Corticotropin releasing factor excites neurons of posterior hypothalamic nucleus to produce tachycardia in rats

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Corticotropin releasing factor (CRF), a peptide hormone involved in the stress response, holds a key position in cardiovascular regulation. Here, we report that the central effect of CRF on cardiovascular activities is mediated by the posterior hypothalamic nucleus (PH), an important structure responsible for stress-induced cardiovascular changes. Our present results demonstrate that CRF directly excites PH neurons via two CRF receptors, CRFR1 and CRFR2, and consequently increases heart rate (HR) rather than the mean arterial pressure (MAP) and renal sympathetic nerve activity (RSNA). Bilateral vagotomy does not influence the tachycardia response to microinjection of CRF into the PH, while β-adrenergic receptor antagonist propranolol almost totally abolishes the tachycardia. Furthermore, microinjecting CRF into the PH primarily increases neuronal activity of the rostral ventrolateral medulla (RVLM) and rostral ventromedial medulla (RVMM), but does not influence that of the dorsal motor nucleus of the vagus nerve (DMNV). These findings suggest that the PH is a critical target for central CRF system in regulation of cardiac activity and the PH-RVLM/RVMM-cardiac sympathetic nerve pathways, rather than PH-DMNV-vagus pathway, may contribute to the CRF-induced tachycardia.

Corticotropin releasing factor (CRF), also known as corticotropin-releasing hormone, is a critical neuropeptide responsible for initiating coordinated autonomic, endocrine and behavioral responses to stress. Both acute and chronic stresses cause changes in heart rate (HR) and arterial pressure (AP), indicating a close relationship between the CRF system and cardiovascular regulation. In fact, intracerebroventricular injection of CRF related peptides increases HR, cardiac output, and mean arterial pressure (MAP), whereas systemic administration of CRF or urocortin 1, an endogenous member of CRF family, remarkably decreases MAP and increases superior mesenteric artery flow. Urocortin has also been shown to induce positive inotropic effect in heart. Thus, CRF related peptides may profoundly modulate cardiovascular activities via both central and peripheral mechanisms.

Two major subtypes of G-protein-coupled receptors for CRF related peptides have been identified, CRFR1 and CRFR2. Both of them are expressed in the central nervous system and periphery and mediate cardiovascular effects of the CRF system. In CRFR1 knockout mice, an abnormally high cardiac noradrenergic activity following stress induced by morphine withdrawal, which is observed on wildtype mice, is inhibited. Moreover, systemic administration of urocortin 1 fails to decrease MAP in CRFR2 knockout mice, whereas wildtype mice show a marked reduction. Although the peripheral effect of CRF on cardiovascular activities primarily mediated by CRFR2 has been well studied, the central pathway and mechanism underlying the modulation of CRF system on cardiovascular activities is still largely unknown.

The posterior hypothalamic nucleus (PH) is an important center for cardiovascular regulation. Electrical or chemical stimulation of the PH increases AP, HR and sympathetic nerve activity. Recently, it has been reported that in patients, chronic deep brain stimulation of the PH is associated with an enhanced sympathoexcitatory drive on the cardiovascular system. Intriguingly, the PH holds a key position in the generation of stress-induced cardiovascular changes. Therefore, in the present study, we examined the contribution of the...
PH in the central effects of CRF on cardiovascular activities and the underlying mechanisms using electrophysiological techniques combined with molecular and immunostaining methods.

Results

Expression of CRFR1 and CRFR2 in the PH. We assessed the expressions of CRFR1 and CRFR2 mRNAs in the PH in rats (n = 5) by quantitative real-time RT-PCR. The rat PH tissues, localized between 3.36 and −4.36 mm from bregma (Fig. 1A), were extracted according to the rat brain atlas. As shown in Fig. 1B, the *crhr1* mRNA % v.s. *gapdh* mRNA was 0.3241 ± 0.0073, whereas the *crhr2* mRNA % v.s. *gapdh* mRNA was 0.1136 ± 0.0038. Thus, both CRFR1 and CRFR2 mRNAs were detected in the rat PH.

