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Corticotropin-releasing factor receptor type 1 colocalizes with type 2 in corticotropin-releasing factor-containing cellular profiles in rat brain

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Abstract

OBJECTIVES: To investigate whether CRHR1 and CRHR2 are colocalized in CRH-specific neurons in rat brain.
METHODS: Double/triple immunofluorescence, and combined in situ hybridization were performed in the PVN, amygdala and hippocampus, and triple immunofluorescence was applied to the median eminence (ME), dorsal raphe (DR) and locus coeruleus (LC).
RESULTS: Both CRHR1 and CRHR2 immunoreactivity were highly coexpressed in the PVN, central nucleus of the amygdala (CeA) and hippocampus. Triple immunofluorescence under confocal microscopy confirmed that CRHR1 and CRHR2 are coexpressed in CRH-producing neurons in these regions. The results of in situ hybridization combined with double immunofluorescence further strengthened the finding that CRHR1 and CRHR2 were coexpressed in CRH-specific neurons in the PVN, CeA and hippocampus. In addition, CRH immunoreactivity signals were evidently distributed in the ME, DR and LC, and were coexpressed with both receptors.
CONCLUSION: CRH receptors colocalize in CRH-containing neurons in the PVN, CeA and hippocampus, and CRH, CRHR1, and CRHR2 coexist in the DR and LC. Our results implicate CRHR1 and CRHR2 in coordinating the regulation of CRH neuronal activity in stress and behavioral responses.

ORIGINAL ARTICLE

INTRODUCTION

Corticotropin-releasing factor (CRH), a 41-amino acid neuropeptide, is a key regulator of the hypothalamic-pituitary-adrenal (HPA) axis, as well as neuroendocrine, autonomic, and behavioral responses to stress (Vale *et al.* 1981). In the rat brain, CRH is produced predominantly in the parvocellular subdivision of the paraventricular nucleus (PVN) of the hypothalamus, from which CRH neurons innervate a multitude of targets throughout the central nervous system (CNS) (Swanson *et al.* 1983; Vale *et al.* 1981). The PVN contains the highest density of CRH-producing cell bodies with the majority projecting to the median eminence (ME). This CRH pathway comprises the hypothalamic component of the HPA axis (Vale *et al.* 1981). However, the production of CRH is not restricted to these neurosecretory neurons. CRH cell bodies and terminals are widely distributed in the CNS, with especially high numbers in the central nucleus of the amygdala (CeA), hippocampus, dorsal raphe (DR) and locus ceruleus (LC) (De Souza *et al.* 1985; Gray and Bingaman 1996; Keegan *et al.* 1994; Li *et al.* 2002; Potter *et al.* 1994; Van Pett *et al.* 2000), which are closely associated with disorders such as anxiety and depression (Dunn and Swiergiel 2008; Koob 1999; Nestler *et al.* 2002; Reyes *et al.* 2006; Stahl and Wise 2008; Sztainberg *et al.* 2010). Although CRH axons from these sites do not project to the ME, they make synaptic connections and are thought to act by neuromodulation or neurotransmission (Gallagher *et al.* 2008).

CRH is functionally bound in the activation of two distinct receptor subtypes, CRH receptor type 1 (CRHR1) and type 2 (CRHR2), which are broadly expressed in the rodent brain and regulate a variety of biological functions (Hauger *et al.* 2006; Hillhouse and Grammatopoulos 2006). CRHR1 is believed to be crucial in stress-induced HPA responsiveness and anxiety-like effects; in contrast, CRHR2 seems to be important in dampening HPA axis activity and mediating anxiolytic-like effects, as implied by the phenotypes of CRHR1 or CRHR2 knockout mice (Bale *et al.* 2000; Bale *et al.* 2002; Smith *et al.* 1998; Timpl *et al.* 1998), which suggest that CRHR2 functions to modulate the stress response associated with CRHR1 activation (Bale 2005). Dysfunction of the CRH and CRH receptor systems is closely linked with psychiatric disorders such as anxiety and depression (Bale 2005; Hauger *et al.* 2006; Hauger *et al.* 2009; Hillhouse and Grammatopoulos 2006). One mechanism is sensitization of the HPA axis and disturbance of homeostasis, which is based upon the strict balance of CRH and CRH receptor systems in the brain (Bale 2005; de Kloet *et al.* 2005). The other is the dysfunction of CRH regulation of major biogenic amine brain systems that have long been implicated in the pathophysiology of anxiety and depression (Dunn and Swiergiel 2008; Koob 1999; Krishnan and Nestler 2008; Lanfumey *et al.* 2008; Porter *et al.* 2004; Stahl and Wise 2008).

There is mounting evidence that CRH and its receptors are present in the same subsets of neurons. For instance, PC12 cells express both CRHR1 and CRHR2 in the membrane (Dermitzaki *et al.* 2007). We previously found, by immunofluorescence microscopy, that CRH and CRHR1 or CRHR2, Urocortin 1 and CRHR1, and Urocortin 3 and CRHR2 are highly colocalized in PVN neurons, and CRH and CRHR1 coexist in LC neurons (Fan *et al.* 2009). Using a similar approach, CRH and CRHR1 were found to colocalize in the basolateral nuclei of the amygdala in mouse (Sherrin *et al.* 2009). A recent study, using postembedding immunogold electron microscopy, showed that CRHR1 and CRHR2 are differentially distributed in the synapses of a select subset of rat DR cells, with CRHR1 being prominent on the plasma membrane and CRHR2 being cytoplasmic (Waselus *et al.* 2009). An understanding of the CRH cell types expressing CRH receptors in the brain is required for developing hypotheses to explain novel roles of CRH receptor signaling, including the mechanisms underlying interactions between the CRH receptors and CRH in stress responses and disorders. However, an important aspect not previously explored is the neuroanatomical correlation of CRHR1 and CRHR2 in CRH-producing cells in the sites involved in stress-related responses and behavior.

