, and
230 cross-links were reversed to free DNA. After DNA purification, normal PCR and quantitative PCR was performed
231 using CRHR1 promoter-specific primers and the binding sites (Supplementary S Table 2 and 3).
232
233 3.0. *Statistical analysis*
234 All studies were conducted by an investigator blind to SGH groups. For CRHR1 gene methylation in embryos and
235 offspring pituitary, that data were analyzed using two-tailed unpaired *t* tests. For Effect of CRH, PDTC, and AP-1
236 on CRHR1 mRNA in vitro were analyzed by using one-way ANOVA. Post hoc comparisons after one-way
237 ANOVA were made using Tukey's post hoc test (GraphPad Prism6). All data are presented as mean ± SD. $P<0.05$
238 was considered statistically significant.
239

240 **3. Results**

241 3.1. *CRHR1 expression and CRHR1 methylation in embryos brain and P1-P90 pituitary after short exposure of*
242 *gestational dams to hypoxia*
243
244 To determine whether gestational short period hypoxia alters CRHR1 promoter methylation, and thereby changes

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245 CRHR1 mRNA, CRHR1 mRNA and CRHR1 promoter methylation were measured. CRHR1 mRNA and CRHR1
246 promoter methylation was not significantly changed in the CpG island 1 of the promoter in the E12 brain
247 (Supplementary Fig. 3A and B). We next investigated the effect of SGH on CRHR1 mRNA expression and
248 methylation of the CRHR1 gene promoter in the pituitary of postnatal male offspring who had not been subjected to
249 a further hypoxia test. CRHR1 mRNA levels were markedly decreased in P1, P7 and P14 offspring (Fig.1A),
250 however on day P21, both control and SGH animals the CRHR1 mRNA levels were dramatically increased to a
251 similar extent in each. DNA methylation levels were markedly reduced at sites -547,-544, -535 within the CRHR1
252 CpG promoter in P1 males (p<0.05; Fig. 1B), but no significant differences in methylation within the CRHR1
253 promoter CpG island 1 were found at P7, P14 or P21 (Fig. 1B). On day P90, there was no significant difference
254 from control and SGH rats in the CRHR1 mRNA levels and CRHR1 DNA methylation levels in pituitary (Fig.
255 1C,D). These data indicate that there is no logical association between CRHR1 mRNA and CRHR1 DNA
256 methylation in postnatal offspring following exposure to gestational hypoxia.
257
258 3.2. *Hypoxia-induced bidirectional expression and regulation of CRHR1 mRNA in rat pituitary*
259
260 Short periods of 1 h and 8 h hypoxia (SH, at altitude of 7000 m) reduced CRHR1 mRNA whereas prolonged
261 hypoxia (PH) of 2 d or 5 d at altitude of 5000 m (4 h hypoxia/day) increased CRHR1 mRNA in adult male rat
262 pituitary (Fig. 2A, B), thus showing a spatio-temporal bidirectional (biphasic) response pattern of CRHR1
263 expression. To further explore the regulatory and transcriptional mechanism underlying the bidirectional response of
264 CRHR1 under the similar hypoxia, SH and PH condition, the adult rats were treated with hypoxia and with or
265 without pretreatment of PDTC (a NF-κB inhibitor), CP154,526 (a CRHR1 antagonist), or Dex (glucocorticoid
266 hormone and suppressant of CRH), respectively (Fig. 2C,D,E, and I). The hypoxia 8 h-reduced pituitary CRHR1

267 mRNA levels, were markedly reversed by pretreatment of CP154,526, an antagonist of CRHR1(Fig. 2D), but not by
268 PDTC, a inhibitor of NF-κB (Fig. 2C), indicative of a role for glucocorticoid negative feedback in the initial phase
269 of CRHR1 suppression during short hypoxia. By contrast, prolonged hypoxia (PH) for 5 or 2 days-increased
270 CRHR1 mRNA expression, was reversed by pretreatment of PDTC, but not by Dex, indicative of a role for NF-κB
271 (Fig. 2E) and ruling out a role for glucocorticoid negative feedback (Fig. 2I). Furthermore, CRH mRNA expression
272 increased markedly at 2, 8, and 24 h of acute short hypoxia (SH), reaching a peak at 8 h (Fig. 2F), and was blocked
273 by pretreatment of PDTC (Fig. 2G) or CP154,526 (Fig. 2H). This raised the possibility that both NF-κB and CRHR1
274 signaling may contribute to local regulation of the rapid increase in CRH mRNA expression in adult rat pituitary
275 cells. However, plasma Corts levels were increased during acute hypoxia at 2, 8, and 24 h and in a time-dependent
276 manner (Fig. 2J), indicating fast negative feedback suppression to CRHR1 gene expression by Corts during short
277 hypoxia. Moreover, EMSA test showed hypoxia increased p65 or p50 protein binding with nuclear protein, and this
278 binding could be blocked by PDTC and Mut NF-κB (Fig. 2K, L). Consideration of these findings as a whole (Fig. 2)
279 reveals that hypoxia delivers bidirectional control of CRHR1 expression, characterized by initially fast suppression
280 by Corts then sustained increase of CRHR1 expression by NF-κB transcription.

281

282 *3.3. Hypoxia increased CRHR1 promoter activity in primary rat pituitary cell and AtT20 cell*

283

284 To address CRHR1 gene transcriptional mechanisms in pituitary cells under hypoxia, the p2161Luc plasmid
285 containing the length of CRHR1 gene promoter region (-2161) was constructed and transfected into primary rat
286 pituitary cell (RPC) cells or AtT20 cells, and dual-luciferase activity assay was performed to test the CRHR1
287 promoter activity. Hypoxia (1% O_2) caused a significant increase in promoter activity of the reporter gene in RPC
288 cells (1.5-fold; $p<0.05$; Fig.3A) and AtT20 cells (2.5-fold; $p<0.05$; Fig. 3B), compared with normoxia (21% O_2). An

289 empty vector (pGL3-basic) was transfected as a control to adjust the contribution of the ligated region. Hypoxia
290 significantly increased rat *CRHR*1 gene transcription in a time-dependent manner within 4-24 h of hypoxia exposure
291 (*p<0.05, ***p<0.001, Fig. 3C).

292

293 *3.4. HIF-1α, NF-κB, and AP-1 involvement in transcription of CRHR1 promoter during hypoxia*

294

295 Bioinformatics analysis predicts that there are four NF-κB, five Jun/AP-1, eight HIF-1α binding sites, and one
296 HAS (HIF-1 ancillary sequence) in the 5' flanking region of rat CRHR1 gene, between -2161 and +360 (Fig. 4A).
297 To distinguish whether these *cis*-elements are responsible for hypoxia-induced CRHR1 promoter activation and the
298 different roles played in transcriptional activity by them, a series of deletion constructs for the CRHR1 promoter
299 (from -p2161Luc to **-**p360Luc through deletion of transcriptional sites selected) were generated according to the
300 distribution of these *cis*-elements. Transcriptional activity of all the constructs of CRHR1 promoter from p2161Luc
301 to p838Luc were increased during hypoxia (1% O_2) when compared to normoxia (p<0.001 and 0.05). However,
302 transcriptional activity of p1218Luc was dramatically increased compared with p2161Luc, after deletion of the
303 adjacent three AP-1 binding sites (triangle) (Fig. 4B, ^{+++}p<0.001), while transcriptional activity of p1218Luc was
304 dramatically decreased relative to that of p838Luc following deletion of the adjacent HIF-1α binding sites (Fig. 4B,
305 ^{+++}p<0.001). Deletion of NF-κB bindings sites (square) from p838Luc resulted in loss of transcriptional activity in
306 p687Luc (p>0.05), and the shortest p360Luc failed to activate the transcription of reporter gene (Fig. 4B).
307 Investigations on AtT20 cells *in-vitro* showed that transcriptional activities of p2161Luc, p1289Luc, and p838Luc
308 increased in a time-dependent manner (4-24 h) under normoxia and hypoxia, but the rate of increase in
309 transcriptional activities was significantly higher during hypoxia when compared with normoxia (Fig. 4C).