To further map the distributions of CRFR1 and CRFR2 in the PH, we performed immunofluorescence of the rat brain slices (n = 5) containing the PH with an antibody against CRFR1 and CRFR2, respectively. We found that both CRFR1 and CRFR2 were not only localized in the PH (Fig. 1C1–C3 and D1–D3), but also co-localized in the same PH neurons (Fig. 1E1, E2 and E3).

CRF remarkably excites PH neurons via CRFR1 and CRFR2. We recorded a total of 37 PH neurons in rats (n = 5) with the input resistance higher than 300 MΩ and the capacitance of 113.5 ± 16.5 pF in the present study by carrying out whole-cell voltage clamp recordings on brain slices. An example illustrating the location (Fig. 2A1) and morphology (Fig. 2A2) of one recorded neuron in the PH was shown in Fig. 2. We found that CRF (300 nM) significantly elicited an inward current (65.6 ± 5.5 pA) on the recorded PH neurons (n = 5; Fig. 2B1). Since there are extensive glutamateric and GABAergic neurotransmissions in the PH, we performed electrophysiological experiments to determine whether the PH contributes to potentiation effect of CRF on HR rather than AP and RSNA.

Microinjection of CRF into the PH significantly increases HR rather than MAP via the activation of CRFR1 and CRFR2. Next, whether the excitatory action of CRF on PH neurons influences cardiovascular activities in vivo was assessed. We found that microinjection of 20, 60 and 200 μM CRF into the rat PH elicited a remarkable increase in average peak HR (6.3 ± 0.8, 13.8 ± 1.7 and 25.8 ± 3.1 bpm, respectively) in a concentration-dependent manner, compared to no change (3.2 ± 0.7 bpm) following injection of normal saline (n = 8, P < 0.01; Fig. 4A1,4A2). However, microinjection of CRF (up to 200 μM) into the PH did not influence MAP (Fig. 4B1 and B2) and RSNA (Fig. 4C1 and C2). The changes in MAP after injection of CRF or vehicle were 2.8 ± 0.8 mmHg and 1.7 ± 0.8 mmHg (n = 8, P = 0.36), respectively. And the change ratios of integrated RSNA were 6.3 ± 2.9% v.s. 6.1 ± 2.4% (n = 8, P = 0.16). The data indicate that the PH contributes to potentiation effect of CRF on HR rather than AP and RSNA.

Given an involvement of both CRF receptors in the excitation of CRF on PH neurons, we further addressed whether CRFR1 and CRFR2 in the PH co-mediate the enhancement effect of CRF on HR. In this experiment, selective CRFR1 antagonist alantiamin hydrochloride or selective CRFR2 antagonist antisauvagine-30 was microinjected into the PH, followed within 2 min by a microinjection of CRF at the same site. As shown in Fig. 5, blockade of either CRFR1 or CRFR2 in the PH partly reduced the tachycardic effect induced by CRF. The increase in average peak HR induced by CRF decreased from 25.8 ± 3.1 bpm to 7.8 ± 2.1 bpm (n = 5, P < 0.05) and 13.8 ± 1.9 bpm (n = 5, P < 0.05), respectively. Moreover, microinjection of broad-spectrum CRF receptor antagonist astressin almost totally blocked the tachycardic response to microinjection of CRF into the PH (the increase in average peak HR decreased to 2.6 ± 1.3 bpm; n = 5, P < 0.01), suggesting that both CRFR1 and CRFR2 in the PH are involved in the tachycardic effect of CRF.