In this study, we determined the distribution of CRHR1 and CRHR2 in the PVN, CeA, hippocampus, ME, DR, and LC. Although the PVN has not been associated with anxiety- and depression-like behavior, it is a critical site for control of the HPA axis (Rivier *et al.* 1983; Vale *et al.* 1981), so it was first to be investigated. The CeA is a major site of extrahypothalamic CRH expression and a key element of the circuitry through which CRH contributes to affective disorders (Asan *et al.* 2005; Bale and Vale 2004; Gray 1993; Reul and Holsboer 2002). The hippocampus has neural interactions with the PVN and amygdala (Berretta *et al.* 2001; Cammarota *et al.* 2008; Sheth *et al.* 2008), and is closely associated with anxiety-like behavior, as well as learning and memory (Sananbenesi *et al.* 2003; Todorovic *et al.* 2009). The DR is a primary source of serotonergic neurons projecting to forebrain and brainstem targets, and CRH regulates serotonin (5-HT) activity, which is closely associated with sensitization of behavioral responses during stressful challenges, and disturbance of 5-HT function plays a key role in the etiology of depression (Lanfumey *et al.* 2008; Porter *et al.* 2004). The LC is linked to the PVN and CeA and has been suggested to be a central integrating site for the autonomic, behavioral and anxiogenic effects of stressful stimuli (Dunn & Swiergiel 2008; Koob 1999; Leonard 2001; Valentino *et al.* 1993). Collectively, the present investigation sought to extend our understanding of the neuroanatomical basis of CRH and CRH receptor involvement in the regulation of the HPA axis activity and stress response.

METHODS AND MATERIALS

Animals

Adult male Sprague-Dawley rats (Certification No. 2001001, Experimental Animal Center, Zhejiang Province, China) weighing 250 ± 10 g were group-housed for at least 7 days after arrival, maintained on a 12:12 hours light/dark cycle (lights on at 06:00) under temperature-controlled (22 ± 1°C) and air-regulated conditions (relative humidity 50–60%), and had access to standard rat pellets and tap water *ad libitum*. The experimental procedures were carried out in accordance with the National Institutes of Health guidelines and China's guidelines for the care and use of laboratory animals, and were approved by the Institutional Animal Care and Use Committee of Zhejiang University. All possible efforts were made to minimize the number of animals used and animal suffering.

Characterization of antibodies

A rabbit antiserum raised against human/rat CRH (H-019-06, Phoenix Pharmaceuticals, Inc., USA) was used for immunolabeling CRH. The 41-amino-acid CRH antigenic peptide sequence is H-Ser-Glu-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Met-Glu-Ile-Ile-NH_2 (Rivier *et al.* 1983). This peptide has 100% homology with CRH (Table 1).

A mouse monoclonal anti-human antiserum to CRHR1 was produced from a hybridoma resulting from the fusion of a mouse myeloma with B cells obtained from a mouse immunized with NS0 cells transfected with human CRHR1. The immunogen for this antiserum corresponds to one of the four alternately spliced forms and has a 29-amino-acid deletion in the first cytoplasmic domain. Human CRHR1 shares 97% amino acid sequence identity with rat CRHR1. The antiserum detects CRHR1 transfectants but not irrelevant transfectants (SN: MAB3930, R&D System, Inc.).

A goat polyclonal antiserum raised against a peptide mapping near the C-terminus of CRHR2 of human origin (C-15, sc-20550; Santa Cruz Biotechnology, Santa Cruz, USA) was used for CRHR2 immunolabeling. The secondary antibodies conjugated to Alexa-488 donkey anti-mouse, Alexa-555 donkey anti-goat, Alexa-555 donkey anti-rabbit, and Alexa-647 donkey anti-goat were from Invitrogen (USA). The nonisotopic digoxigenin-labeled cDNA probe with an *in situ* hybridization (ISH) kit for CRH mRNA was provided by Boster Biotechnology Co. Ltd. (Wuhan, China).

Double immunofluorescence for CRHR1 and CRHR2

Double immunofluorescence was used to study the colocalization of CRHR1 and CRHR2 in sections from the PVN, amygdala and hippocampus. The rats were sacrificed rapidly (<30 s) by decapitation. Brains were quickly removed, frozen in liquid nitrogen, and stored at –80 °C until further analysis. The frozen brain was mounted on a microtome (Cryostat Microtome HE 505, Germany) and 20 μm coronal sections containing bilateral PVN, amygdala and hippocampus were cut at –20°C (PVN and amygdala, bregma –1.68 to –1.80 mm; hippocampus, bregma –3.00 to –3.12 mm), according to the atlas of Paxinos and Watson (Paxinos and Watson 2007). The sections were thaw-mounted onto gelatin-coated slides, desiccated under vacuum overnight, and stored at –80 °C until use. The process was as described previously (Fan *et al.* 2009). Briefly, sections were fixed in 4% buffered paraformaldehyde, incubated in 0.4% Triton X-100 in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 30 minutes, and blocked with 3% bovine serum albumin in 0.4% Triton X-100 in PBS, pH 7.4, for 60 minutes at 37 °C. Consecutive sections were incubated with primary antibodies of CRHR1 (1:25) and CRHR2 (1:25) overnight at 4 °C. After incubation, sections were rinsed 3 times for 10 minutes with 0.01 M PBS, pH 7.4, and incubated with secondary antibodies conjugated to Alexa-488 donkey anti-mouse and Alexa-555 donkey anti-goat (1:100) for

Tab. 1. Antibodies used for immunolabeling.

