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The integration of multiple signaling pathways provides for bidirectional control of *CRHR1* gene transcription in rat pituitary cell during hypoxia

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Abstract

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

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

1. Introduction

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

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

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

2. Materials and methods

2.1. *Animals*

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

sperm was microscopically observed in vaginal smears was designated as embryonic day 0 (E0). The pregnant rats were randomly allocated to gestational dams to hypoxia (SGH) and control groups. Rats were housed individually 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 provided *ad libitum* and the cages were cleaned twice weekly. All experiments were conducted accordance with the NIH laboratory animal care guidelines. All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee of School of Medicine, Zhejiang University (ZJU201304-1-01-025).

2.2. *Prenatal hypoxia stress*

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

2.3. *Adult hypoxia stress*

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

China) weighing 180 ± 20 g were group-housed in the behavior lab 7 days for environmental adaptation before experiments. Rats in the hypoxia group were placed in a hypobaric chamber and exposed to hypobaric hypoxia of 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 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 (Control, Con) was placed in the same chamber set at sea level (~ 21 % O_2). Rats were randomized into different groups. 1. The Control group was injected (ip) with 0.9% saline. 2. The Hypoxia group was injected with vehicle (0.9% saline) before hypoxia stress. 3. Dex group was injected with Dexamethasone (Dex, 500 μg/kg, ip) for 2 days (4 h hypoxia /day, Fig.2I). 4. PDTC group was injected with PDTC (The pyrrolidine dithiocarbamate, an inhibitor of 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% O_2, Fig.2B,G). 5. An antagonist group (Fig.2C,H) was treated with CP154,526 (an antagonist of CRHR1, 30 mg/kg, kindly donated by Pfizer Inc.USA). After exposure, rats were rapidly decapitated within half an hour at 14:00 -14:30 to minimize circadian rhythm effects.

2.4. *Sample collection*

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

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

Plasma corticosterone (Cayman Chemical) was estimated with commercial ELISA kits for rats. The sensitivity of the assay was 0.40 ng/mL, and interassay and intraassay coefficients of variation were 6.5% and 4.5%, respectively. The antibody cross-reacted 100% with corticosterone and <0.5% with other steroids.

2.5. *Real-time qPCR and DNA methylation analysis of the CRHR1 promoter*

Total RNA was reverse-transcribed to cDNA using TransScript^{TM} First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Changes in human CRHR1 (NM_001145146.1) and rat CRH (NM_031019.1), CRHR1 (NM_030999.3), were assessed using SYBR Premix Ex Taq^{TM} (TaKaRa Biotechnology Co., Ltd., Dalian, China). In addition, rat 18S ribosomal RNA was amplified for each sample as an endogenous control, and the cycle threshold was subtracted from the target threshold value. All samples and negative controls were prepared in duplicate wells of a 384-well plate and analyzed using the PRISM7900HT real-time PCR system (Applied Biosystems, Foster City, CA, USA). The cycle number at threshold (CT value) was used to calculate the relative amount of mRNA. The CT value of each target was normalized by subtraction of the CT value of 18 s. Primers were used in qPCR are shown in the supplementary material (Table S1).

Transcription factor binding sites were predicted using the MATCH software (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi) and http://jaspar.genereg.net/, with cut-off selection for matrix to minimize the false negatives (Supplementary Fig. 1 and Fig. 2 for transcription factor binding sites of mouse and rat in the region of the *CRHR1* promoter). CpG island status within the promoter region of *CRHR1* (NC_005109.2) and bisulfite DNA sequencing PCR (BSP) primer were both analyzed using MethPrimer-Design Primers for Methylation PCRs (http://www.urogene.org/methprimer/index1. html) (Wang et al., 2013; Li and Dahiya, 2002). DNA was isolated from the E12 brain and hypothalamus of E19 embryos and pituitary in offspring. Genomic DNA (500 ng) was bisulfite-converted using the EZ-DNA Methylation-Gold Kit^{TM} (Zymo Research Corp., CA, USA) according to the manufacturer's instructions. Bisulfite treatment of genomic DNA converts cytosine to uracil,

but leaves methylated 5' cytosine unchanged. The BSP primer pairs used for the assessment of the CRHR1 CpG islands (106 bp) were shown in the supplementary material (Table S1).