310

311 *3.5. AP-1involvment in transcriptional suppression of CRHR1 gene under hypoxia and normoxia*

312 To determine whether AP-1 exerted a positive or negative influence on transcriptional activity of the *Crhr1*
313 promoter p2161Luc to p1218Luc, we measured the transcriptional activity of p2161 Luc (the full length, including
314 three AP-1 sites), p1218Luc (deleted the three of AP-1, but all HIF-1α contained), and p838Luc (deleted three HIF-
315 1α, but one NF-κB contained) in cultured AtT20 cells under both hypoxia (1% O_2) and normoxia (21% O_2). We
316 found that deletion of the adjacent three AP-1 bindings sites (from p2161Luc shortened to p1218Luc) resulted in
317 dramatically increased transcription, which strongly suggests that AP-1 exerts an inhibitory influence on expression.
318 However, deletion of the adjacent three HIF-1α bindings sites (p1218Luc shortened to p838Luc) resulted in
319 dramatic decreases in transcription, indicative of a positive influence on CRHR1 transcription (Fig. 5A), $^{###}p$<0.001
320 and ^{+++}p<0.001, compared under normoxia and hypoxia, respectively. Furthermore, using cultured AtT20 cells,
321 CRHR1 mRNA level was measured in the presence and absence of CRH (10 nM) or an AP-1 inhibitor (SR11302, 10
322 μM) in the culture media under normoxia (21% O_2) or hypoxia (1% O_2). CRH decreased CRHR1 mRNA levels and
323 this decrease was reversed by the AP-1 inhibitor under normoxia, $^{**}p$<0.01, ^{++}p<0.01 (Fig. 5B). However, 1% O_2
324 increased CRHR1 mRNA and CRH induced a further increase which remained unaffected in the presence of the AP-
325 1 inhibitor, $^{##}p$<0.01, $^{@@}p$<0.01 (Fig. 5B). EMSA experiments identified AP-1 binding under normoxia (lane 2 and 4)
326 and relative increases AP-1 binding during hypoxia (lane 1 and 3). Mutated AP-1 resulted in loss of AP-1 binding
327 (lane 5). An unlabeled probe, AP-1 competitor was used (Lane 6). Addition of a Jun/AP-1 antibody (lane 7)
328 eliminated AP-1 binding. An uncorrelated antibody (NA, lane 8) had no such effect (Fig. 5C). These results indicate
329 that hypoxia induces increases of Jun/AP-1 binding relative to normoxia, and that AP-1 inhibits transcriptional by
330 binding to the CRHR1 promoter at sites p2161-p1289 and also AP-1 is responsible for CRH-induced reductions in
331 CRHR1mRNA expression under normoxia. Under hypoxia, however, the inhibitory influence of AP-1 appears to be
332 overcome by transcription activation through HIF-1α and NF-κB.

333

334 3.6. *NF-κB involvement in hypoxia-activated CRHR1 promoter transcription*

335

336 To determine whether or not the NF-κB binding site (CGGAGACTCC) positioned at p838 within the CRHR1
337 promoter is responsible for the increased CRHR1 promoter activity during hypoxia (1% O_2, 24 h), we assessed the
338 relative luciferase activity in cultured AtT20 cells with or without 10 μM PDTC. We found that hypoxia increased
339 transcriptional activity of p838Luc CRHR1 promoter, and this effect was abolished by pretreatment with PDTC.
340 Moreover mutation of p838Luc (Mut-p838) blocked the increases in transcription via the CRHR1promoter during
341 hypoxia (p<0.01, Fig. 6A, B). Moreover, CRH (10 nM) decreased CRHR1 mRNA in cultured AtT20 cells under
342 normoxia (21% O_2)($^{**}p$<0.01), and this effect was not reversed by incubation with PDTC (NF-κB inhibitor, 10 μM;
343 ^{++}p<0.01, Fig. 6C). However, hypoxia (1% O_2) alone increased CRHR1 mRNA expression when compared to
344 normoxia ($^{***}p$<0.001), and CRH (10 nM) + hypoxia further increased CRHR1 mRNA expression ($^{\#\#}p$<0.01). This
345 latter effect was blocked by co-incubation of cells with CRH (10 nM) and PDTC (NF-κB inhibitor, 10 μM;
346 $^{@@}p$<0.01, Fig. 6C), which suggests that CRH suppresses CRHR1 mRNA expression by AP-1-dependent inhibition
347 of transcription during normoxia and in a manner that is overcome by increased expression driven by HIF-1α and
348 NF-κB during hypoxia. EMSA experiments showed p65/p50 binding to nuclear protein under hypoxia in AtT20
349 (lane 4, Fig. 6D). An excess amount (100-fold) of unlabeled probe (NF-κB competitor) resulted in loss of hypoxia-
350 induced p65 binding (lane 5, Fig. 6D). Mutated NF-κB also resulted in loss of hypoxia-induced p65 binding (lane 6,
351 Fig. 6d) and p65/p50 antibody abolished the binding (lane 7, Fig. 6D), while non relative antibody (NA) was unable
352 to block the p65 binding (lane 8, Fig.6D). The PDTC (NF-κB inhibitor) also eliminated binding (lane 9, vs. lane 4 or
353 vs. lane 10, normoxia). These results strongly suggest that NF-κB binding at site p838 of the *Crhr1* promoter plays a
354 key role in transcriptional activation of CRHR1 expression by hypoxia.

355

356 *3.7 Transcription factor Jun/AP-1 and NF-κB binds at the region of the CRHR1 promoter*

357

358 Bioinformatics analysis predicts that there are twelve Jun/AP-1 and twelve NF-κB binding sites in the 5' flanking
359 region of mouse CRHR1 gene, between -2700 and +1 (Supplementary, Fig.1), and eleven Jun/AP-1 and six NF-κB
360 binding sites in the 5' flanking region of rat CRHR1 gene, between -2700 and +1" (Supplementary Fig. 2). We have
361 identified five Jun/AP-1 and eight NF-κB binding sites in the 5' flanking region of mouse CRHR1 gene by CHIP-
362 PCR in AtT-20 cells (Supplementary Fig. 4, original bands). In normoxia, CRH treatment induced an increased
363 binding at the region of the CRHR1 promoter (representative bands for AP-1 binding site 1, AP-1-1) and NF-κB
364 binding site 1, NF-κB-1), the increased binding can be decreased by the inhibitor of AP-1(SR11302) or NF-κB
365 (PDTC) respectively (Fig.7A, B), under hypoxia, the increased binding can be blocked by the inhibitor of AP-1 or
366 NF-κB respectively. CRH induced-increased transcription of the CRHR1 promoter markedly blocked after
367 incubation with the inhibitor of AP-1 or NF-κB (Fig.7C, D) in normoxia and hypoxia respectively.

368

369 **4. Discussion**

370

371 We have reported that hypobaric hypoxia causes an activation of the HPA axis and stimulates the brain-
372 neuroendocrine-immune network systems, leading to physiological dysfunction and consequent behavioral
373 abnormality as well as acute mountain sickness in which CRH and its CRHR1 play a crucial role (Chen et al., 2012;
374 Fan et al., 2009; Fan et al., 2013; Wang et al., 2013; Wang et al., 2004; Xu et al., 2006; Chen et al., 2014; Song et al.,
375 2016). The pituitary is the major component of the HPA axis, delivering central neuroendocrine regulation in a
376 manner determined, in great part, by CRH release and CRHR1 expression at the level of the hypothalamus and

377 pituitary (Chen et al., 2012; Fan et al., 2009; Fan et al., 2013; Wang et al., 2013; Wang et al., 2004; Xu et al., 2006;
378 Chen et al., 2014; Pournajafi-Nazarloo et al., 2011). Although CRHR1 mRNA changes by hypoxia have been shown
379 in the pituitary (Wang et al., 2004) the cellular and molecular mechanisms involved have not been addressed. The
380 present study reveals that hypoxia induces an initial fast decrease of CRHR1 mRNA expression in the pituitary that
381 is followed by a delayed increase in expression. This is associated with negative transcriptional control of CRHR1
382 promoter by CRHR1 triggered signaling and transcriptional factor AP-1 as well as positive transcriptional activation
383 by NF-κB, and HIF-1α respectively (Fig.8).

