The Involvement of Retinoic Acid Receptor-α in Corticotropin-Releasing Hormone Gene Expression and Affective Disorders

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Background: Corticotropin-releasing hormone (CRH) is considered the central driving force in the stress response and plays a key role in the pathogenesis of depression. Retinoic acid (RA) has been suggested by clinical studies to be associated with affective disorders.

Methods: First, hypothalamic tissues of 12 patients with affective disorders and 12 matched control subjects were studied by double-label immunofluorescence to analyze the expression of CRH and retinoic acid receptor-α (RAR-α). Second, critical genes involved in the RA signaling pathways were analyzed in a rat model of depression. Finally, the regulatory effect of RAR-α on CRH gene expression was studied in vitro.

Results: We found that the expression of RAR-α was colocalized with CRH neurons in human hypothalamic paraventricular nucleus (PVN). The density of RAR-α-immunoreactive neurons and CRH-RAR-α double-staining neurons was significantly increased in the PVN of patients with affective disorders. The ratio of the CRH-RAR-α double-staining neurons to the CRH-immunoreactive neurons in affective disorder patients was also increased. Recruitment of RAR-α by the CRH promoter was observed in the rat hypothalamus. A dysregulated RA metabolism and signaling was also found in the hypothalamus of a rat model for depression. Finally, in vitro studies demonstrated that RAR-α mediated an upregulation of CRH gene expression.

Conclusions: These results suggest that RAR-α might contribute to regulating the activity of CRH neurons in vivo, and the vulnerable character of the critical proteins in RA signaling pathways might provide novel targets for therapeutic strategies for depression.

Key Words: Chronic stress, corticotropin-releasing hormone, depression, gene regulation, hypothalamus, retinoic acid receptor

Corticotropin-releasing hormone (CRH), a 41-aminoacid peptide that is mainly produced in the hypothalamic paraventricular nucleus (PVN), plays a crucial role in stress response and is considered the central driving force in the activity of hypothalamic–pituitary–adrenal (HPA) axis (1,2). The HPA axis is generally regarded by many as the final common pathway for a number of signs and symptoms of depression (3,4). A number of studies have pointed to the involvement of the steroid/thyroid receptor family in the regulation of the activity of CRH neuron in the hypothalamic PVN, such as the receptor for estrogens (ER), androgens (AR), and glucocorticoids (GR) (5–8), which might play an important role in the pathogenesis of affective disorders.

It is of interest to notice, however, that another important member of the steroid/thyroid receptor family, the retinoic acid receptor (RAR), has been rarely mentioned in the etiology of depression, although as a matter of fact some studies have suggested an association between retinoic acid (RA) and affective disorders (9,10). Retinoic acid has been used as a routine stimulus to induce CRH expression in cell lines for the study of regulation of CRH in vitro (11,12). Clinical case reports have described the development of affective disorders and suicide in some acne patients treated with isotretinoin (13-cis-RA) (13–17), although with some controversies. Furthermore, depression associated with vitamin A intoxication was also reported (18). In addition, a positron emission tomography (PET) study showed decreased brain metabolism in the orbitofrontal cortex, a brain area known to mediate symptoms of depression (19), in acne patients treated with isotretinoin (20). The cellular machinery required for transducing the RA signaling pathway (e.g., the nuclear receptors [RARs], metabolic enzymes, and binding proteins) are found to be expressed in the rodent hypothalamus. More precisely, first, the expression of two subtypes of RARs, RAR-α and RAR-β, has been reported in the rodent hypothalamus (21,22). Second, retinal dehydrogenase (RALDH) 1 and 3, the critical enzymes for the synthesis of RA are also found in the mousehypothalamus (23). At last, the cellular retinoic acid-binding protein 1 (CRABP1), which seems to function to decrease cellular responses to all-trans RA (24,25), has been observed in the rat hypothalamus (21). However, to our knowledge, so far there is no study on CRH neurons in the hypothalamic PVN as a possible target for RA in the stress response or affective disorders.

We aimed, on the basis of the aforementioned observations: 1) to investigate whether the activity of the CRH neurons in affective disorders might be modulated by a direct effect of RAR-α; 2) if so, in an animal model for depression, to study whether RA metabolism and signaling is disturbed in the hypothalamus of living animals coping with stress, and 3) in a cell line, to investigate whether RAR-α regulates CRH gene expression. Because RAR-β seems to be expressed in areas other than the PVN of the hypothalamus (22), we focused on the possible function of RAR-α in this study. Our hypothesis was that a disturbed equilibration of RA signaling might contribute to the etiology of affective disorders and RAR-α can thus be a novel link between RA and affective disorders.