The BSP products were sequenced using the forward primer by Genscript Biotechnology Co. (Nanjing, China), two samples for each E12 brain and pituitary was sequenced. The first CpG island between -609 and -502bp functions is the major regulatory domain of CRHR1 transcription activity (Wang et al., 2013), methylation of each CpG site within the region were tested. The percentage methylation of each CpG site within the region amplified was determined by the ratio between the peak values of C and T (C/[C+T]), and these levels were determined using Chromas software 2.31.

2.6. *Plasmid construction and site-directed mutagenesis*

Genomic DNA was isolated from the anterior pituitary of male Sprague Dawley rats, and used as a template to amplify the 5'-flanking region of the CRHR1 gene ranging from -2161 to +347 by using a primer set (supplementary Table S1). Primers were designed based on Rattus norvegicus genome data resources (NW_047340.1, Rn10_WGA1860_4:1983790-1984316). The amplified PCR product was subcloned into pMD18-T vector (Takara) and sequenced, The PCR fragment was isolated again by digesting of XhoI and HindIII, and then subcloned into a promoter less luciferase vector (pGL3-basic; Promega) in the sense orientation to generate p2161Luc. A series of truncated pGL3-basic plasmids containing the 5'-flanking region of the rat CRHR1 gene (-2161/+347, -1833/+347, -1795/+347, -1692/+347, -1289/+347, -1248/+347, -1218/+347, -1140/+347, -838/+347, -687/+347 and -360/+347) were constructed in the similar manner. Two sets of mutants with the NF-κB site positioned at -809~-800 (p838) mutation and c-Jun(AP-1) mutation were constructed by site-directed mutagenesis to create Mutated Luc using primers (supplementary Table S1). The full open reading frame of rat c-Jun gene was amplified by PCR from rat cDNA based on published sequence (1,005 bp; NM_021835.3) by using forward primer

(supplementary Table S1). pcDNA3.1-c-Jun was generated by inserting the PCR fragment into the pcDNA3.1 vector (Invitrogen) and sequencing.

2.7. *Cell culture and transfection and Cell treatment*

We established an *in vitro* cell culture models. AtT20[mouse pituitary tumor cells AtT20, American Type Culture Collection (ATCC) CCL-89™]cell lines were grown in RPMI medium 1640 (Gibco) or DMEM (Gibco), respectively, containing 10% (vol/vol) FBS, and 100 U/ml penicillin at 37 °C in a humidified incubator with 5% (vol/vol) CO_2. AtT20 cells were plated into 12-well plates with approximately 80% confluence. Two days after plating, cells were transiently transfected with 1 μg reporter construct using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. For cotransfection experiments, 1 μg reporter plasmid was cotransfected with 1 μg pcDNA 3.1-c-Jun/AP-1 or the empty pcDNA 3.1 vector. All analyses were performed 24-48 h after transfection.

For primary pituitary cell (RPC) culture, the anterior pituitary glands from male Sprague Dawley rats (male, 180-200 g) were quickly removed, and then chopped into little pieces (about 1×1 mm) with a small dissecting scissor, and dispersed by incubation with 1 mg/ml trypsin (Sigma, Madrid, Spain) in Hank's balanced salt solution (Life Technologies, Inc., Paisley, UK) at 37°C for 15 min. The primary anterior pituitary cells were cultured in DMEM containing 100 U/ml penicillin G potassium, 1 mg/ml streptomycin sulfate and 10% FBS. The cells were maintained 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 primary anterior pituitary cells were nucleofected (Nucleofector, Amaxa Biosystems GmbH, Cologne, Germany) in an electroporation cuvette along with nucleofector solution R and 2 μg plasmids using the programme A-023. Cells were transferred into fresh pre-warmed media with 10% FBS and incubated for 24 h. Dual-luciferase reporter assays were performed using a dual-luciferase reporter assay system (Promega Corp., WI, USA).

The hypoxia treatment was performed using the Proox Model P110 and ProCO2 Model P120 hypoxia systems (BioSpherix, USA). AtT20 and RPC were moved to the hypoxia incubator in which the oxygen level was set as 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 al., 2016; Zhao et al., 2013; Zhang et al., 2013). AtT20 cells were treated with CRH(10 nM, Tocris Bioscience), PDTC (10 or 100 μM), antibody (NF-κB or AP-1) , or AP-1 inhibitor (SR11302,1 or 10 μM, Tocris Bioscience) for 24 h.