Effect of vagotomy and blockade of CSNA on the CRF-induced tachycardia. To clarify the peripheral mechanism underlying the tachycardia effect of CRF mediated by the PH, we determined whether the vagus nerve or cardiac sympathetic nerve was involved in. As shown in Fig. 6, bilateral vagotomy increased the baseline of HR; however, it only slightly reduced the tachycardic response elicited by microinjection of CRF into the PH. The increases in HR induced by microinjection of CRF before and after the vagotomy were 25.8 ± 3.1 bpm and 22.0 ± 2.1 bpm, respectively (n = 5, P = 0.56). On the other hand, blocking CSNA by intravenous injection of propranolol almost abolished the tachycardic response elicited by microinjection of CRF into the PH. The increase
Figure 1. Quantitative real-time RT-PCR and immunofluorescence results showing that CRFR1 and CRFR2 were expressed in the rat PH. (A) Sagittal view of the rat brain (Paxinos and Watson, 2007), localizing the PH between −3.36 and −4.36 mm from the bregma. (B) Bar graphs showing relative expression of CRFR1 and CRFR2 in the rat PH. (C1–C3) CRFR1 was present in the rat PH. (D1,D2) CRFR2 was present in the PH. (E1,E2) CRFR1 and CRFR2 were co-localized in the same PH neurons. Scale bars: (C1), (D1) and (E1), 550 μm; (C2), (D2) and (E2), 160 μm; (C3), (D3) and (E3), 20 μm. 3V, 3rd ventricle; mt, mammillothalamic tract; PH, posterior hypothalamic nucleus.
in HR induced by microinjection of CRF after the blockade of CSNA reduced to 1.4 ± 1.0 bpm (n = 5, P < 0.01; Fig. 6). These results indicate that it is the cardiac sympathetic nerve rather than vagus mediates the tachycardia effect induced by microinjection of CRF into the PH.

Figure 2. CRF excited PH neurons with a direct postsynaptic manner. (A1,A2) Graph of brain section shows the area of PH investigated in this study. (B1,B2) CRF still induce an inward current when perfusing the slice with TTX. (B3) Group data of the 5 recorded PH neurons in rats. (C1) CRF concentration-dependently excited PH neurons. (C2) Concentration-response curves for CRF on the 7 recorded PH neurons in rats shows the mean EC50 of 176.5 nM. In this and the following figures, the short horizontal bars above the data indicate the period of application of CRF or CRF receptor agonist, and the long horizontal bars indicate the exposure of the slice to TTX and CRF receptor antagonist.
Responses of RVLM, RVMM, and DMNV neurons to microinjection of CRF into the PH.

Considering that the RVLM and RVMM in the brainstem are two most important areas of presympathetic neurons, whereas the DMNV and nucleus ambiguus are two nuclei gathering vagal preganglionic neurons, we conducted extracellular recordings in the RVLM, RVMM and DMNV (the nucleus ambiguus neurons were not recorded since they are intrinsically silent) in vivo to clarify which structures were involved in the tachycardic effect elicited by microinjection of CRF into the PH. We recorded 80 RVLM, 72 RVMM and DMNV neurons in rats (n = 12). Among them, 37 (37/80, 46.3%) RVLM neurons and 32 (32/72, 44.4%) RVMM neurons increased their firing rates from 9.3 ± 1.2 spikes/s and 12.8 ± 1.9 spikes/s to 15.7 ± 1.8 spikes/s (P < 0.01) and 21.3 ± 2.5 spikes/s (P < 0.01), respectively, in response to microinjection of CRF into the PH (Fig. 7). In addition, 4 (4/80, 5.0%) RVLM neurons and 3 (3/72, 4.2%) RVMM neurons decreased their firing rates from 21.0 ± 2.8 spikes/s and 23.8 ± 3.8 spikes/s to 12.3 ± 2.0 spikes/s (P < 0.05) and 12.8 ± 2.4 spikes/s (P < 0.05), respectively, to microinjection of CRF into the PH (Fig. 7), and the rest 39 RVLM neurons and 37 RVMM neurons showed no response. On the other hand, all of the 14 recorded DMNV neurons had no response (1.9 ± 0.3 spikes/s vs. 1.7 ± 0.2 spikes/s, P = 0.58) to microinjection of CRF into the PH (Fig. 7). The results suggest that it is the RVLM and RVMM but not DMNV mediate the tachycardia effect induced by microinjection of CRF into the PH.