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Table 1. Brain Material of Patients with Mood Disorders and Control Subjects

Code	Age	Gender	PMD
Mood Disorders			
D1	45	M	7:00
D2	55	F	4:54
D3	61	M	40:20
D4	71	M	16:15
D5	72	F	22:00
D6	72	F	28:25
D7	74	M	62:55
D8	79	M	21:10
D9 b	68	M	5:55
D10 b	70	M	4:50
D11 b	73	M	5:15
D12 b	75	F	4:00
Mean ± SD	68 ± 10		18:34 ± 18:08
Control			
C1	44	M	10:00
C2	46	F	10:25
C3	47	M	29:13
C4	61	F	17:45
C5	66	M	41:00
C6	70	M	9:00
C7	74	M	8:00
C8	76	M	17:55
C9	77	F	2:40
C10	78	M	53:00
C11	78	M	4:20
C12	83	F	7:45
Mean ± SD	67 ± 14		17:35 ± 15:41

b, bipolar disorder; PMD, postmortem delay in hours: minutes.

Methods and Materials

Part 1: Immunohistochemistry Study in Human Brain Material

Human Brain Material. Postmortem brain samples were studied, from 12 patients with affective disorders and 12 matched control subjects (Table 1). Matched affective disorder patient and control human brains were obtained by autopsy within the framework of the Netherlands Brain Bank. Permission was obtained for a brain autopsy and for the use of the material and medical records for research purposes. The DSM-III-R/DSM-IV criteria were used for the diagnosis of major depression (MD)/major depressive disorder (MDD) or bipolar disorder (BD) at any time during life. The criteria for the presence, duration, and severity of symptoms of either MD/MDD or BD as well as the exclusion of other psychiatric and neurological disorders were systematically scored by two qualified psychiatrists (Dr. W.J.G. Hoogendijk or Dr. E. Vermette). Eight patients fulfilled the criteria for MD/MDD, and four fulfilled the criteria for BD. For detailed information, see Table 1 in Supplement 1.

Immunohistochemistry and Image Acquisition. For the immunohistochemical staining for RAR-α, the sections from human PVN region were incubated with anti-RAR-α antibody (SC-551, Santa Cruz, California). The specificity of this antibody for immunohistochemistry has been proved by previous studies (26,27). We also confirmed the specificity of the antibody by western blotting (Figure 1 in Supplement 1). Generally, the sections were sequentially incubated with the anti-RAR-α antibody, biotinylated secondary goat anti-rabbit antibody (Vector, Burlingame, California), and avidin-biotin complex (Vector) and finally exposed to the substrate. Photographs were collected with a Nikon E800u microscope (Nikon, Tokyo, Japan).

Immunofluorescence and Confocal Laser Scanning Microscopy. The immunofluorescence staining for CRH and RAR-α was performed on every 100th section taken along the rostrocaudal axis throughout the PVN region. Generally, the sections were sequentially incubated with the anti-RAR-α antibody and anti-CRH antibody (PFU83) (6), FITC-labeled goat anti-rabbit antibody (Southern Biotech, Birmingham, Alabama) and biotinylated anti-rat antibody (Vector), and finally Cy3-labeled streptavidin (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland).

A confocal laser scanning microscope (LSM 510, Carl Zeiss, Göttingen, Germany) was used for the determination of fluorescence. Briefly, the contour of the entire PVN field, defined by CRH immunostained cells, was manually outlined at low magnification (10× objective), and subsequently all the fields within the PVN region were scanned one by one (from dorsal to ventral and from medial to lateral) with the 40× objective.

For each section, 50% of the photographs were selected randomly. In each photo, all cellular profiles showing immunoreactivity for either only CRH or RAR-α or double-stained were

Figure 1. Retinoic acid receptor (RAR)-α immunoreactivity in the human hypothalamic paraventricular nucleus (PVN). **(A–D)** The RAR-α immunoactivity in the PVN of a control **(A,C)** and a patient with affective disorder **(B,D)**. **C** and **D** represent a 4× higher magnification of the framed field of **A** and **B**. For **A** and **B**, bar in the lower right corner = 80 μm; for **C** and **D** bar in the lower right corner = 20 μm.

counted (the area of one photo is approximately .1 mm^2, representing a volume of .0006 mm^3 because the sections' thickness is 6 μm). A cellular profile was only counted if at least part of the nucleus was visible. By summating the number of the immunoreactive (IR) cell profiles in all the selected photos from all the slices of one subject, the total number of IR cell profiles was obtained. The density of the cell profiles for each subject was obtained by dividing the total number by the total volumes counted.