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

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

2.9 *Chromatin Immunoprecipitation (ChIP) Assay*

The chromatin immunoprecipitation (ChIP) assay was performed using ChIP kit according to the manufacturers' instructions (EZ ChIP™-Catalog # 17-371, Millipore, USA). AtT-20 cells were seeded in 60-mm dishes, after treatment for 24 h, AtT-20 cells were fixed with 1% formaldehyde at room temperature for 10 minutes and terminated with glycine. The fixed cells were harvested with ice-cold PBS containing Protease Inhibitor Cocktail II, and the pellet was resuspended in SDS lysis buffer. Then, the DNA was sheared to 200- to 1000-bp fragments by sonication. Immunoprecipitation was performed using c-Jun antibody (1:50, Cell Signaling) or NF-κB p65 antibody (1:100, Cell Signaling) with rotation overnight at 4°C. Protein/DNA complexes were captured in elution buffer, and cross-links were reversed to free DNA. After DNA purification, normal PCR and quantitative PCR was performed using CRHR1 promoter-specific primers and the binding sites (Supplementary S Table 2 and 3).

3.0. *Statistical analysis*

All studies were conducted by an investigator blind to SGH groups. For CRHR1 gene methylation in embryos and offspring pituitary, that data were analyzed using two-tailed unpaired *t* tests. For Effect of CRH, PDTC, and AP-1 on CRHR1 mRNA in vitro were analyzed by using one-way ANOVA. Post hoc comparisons after one-way ANOVA were made using Tukey's post hoc test (GraphPad Prism6). All data are presented as mean ± SD. $P<0.05$ was considered statistically significant.

3. Results

3.1. *CRHR1 expression and CRHR1 methylation in embryos brain and P1-P90 pituitary after short exposure of gestational dams to hypoxia*

To determine whether gestational short period hypoxia alters CRHR1 promoter methylation, and thereby changes

CRHR1 mRNA, CRHR1 mRNA and CRHR1 promoter methylation were measured. CRHR1 mRNA and CRHR1 promoter methylation was not significantly changed in the CpG island 1 of the promoter in the E12 brain (Supplementary Fig. 3A and B). We next investigated the effect of SGH on CRHR1 mRNA expression and methylation of the CRHR1 gene promoter in the pituitary of postnatal male offspring who had not been subjected to a further hypoxia test. CRHR1 mRNA levels were markedly decreased in P1, P7 and P14 offspring (Fig.1A), however on day P21, both control and SGH animals the CRHR1 mRNA levels were dramatically increased to a similar extent in each. DNA methylation levels were markedly reduced at sites -547,-544, -535 within the CRHR1 CpG promoter in P1 males ($p<0.05$; Fig. 1B), but no significant differences in methylation within the CRHR1 promoter CpG island 1 were found at P7, P14 or P21 (Fig. 1B). On day P90, there was no significant difference from control and SGH rats in the CRHR1 mRNA levels and CRHR1 DNA methylation levels in pituitary (Fig. 1C,D). These data indicate that there is no logical association between CRHR1 mRNA and CRHR1 DNA methylation in postnatal offspring following exposure to gestational hypoxia.

3.2. *Hypoxia-induced bidirectional expression and regulation of CRHR1 mRNA in rat pituitary*

Short periods of 1 h and 8 h hypoxia (SH, at altitude of 7000 m) reduced CRHR1 mRNA whereas prolonged hypoxia (PH) of 2 d or 5 d at altitude of 5000 m (4 h hypoxia/day) increased CRHR1 mRNA in adult male rat pituitary (Fig. 2A, B), thus showing a spatio-temporal bidirectional (biphasic) response pattern of CRHR1 expression. To further explore the regulatory and transcriptional mechanism underlying the bidirectional response of CRHR1 under the similar hypoxia, SH and PH condition, the adult rats were treated with hypoxia and with or without pretreatment of PDTC (a NF-κB inhibitor), CP154,526 (a CRHR1 antagonist), or Dex (glucocorticoid hormone and suppressant of CRH), respectively (Fig. 2C,D,E, and I). The hypoxia 8 h-reduced pituitary CRHR1

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

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

To address CRHR1 gene transcriptional mechanisms in pituitary cells under hypoxia, the p2161Luc plasmid containing the length of CRHR1 gene promoter region (-2161) was constructed and transfected into primary rat pituitary cell (RPC) cells or AtT20 cells, and dual-luciferase activity assay was performed to test the CRHR1 promoter activity. Hypoxia (1% O_2) caused a significant increase in promoter activity of the reporter gene in RPC 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