384 Increasing evidence suggests that stress alters the methylation status of CRH and/or CRHR1 DNA in the brain
385 and that this is associated with both changes in CRHR1 mRNA expression and behavioral dysfunction (Wang et al.,
386 2013; Wang et al., 2014; Sotnikov et al., 2014; Elliott et al., 2010; Mueller and Bale, 2008; Jaenisch and Bird, 2003;
387 de Kloet et al., 2005). Importantly, methylation is known to repress gene transcription by blocking the binding of
388 transcription factors to double-stranded DNA (Kass et al., 1997). Early life stress, such as postnatal maternal
389 separation, increases hippocampal CRH expression, while blockade of CRHR1 signaling ameliorates the
390 hippocampal synaptic dysfunction and memory defects that accompany decreased methylation of the CRH promoter
391 (Wang et al., 2014). Epigenetic regulation of CRHR1 expression plays a critical role in trait anxiety, with
392 bidirectional changes in its expression in the basolateral amygdala having been noted in response to environmental
393 cues and linked to increased methylation status of the CRHR1 promoter (Sotnikov et al., 2014). We have previously
394 reported that gestational hypoxia induced a decrease in CRHR1 promoter methylation within CpG island region in
395 the hypothalamus, which was associated with gender-biased anxiety-like behavior in male offspring (Wang et al.,
396 2013). A short exposure of gestational dams to hypoxia (SGH) decreased the levels of DNA methylation at specific
397 CpG sites (-535) within the CRHR1 promoter in the hypothalamus of E19 embryos (Wang et al., 2013), whilst there
398 was increased CRHR1 protein and mRNA expression, suggesting that decrease of DNA methylation of CRHR1

399 seems to be associated with positive CRHR1 transcription in the hypothalamus of E19 embryos (Wang et al., 2013).
400 However, the methylation levels of the CRHR1 promoter in pituitary of the male offspring appear to be not
401 associated with CRHR1 mRNA expression, because the methylation at specific CpG sites (-547,-544, -535) of the
402 CRHR1 promoter was decreased in P1 male pituitary and no change in P7, P14, and P21 male offspring (Fig. 1A,B),
403 but CRHR1 mRNA expression was also decreased in P1 and P14 pituitary. This non-causality may be associated
404 with a lower response pattern during the early developmental period of neonatal offspring, as the baseline of Corts is
405 lower in P2-P12 pups (Chintamaneni et al., 2013). Exposure of P8 rat to hypoxia (8% inspired O_2 for 4 h) resulted
406 in a decrease CRHR1 mRNA expression in anterior pituitary (Bruder et al., 2008), which seems to be similar to the
407 SGH induced changes in our offspring (Fig.1). Surprisingly, CRHR1 mRNA expression was significantly decreased
408 in P1 and P14 pituitary of all offspring exposed to SGH, but was dramatically increased in pituitary of P21 offspring
409 under both normoxia and hypoxia (Fig. 1A). This is likely associated with offspring isolation from the mother
410 during weaning, feeding and metabolic demands of body development, and the new environment at P21. In this
411 respect it is notable that reduced methylation of the CRHR1 promoter in the PVN is likely associated with both
412 increased CRHR1 expression and anxiety-like behavior in P90 male offspring following exposure to gestational
413 hypoxia (Wang et al., 2013). By contrast, no such association was observed with respect to the methylation status of
414 CRHR1 promoter in the pituitary at early period of neonatal developing of offspring rats. This change of CRHR1
415 DNA methylation in offspring pituitary seems not be associated with the development of anxiety-like behavior
416 because of maternal protective effect for fetus.

417 Hypoxia in tissues or cells occurs during a diverse array of diseases, including inflammation, cancer disease
418 (Rocha, 2007; Cummins and Taylor, 2005; Seta and Millhorn, 2004; Bruning,2012) and acute mountain sickness
419 (Chen et al., 2014; Song et al., 2016; Hao et al., 2015). Gene array analysis has revealed global changes in the
420 transcriptome during hypoxia. A cohort of alternatively regulated genes, including those for the glucocorticoid

421 receptor (GR) and transcription factors CREB, AP-1, HIF-1α, NF-κB, may therefore contribute to hypoxia-induced
422 changes in transcriptional activity and cell phenotype that are both cell-type and cell-stage specific (Rocha, 2007;
423 Cummins and Taylor, 2005; Stem et al., 2011; Bandyopadhyay et al., 1995). HIF-1α activity was induced during the
424 early phase of hypoxia, while NF-κB was activated during the later phase, and synergistic behaviour of HIF and NF-
425 κB during hypoxic inflammation (Bruning et al., 2012; Nakayama, 2013; Walmsley et al., 2005). In the present
426 study, we showed that exposure of the adult rat pituitary to a short hypoxia induced a fast phase of suppressed
427 CRHR1 mRNA expression which was switched to an increase when exposed to a prolonged hypoxia, this biphasic
428 effect involved CRHR1 signaling and NF-κB as it could be blocked by a CRHR1 antagonist and an NF-κB inhibitor,
429 respectively (Fig. 2). Since local CRH mRNA expression was simultaneously increased in the pituitary via NF-κB
430 (Fig. 2G) and CRHR1 signaling-activated transcription of CRH promoter (Fig.2H), which is supported by cAMP-
431 PKA activated CREB of CRH promoter (Kageyama and Suda, 2010), thereby CRH might also be involved in the
432 fast suppression of CRHR1 mRNA expression in the pituitary. Activating CRH causes a positive feedback control of
433 CRHR1 promoter activity via PKA and PKC pathway in a primary culture of human pregnant myometrial cells
434 (Parham et al., 2004). This distinct effect may be due to a tissue and cell specificity and stressor used. Given that
435 hypoxia-activated local changes in CRHR1 expression and pituitary activities, an autocrine and/ or paracrine
436 pathways, likely deliver changes in CRHR1 gene transcription events through CRHR1-signaling CREB, the cyclic
437 AMP response element binding protein, through PKA or Calmodulin (CaM) kinase (Mayr and Montmin, 2001) and
438 NF-κB action. Our proposal gains support from the findings that hypoxia induced activation of both Jun/AP-1 and
439 NF-κB in pituitary by EMSA test and CHIP-PCR.

440 To determine the mechanisms underpinning changes in CRHR1 gene expression by Jun/AP-1, NF-κB, and HIF-
441 1α, a series of truncated pGL3-basic plasmids that excluded the binding sites for these transcription factors were
442 constructed and transfected into AtT20 cells, and their transcriptional activity was tested. We found that 1% hypoxia

443 activated the transcriptional activity of CRHR1 promoter (the length of p2161Luc) in AtT20 and primary rat
444 pituitary cells, and that activity increased in a time-dependent manner over 24 h (Fig. 3). Besides the transcription
445 activity of p2161Luc was dramatically enhanced after the three Jun/AP-1 binding sites were deleted, which shows
446 that AP-1 acts to suppress CRHR1 transcription under normoxia and hypoxia. Furthermore, when deletion of the
447 adjacent three HIF-1α (including HAS) binding sites dramatically reduced transcription activity in p838Luc of
448 CRHR1 promoter under normoxia and hypoxia (Fig. 4B, $p<0.001$), suggesting that HIF-1α mediates activation of
449 CRHR1 transcription. Deletion of the last HIF-1α site alone resulted in loss of any transcription of CRHR1
450 promoter at p687Luc during hypoxia (Fig. 4B, $p<0.001$), which is consistent with NF-κB acting as a positive
451 regulator of CRHR1 transcription. In addition, NF-κB mediated regulation of DNA-binding affinity in pituitary
452 POMC gene by CRH (Karalis et al., 2004), while in AtT-20 cells CRH increases in AP-1-DNA (Autelitano and
453 Cohen, 1996). Therefore, by differential regulation of the activity of NF-κB and AP-1, CRH may act in a classic
454 physiological feedback loop to exquisitely regulate its own expression and that of CRHR1 in order to appropriately
455 tune the response of the HPA axis.

456

457 **5. Conclusion**

458 In summary (Fig. 8), this study revealed that hypoxia-induced multimodal expression of rat CRHR1 gene in
459 pituitary cells, is through local activation of CRH by autocrine and/or paracrine mechanisms. This occurs via the
460 integration of signals carried by multiple transcription factors with Corts (via GR), and the Jun/AP-1 presenting
461 negative control and with HIF-1α and NF-κB providing positive control. Therefore, the present study provides a
462 novel insight into the molecular mechanisms of CRHR1 transcriptional control by hypoxia.

463

464 **Conflict of interests:** The authors declare that they have no conflict of interests.