Part 2: Studies in the Chronic Unpredictable Mild Stress Animal Model

Chronic Unpredictable Mild Stress Protocol and Open-Field Tests. Eighteen rats were randomly assigned to an unhandled control ($n = 7$) or a chronic stress group ($n = 11$), matched for weight and locomotion behavior in an open-field test before the onset of chronic unpredictable mild stress (CUMS). The CUMS paradigm consisted of daily exposure to alternating stressors along with occasional overnight stressors for 3 weeks.

Open-field tests were conducted after each week's CUMS. The open-field apparatus consisted of a black square arena 100 cm × 100 cm, with a black wall 30 cm high. The floor was marked with a grid dividing the floor into 16 equal-size squares. Each rat was placed in the central square and observed for 5 min. A record was kept for the latency each rat left the central four squares, the time it spent in the central four squares, the numbers of rearing (defined as it standing upright on its hind legs) and the numbers of grid lines it crossed with all paws.

Hypothalamus Dissection and Sample Preparation. All the rats were decapitated after the last open-field tests. Blood samples were collected in tubes containing heparin sodium as an anticoagulant and centrifuged at 4°C, and after separation the plasma was stored at −70°C until assayed with Rat corticosterone enzyme-linked immunosorbent assay (ELISA) kit (RapidBio Lab, Calabasas, California). The brain was removed immediately, and a hypothalamic block was dissected. Tissue samples were homogenized in cold Trizol (Invitrogen, Carlsbad, California) for further RNA and protein extraction according to the manufacturer's instructions.

Western Blotting. Hypothalamic protein samples were detected with antibodies against RAR-α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Western blotting experiments were performed as described previously (28) with minor modifications.

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction. Hypothalamic RNA samples were reverse transcribed and analyzed. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to analyze CRH, arginine vasopressin (AVP), RAR-α, CRABP1, RALDH1, and RALDH3 messenger RNA (mRNA) expressions in the rat hypothalamus. The β-actin mRNA expression was analyzed as the internal control. See Table 2 in Supplement 1 for primer pair sequences.

Chromatin Immunoprecipitation Assay. Chromatin immunoprecipitation (ChIP) assays were performed to determine whether RAR-α can bind to the CRH promoter in the rat hypothalamus. For ChIP assay, male Sprague Dawley rats were decapitated, and the hypothalami were removed as described in the preceding text. Chromatin solutions from rat hypothalamus were immunoprecipitated with the anti-RAR-α antibody (SC-551, Santa Cruz). For the negative control, no antibody was added. Final DNA extraction was amplified with primers designed to amplify the 320-bp rat CRH promoter region.

Figure 2. Confocal laser scanning images of corticotropin-releasing hormone (CRH) (red) and RAR-α (green) in the PVN of a control **(A–C)** and a patient with affective disorder **(D–F)**. Both sections show the central part (midlevel) of the PVN and contain the largest number of stained neurons. Each picture was made by sequential excitation of the same optical section with the 543- and 488-nm laser. Subsequently, the obtained CRH (Cy3, red) (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) and corresponding RAR-α image (FITC, green) (Southern Biotech, Birmingham, Alabama) were superimposed. The yellow-stained cell profiles point to CRH-RAR-α double-staining cells. Bar = 30 μm. Other abbreviations as in Figure 1.

Part 3: In Vitro Studies in Cell Line

Cell Culture. The human neuroblastoma cell line BE(2)-C, which has been reported to express endogenous CRH mRNA upon the treatment of RA (29), were cultured with DMEM/F12 (DF) (Sigma, St. Louis, Missouri) supplied with 10% fetal bovine serum (FBS) (Gibco, Grand Island, New York).

ChIP. For ChIP assay, BE(2)-C cells were cultured in 100-mm dishes and pretreated with 1 μmol/L RA (Sigma) for 24 hours before harvest. The ChIP assays were performed as described in the preceding text with the anti-RAR-α antibody.

Transient Transfection in BE(2)-C Cells. pcDNA4-RAR-α plasmid was constructed by inserting the human RAR-α complementary DNA into the pcDNA4 vector. For the transient transfection of RAR-α, 1 μg of pcDNA4-RAR-α or pcDNA4 were transfected with Lipofectamine2000 (Invitrogen) in 6-well culture plates. Four hours after transfection, culture media were changed to phenol red-free DF containing 10% FBS and 1 μmol/L RA. The cells were harvested 24 hours later for RNA analyses.

RNA Interference. For RNA interference transfection in BE(2)-C cells, cells were transfected with 1.5 μg each small interfering RNA duplexes (Gene Pharma, Shanghai, China) in 12-well plates with RNAi-Mate (Gene Pharma). Six hours later, the medium were replaced by DF containing 10% FBS and 1 μmol/L retinoic acid and harvested 24 hours later for RNA analyses.