Antibody	Antigen	Host	Type	Company	Catalog No.	Dilution
CRH						
primary	Anti-human/rat serum	Rabbit	Polyclonal IgG	Phoenix	H-019-06	1:100
secondary	Anti-rabbit IgG (H+L)	Donkey	Alexa Fluor 555 conjugated	Invitrogen	A31572	1:200
CRHR1						
primary	Anti-human CRHR1 peptide	Mouse	Monoclonal IgG	R & D	MAB3930	1:100
secondary	Anti-mouse IgG (H+L)	Donkey	Alexa Fluor 488 conjugated	Invitrogen	A21202	1:200
CRHR2						
primary	Anti-human CRHR2 peptide	Goat	Polyclonal IgG	Santa Crutz	SC-20550	1:100
secondary	Anti-goat IgG (H+L)	Donkey	Alexa Fluor 555 conjugated	Invitrogen	A21432	1:200
	Anti-goat IgG (H+L)	Donkey	Alexa Fluor 647 conjugated	Invitrogen	A21447	1:200

1 hour at 37 °C. After removal of the secondary antibody, sections were washed 3 times for 10 minutes with PBS, pH 7.4. The slides were then dehydrated in a series of alcohols, soaked in xylene and coverslipped.

Combined in situ hybridization for CRH and double immunofluorescence for CRHR1 and CRHR2

For this purpose, another separate cohort of sections was studied first to detect CRH mRNA expression in the PVN, amygdala and hippocampus by nonisotopic digoxigenin-labeled cDNA probe with an ISH kit. The following hybridization procedure was based on that of a previous report (He *et al.* 2008). Briefly, before hybridization, sections were air-dried and fixed in 4% paraformaldehyde for 30 minutes, washed three times for 5 minutes each with 0.01 M PBS (pH 7.4), and digested by proteinase K (1 μg/ml) at 37 °C for 30 minutes, and then the slides were incubated with 0.1 M glycine (0.1 M PBS, pH 7.2) at room temperature for 5 minutes. After fixation with 4% paraformaldehyde at room temperature to end the reaction with proteinase K, sections were washed with PBS and 20 μl of the prehybridization solution was added to each section for 30 minutes at 37 °C. Then 20 μl of the hybridization mixture containing digoxigenin-labeled cDNA probe for CRH mRNA (5'-CATGCGGCTGCGGCTGCTGGTGTCCGCGGG-3') was added to the section, covered with parafilm and incubated in a moist chamber at 4 °C overnight (16–18 hours) with 5× standard saline citrate (SSC). The slides were rinsed in 4× SSC at 37 °C for 15 minutes, sections were digested by RNase A (20 μg/ml, Promega) at 37 °C for 30 minutes, and rinsed in descending concentrations of SSC (2×, 1× and 0.5× SSC, 37 °C). After that, sections were incubated with serum for 30 minutes at 37 °C and then incubated with horseradish peroxidase streptavidin-conjugated anti-digoxigenin antiserum (from the ISH kit) and washed with 0.02 M PBS. All sections were incubated with avidin biotin peroxidase complex (from the ISH kit) for 20 minutes at 37 °C and washed again with 0.02 M PBS. The sections were incubated in dark moist chambers at room temperature for 10 minutes in 3,3'-diaminobenzidine (Beijing Zhongshan Biotechnology Co. Ltd., China). Finally, color development was terminated by 0.02 M PBS. The specificity of probes was tested by two types of control experiment. First, sections were pretreated with RNaseA (20 μg/ml) for 30 minutes at 37 °C, then washed three times with PBS/diethylpyrocarbonate. These sections were then hybridized in the normal hybridization buffer. Second, sections were incubated with hybridization solution without probe. Neither of the control experiments demonstrated a detectable hybridization signal.

Combined *in situ* hybridization and double-labeling immunofluorescence was carried out to further directly study whether the colocalization of CRHR1 and CRHR2 in the PVN, amygdala and hippocampus is in CRH neurons. For this purpose, ISH experiments for CRH mRNA expression were carried out first, and the double immunofluorescence process for CRHR1 and CRHR2 was performed after the ISH protocol, as described in detail above.

Triple immunofluorescence for CRH, CRHR1 and CRHR2

To determine whether CRH, CRHR1 and CRHR2 coexist in the PVN, amygdala, ME, hippocampus, DR and LC, a separate cohort of sections was used for triple immunofluorescence. The sections were cut at –20 °C according to the atlas of Paxinos and Watson (Paxinos and Watson 2007) (PVN and amygdala, bregma –1.68 to –1.80 mm; hippocampus and ME, –3.00 to –3.12 mm; DR, –6.96 to –7.08 mm; LC, –9.48 to –9.60 mm). Briefly, after blocking, the sections were incubated with primary antibodies to CRH (1:25), CRHR1 (1:25) and CRHR2 (1:25) overnight at 4 °C. After incubation, sections were rinsed 3 times for 10 minutes with PBS, pH 7.4, and incubated with secondary antibodies conjugated to Alexa-488 donkey anti-mouse, Alexa-555 donkey anti-rabbit, and Alexa-647 donkey anti-goat (1:100) for 1 hour at 37 °C. The sections were then processed for immunostaining as described above.