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

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

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

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

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

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

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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

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

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

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

5. Conclusion

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

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

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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

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

p2161Luc (the length of CRHR1 promoter) and pRL-TK plasmids and pRL-TK plasmids (empty vector pGL3-basic, as a control) respectively. **A** Hypoxia (1% O_2) increased transcription of the rat CRHR1 gene in cultured PRC cells. **B** Hypoxia (1% O_2) increased CRHR1 transcription in AtT20 cell. **C** Hypoxia (1% O_2) time-dependently increased Transcription of CRHR1 promoter (p2161Luc) in AtT20 cell. All data are presented as means ± SD. n=3-4 in each group, *p<0.05, ***p<0.001, vs. normoxia control (21% O_2) at the time point indicated.

Fig. 4. Transcriptional factors, HIF-1α, NF-κB, and AP-1 were involved in the hypoxia-induced CRHR1 promoter activation. **A** Predicted binding sites distribution of hypoxia responsive cis-regulatory element in rat CRHR1 promoter region, including four NF-κB (rectangle), five Jun /AP-1(triangle), eight HIF-1α (vertical line) binding sites, and one HAS(circle) as indicated in the 5' flanking region from -2161 to +360. **B** A series of deletion (truncated reporter) constructs were generated according to the predicted distribution of the transcriptional factors at CRHR1 promoter region and comparison of those transcriptional activities of p2161Luc, p1218Luc, and p360Luc were taken respectively. Under both hypoxia and normoxia, the transcription activity of p1218Luc was higher than that of p2161Luc and p838Luc, due to AP-1 inhibitory effect on transcription activation by HIF-1α. The p838Luc showed a lower transcription. Most shortened p360Luc had no transcriptional activity. All data were compared between normoxia and hypoxia as well as among p2161Luc, p1218Luc, and p360Luc. All data are presented as means ± SD. n=3-4 for each group, *p<0.05, **p<0.01, ***p<0.001, between normoxia and hypoxia, respectively, and $^{+++}$p<0.001, p1218Luc vs. p2161Luc or p1218Luc vs. p838Luc. **C** Hypoxia (1% O_2)-time course (1 to 24 h)-dependent increase in the transcriptional activity among p2161Luc, p1289Luc, and Luc p838Luc of CRHR1 gene in AtT20 cell, $^{++}$p<0.01, $^{+++}$p<0.001, for p1289Luc between normoxia and hypoxia (two black line); **p<0.01, ***p<0.001, for p2161Luc between normoxia and hypoxia (two red line); $^{\#\#}$p<0.01, for p838Luc between normoxia 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