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469

470 **References**

471 Autelitano, D.J., Cohen, D.R., 1996. CRF stimulates expression of multiple fos and jun related genes in the AtT-20
472 corticotroph cell. Mol. Cell. Endocrinol. 119, 25-35.
473 Bandyopadhyay, R.S., Phelan, M., Faller, D.V., 1995. Hypoxia induces AP-1-regulated genes and AP-1 transcription
474 factor binding in human endothelial and other cell types. Biochim. Biophys. Acta. 1264, 72-8.
475 Bruder, E.D., Taylor, J.K., Kamer, K.J., Raff, H., 2008. Development of the ACTH and corticosterone response to
476 acute hypoxia in the neonatal rat. Am. J. Physiol. Regul. Integr. Comp. Physiol. 295, R1195-203.
477 Bruning, U., Fitzpatrick, S.F., Frank, T., Birtwistle, M., Taylor, C.T., Cheong, A., 2012. NFkappaB and HIF display
478 synergistic behaviour during hypoxic inflammation. Cell. Mol. Life. Sci. 69, 1319-29.
479 Carlin, K.M., Vale, W.W., Bale, T.L., 2006. Vital functions of corticotropin-releasing factor (CRF) pathways in
480 maintenance and regulation of energy homeostasis. Proc. Natl. Acad. Sci. U S A. 103, 3462-7.
481 Chen, S.J., Yang, J.F., Kong, F.P., Ren, J.L., Hao, K., Li, M., Yuan, Y., Chen, X.C., Yu, R.S., Li, J.F., Leng, G., Chen,
482 X.Q., Du, J.Z., 2014. Overactivation of corticotropin-releasing factor receptor type 1 and aquaporin-4 by
483 hypoxia induces cerebral edema. Proc. Natl. Acad. Sci. U S A. 111, 13199-204.
484 Chen, X.Q., Kong, F.P., Zhao, Y., Du, J.Z., 2012. High-altitude hypoxia induces disorders of the brain-endocrine-
485 immune network through activation of corticotropin-releasing factor and its type-1 receptors. Zhongguo
486 Ying Yong Sheng Li Xue Za Zhi. 28, 481-7.
487 Chen, W., Han, C., Xie, B., Hu, X., Yu, Q., Shi, L., Wang, Q., Li, D., Wang, J., Zheng, P., Liu, Y., Cao, X., 2013.

488 Induction of Siglec-G by RNA viruses inhibits the innate immune response by promoting RIG-I degradation.
489 Cell, 152, 467-78.

490 Chintamaneni, K., Bruder, E.D., Raff, H., 2013. Effects of age on ACTH, corticosterone, glucose, insulin, and
491 mRNA levels during intermittent hypoxia in the neonatal rat. Am. J. Physiol. Regul. Integr. Comp. Physiol.
492 304, R782-9.

493 Cummins, E.P., Taylor, C.T., 2005. Hypoxia-responsive transcription factors. Pflugers. Arch. 450, 363-71.

494 de Kloet, E.R., Joels, M., Holsboer, F., 2005. Stress and the brain: from adaptation to disease. Nat. Rev. Neurosci. 6,
495 463-75.

496 Elliott, E., Ezra-Nevo, G., Regev, L., Neufeld-Cohen, A., Chen, A., 2010. Resilience to social stress coincides with
497 functional DNA methylation of the Crf gene in adult mice. Nat. Neurosci. 13, 1351-3.

498 Fan, J.M., Chen, X.Q., Jin, H., Du, J.Z., 2009. Gestational hypoxia alone or combined with restraint sensitizes the
499 hypothalamic-pituitary-adrenal axis and induces anxiety-like behavior in adult male rat offspring.
500 Neuroscience. 159, 1363-73.

501 Fan, J.M., Wang, X., Hao, K., Yuan, Y., Chen, X.Q., Du, J.Z., 2013. Upregulation of PVN CRHR1 by gestational
502 intermittent hypoxia selectively triggers a male-specific anxiogenic effect in rat offspring. Horm. Behav. 63,
503 25-31.

504 Hao, K., Kong, F.P., Gao, Y.Q., Tang, J.W., Chen, J., Evans, A.M., Lightman, S.L., Chen, X.Q., Du, J.Z., 2015.
505 Inactivation of corticotropin-releasing hormone-induced insulinotropic role by high-altitude hypoxia.
506 Diabetes. 64, 785-95.

507 Hillhouse, E.W., Grammatopoulos, D.K., 2006. The molecular mechanisms underlying the regulation of the
508 biological activity of corticotropin-releasing hormone receptors: implications for physiology and
509 pathophysiology. Endocr. Rev. 27, 260-86.

ACCEPTED MANUSCRIPT

510 Hoffmann, A., Natoli, G., Ghosh, G., 2006. Transcriptional regulation via the NF-kappaB signaling module.
511 Oncogene. 25, 6706-16.
512 Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and
513 environmental signals. Nat. Genet. 33 Suppl, 245-54.
514 Kageyama, K., Suda, T., 2010. Transcriptional regulation of hypothalamic corticotropin-releasing factor gene. Vitam.
515 Horm. 82, 301-17.
516 Karalis, K.P., Venihaki, M., Zhao, J., van Vlerken, L.E., Chandras, C., 2004. NF-kappaB participates in the
517 corticotropin-releasing, hormone-induced regulation of the pituitary proopiomelanocortin gene. J. Biol.
518 Chem. 279, 10837-40.
519 Kass, S.U., Pruss, D., Wolffe, A.P., 1997. How does DNA methylation repress transcription? Trends Genet. 13, 444-
520 9.
521 Klenerova, V., Sery, O., Hynie, S., 2008. Corticotropin-releasing hormone receptor subtypes in the rat anterior
522 pituitary after two types of restraint stress, Ann. N. Y. Acad. Sci. 1148, 415-20.
523 Kolasa, M., Faron-Gorecka, A., Kusmider, M., Szafran-Pilch, K., Solich, J., Zurawek, D., Gruca, P., Papp, M.,
524 Dziedzicka-Wasylewska, M., 2014. Differential stress response in rats subjected to chronic mild stress is
525 accompanied by changes in CRH-family gene expression at the pituitary level. Peptides. 61, 98-106.
526 Li, L.C., Dahiya, R., 2002. MethPrimer: designing primers for methylation PCRs. Bioinformatics. 18, 1427-31.
527 Mayr, B., Montminy, M., 2001. Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat. Rev.
528 Mol. Cell Biol. 2, 599-609.
529 Mueller, B.R., Bale, T.L., 2008. Sex-specific programming of offspring emotionality after stress early in pregnancy.
530 J. Neurosci. 28, 9055-65.
531 Nakayama, K., 2013. cAMP-response element-binding protein (CREB) and NF-kappaB transcription factors are

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532 activated during prolonged hypoxia and cooperatively regulate the induction of matrix metalloproteinase
533 MMP1. J. Biol. Chem. 288, 22584-95.
534 Nikodemova, M., Diehl, C.R., Aguilera, G., 2002. Multiple sites of control of type-1 corticotropin releasing hormone
535 receptor levels in the pituitary. Arch. Physiol. Biochem. 110, 123-8.
536 O'Dea, E., Hoffmann, A., 2010. The regulatory logic of the NF-kappaB signaling system. Cold Spring Harb.
537 Perspect. Biol. 2, a000216.
538 Parham, K.L., Zervou, S., Karteris, E., Catalano, R.D., Old, R.W., Hillhouse, E.W., 2004. Promoter analysis of
539 human corticotropin-releasing factor (CRF) type 1 receptor and regulation by CRF and urocortin.
540 Endocrinology. 145, 3971-83.
541 Potter, E., Sutton, S., Donaldson, C., Chen, R., Perrin, M., Lewis, K., Sawchenko, P.E., Vale, W., 1994. Distribution
542 of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. Proc. Natl. Acad.
543 Sci. U S A. 91, 8777-81.
544 Pournajafi-Nazarloo, H., Partoo, L., Yee, J., Stevenson, J., Sanzenbacher, L., Kenkel, W., Mohsenpour, S.R.,
545 Hashimoto, K., Carter, C.S., 2011. Effects of social isolation on mRNA expression for corticotrophin-
546 releasing hormone receptors in prairie voles. Psychoneuroendocrinology. 36, 780-9.
547 Ramot, A., Jiang, Z., Tian. J.B., Nahum, T., Kuperman. Y., Justice, N., Chen, A., 2017. Hypothalamic CRFR1 is
548 essential for HPA axis regulation following chronic stress. Nat. Neurosci. 20,385-88.
549 Ren, H., Accili, D., Duan, C., 2010. Hypoxia converts the myogenic action of insulin-like growth factors into
550 mitogenic action by differentially regulating multiple signaling pathways. Proc. Natl. Acad. Sci. U S A. 107,
551 5857-62.
552 Refojo, D., Schweizer, M., Kuehne, C., Ehrenberg, S., Thoeringer, C., Vogl, A.M., Dedic, N., Schumacher, M., von
553 Wolff, G., Avrabos, C., Touma, C., Engblom, D., Schutz, G., Nave, K.A., Eder, M., Wotjak, C.T., Sillaber, I.,