Visualization and image acquisition

Labeling for double immunofluorescence was visualized by confocal microscopy (TE-2000, Nikon, Japan; UltraViewERS, PerkinElmer Co., USA) and images were captured using Volocity 4D image analysis software (USA). Labeling for triple immunofluorescence was visualized with Olympus Fluoview FV1000 (Japan) and images were captured using Olympus Fluoview Ver. 1.5.0.14 image analysis software (Japan). AlexaFluor488, AlexaFluor555 or AlexaFluor647 was excited with a 488-, 543- or 633-nm laser beam and observed through a 505–530, 560–590 or 645–650 nm emission prism window, respectively. Each of the antibodies was visualized separately and then merged to form the final image. Images were captured at 10–100× magnification and saved as TIFF files. Optimal adjustment of brightness and contrast were made in Adobe Photoshop 7.0 (±30% contrast and brightness enhancement).

RESULTS

CRHR1 and CRHR2 are colocalized in CRH-producing neurons in rat PVN

Previous work from our laboratory reported in detail that CRH and CRHR1, and CRH and CRHR2 are highly colocalized in the PVN (Fan *et al.* 2009). To determine the putative colocalization of CRHR1 and CRHR2 in CRH-producing neurons in the PVN, we first used a double immunofluorescence protocol with CRHR1 and CRHR2 primary antibodies. CRHR1- and CRHR2-immunoreactive neurons were distributed throughout the PVN, including parvocellular and magnocellular neurons, and no immunoreactive neurons

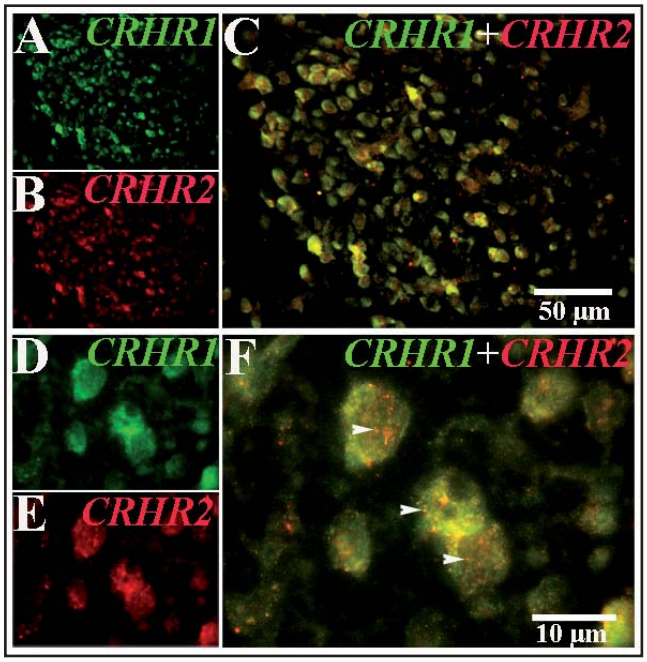

Figure 1. Double immunofluorescence labeled images of CRHR1 and CRHR2 in the PVN. A and B are single channel images showing CRHR1 (green) and CRHR2 (red) immunoreactivity, respectively. Colocalization of CRHR1 and CRHR2 signals (arrows) occurred in the PVN, as evident in C (yellow). D, E and F are magnified from the images A, B and C, respectively.

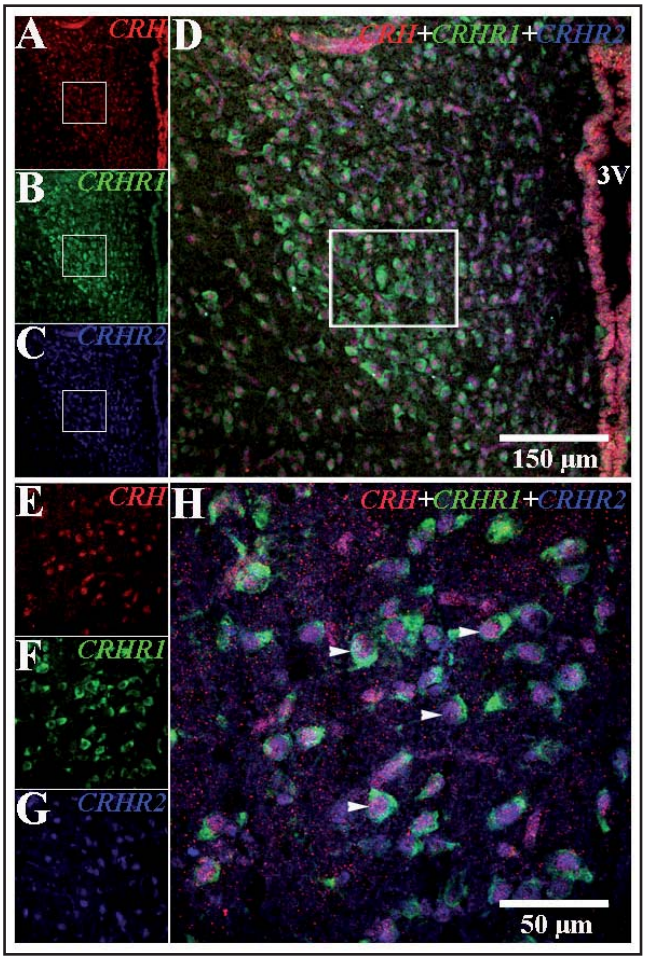

Figure 2. Triple immunofluorescence labeled images of CRH, CRHR1 and CRHR2 in the PVN. A, B and C are single channel images showing CRH (red), CRHR1 (green), and CRHR2 (blue) immunoreactivity, respectively. Colocalization of CRHR1 and CRHR2 signals (arrows) occurred in CRH-producing neurons in the PVN, as evident in D (magenta). E, F, G and H are magnified from the outlined areas in images A, B, C and D, respectively. 3V, third ventricle.