Real-Time Quantitative RT-PCR. Total RNA from BE(2)-C cells was extracted, reverse transcribed, and analyzed by real-time PCR. The CRH, RAR-α, and β-actin expressions were analyzed. Primers used were also shown in Table 2 in Supplement 1.

Statistical Analyses

Throughout this study, values are expressed as mean ± SEM. Statistical analysis was performed by SPSS software (SPSS, Chicago, Illinois). Difference between the control subjects and affective disorder cases was evaluated by nonparametric Mann–Whitney *U* test. Differences in clock time of death and month of death (circular parameters) between control subjects and patients with affective disorders were tested with the Mardia–Watson–Wheeler test (30). For the Western blotting, quantitative polymerase chain reaction (Q-PCR) study differences between the groups were tested with the two-sample *t* tests. Tests were two-tailed. Values of $p < .05$ were considered significant.

See Methods in Supplement 1 for detailed description of the Methods and Materials.

Results

RAR-α-Immunoreactivity in the Human Hypothalamic PVN in Affective Disorders

We first determined whether RAR-α is expressed in the human hypothalamic PVN by immunohistochemistry in the human brain slices. Large population of RAR-α IR cells was observed in the PVN of both control subjects (Figures 1A and 1C) and affective disorder patients (Figures 1B and 1D). The RAR-α-IR cells were polymorphic and variable in size (Figures 1A–1D), distributing widely from rostral to caudal PVN.

The involvement of RAR-α with the CRH neurons in the hypothalamic PVN was studied in the control subjects and patients with affective disorders by immunofluorescence. The gender, age ($z = -.318$, $p = .751$), season and clock time at death ($F = 2$, $\chi^2 = .710$, $p = .701$; and $F = 2$, $\chi^2 = 1.307$, $p = .520$; respectively), brain weight ($z = -.635$, $p = .525$), postmortem delay ($z = -.289$, $p = .773$), and fixation time ($z = -1.330$, $p = .184$) were well-matched for the affective disorders and control group (Table 1 in Supplement 1).

The density of CRH-IR neurons, RAR-α-IR neurons, and CRH-RAR-α double-staining neurons were found to be significantly higher in the PVN in the affective disorder group (Figures 2A–2F). The mean density of CRH-IR neurons in the PVN was approximately 1.6 times higher than that of the control subjects ($z = -3.060$, $p = .002$, Figure 3A). The mean density of RAR-α-IR neurons in the PVN was approximately 2 times higher than that in the control subjects ($z = -3.580$, $p < .001$, Figure 3B). The mean density of CRH-RAR-α double-staining neurons was approximately 2.5 times higher than that of the control subjects ($z = -3.926$, $p < .001$, Figure 3C). In patients with affective disorders, the mean proportion of the density of CRH-RAR-α double-staining neurons of the density of CRH neurons was also significantly higher than that in the control subjects (81% vs. 54%) ($z = -3.522$, $p < .001$, Figure 3D).

No significant differences were found between MD/MDD ($n = 8$) and BD subjects ($n = 4$) ($p \geq .497$) in any one of the aforementioned parameters (i.e., the density of CRH neurons, RAR-α neurons and CRH-RAR-α double-staining neurons, the proportion of the density of CRH-RAR-α double-staining neurons of the density of CRH neurons).

Open-Field Tests and CRH-, AVP-mRNA Expression in the CUMS Rats

To further study the involvement of RA signaling in affective disorders, a CUMS rat model for depression was established, and open-field tests were performed to detect the depression-related behavior in the CUMS rats and control subjects. The average