Figure 3. CRF excited PH neurons via CRFR1 and CRFR2. (A) CRF excited PH neurons, and highly selective agonists for CRFR1 and CRFR2, stressin I and urocortin II, respectively, mimicked the excitatory effect of CRF on PH neurons. (B) Selective CRFR1 antagonist antalarmin and selective CRFR2 antagonist antisavagine-30 partly blocked the excitation of PH neurons induced by CRF, and in combination with antalarmin and antisavagine-30 totally blocked the neuronal excitation induced by CRF. (C) Group data of the tested PH neurons in rats. Statistical significance was determined using Student's t test. **P < 0.01.
and co-mediate the CRF-induced excitation on PH neurons (Figs 1–3). Since either excitation by electrical stimulation or disinhibition by microinjection GABA receptor antagonist in the PH significantly increased AP and HR\(^{39,40}\), our result that CRF excited the PH neurons suggests a possibility that PH may contribute to central regulation of CRF on cardiovascular activities. Intriguingly, microinjection of CRF into the PH significantly increased HR but not MAP and RSNA (Fig. 4), suggesting that by activating PH neurons, CRF may actively affect cardiac activity. Furthermore, selective CRFR1 antagonist antalarmin or selective CRFR2 antagonist antisauvagine-30 partly decreased tachycardiac response to microinjecting CRF into the PH, while broad-spectrum CRF receptor antagonist astressin totally blocked the response (Fig. 5), demonstrating that the tachycardiac response to CRF microinjected into the PH is mediated by activation of both CRFR1 and CRFR2. Nonetheless, microinjection of selective antagonist for CRFR1 or CRFR2 or broad-spectrum CRF receptor antagonist into the PH before CRF injection did not elicit any changes in basal HR (Fig. 5A). Thus, we speculate that under normal physiological conditions, CRF receptors in the PH may be not under a status of tonic control of endogenous CRF to modulate cardiovascular activity.

Microinjection of CRF into the PH only elevated HR but did not influence AP and RSNA suggests that the regulation of cardiac activity by CRF may be mediated by autonomic cardiovascular centers. On the basis of current knowledge regarding the pathway underlying the PH control of cardiac function, activation of cardiac sympathetic nerve or inhibition of cardiac vagal nerve may be responsible for tachycardiac effect followed by microinjection of CRF into the PH. In this study, we found that vagotomy did not affect the tachycardiac response by CRF, while blockade of CSNA with propranolol almost totally abolished the tachycardiac response (Fig. 6).
These results reveal that the tachycardia response is not an effect of reduced vagus nerve activity elicited by microinjecting CRF into PH, but due to an increase in CSNA. This result accords with the previous reports that activation of the PH increased HR by increasing sympathetic nerve activity\(^*\). It is well known that the PH

Figure 5. The effect of microinjection of antalarmin, antisauvagine-30, and astressin into the PH on the increased HR response by microinjection of CRF into the PH. (A) Changes in HR, evoked by microinjection of CRF into the PH, and the CRF-induced changes after microinjection of antalarmin, antisauvagine-30, and astressin. (B) Group data showing the CRF-induced changes in average peak HR in rats before and after microinjection of CRF receptor antagonists. Statistical significance was determined using Student’s t test. *P < 0.05, **P < 0.01.

Figure 6. Effect of vagotomy and blockade of cardiac sympathetic nerve activity (CSNA) on the increased HR response by microinjection of CRF into the PH. (A) Changes in HR, evoked by microinjection of CRF into the PH, before and after intravenous administration of vagotomy and propranolol (blockade of CSNA). (B) Group data showing the CRF-induced changes in average peak HR in rats before and after vagotomy and blockade of CSNA. Statistical significance was determined using Student’s t test. n.s. no significance, **P < 0.01.
neurons project to various areas/structures in the brainstem, which are involved in regulation of cardiovascular activity, including the RVLM and RVMM. The RVLM and RVMM are the key centers providing synaptic inputs to sympathetic preganglionic neurons and directly regulating sympathetic activity to influence cardiovascular function. Excitation of RVLM and RVMM increases HR and AP by strengthening sympathetic nerve activity which output through the sympathetic preganglionic neurons of intermediolateral cell column in spinal cord. Thus, the tachycardiac effect induced by microinjection of CRF in the PH may be mediated by the excitation of RVLM and RVMM neurons. In fact, in this study, we found that it is the RVLM and RVMM neurons rather than DNV neurons controlling vagus activity responsive to microinjecting CRF into the PH. Therefore, we speculate that the pathways from the PH to the RVLM and RVMM may contribute to the modulation of central CRF system on sympathetic outputs to the heart and the consequent HR regulation.