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

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

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

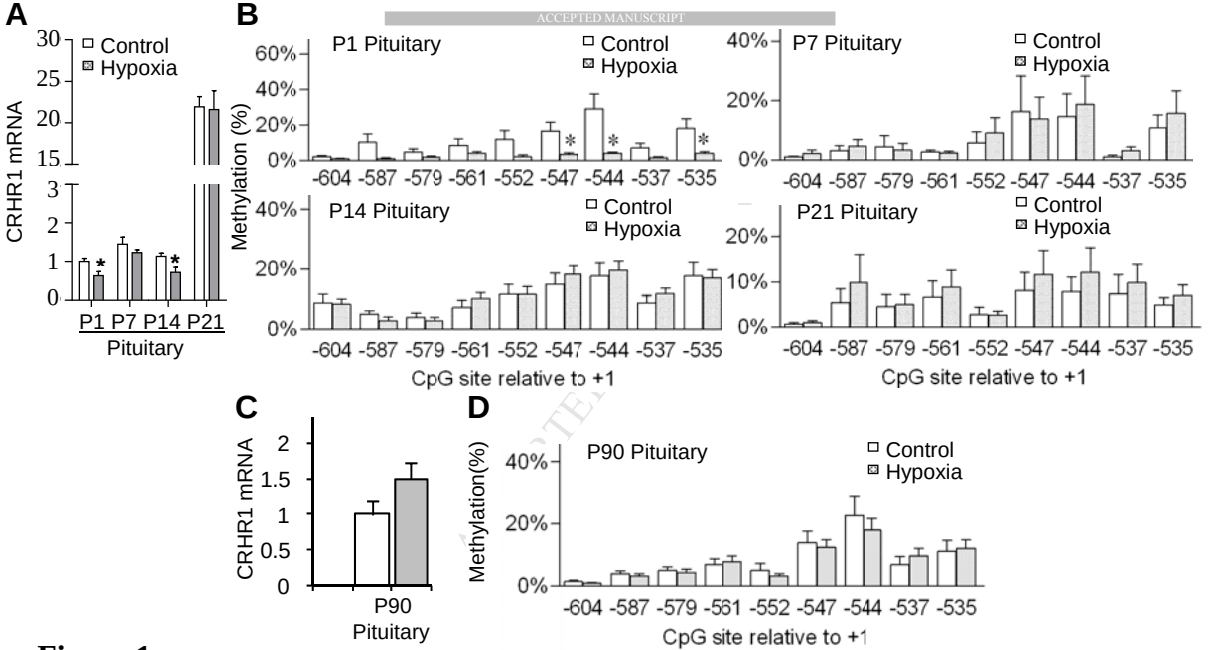

Figure 1.

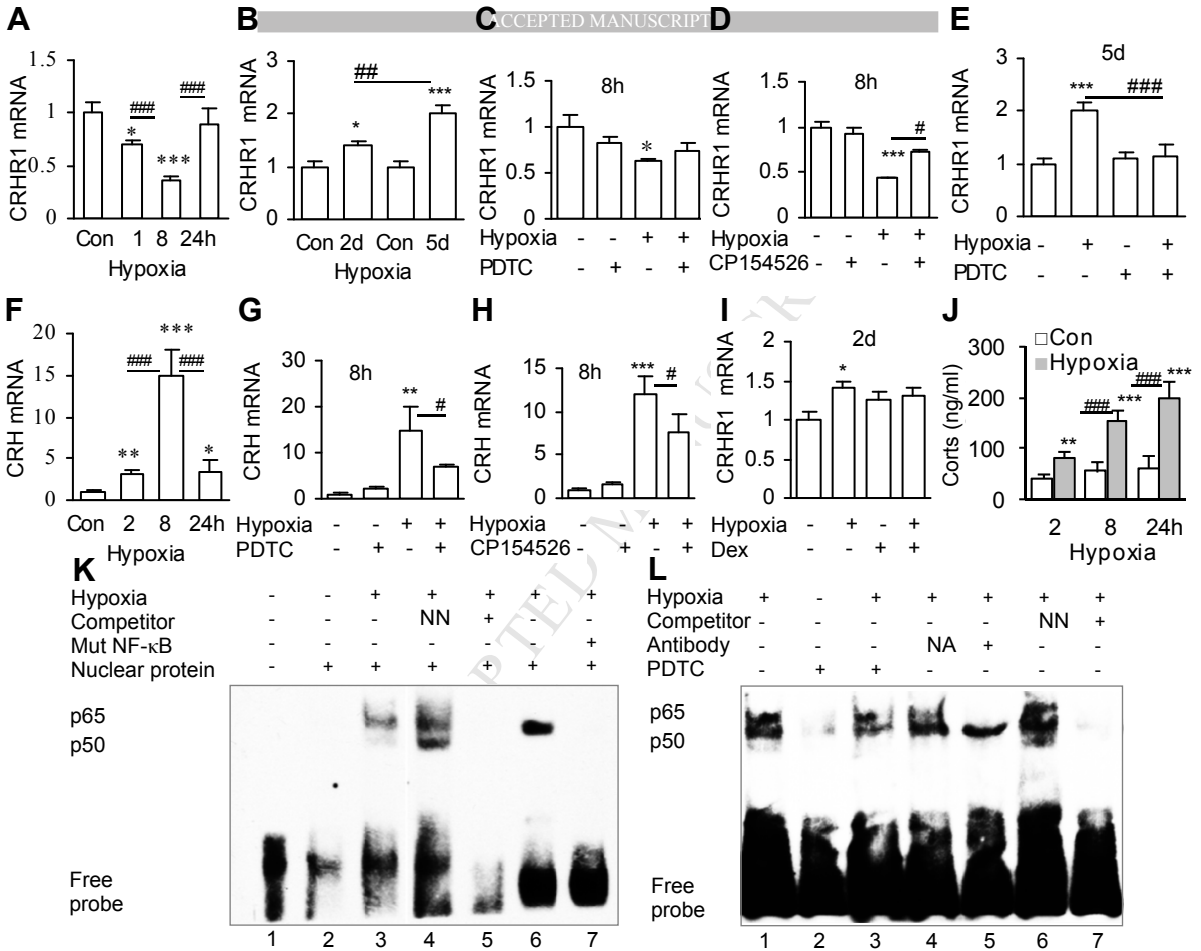

Figure 2.