were scattered along the ventral part of the third ventricle (Figure 1A, B, D, E). The observed distribution pattern of CRHR1- and CRHR2-expressing cells is in agreement with the results of our previous study (Fan *et al.* 2009). By double immunofluorescence confocal microscopy, we found that CRHR1 and CRHR2 were highly colocalized (Figure 1C, F).

To determine the specific cell types in which CRHR1 and CRHR2 are coexpressed in the PVN, the triple immunofluorescence study was performed using a specific antibody to CRH. We found the PVN contained numerous dense, topographically distributed fibers immunoreactive for CRH and these fibers displayed a distribution consistent with our previous immunohistochemical results (Fan *et al.* 2009; He *et al.* 2008; Xu *et al.* 2005). Moreover, not only in the parvocellular, predominantly CRH-containing cellular profiles, but also in the magnocellular subdivision, CRHR1 and CRHR2 were highly colocalized in CRH-specific neurons (Figure 2 D, H).

To further confirm that CRHR1 and CRHR2 are colocalized in the CRH-producing cells in the PVN, we labeled with ISH using a digoxigenin-labeled probe for CRH- expressing neurons and CRHR1- and CRHR2-containing neurons by immunofluorescence using specific antibodies to CRHR1 and CRHR2. The nonisotopic ISH protocol used here confirmed our previously reported CRH mRNA expression in the PVN of male rats (He *et al.* 2008; Xu *et al.* 2005). Expression of CRH mRNA was observed in numerous cells in the PVN (Figure 3A, D), and was consistent with the expression of CRH-immunoreactivity, while no hybridization signal was present in sections pretreated with RNase A or incubated with hybridization solution without probe (data not shown). Moreover, CRHR1- and CRHR2-positive-like staining was also observed in the PVN (Figure 3B, C, E, F). By combining ISH and double immunofluorescence with 3-dimensional reconstruction, we found that the colocalization of CRHR1 and CRHR2 was precisely in CRH-producing neurons in the PVN (Figure 3G).

CRHR1 and CRHR2 are colocalized in CRH-producing neurons in rat CeA and hippocampus

By triple immunofluorescence, we found that the immunoreactivity signals for CRH, CRHR1 and CRHR2 in the amygdala were mainly confined to the CeA (Figure 4A1–C1), with slight or no labeling signal

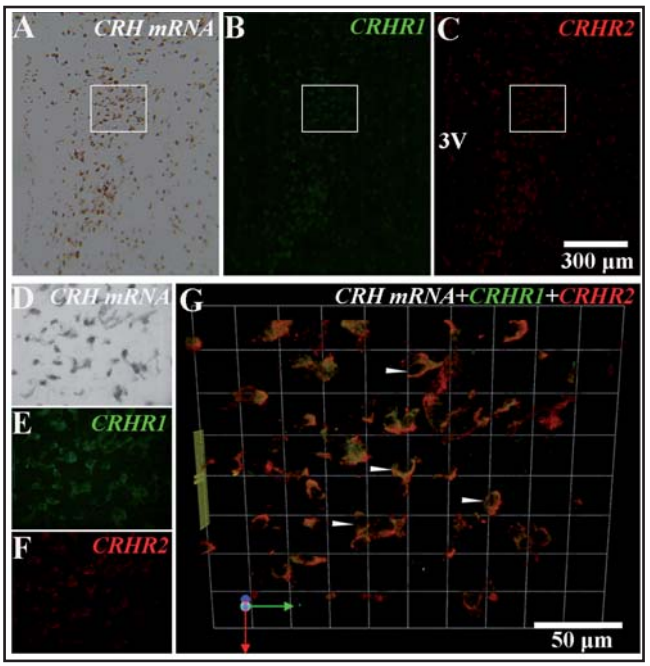

Figure 3. *In situ* hybridization immunofluorescence images for CRH mRNA, CRHR1 and CRHR2 immunoreactivity in the PVN. A, B and C are single channel images showing CRH mRNA (light microscope), CRHR1 (green), and CRHR2 (red) immunoreactivity, respectively. D, E and F are magnified from the outlined areas in images A, B and C, respectively. G (magenta) shows colocalization of CRHR1 and CRHR2 signals (arrows) in CRH-producing neurons in the PVN. 3V, third ventricle.