554 Holsboer, F., Wurst, W., Deussing, J.M., 2011. Glutamatergic and dopaminergic neurons mediate
555 anxiogenic and anxiolytic effects of CRHR1. Science. 333, 1903-7.
556 Rocha, S., 2007. Gene regulation under low oxygen: holding your breath for transcription. Trends Biochem. Sci. 32,
557 389-97.
558 Semenza, G.L., 2009. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology (Bethesda). 24,
559 97-106.
560 Seta, K.A., Millhorn, D.E., 2004. Functional genomics approach to hypoxia signaling. J. Appl. Physiol. (1985). 96,
561 765-73.
562 Song, T.T., Bi, Y.H., Gao, Y.Q., Huang, R., Hao, K., Xu, G., Tang, J.W., Ma, Z.Q., Kong, F.P., Coote, J.H., Chen,
563 X.Q., Du, J.Z., 2016. Systemic pro-inflammatory response facilitates the development of cerebral edema
564 during short hypoxia. J. Neuroinflammation. 13, 63.
565 Sotnikov, S.V., Markt, P.O., Malik, V., Chekmareva, N.Y., Naik, R.R., Sah, A., Singewald, N., Holsboer, F., Czibere,
566 L., Landgraf, R., 2014. Bidirectional rescue of extreme genetic predispositions to anxiety: impact of CRH
567 receptor 1 as epigenetic plasticity gene in the amygdala. Transl. Psychiatry. 4, e359.
568 Stern, C.M., Luoma, J.I., Meitzen, J., Mermelstein, P.G., 2011. Corticotropin releasing factor-induced CREB
569 activation in striatal neurons occurs via a novel Gbetagamma signaling pathway. PLoS One. 6, e18114.
570 Walmsley, S.R., Print, C., Farahi, N., Peyssonnaux, C., Johnson, R.S., Cramer, T., Sobolewski, A., Condliffe, A.M.,
571 Cowburn, A.S., Johnson, N., Chilvers, E.R., 2005. Hypoxia-induced neutrophil survival is mediated by
572 HIF-1alpha-dependent NF-kappaB activity. J. Exp. Med. 201, 105-15.
573 Wang, A., Nie, W., Li, H., Hou, Y., Yu, Z., Fan, Q., Sun, R., 2014. Epigenetic upregulation of corticotrophin-
574 releasing hormone mediates postnatal maternal separation-induced memory deficiency. PLoS One. 9,
575 e94394.

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576 Wang, T.Y., Chen, X.Q., Du, J.Z., Xu, N.Y., Wei, C.B., Vale, W.W., 2004. Corticotropin-releasing factor receptor
577 type 1 and 2 mRNA expression in the rat anterior pituitary is modulated by intermittent hypoxia, cold and
578 restraint. Neuroscience. 128, 111-9.

579 Wang, X., Meng, F.S., Liu, Z.Y., Fan, J.M., Hao, K., Chen, X.Q., Du, J.Z., 2013. Gestational hypoxia induces sex-
580 differential methylation of Crhr1 linked to anxiety-like behavior. Mol. Neurobiol. 48, 544-55.

581 Westphal, N.J., Evans, R.T., Seasholtz, A.F., 2009. Novel expression of type 1 corticotropin-releasing hormone
582 receptor in multiple endocrine cell types in the murine anterior pituitary. Endocrinology. 150, 260-7.

583 Xu, J.F., Chen, X.Q., Du, J.Z., 2006. CRH receptor type 1 mediates continual hypoxia-induced changes of
584 immunoreactive prolactin and prolactin mRNA expression in rat pituitary. Horm. Behav. 49, 181-9.

585 Zhang, C., Samanta, D., Lu, H., Bullen, J.W., Zhang, H., Chen, I., He, X., Semenza, G.L., 2016. Hypoxia induces
586 the breast cancer stem cell phenotype by HIF-dependent and ALKBH5-mediated m(6)A-demethylation of
587 NANOG mRNA. Proc. Natl. Acad. Sci. U S A. 113, E2047-56.

588 Zhang, S., Zhao, Y., Hu, X., Liu, Z., Chen, X., Du, J., 2013. Distinct post-transcriptional regulation of Igfbp1 gene
589 by hypoxia in lowland mouse and Qinghai-Tibet plateau root vole Microtus oeconomus. Mol. Cell.
590 Endocrinol. 376, 33-42.

591 Zhao, Y., Ren, J.L., Wang, M.Y., Zhang, S.T., Liu, Y., Li, M., Cao, Y.B., Zu, H.Y., Chen, X.C., Wu, C.I., Nevo, E.,
592 Chen, X.Q., Du, J.Z., 2013. Codon 104 variation of p53 gene provides adaptive apoptotic responses to
593 extreme environments in mammals of the Tibet plateau. Proc. Natl. Acad. Sci. U S A. 110, 20639-44.

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595

596 **Figs 1-8 and Figure legend**

597 **Fig. 1.** CRHR1 mRNA expression and promoter methylation changes in pituitary (P1-P90) of SGH-treated male rat

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598 offspring. A CRHR1 mRNA expression in P1 and P14 pituitary. B Alterations in DNA methylation of CRHR1
599 promoter in P1-P21 pituitary. C CRHR1 mRNA expression in P90 pituitary. D Alterations in DNA methylation of
600 CRHR1 promoter in P90 pituitary. All data are presented as means ± SD. n=8-10, *p<0.05 vs. control

601

602 **Fig. 2.** Hypoxia induced a bidirectional regulation of CRHR1 mRNA expression in adult male rat pituitary, and
603 involvement of NF-κB and CRHR1 pathway. **A** Short hypoxia (SH, 8.2 % O_2, 1 or 8 h) decreased CRHR1 mRNA
604 expression ,*p<0.05, ***p<0.001 vs. control(Con), $^{\#\#\#}p$<0.001 vs. 8 h. **B** Prolonged hypoxia (PH,10.8% O_2, 4 h/d,
605 2 or 5 d) increased CRHR1 mRNA expression, *p<0.05, ***p<0.001 vs. Con, $^{\#\#}p$<0.01 vs. 2d. **C, D** SH(8.2 % O_2,
606 8 h)-decreased CRHR1 mRNA was not reversed by PDTC treatment(**c**), but reversed by CRHR1 antagonist (CP
607 154,526) treatment (**D**) *p<0.05, ***p<0.001, vs. hypoxia(-), $^{\#}p$<0.05, hypoxia+CP154,526 vs. hypoxia. **E** PH
608 (10.8% O_2, 4h/d, 5d) increased-CRHR1 mRNA was reversed by PDTC treatment, ***p<0.001, vs. hypoxia(-),
609 $^{\#\#\#}p$<0.001, hypoxia+PDTC vs. hypoxia. **F** SH (8.2% O_2, 8h) increased CRH mRNA expression in pituitary,
610 *p<0.05, **p<0.01,***p<0.001, vs. Con, $^{\#\#\#}p$<0.001, vs. hypoxia 2h and 24h. **G, H** SH increased-CRH mRNA was
611 blocked by PDTC (**G**), and partly blocked by CRHR1 antagonist (CP154,526) **(H)**, **p<0.01, ***p<0.001, vs.
612 hypoxia(-) ; $^{\#}p$<0.05, hypoxia+PDTC, vs. hypoxia; **I** PH (10.8% O_2, 4h/d, 2d) increased-CRHR1 mRNA was not
613 reversed by Dex treatment. **J** SH enhanced plasma corticosterone levels, **p<0.01,***p<0.001, vs. its own control,
614 respectively, mean ± SD, n=7 in each group. **K, L** Hypoxia induced NF-κB binding affinity tested by EMSA in rat
615 pituitary. PH (10.8% O_2, 4h/d, 5 d) increased p65 or p50 protein expression (Fig. 2 k, lane 3 vs. 2), which was
616 abolished by Mut NF-κB (Fig.2 k, lane7 vs. 6) and partly by PDTC (Fig. 2 l, lane 3 vs. 1) (N=3-4 in each group).
617 (NN= Non-relative probe; NA= Non-relative antibody; Competitor=NF-κB competitor; Antibody=p65/p50 antibody)

618

619 **Fig. 3.** Hypoxia increased rat CRHR1 promoter activity in RPC and AtT20 cells. Cells co-transfected with 1μg

620 p2161Luc (the length of CRHR1 promoter) and pRL-TK plasmids and pRL-TK plasmids (empty vector pGL3-basic,

621 as a control) respectively. **A** Hypoxia (1% O_2) increased transcription of the rat CRHR1 gene in cultured PRC cells.