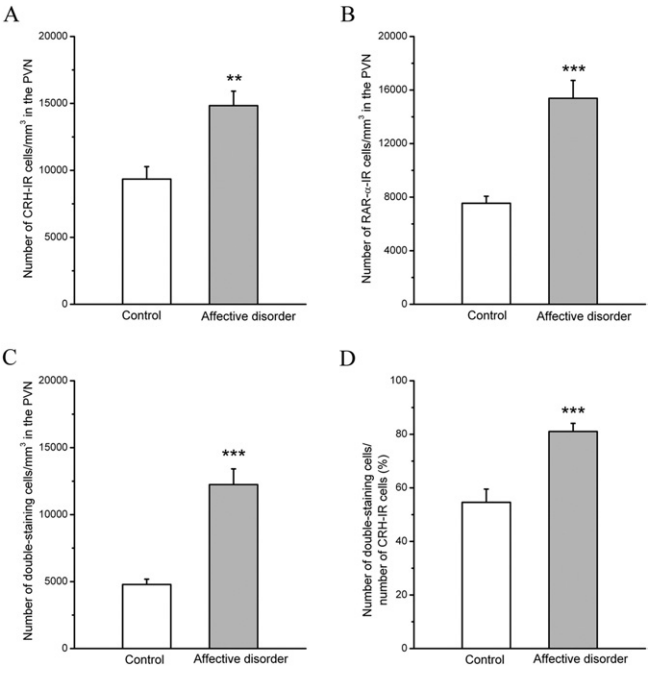

Figure 3. Comparison of CRH and RAR-α immunoreactivity in the PVN of affective disorder patients ($n = 12$) and matched control subjects ($n = 12$). **(A–C)** Comparison of cellular density of CRH-immunoreactive (IR) neurons **(A)**, RAR-α-IR neurons **(B)**, and CRH-RAR-α double-staining neurons **(C)** in the PVN. **(D)** Comparison of the proportion of CRH-RAR-α double-staining neurons of the CRH-IR neurons. For **(A–D)** data are mean ± SEM. $^{**}p < .01$; $^{***}p < .001$; two-tailed nonparametric Mann–Whitney *U* test. Other abbreviations as in Figures 1 and 2.

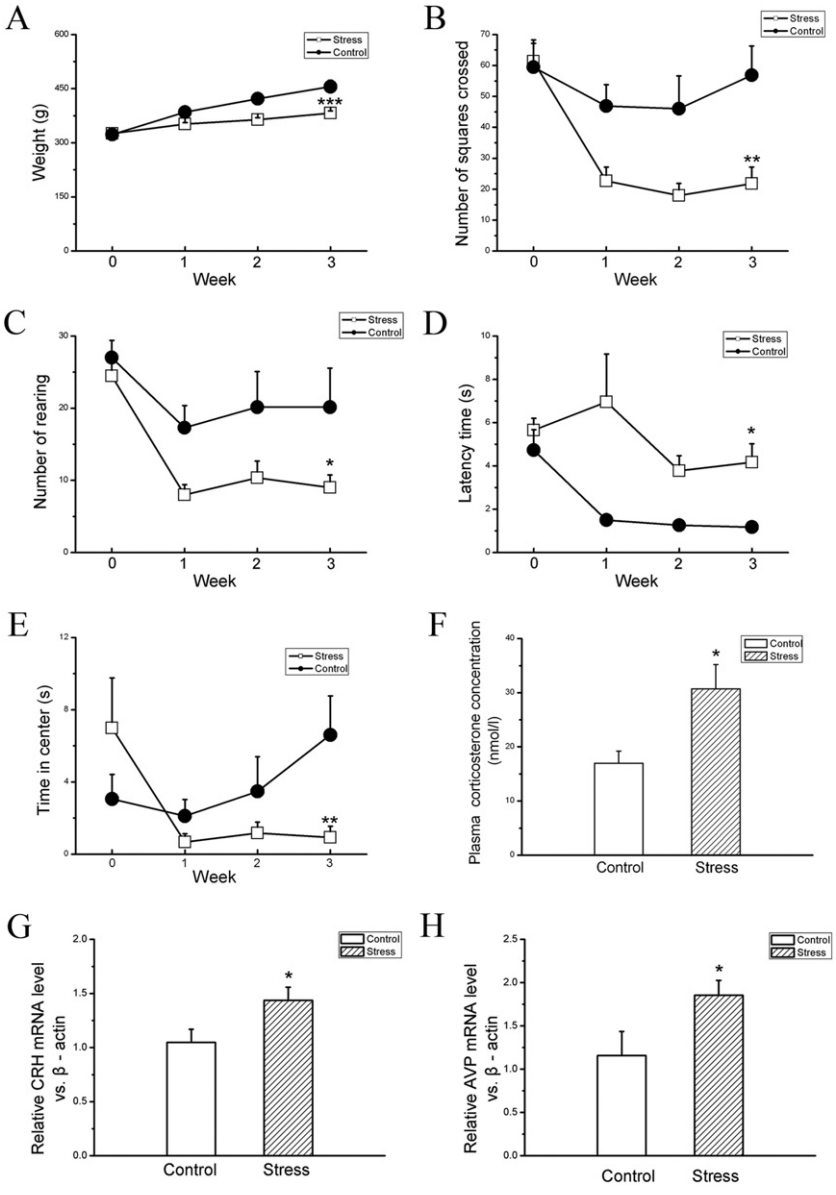

Figure 4. The chronic unpredictable mild stress (CUMS) rat model for depression. Body weight **(A)** and open-field tests data **(B–E)** of the CUMS rats and control subjects. Plasma corticosterone concentration **(F)**, relative CRH **(G)**, and arginine vasopressin (AVP) **(H)** messenger RNA (mRNA) expression in the hypothalamus of the CUMS rats and the control subjects. All data are mean ± SEM. $*p < .05$; $**p < .01$; $***p < .001$, compared with the control group; two-tailed unpaired Student *t* test; $n = 11$ for the CUMS group and $n = 7$ for the control subjects. Other abbreviations as in Figures 1 and 2.