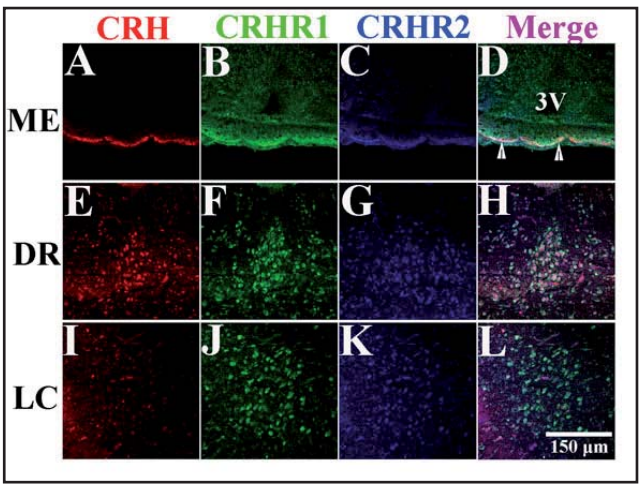

Figure 5. Triple immunofluorescence labeled images of CRH, CRHR1 and CRHR2 in the outlayer of median eminence (arrows) (A-D), dorsal raphe (DR) (E-H), and locus coeruleus (LC) (I-L). A, E and I; B, F and J; and C, G and K are single channel images showing CRH (red), CRHR1 (green), and CRHR2 (blue) immunoreactivity, respectively. Colocalization of CRHR1 and CRHR2 signals occurred in CRH-producing neurons in the ME, DR, and LC, as evident in D, H and L (magenta), respectively.

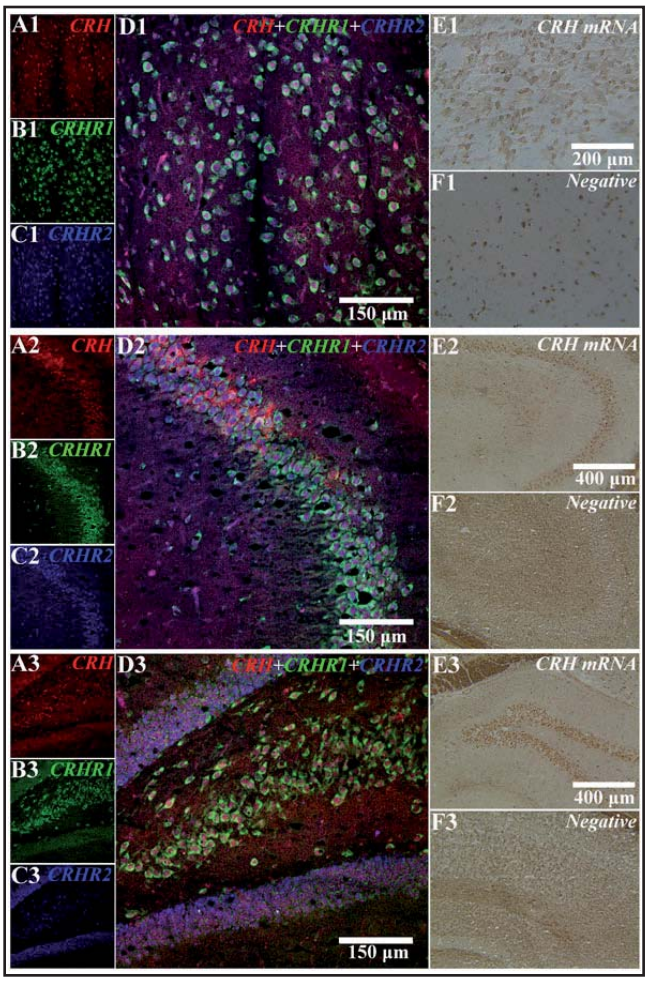

Figure 4. Triple immunofluorescence labeled images of CRH, CRHR1 and CRHR2 in the central nucleus of the amygdala (A1-D1), hippocampus (A2-D2), and CA3 and dentate gyrus (A3-D3). A1-A3, B1-B3 and C1-C3 are single channel images showing CRH (red), CRHR1 (green), and CRHR2 (blue) immunoreactivity, respectively. Colocalization of CRHR1 and CRHR2 signals occurred in CRH-producing neurons in the CeA, CA1, CA3 and DG, as evident in D1-D3 (magenta). E1, E2 and E3 are single channel images showing CRH mRNA (light microscope), and F1, F2 and F3 are the corresponding negative controls for E1, E2 and E3.

in the medial or basolateral nuclei (data not shown). In the hippocampus, the density of immunoreactivity to CRH and CRHR1 in CA1 (Figure 4A2–C2), CA3 and the dentate gyrus (Figure 4A3–C3) was strong while CRHR2 immunoreactivity was relatively weak. By triple immunofluorescence confocal microscopy, we found that CRHR1 and CRHR2 were highly colocalized in CRH-producing neurons in the CeA (Figure 4D1), and in CA1 (Figure 4D2), CA3 and the dentate gyrus (Figure 4D3).

Expression of CRH mRNA was also observed in numerous cells in the CeA (Figure 4E1), and in CA1 (Figure 4E2), CA3 and the dentate gyrus (Figure 4E3), and was consistent with the expression of CRH-immunoreactivity, while no hybridization signal was present in sections pretreated with RNaseA or incubated with hybridization solution without probe (Figure 4F1, F2, F3). Moreover, CRHR1- and CRHR2-positive-like staining was also observed in CRH-producing neurons in these regions.

CRH, CRHR1 and CRHR2 are colocalized in rat ME, DR and LC

We also investigated whether CRH, CRHR1 and CRHR2 are coexpressed in ME, DR and LC by triple immunofluorescence. In the ME, immunoreactivity was seen mainly in the outer but not the inner layer below the third ventricle (Figure 5A–D). In the DR, the immunoreactivity was localized to dorsal, ventral and lateral areas, the major 5-HT sources, with scattered signals in the caudal and interfascicular subregions (Figure 5E–H). In the LC, immunoreactivity was seen throughout (Figure 5I–L).