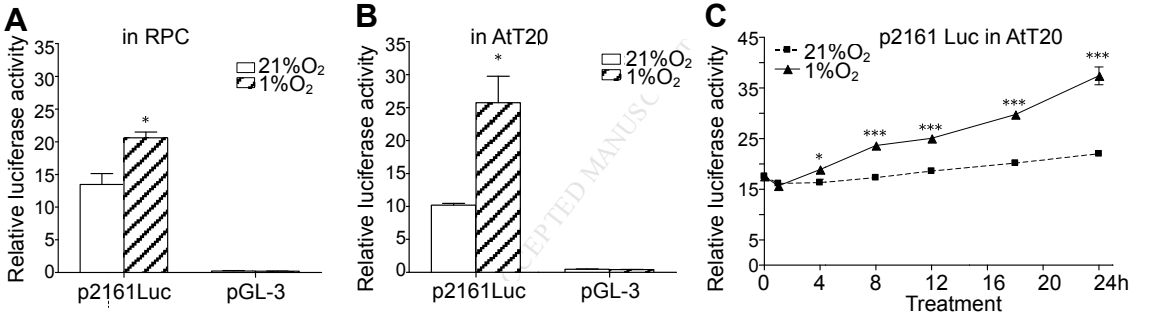

Figure 3.

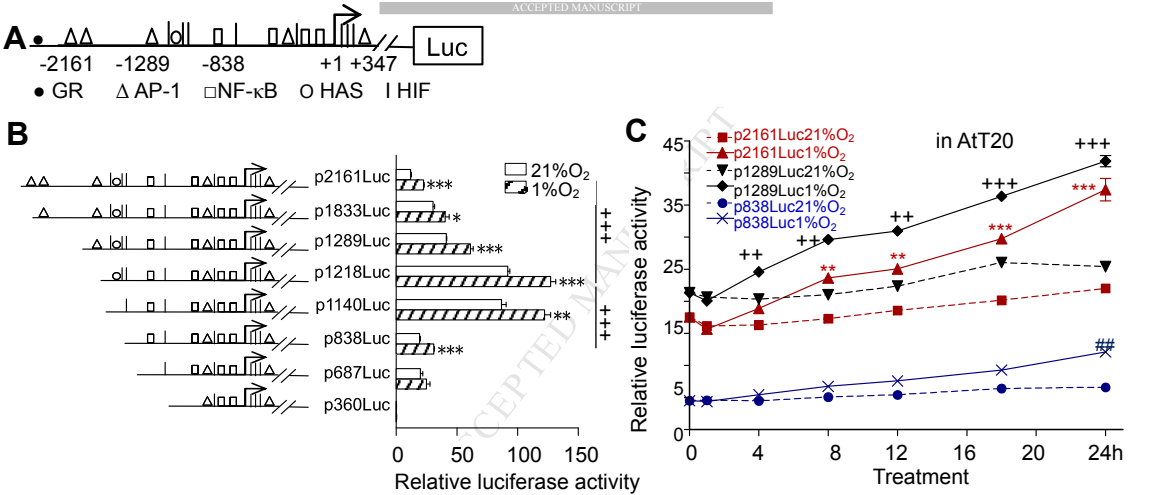

Figure 4.