body weights of rats in the two groups did not differ significantly before CUMS [325.5 g ± 5.2 g vs. 322.9 g ± 8.3 g, $F(1.16) = .170$, $p = .775$, Figure 4A]. After the 3 weeks of CUMS, the average body weight increased in both groups, but the weight gain was significantly less in the stressed group compared with control subjects [382.3 g ± 5.6 g vs. 455.3 g ± 17.6 g, $F(1.16) = 3.978$, $p < .001$, Figure 4A]. The CUMS rats showed significantly less horizontal locomotion [21.8 ± 5.3 vs. 56.8 ± 9.4, $F(1.16) = .862$, $p = .003$, Figure 4B], rearing [9 ± 2.3 vs. 20.1 ± 5.4, $F(1.16) = 6.880$, $p = .033$, Figure 4C], significantly longer start latencies [4.17 sec ± .85 sec vs. 1.18 sec ± .13 sec, $F(1.16) = 10.663$, $p = .014$, Figure 4D], and less time in the center squares [.93 sec ± .62 sec vs. 6.60 sec ± 2.16 sec, $F(1.16) = 3.660$, $p = .008$, Figure 4E] compared with the control subjects. The CUMS rats also had a higher corticosterone concentration [30.69 nmol/L ± 3.50 nmol/L vs. 16.97 nmol/L ± 2.21 nmol/L, $F(1.16) = 5.638$, $p = .035$, Figure 4F].

The hypothalamic AVP and CRH level of the CUMS and control rats were analyzed by Q-PCR. After 3 weeks of stress, the CUMS rats showed a 37% increase in CRH mRNA [$F(1.16) = .471$, $p = .047$, Figure 4G] and 60% increase in AVP mRNA [$F(1.16) = .313$, $p = .038$, Figure 4H] in the hypothalamus compared with control subjects.

RAR-α Is Recruited by the CRH Promoter in the Rat Hypothalamus and RAR-α Expression Level Is Increased in the CUMS Rats

To determine whether RAR-α can be recruited by the CRH promoter in vivo, we performed a ChIP assay with the rat hypothalamus tissue. The occupancy of rat CRH promoter by RAR-α was observed when immunoprecipitated by RAR-α antibody (Figure 5A). In the negative control, no PCR band was present (Figure 5A).

Western blotting of RAR-α in the rat hypothalamus demonstrated that the RAR-α protein expression increased approximately threefold in the hypothalami of CUMS rats, compared with that in the control group [Figures 5B, $F(1.16) = 3.393$, $p = .028$], and they also had a 1.45-fold increase of RAR-α mRNA

expression in the hypothalamus [Figures 5C, $F(1.16) = .608$, $p = .048$]. A representative western blot profile of hypothalamic RAR-α and GAPDH expression of a CUMS rat and a control rat was shown in Figure 2 in Supplement 1.

CRABP1, RALDH1, and RALDH3 Expression Levels in the Hypothalamus of Control and CUMS Rats

We then detected the mRNA expression of some other critical proteins involved in transducing RA signaling pathway in rat hypothalamus, including RA binding protein (CRABP1) and enzymes for RA synthesis (RALDH1 and RALDH3). The CRABP1 mRNA expression was decreased by 30% in the hypothalamus of the CUMS rats compared with control subjects [Figure 5F, $F(1.16) = 6.325$, $p = .002$], whereas the RALDH3 mRNA expression increased by 60% in CUMS rats [Figure 5E, $F(1.16) = .011$, $p = .027$]. The CUMS had no significant effect on RALDH1 mRNA expression in the hypothalamus [Figure 5D, $F(1.16) = 2.117$, $p = .105$].

RAR-α Is Recruited by the CRH Promoter in BE(2)-C Cells

We used the RA-treated BE(2)-C cells as a model to mimic the CRH-expressing neurons under effect of RA. The BE(2)-C cells expressed low-level endogenous RAR-α (Figure 1 in Supplement 1). We performed a ChIP assay in the BE(2)-C cell line and also observed CRH promoter occupancy by endogenous RAR-α in this CRH-expressing human neuroblastoma BE(2)-C cell line when immunoprecipitated by RAR-α antibody (Figure 6A). In the negative control, no PCR band was present (Figure 6A).