622 **B** Hypoxia (1% O_2) increased CRHR1 transcription in AtT20 cell. **C** Hypoxia (1% O_2) time-dependently increased

623 Transcription of CRHR1 promoter (p2161Luc) in AtT20 cell. All data are presented as means ± SD. n=3-4 in each

624 group, *p<0.05, ***p<0.001, vs. normoxia control (21% O_2) at the time point indicated.

625

626 **Fig. 4.** Transcriptional factors, HIF-1α, NF-κB, and AP-1 were involved in the hypoxia-induced CRHR1 promoter

627 activation. **A** Predicted binding sites distribution of hypoxia responsive cis-regulatory element in rat CRHR1

628 promoter region, including four NF-κB (rectangle), five Jun /AP-1(triangle), eight HIF-1α (vertical line) binding

629 sites, and one HAS(circle) as indicated in the 5' flanking region from -2161 to +360. **B** A series of deletion

630 (truncated reporter) constructs were generated according to the predicted distribution of the transcriptional factors at

631 CRHR1 promoter region and comparison of those transcriptional activities of p2161Luc, p1218Luc, and p360Luc

632 were taken respectively. Under both hypoxia and normoxia, the transcription activity of p1218Luc was higher than

633 that of p2161Luc and p838Luc, due to AP-1 inhibitory effect on transcription activation by HIF-1α. The p838Luc

634 showed a lower transcription. Most shortened p360Luc had no transcriptional activity. All data were compared

635 between normoxia and hypoxia as well as among p2161Luc, p1218Luc, and p360Luc. All data are presented as

636 means ± SD. n=3-4 for each group, *p<0.05, **p<0.01, ***p<0.001, between normoxia and hypoxia, respectively,

637 and $^{+++}$p<0.001, p1218Luc vs. p2161Luc or p1218Luc vs. p838Luc. **C** Hypoxia (1% O_2)-time course (1 to 24 h)-

638 dependent increase in the transcriptional activity among p2161Luc, p1289Luc, and Luc p838Luc of CRHR1 gene in

639 AtT20 cell, $^{++}$p<0.01, $^{+++}$p<0.001, for p1289Luc between normoxia and hypoxia (two black line); **p<0.01,

640 ***p<0.001, for p2161Luc between normoxia and hypoxia (two red line); $^{\#\#}$p<0.01, for p838Luc between normoxia

641 and hypoxia (two blue line).

642

643 **Fig. 5.** Transcriptional factor AP-1 was involved in hypoxia-reduced CRHR1 transcription. The transcriptional
644 activity measured in cultured AtT20 cell transfected with p2161Luc, p1218Luc, and p838Luc respectively under
645 normoxia or hypoxia. **A** The transcriptional activity of p2161Luc, p1218Luc, and p838Luc. The data are presented
646 as means ± SD, *** $p<0.001$, hypoxia vs. normoxia; ### $p<0.001$, p1218Luc vs. p2161Luc and p838Luc during
647 normoxia, +++ $p<0.001$, p1218 vs. p2161 or p838 during hypoxia. **B** CRH used in AtT20 cell to mimic CRH release
648 under normaxia or hypoxia in intact rats. CRH (10 nM) induced a decreased CRHR1 mRNA during normoxia
649 (21% O_2) (** $p<0.0$ 1, CRH vs. CRH (-), and this effect was reversed by AP-1 inhibitor (SR11302, 1 μM) (++ $p<0.01$,
650 CRH vs. CRH+AP-1 inhibitor). 1% O_2 hypoxia enhanced CRHR1 mRNA (** $p<0.0$ 1, vs. normoxia control (CRH
651 (-), CRH (10 nM) could further increase hypoxia-increased CRHR1 mRNA (## $p<0.01$, CRH+1% O_2 vs. 1% O_2 +
652 CRH (-), and this could not be abolished by AP-1 inhibitor (@@ $p<0.01$, AP-1 inhibitor vs. CRH+AP-1 inhibitor). **C**
653 EMSA experiments showed hypoxia increased Jun (AP-1) expression (lane 1 and 3, vs. normoxia lane 2 and 4.
654 Mutation of AP-1 resulted in loss of Jun (AP-1) binding band (lane 5). Unlabeled probe (competitor of AP-1, lane 6)
655 markedly displaced the binding band. Jun(AP-1) antibody eliminated the binding (lane 7), but non relative antibody
656 (NA, lane 8) has no such effect. All data are presented as means ± SD. n=3-4 in each group.

657

658 **Fig. 6.** NF-κB (binding site 838) was involved in increased transcription of CRHR1 gene during hypoxia. The
659 transcriptional activity measured in cultured AtT20 cell transfected with p838Luc or mutated p838Luc, respectively
660 during normoixa or hypoxia. **A** Hypoxia (1% O_2) increased transcriptions of p838Luc of CRHR1 promoter in AtT20
661 cell, and the effect was abolished by PDTC or mutated NF-κB, ** $p<0.01$, vs. hypoxia(-); ## $p<0.01$, hypoxia vs.
662 PDTC+hypoxia. **B** Hypoxia activated transcription of p838Luc (NF-κB binding sites) , *** $p<0.001$, vs. normoxia at
663 each time point indicated. **C** CRH (10 nM) decreased CRHR1 mRNA expression during normoxia, which was not

664 reversed by PDTC treatment, while under hypoxia (1% O_2) CRHR1 mRNA increased, which was further increased
665 by CRH (10 nM), and this effect was blocked by PDTC treatment, $^{**}p<0.01$, $^{***}p<0.001$, vs. normoxia control
666 (CRH(-); $^{++}p<0.01$, PDTC vs. PDTC+CRH; $^{\#\#}p<0.01$, CRH vs. CRH(-), $^{@@}p<0.01$ CRH vs. CRH+PDTC. **D**
667 EMSA was performed using nuclear extracts from AtT20 cells under 1% O_2 hypoxia for 24 h. The sequences
668 (CGGAGACTCC) specifically bind NF-κB. The p65 antibody was added into the binding reaction mixture with
669 equal amount of AP-1 antibody used as a control. Lane 1, 2 and 3 as controls; lane 4: hypoxia induced p65
670 expression that was abolished by NF-kB competitor (Lane 5), Mut NF-κB (Lane 6), NF-κB antibody (Lane 7), partly,
671 non-relative antibody (NA) (Lane 8), and PDTC (lane 9), n=3-4 in each group.

672

673 **Fig. 7.** Transcription factor of AP-1 and NF-κB binds at the region of the CRHR1 promoter in AtT20 cell. **A**, **B**
674 Inhibitor of AP-1 (A) or NF-κB (B) decreased or blocked the binding at the region of the CRHR1 promoter during
675 normoxia (21%O_2) and hypoxia (1%O_2). **C**, **D** Inhibitor of AP-1(C) or NF-κB(D) blocked the transcription of the
676 CRHR1 promoter during normoxia (21%O_2) and hypoxia (1%O_2). **C**, $^{**}p<0.0$ 1, CRH vs. CRH +inhibitor of AP-1
677 under normoxia, $^{\#}p<0.05$, CRH vs. CRH+inhibitor of AP-1 under hypoxia. **D**, $^{+}p<0.0$ 1, CRH vs. CRH +inhibitor of
678 NF-κB under normoxia, $^{@}p<0.05$, CRH vs. CRH+inhibitor of NF-κB under hypoxia. DAPDH is included as an
679 internal control, IgG is negative control. All data are presented as means ± SD, n = 5 (Fig. C) and n = 8 (Fig. D).

680

681 **Fig. 8.** Biphasic model of transcript of CRHR1 by Corts, Jun/AP-1, NF-κB, and HIF-1α during short and prolonged
682 hypoxia in pituitary cell of adult rat.