Triple immunofluorescence images showed that virtually all CRH-immunoreactive cells exhibited strong double-labeled signals for CRHR1 and CRHR2 in the ME, DR and LC (Table 2).

DISCUSSION

The results of the present study provide anatomical evidence for the high colocalization of CRHR1 and CRHR2 with CRH-containing cellular profiles in rat PVN, CeA and hippocampus, and CRH, CRHR1 and CRHR2 coexist in the ME, DR and LC. To our knowledge, this is the first report of the anatomical relationship of CRHR1 and CRHR2 with CRH in rat brain. The colocalization of CRH with both CRHR1 and CRHR2 in the same neurons has functional implications, as the activation of CRH receptors by CRH is facilitated in stress. Our present findings lead us to speculate that this anatomical connection may underly the synergism of CRHR1 with CRHR2 in their physiological effects on CRH neurons, and have implications for CRHR1 and CRHR2 mediation of responses to stress.

Colocalization of CRHR1 and CRHR2 in CRH-producing neurons in PVN, CeA and hippocampus

The PVN is critically involved in the integration of the neural circuitry controlling stress. In the brain, CRH is produced predominantly in the medial parvocellular subdivision of the PVN. These CRH-producing cells are hypophysiotrophic and mainly involved in the regulation of HPA activity in response to stress (Swanson *et al.* 1983; Vale *et al.* 1981). Brain regions such as the CeA, hippocampus, DR and LC also contain large numbers of CRH cells and terminals (Commons *et al.* 2003; Gray and Bingaman 1996; Swanson *et al.* 1983). Especially, CRH-containing cell bodies are found in monoamine-containing nuclei of the brainstem such as the LC (Swanson *et al.* 1983) and in the major serotonergic cells of the DR (Commons *et al.* 2003). Immunohistochemical studies have demonstrated a substantial level of CRHR1-like immunoreactivity in the DR of both the mouse (Chen *et al.* 2000) and the rat (Roche *et al.* 2003). CRH receptor mRNA is detectable in LC tissue by reverse transcription-PCR and the presence of the CRH receptor proteins within LC neurons has been confirmed by immunohistochemistry at the light and electron microscopy levels (Bangasser *et al.* 2010; Reyes *et al.* 2006; Sauvage and Steckler 2001). This points to a role for CRH in modulating these monoaminergic systems and may involve inducing neuroplasticity of the neuroendocrine stress system and behavioral responses by activating CRHR1 and CRHR2. In a previous study, we found that CRH and CRHR1 are coexpressed in the PVN and LC, and CRH and CRHR2 coexist in the PVN, suggesting a possible neural basis for CRHR1 and CRHR2 in regulating CRH neuronal activity in the anxiety-like behavior induced in male rat offspring by prenatal intermittent hypoxia (Fan *et al.* 2009). CRH and CRHR1 are also colocalized in the basolateral nuclei of the amygdala in mouse (Sherrin *et al.* 2009). A recent study revealed that CRHR1 and CRHR2 are differentially distributed within DR cells, with CRHR1 being prominent on the plasma membrane and CRHR2 being cytoplasmic, and stress experience reverses this distribution, when CRHR2 is recruited to the plasma membrane and CRHR1 tends to internalize. This stress-triggered reversal in receptor localization provides a cellular mechanism for switching behavioral strategies for coping with stressors (Waselus *et al.* 2009). However, whether CRHR1 and CRHR2 are colocalized in CRH-containing cellular profiles in rat brain was not clear. Our present results are in accord with these previous observations. CRH neurons were found to constitute an apparently continuous cell group in the PVN, CeA and hippocampus. Importantly, this is the first study to demonstrate that the strongly CRH-producing

Tab. 2. Coexpression coefficients of CRH with CRH receptors in the PVN, CeA, hippocampus, ME, DR and LC of rat brain.

Brain site	PVN	Hippocampus			ME	DR	LC
		CeA	CA1	CA3 and DG			
CRH/CRHR1	0.932	0.904	0.904	0.923	0.928	0.902	0.919
CRH/CRHR2	0.972	0.968	0.943	0.942	0.935	0.970	0.962
CRHR1/CRHR2	0.931	0.909	0.917	0.911	0.962	0.936	0.958

The relative strength of coexpression of CRH with CRH receptors in a given brain site was rated by two independent observers. At least two relevant separate positive areas for each site were selected and two consecutive sections were examined for each animal.
PVN, paraventricular nucleus. CeA, central nucleus of the amygdala. DG, dentate gyrus. ME, median eminence.
DR, dorsal raphe. LC, locus coeruleus.

neurons in these sites coexpressed CRHR1 and CRHR2, suggesting possible roles for both receptors in coordinating the regulation of CRH neuronal activity in stress and behavioral responses in these regions.

Functional implications: A regulation of CRHR1 and CRHR2 on CRH neuron in PVN

In the basal state, CRHR1 mRNA expression within the PVN is extremely low, but expression of both CRH and CRHR1 mRNA is substantially and rapidly increased after stress and CRH administration. This is attenuated by pretreatment with a selective antagonist of CRHR1, CP-154,526 (Imaki *et al.* 2001; Imaki *et al.* 1996; Makino *et al.* 1995; Rivest *et al.* 1995; Van Pett *et al.* 2000). CRH directly affects CRH-producing neurons of the PVN to increase CRHR1 mRNA expression (Konishi *et al.* 2003). Chronic hypoxia (5 km altitude) induces increases in CRH and CRH mRNA in the rat PVN, and this is completely blocked by the CRHR1 antagonist CP-154,526 (He *et al.* 2008; Xu *et al.* 2005). These results suggest a stimulatory role for CRHR1 in the activation of CRH neurons in the PVN during stressful stimuli, and CRHR1 in the PVN may be responsible for the stress-induced CRH increase and HPA axis activation through positive feedback (He *et al.* 2008; Imaki *et al.* 2001; Xu *et al.* 2005).