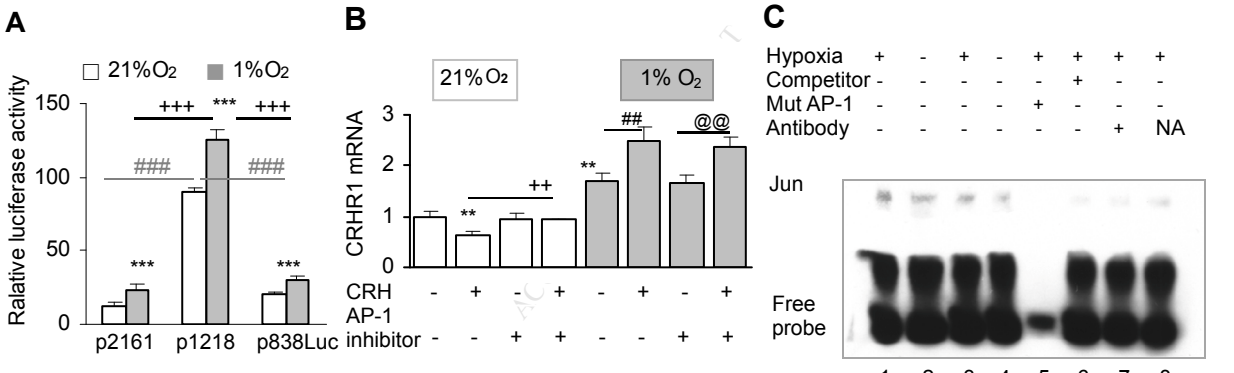

Figure 5.

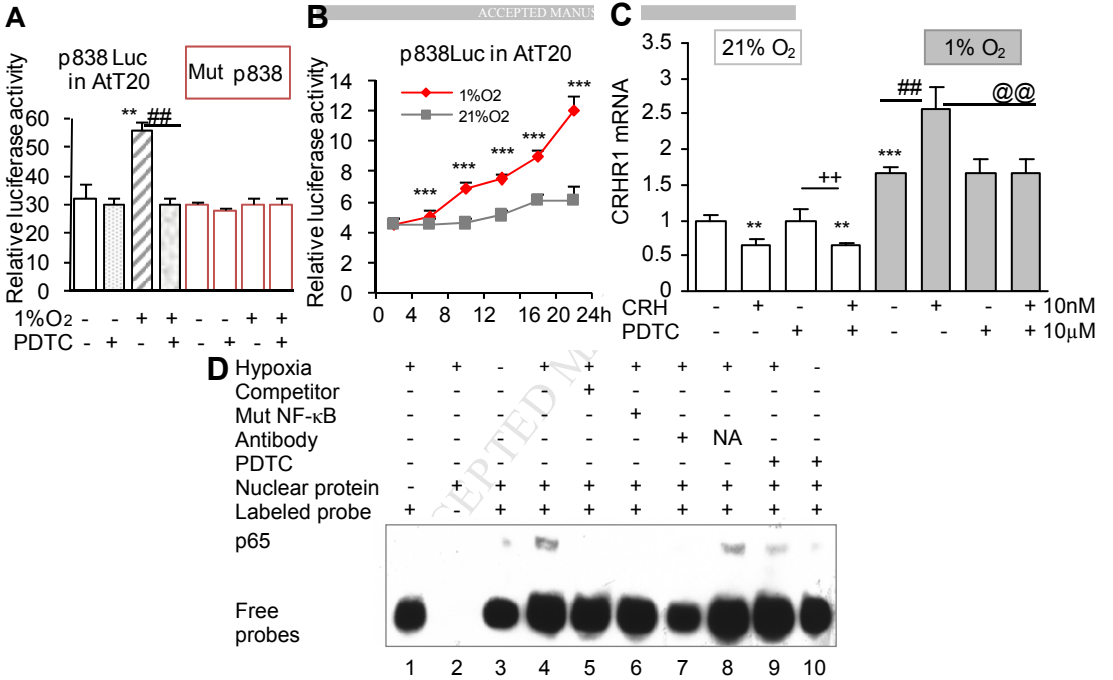
A
p838 Luc in AtT20
Mut p838
Relative luciferase activity
1%O2 - - + + - - + +
PDTC - + - + - + - +
B
p838Luc in AtT20
1%O2
21%O2
Relative luciferase activity
0 4 8 12 16 20 24h
C
21% O2
1% O2
CRHR1 mRNA
CRH - + - + - + - + 10nM
PDTC - - + + - - + + 10μM
D
Hypoxia + + - + + + + + -
Competitor - - - + - - - - -
Mut NF-κB - - - - + - - - -
Antibody - - - - - + NA - -
PDTC - - - - - - - + +
Nuclear protein - + + + + + + + +
Labeled probe + - + + + + + + +
p65
Free probes
1 2 3 4 5 6 7 8 9 10

Figure 6.