RAR-α Regulates CRH mRNA Expression in BE(2)-C Cells

We used the BE(2)-C cell line to check whether RAR-α could act on CRH mRNA expression. Quantitative PCR analysis showed that CRH mRNA expression doubled after RAR-α transfection [$F(1.10) = 9.983$, $p = .02$, Figure 6B]. To examine whether reduction of RAR-α function would alter CRH mRNA expression in BE(2)-C cells, RAR-α was silenced by RNA interference technique. Quantitative RT-PCR analysis demonstrated that RAR-α was silenced to <30% of the control at the mRNA level [Figure 6C, $F(1.10) = 10.642$, $p < .001$]. Endogenous CRH mRNA level was decreased to 33% [Figure 6D, $F(1.10) = .807$, $p < .001$] after the RAR-α mRNA expression was silenced.

Discussion

Possible involvement of RA signaling in affective disorders has been proposed by various authors (9,31,32). Yet the direct involvement of RAR-α in affective disorders has not been reported. Our study is the first to find increased RAR-α-IR density in the hypothalamic PVN of patients with affective disorders. Considering the affective disorder patients we studied contain both MD/MDD and BD patients, we compared the RAR-α expression between the two subgroups but did not find any difference. Thus, the patients with MD/MDD and BD were pooled into one group. In addition, the expression of RAR-α was also significantly increased in the eight patients with MD/MDD compared with their control group ($p = .002$). The similarity to RAR-α was that we did not find any significant difference of CRH expression between these two subgroups. Similarity of CRH expression between MD/MDD and BD patients is consistent with our previous studies (6,8,33). It should be noted that, although no significant differences were found between the subgroups, the numbers of each subgroup are small. Furthermore, the colocalization of RAR-α with CRH offers the first evidence for a direct involvement of RAR-α with the hypothalamic CRH neurons. Our results that both the density of CRH-RAR-α double staining neurons and its ratio to the density of CRH neurons were increased in the patients with affective disorders suggest that RAR-α might contribute to CRH activation. Of all the patients with affective disorders, there was no difference when we tested only the patients that died during a mood disorder and their appropriate control subjects in any one of the aforementioned parameters ($p \geq .099$), which indicates that for our findings the trade of the mood disorder is more important than the state. Considering that the effects of medications on any observed protein expression are always a limitation of the use of human postmortem brains, the effect of medications on expression of RAR-α was analyzed. We did not find any difference in the density of RAR-α profiles between six subjects who had taken selective serotonin reuptake inhibitors during the last month before death (D1, D2, D7, D8, D11, and D12) and the other subjects with affective disorders ($p = .873$). In addition, there was no difference in the density of RAR-α profiles between the subjects who took tricyclic antidepressant in the past (D2, D3, D4, D6, D9, and D12; D9 and D12 took tricyclic antidepressant in the last month before death) and the other subjects ($p = .570$). However, we did not include these points in the Results section, because the statistical analyses were doubtful due to the small sub-samples and the overlap in the use of these drugs among

Figure 5. Disturbed retinoic acid (RA), metabolism, and signaling in the CUMS rat model for depression. **(A)** Chromatin immunoprecipitation assay (ChIP) showing the recruitment of RAR-α by the rat CRH promoter. Chromatin solutions from rat hypothalamus were immunoprecipitated with anti-RAR-α antibody, and final DNA extraction were amplified with primers that cover the regions of rat CRH gene promoter as indicated. Similar results were observed in two independent experiments. **(B)** Relative RAR-α protein level in the CUMS rats and the control subjects. Relative messenger RNA (mRNA) expression of RAR-α **(C)**, retinal dehydrogenase (RALDH) 1 **(D)**, RALDH 3 **(E)**, and cellular retinoic acid-binding protein (CRABP) 1 **(F)** in the hypothalamus of CUMS rats and the control subjects. All data are mean ± SEM. *$p < .05$; **$p < .01$, compared with the control group; two-tailed unpaired Student *t* test; $n = 11$ for the CUMS group and $n = 7$ for the control subjects. Other abbreviations as in Figures 1 and 2.