Tachycardia is an important component and feature of stress-induced cardiovascular responses and dysfunctions. We demonstrate that CRF, a critical neuropeptide responsible for initiating and integrating stress responses, induces a remarkable tachycardia by exciting PH neurons, which has been proposed to participate in the generation of stress-induced tachycardia, through both CRFR1 and CRFR2. Pathways from the PH to the RVLM and RVMM, which subsequently regulate sympathetic outflow, may contribute to the tachycardia induced by activation of PH by CRF. The findings not only help to understand the effect and mechanism of central CRF on cardiovascular regulation, but also provide a new insight into the stress-related cardiovascular dysfunctions and disorders.

Methods
Animals. We used male Sprague-Dawley rats (375 ± 50 g), housed under controlled conditions with a lighting schedule of 12 h light and 12 h dark at a temperature of 22 ± 2 °C. Standard food and water were provided ad libitum. All experiments, approved by the Experimental Animal Care and Use Committee of Nanjing University, were treated in accordance with U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 2011) and were reported in accordance with the ARRIVE guidelines. All efforts were made to minimize the number of animals used and their suffering.

Quantitative real-time RT-PCR. For this experiment, three independent groups of RNA pools each from 5 rats, deeply anaesthetized with urethane (1600 mg/kg) plus α-chloralose (130 mg/kg) given intraperitoneally, were used as biological replicates. The PH tissue punches were collected from coronal brain slices of rats according to the rat brain atlas of Paxinos and Watson and pooled (5 animals in each pool). RNA extraction was done using an RNA isolation kit (Invitrogen, USA) according to the manufacturer’s instructions. The reaction was carried out in an Applied Biosystem 7300 Real-time PCR System using the following parameters: 47 cycles at 95°C
for 5 s, 60 °C for 30 s, 95 °C for 15 s, 55 °C for 30 s and 95 °C for 15 s. The PCR program was completed by a melting temperature analysis. For quantification, the quantity of the target gene (crfr1 and crfr2) was expressed relative to the amount of the reference gene (gapdh) to obtain a normalized target expression value.

The primer sequences were as follows: crfr1: forward, 5′-TTCTACGGTGTGCAGCTAC-3′ and reverse, 5′-CGAGATGGTTGCTACAGCTC-3′; crfr2: forward, 5′-CCTGCCATATCCTTTCTG-3′ and reverse, 5′-CTCTCACGTGGCTACGTC-3′; gapdh: forward, 5′-TTACCAGGCCAACATGCGAACG-3′ and reverse, 5′-CTCAGCCACCAGCTACC-3′.

Immunofluorescence. The experimental procedures for immunostaining followed our previous reports47,48. Briefly, rats (n = 5) were deeply anesthetized and the rat brain was removed and fixed in the 4% paraformaldehyde in 0.1 M phosphate buffer at 12 h at 4 °C, and then cryoprotected with 30% sucrose for 48 h. Frozen coronal sections (25 μm thickness) containing the PH were obtained by using a freezing microtome (CM 1850, Leica, Germany). The slices were rinsed in phosphate-buffered saline containing 0.1% Triton X-100 (PBST) (Sigma, USA) and then incubated in 10% normal bovine serum (Millipore, USA) in PBST for 30 min. Sections were incubated overnight at 4 °C with primary antibodies to CRFR1 (a rabbit anti-CRFR1 polyclonal antibody, 1:200; Abcam, USA) and CRFR2 (a goat anti-CRFR2 polyclonal antibody, 1:200; Abcam). After a complete wash in PBS, sections were incubated in the Alexa 594-conjugated donkey anti-rabbit (1:2,000; Invitrogen) and Alexa 647-conjugated donkey anti-goat (1:2,000; Invitrogen) for 2 h at room temperature in the dark. The slides were washed and mounted in UltraCruz mounting medium (Santa Cruz Biotechnology, USA). All micrographs were taken with an inverted laser scanning confocal microscope (FV1000; Olympus, Japan).