Although CRH is selective for binding to CRHR1 over CRHR2, a considerable body of evidence suggests that CRH in the PVN plays diverse roles in mediating the stress-induced response through its action on both CRHR1 and CRHR2 (Koob 1999; Schwartz & Seeley 1997). This implies that stress-induced activity of CRH neurons in the PVN is mediated through CRHR1, but synergy with CRHR2 cannot be ruled out. The PVN contains mRNA for both CRHR1 and CRHR2, although the hybridization signal for CRHR1 is stronger than that for CRHR2. We speculate that stress-induced CRHR1 within the PVN is a crucial mechanism in restoring and preparing neuroendocrine CRH cells for subsequent challenges, and that such a phenomenon is essential in novel and unexpected conditions. The counteracting effect of CRHR2 on CRHR1 cannot be neglected, as the increase in CRHR1 mRNA expression in the PVN occurs long after the peak in ACTH and corticostesone (CORT) responses to stress (Imaki *et al.* 1996). We have previously reported that prenatal stress significantly increases CRH and CRHR1 but decreases CRHR2 immunoreactivity in the PVN, along with increased plasma ACTH and CORT levels (Fan *et al.* 2009). Indeed, recent studies have revealed that CRHR2 mediates HPA axis activation, as central administration of urocortin 2 or urocortin 3 activates the HPA axis (Jamieson *et al.* 2006; Maruyama *et al.* 2007), and pretreatment with the selective CRHR2 antagonist ASV-30 attenuates the stimulatory effects of urocortin 2 and urocortin 3 in male Wistar rats (Maruyama *et al.* 2007). Therefore, it is tempting to speculate that there is a synergistic interaction between CRHR1 and CRHR2 to selectively regulate hypophysiotropic and/or autonomic-related CRH neurons. As expected, our present results are largely in agreement with previous reports which revealed that CRH mRNA, and CRH, CRHR1 and CRHR2 immunoactivity are widely distributed in the PVN. Understanding the normal localization seems an essential prerequisite for evaluating the mutual effects of CRH and CRH receptors. However, what is the anatomical relationship of CRHR1 and CRHR2 with CRH in PVN neurons? To answer this question, we first determined by double immunolabeling whether CRHR1 and CRHR2 are colocalized in PVN neurons, and found they were highly colocalized. To identify whether CRHR1 and CRHR2 are coexpressed in CRH-producing neurons, we used a CRH-specific antibody and triple immunolabeling to show that CRHR1 and CRHR2 were colocalized mostly in these neurons. This result was further confirmed by *in situ* hybridization for CRH combined with double immunolabeling for CRHR1 and CRHR2. The distribution of cells expressing CRHR1 and CRHR2 within the PVN coincided with the cellular distribution of CRH mRNA, further suggesting a synergy between CRHR1 and CRHR2 in controlling the activity of CRH neurons, and also illuminating the role of CRHR2 in modulating the action of CRHR1 in HPA axis activity during stress.

From the neurochemical and behavioral aspects, numerous studies have reported that CRH has dose-dependent opposing effects on the pathophysiology of affective behaviors. These effects have been hypothesized to be differentially mediated by CRHR1 and CRHR2. For instance, infusion of CRH into the DR has dose-dependent opposite effects on serotonergic activity, with 100 ng decreasing accumbens 5-HT levels which is abolished by the CRHR1 antagonist antalarmin, while 500 ng significantly increases accumbens 5-HT levels, and is blocked by the CRHR2 antagonist ASV-30, suggesting that the opposing effects of CRH on 5-HT release in the DR are dependent on differential activation of CRHR1 and CRHR2 (Lukkes *et al.* 2008). Electrophysiological data demonstrate that CRHR1 activation in the amygdala contributes to pain-related synaptic facilitation while CRHR2-mediated synaptic transmission is inhibitory (Fu and Neugebauer 2008). CRH application to the LC increases discharge rate and NE release in LC targets (Curtis *et al.* 1997), and this is blocked by a selective CRHR1 antagonist. Our present results on the localization of CRHR1 and CRHR2 within the PVN, CeA, hippocampus, ME, DR and LC where CRH is also located, suggest a possible autoreceptor role for CRHR1 and CRHR2 in regulating CRH involvement in the regulation of excitatory and inhibitory neurotransmitters in these sites. The ultrashort feedback of projection from CRH neuron to CRH neuron with both CRHR1 and CRHR2 in PVN, and short feedback of projection to the another local CRH neuron in PVN, to activate CRH neuron with both CRFR1 and CRFR2

release and CRHR1 and CRHR1mRNA upregulation, to further amplify the activity of HPA axis.

In conclusion, our results demonstrate that CRHR1 and CRHR2 are highly coexpressed in CRH-containing cellular profiles in rat PVN, CeA and hippocampus, and CRH, CRHR1 and CRHR2 coexist in ME, DR and LC. Our results implicate that CRHR1 and CRHR2 in coordinating the regulation of CRH neuronal activity in stress and behavioral responses.

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