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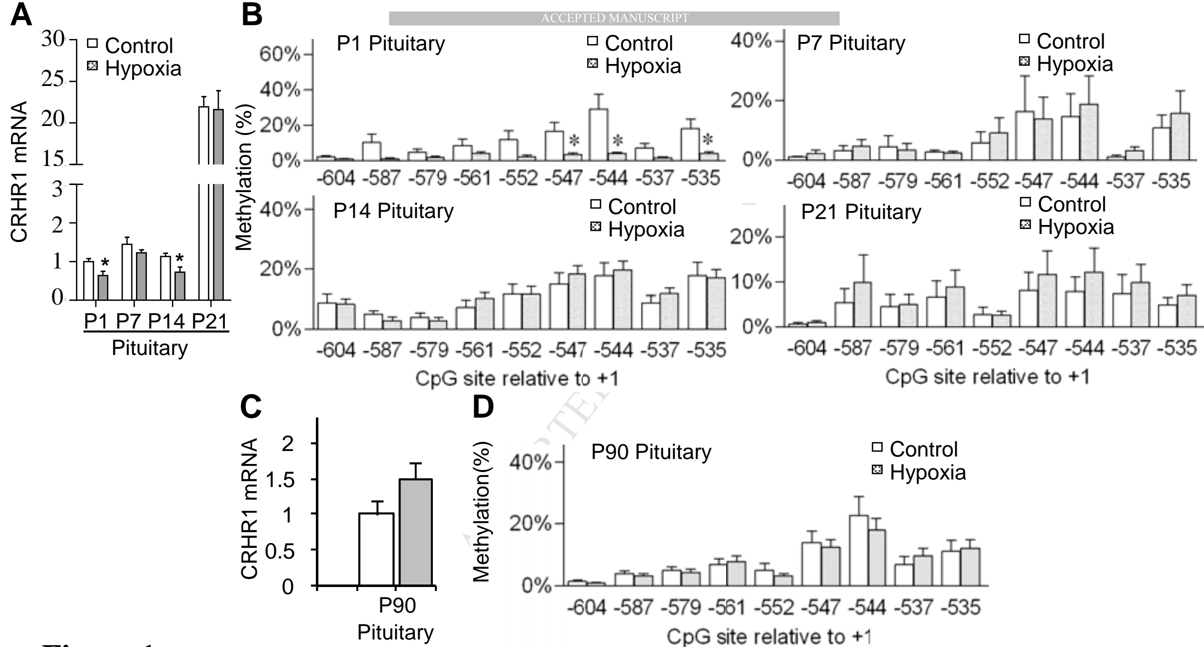

Figure 1.

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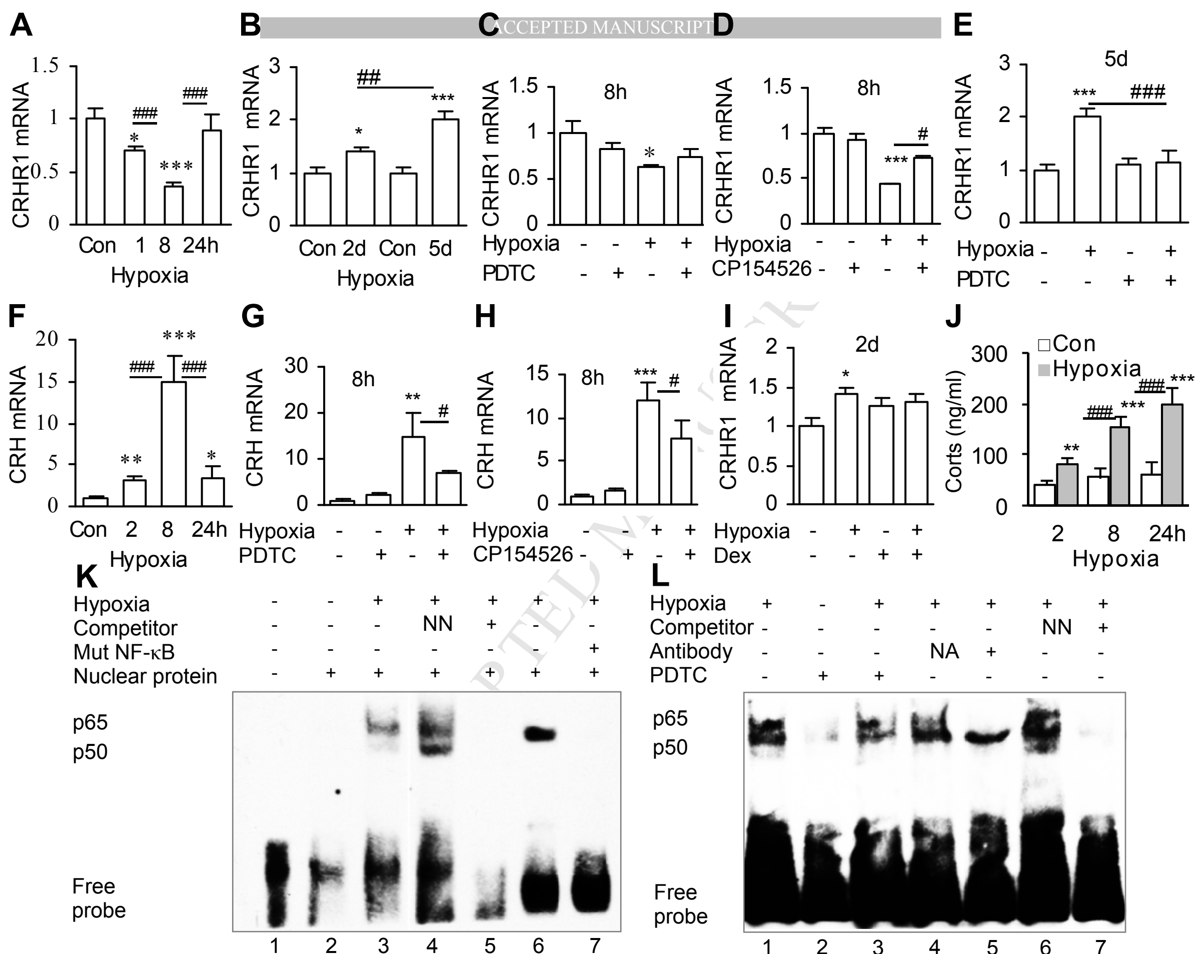

Figure 2.

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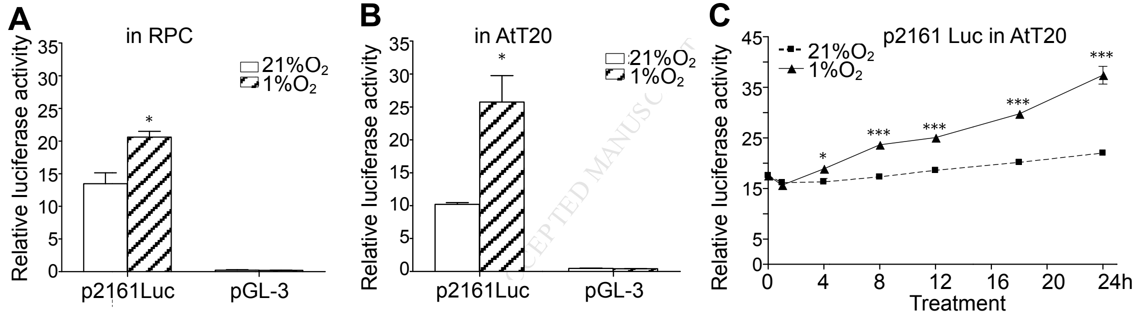

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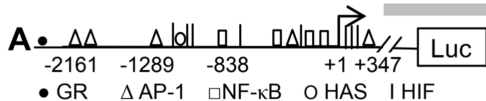