Patch clamp recordings on brain slices. Rats aged 14–21 days were deeply anaesthetized and coronal brainstem slices (300 μm in thickness) containing the PH were prepared with a vibrissocler (VT 1200 S, Leica), according to the rat brain atlas25. The slices were incubated in artificial cerebrospinal fluid (ACSF, composition in mM: 124 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1.3 MgSO4, 26 NaHCO3, 2 CaCl2 and 10 D-glucose) equilibrated with 95% O2 and 5% CO2 at 37 °C. The trachea was intubated for artificial respiration. The right carotid artery was cannulated and the blood pressure was monitored using a transducer (SP844, ADInstruments). MAP and HR were measured using PowerLab (ADInstruments). LabChart 7 software was used to rectify and integrate the renal sympathetic nerve signals.

Bilateral cervical vagus nerves were isolated and tied with silk threads. The vagus nerves were cut by carefully pulling on the silk loop to detect the CRF-induced changes in HR after bilateral vagotomy followed a stabilization period of 15–20 min. To assess role of cardiac sympathetic nerve in the CRF-induced changes in HR, the jugular vein was cannulated for administering propranolol (1 mg/kg, Sigma) to block the cardiac sympathetic nerve activity (CSNA).

Microinjection in the PH. The rat was mounted on a stereotaxic frame (1404, David Kopf Instruments, USA). The craniotomy was made above the PH. According to the rat brain atlas25, the stainless-steel injection cannulae (length 10 mm, o.d. 0.5 mm, i.d. 0.3 mm) were inserted into the PH (A = 3.60 to −3.96, L 0.5, H 7.75) for microinjection of CRF (20–200 μM), astressin (300 μM), antalarmin (500 μM), antivasivane-30 (300 nM, Tocris), and saline (0.9% NaCl) using Hamilton syringes (0.5 μl lasting 2 min). The effective extent of the drug diffusion in the present study was restricted in the PH according to the estimate by using extracellular electrophysiological recording neurons 0.5 mm away from the injection site (the detailed descriptions of methodology can be obtained from our previous reports47,48). The effects of CRF on HR, MAP and RSNA before and after microinjections were detected.
Extracellular recording in brainstem in vivo. Single neuronal discharges were recorded from the RVLM (A = 12.00 to −12.48, L 1.8 to 2.4, H 8.0 to 8.5), RVMM (A = −10.00 to −12.20, L 0.0 to 0.6, H 8.5 to 9.5), and DMNV (A = −13.08 to −13.20, L 0.5 to 1.0, H 5.2 to 5.5), according to the rat brain atlas25, by using a glass electrode filled with pontamine sky blue in 0.5 M sodium acetate (DC resistance 5–10 MΩ). The neuronal discharges were conventionally amplified, displayed on an oscilloscope, and simultaneously fed into a window discriminator. The standard rectangular pulses (1.0 ms, 5 V) triggered from the spikes were sent through an A/D interface (Power 1401, CED, UK) into a laboratory computer.

The peristimulus time histogram (sampling interval 1 sec, sampling length 1,800 sec, single trial) of neuronal discharges were generated by the software Spike 2 (CED) to assess the effects of microinjection of CRF into PH on neuronal discharges of RVLM, RVMM, and DMNV, respectively. According to the previous established criteria49–50, any significant change in neuronal discharge rate, reproducibly evoked by the microinjection, with P < 0.05 was considered as an excitatory or inhibitory response. After experiments, microinjection sites in the PH and recording sites in the RVLM, RVMM, and DMNV were histologically identified as we previously reported49–50. Data from rats in which the injection and the recording sites were out of the target nucleus were excluded from further analysis.

Statistical analysis. All data are presented as mean ± SEM. Preliminary tests for normality of each variable were executed. The Student’s t test was employed for statistical analysis. P < 0.05 was considered significant.

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Author Contributions
J.-N.Z. and J.-J.W. designed research; H.-R.G., Q.-X.Z. and B.L. performed electrophysiological, qPCR and immunostaining experiments and analyzed data; H.-Z.L. performed electrophysiological experiments; Z.-P.C. performed immunostaining experiments; H.-R.G., Q.-X.Z. and B.L. prepared figures and drafts; J.-N.Z. and J.-J.W. wrote the paper.

Additional Information
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