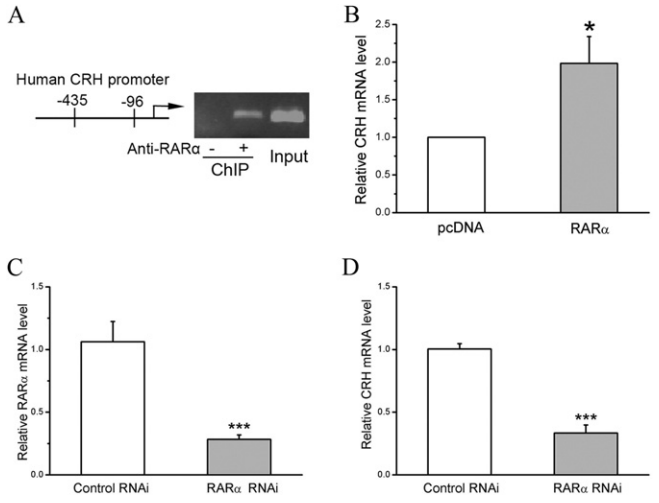

Figure 6. The RAR-α upregulates CRH gene expression. **(A)** Chromatin immunoprecipitation assay (ChIP) showing the recruitment of RAR-α to the human CRH promoter. Chromatin solutions from the BE(2)-C cells were immunoprecipitated with anti-RAR-α antibody, and final DNA extraction were amplified with primers that cover the regions of human CRH gene promoter as indicated. Similar results were observed in two independent experiments. **(B)** Relative CRH messenger RNA (mRNA) expression in RAR-α-transfected cells and control cells. The RAR-α mRNA **(C)** and CRH mRNA **(D)** expression relative to the control after RNA interference (RNAi) against RAR-α. For **(B–D)** all data are mean ± SEM; $*p < .05$; $***p < .001$, compared with the control group; two-tailed unpaired Student *t* test; $n = 6$ in each group. Other abbreviations as in Figures 1 and 2.

these patients. The inherent limitations of the postmortem tissues make the use of our CUMS rats so important, which were not treated with any antidepressants. Moreover, it has been reported that the expression of RAR-α in the neocortex and striatum of the rat brain is not affected by antipsychotic drug administration (34).

Because the HPA axis is considered the "final common pathway" in the stress response and pathogenesis of depression (3,4), our results raise the possibility that RAR-α is involved in regulating the activity of CRH neurons, thus influencing the risk for affective disorders. Our results also show RAR-α expression in the magnocellular neurons of the PVN (Figures 1 and 2), many of which are known to be vasopressin neurons (35). The possible role of vasopressin in the pathogenesis of depression deserves further investigation. Besides the expressing in the PVN of the hypothalamus, RAR-α was also found in the amygdala of the rodents (21,22), which plays an important role in the mediation of behavioral responses to stress (36,37). Given that the amygdala could regulate the HPA axis activity through direct CRH projections to the parvocellular regions of the PVN (38), the possible role of amygdala RAR-α in stress responses deserves further study.

Consistent with the findings in the human subjects, elevated hypothalamic RAR-α expression was observed at both the protein and mRNA level in the CUMS animal model, which has been widely used and is a classic model for depression (39). As one of the most valid animal models of depression, the CUMS model mimics both symptoms of human depression (such as a decrease in locomotor activity and weight loss) and the hyperactivity of the HPA axis in depression (40). Our CUMS rats demonstrated anxiety-like behaviors (decreased time spent in the center part) and decreased locomotor/exploratory activity, which are consistent with most of the reported data (39–42). Furthermore, we also detected elevated plasma corticosterone concentration, increased level of CRH, and AVP expression in the hypothalamus of the CUMS rats, which agrees with the hyperactive HPA axis in the depressed patients (3,4). In addition, our observation that RAR-α can be recruited to the CRH promoter in the rat hypothalamus provided another in vivo demonstration for RAR-α to regulate CRH gene expression.

Although patients experiencing high vitamin A might suffer from depression (18), it is hard to believe that excess vitamin A would occur during daily life. Considering the high frequency of depression, metabolic disturbances in the retinoid signaling pathway might be hypothesized. The possibility of abnormal RA signaling in depression was tested in the CUMS animal model. Our results in the CUMS rats revealed an increased RALDH1 and a decreased CRABP1 expression, which indicate an enhanced RA signaling in the hypothalamus of the CUMS rats (Figure 3 in Supplement 1). The participation of a dysregulated RA signaling in the pathophysiology of affective disorders can be speculated on the basis of these intriguing data. The hypothesis that RAR-α might be the novel link between RA signaling and affective disorders is further supported by our data conducted in the BE(2)-C cell line, which show the direct binding and upregulation of RAR-α on the CRH gene.

We found that, taken together, RAR-α might contribute to regulating the activity of CRH neurons and the dysregulation of RA signaling in the hypothalamic PVN that might influence the CRH expression in affective disorders (Figure 3 in Supplement 1). The vulnerable character of the critical proteins in RA signaling pathways might be targets for novel therapeutic strategies for affective disorders. In addition, our results provide a possible explanation for the adverse psychiatric effects of retinoids and shed light on the complex RA signaling in the adult brain.

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Supplementary material cited in this article is available online.

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