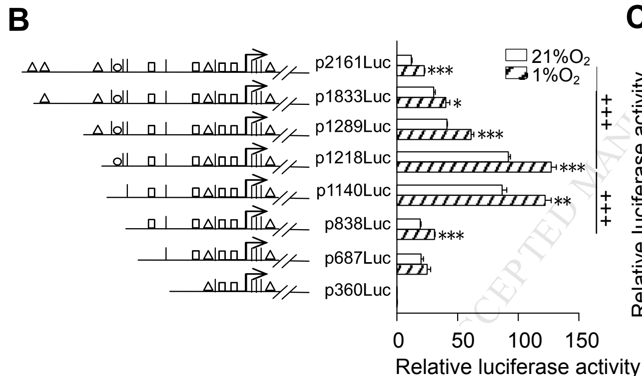

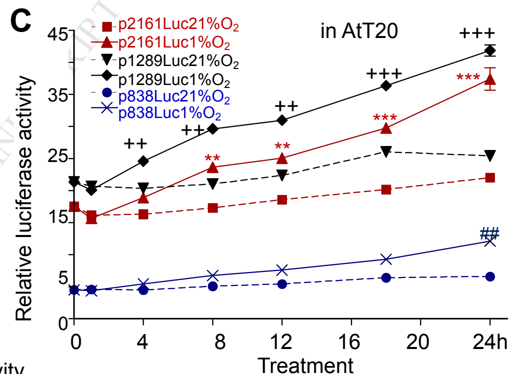

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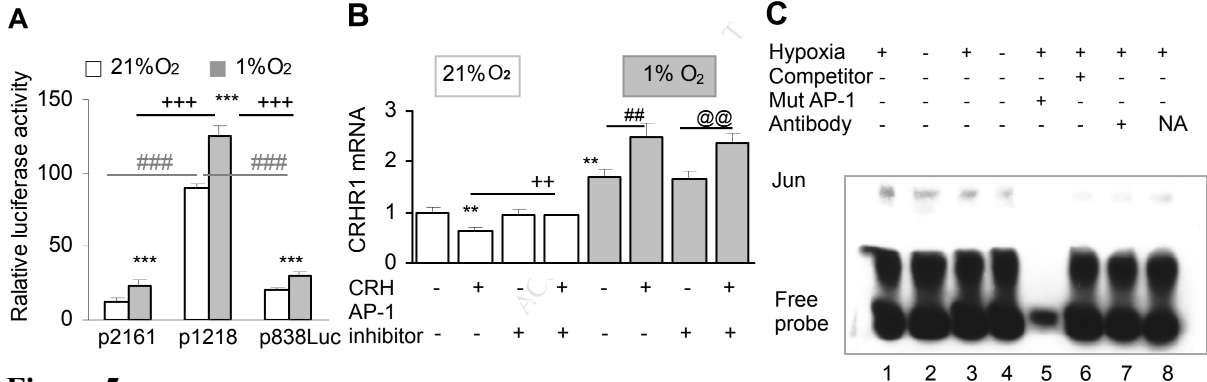

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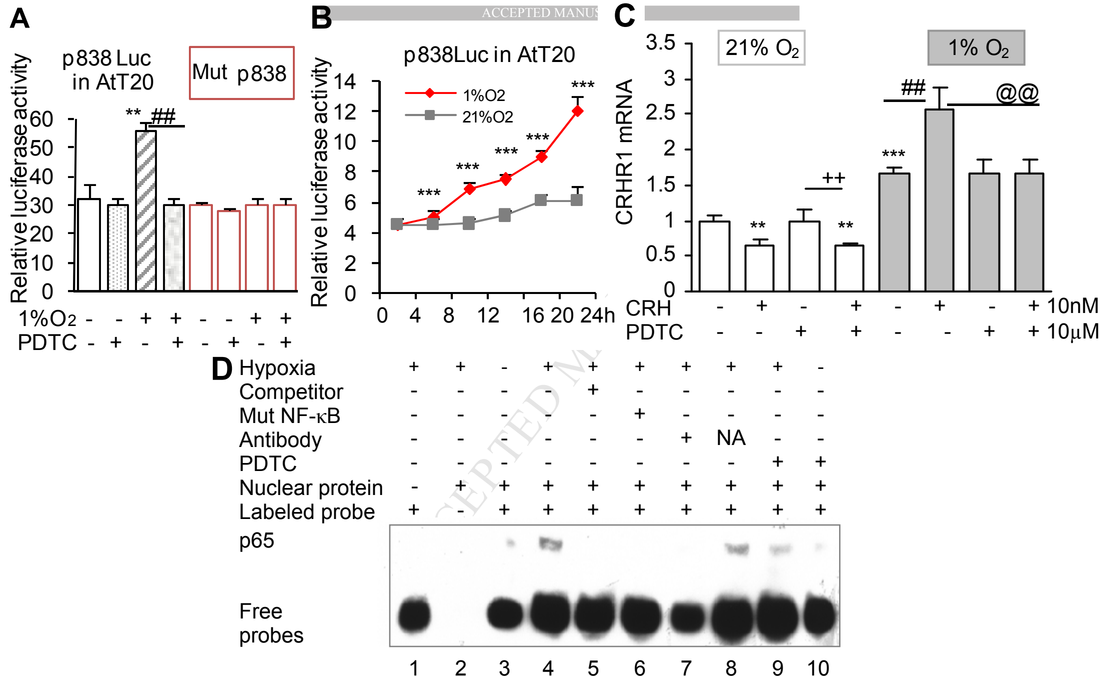

Figure 6.

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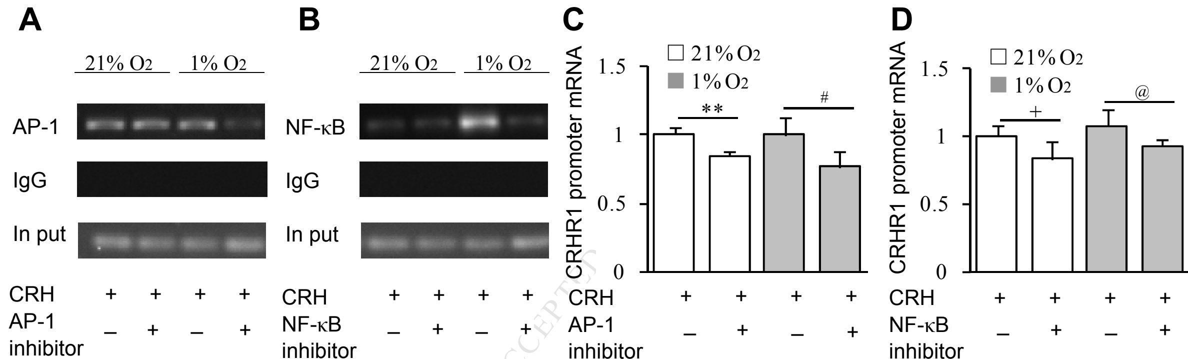

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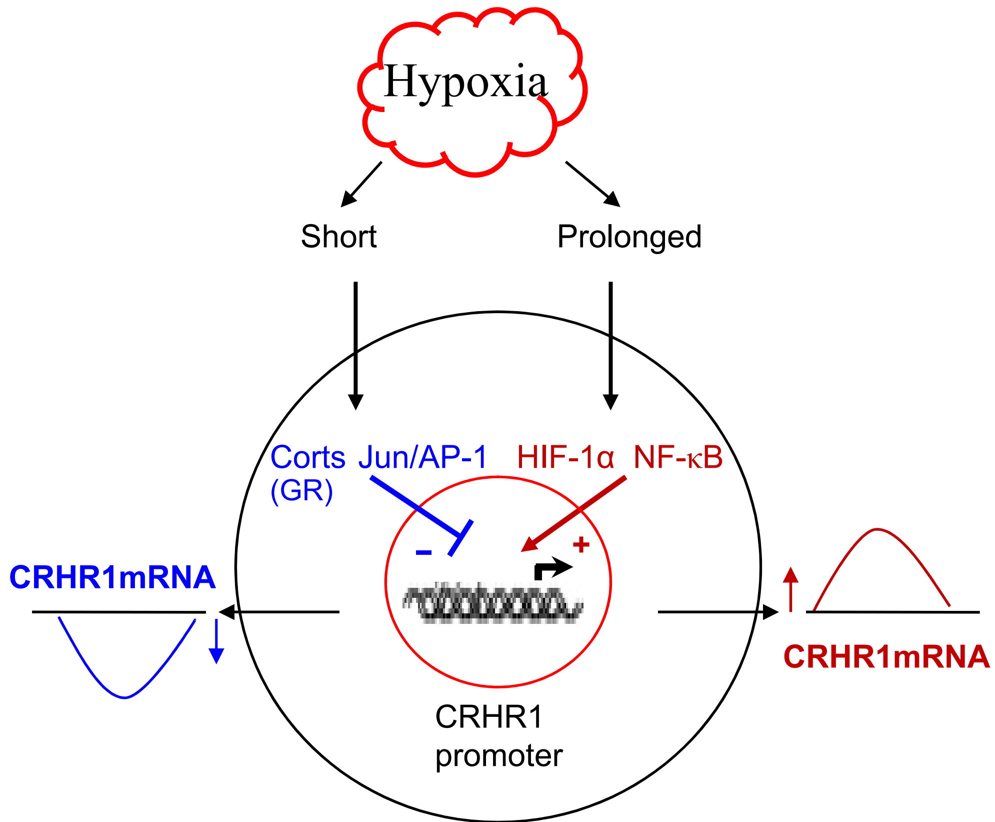

Figure 8.

Highlights

- ●*CRHR1* mRNA response to hypoxia is spatio-temporal and developing stage dependent.
- ●Hypoxia induces a biphasic expression of *CRHR1* mRNA in adult rat pituitary.
- ●c-Jun/AP-1 exerts a negative control at *CRHR1* promoter,-p2161 to -p1289.
- ● HIF-1α exerts a positive control at *CRHR1* promoter,-p1218 to -p1140 and NF-κB,-p838.
- ●Integration of negative and positive input is required in *CRHR1* transcription during hypoxia.

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