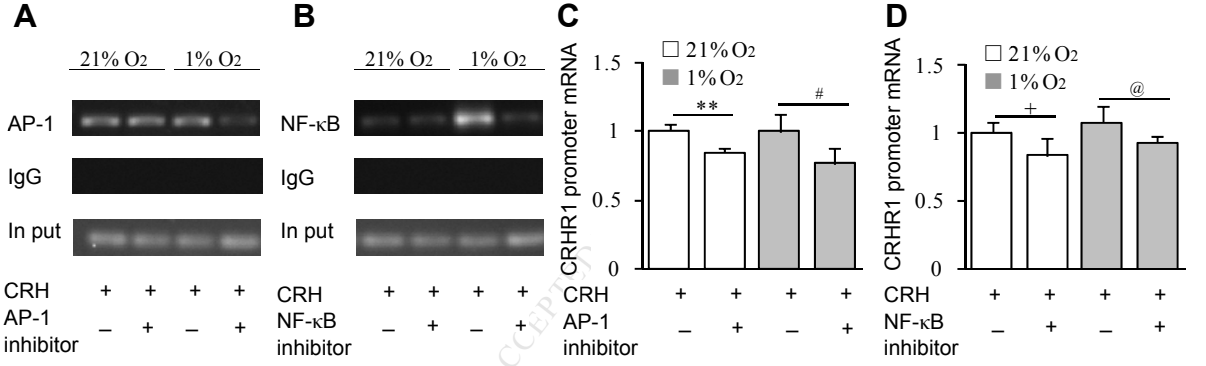

Figure 7.

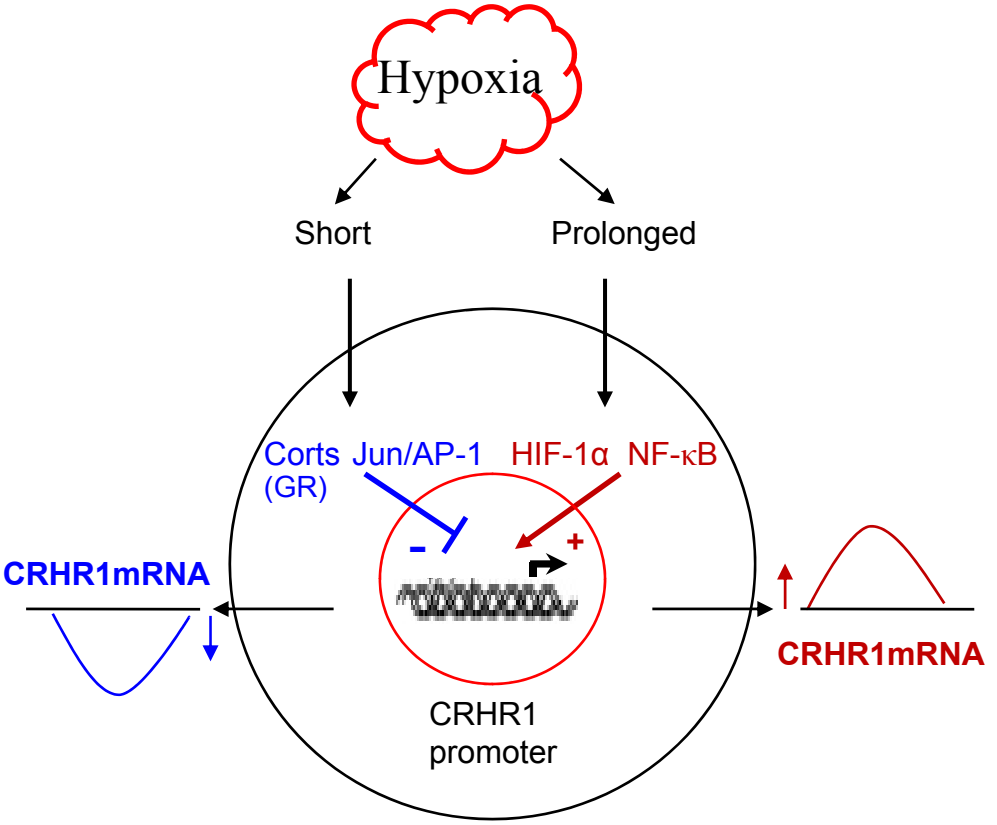
Hypoxia
Short
Prolonged
Corts
(GR)
Jun/AP-1
HIF-1α
NF-κB
-
+
CRHR1mRNA
CRHR1mRNA
CRHR1
promoter

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.