ESC European Society of Cardiology

Cardiovascular Research (2023) **119**, 1751–1762
https://doi.org/10.1093/cvr/cvad056

Corticotropin-releasing hormone neurons in the central nucleus of amygdala are required for chronic stress-induced hypertension

Zhao-Fu Sheng[1†], Hua Zhang [1†], Jeffery G. Phaup[1], PeiRu Zheng[1], XunLei Kang[1], Zhenguo Liu[1], Hui-Ming Chang[2,3,4], Edward T.H. Yeh[2,3,4], Alan Kim Johnson[5], Hui-Lin Pan[6], and De-Pei Li [1*]

[1]Center for Precision Medicine, Department of Medicine, School of Medicine University of Missouri, One Hospital Drive, Columbia, MO 65212, USA; [2]Department of Pharmacology, The University of Arkansas for Medical Sciences, 4301 West Markham Street, Little Rock, AR 72205, USA; [3]Department of Toxicology, The University of Arkansas for Medical Sciences, 4301 West Markham Street, Little Rock, AR 72205, USA; [4]Department of Internal Medicine, The University of Arkansas for Medical Sciences, 4301 West Markham Street, Little Rock, AR 72205, USA; [5]Department of Psychological and Brain Sciences, The University of Iowa, G60 Psychological and Brain Sciences Building, Iowa City, IA 52242, USA; and [6]Department of Anesthesiology and Perioperative Medicine, The University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

Received 1 May 2022; revised 30 November 2022; accepted 8 April 2023; online publish-ahead-of-print 12 April 2023

Time of primary review: 65 days

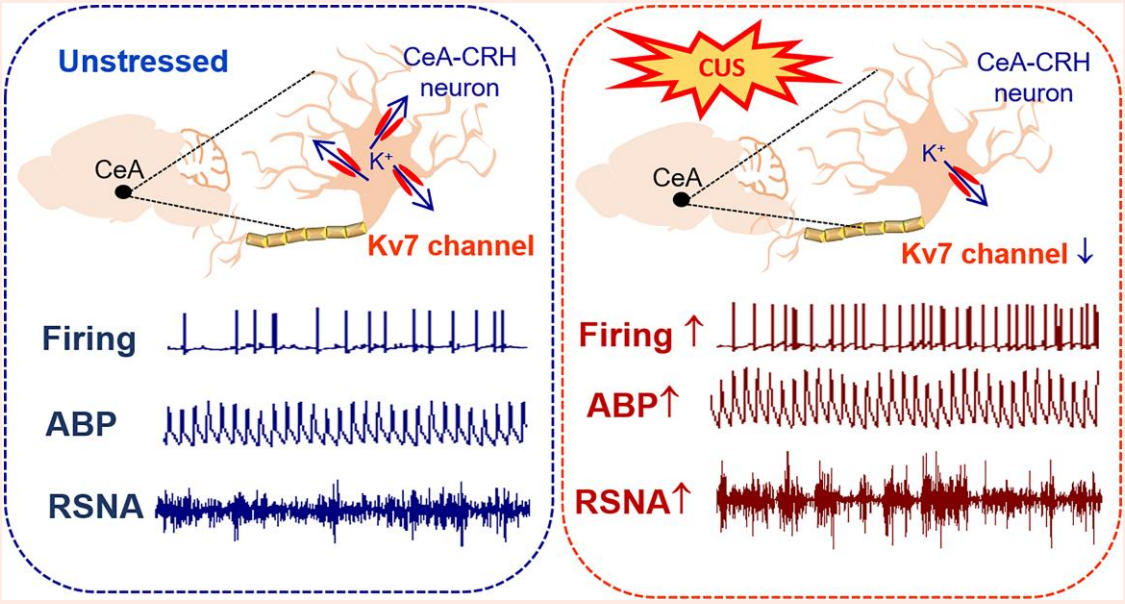
Aims Chronic stress is a well-known risk factor for the development of hypertension. However, the underlying mechanisms remain unclear. Corticotropin-releasing hormone (CRH) neurons in the central nucleus of the amygdala (CeA) are involved in the autonomic responses to chronic stress. Here, we determined the role of CeA-CRH neurons in chronic stress-induced hypertension.

Methods and results Borderline hypertensive rats (BHRs) and Wistar-Kyoto (WKY) rats were subjected to chronic unpredictable stress (CUS). Firing activity and M-currents of CeA-CRH neurons were assessed, and a CRH-Cre-directed chemogenetic approach was used to suppress CeA-CRH neurons. CUS induced a sustained elevation of arterial blood pressure (ABP) and heart rate (HR) in BHRs, while in WKY rats, CUS-induced increases in ABP and HR quickly returned to baseline levels after CUS ended. CeA-CRH neurons displayed significantly higher firing activities in CUS-treated BHRs than unstressed BHRs. Selectively suppressing CeA-CRH neurons by chemogenetic approach attenuated CUS-induced hypertension and decreased elevated sympathetic outflow in CUS-treated BHRs. Also, CUS significantly decreased protein and mRNA levels of Kv7.2 and Kv7.3 channels in the CeA of BHRs. M-currents in CeA-CRH neurons were significantly decreased in CUS-treated BHRs compared with unstressed BHRs. Blocking Kv7 channel with its blocker XE-991 increased the excitability of CeA-CRH neurons in unstressed BHRs but not in CUS-treated BHRs. Microinjection of XE-991 into the CeA increased sympathetic outflow and ABP in unstressed BHRs but not in CUS-treated BHRs.

Conclusions CeA-CRH neurons are required for chronic stress-induced sustained hypertension. The hyperactivity of CeA-CRH neurons may be due to impaired Kv7 channel activity, which represents a new mechanism involved in chronic stress-induced hypertension.

* Corresponding author. Tel: +1 (573) 884 1437; fax: +1 (573) 884 4533, E-mail: lidep@health.missouri.edu
† These two authors contribute equally to this study.

Graphical Abstract



Keywords Corticotropin-releasing hormone • Chronic stress • Central nucleus of the amygdala • Hypertension • Kv7 channel

1. Introduction

Essential hypertension is a multifactorial pathological condition in which genetic-environment interaction may play a significant role.[1] Individuals with genetic susceptibility to hypertension are prone to develop sustained hypertension under chronic stress conditions.[2] Prolonged psychosocial[3] or physiological stress[4] contributes to the development and maintenance of hypertension. Borderline hypertension is highly prevalent in humans (14.5–58.7% in different races worldwide) and is a major risk factor for the development of sustained hypertension.[5,6] However, the mechanisms through which chronic stress causes sustained hypertension remain unknown. Borderline hypertensive rats (BHRs) are first-generation offspring of crossbreeding between the spontaneously hypertensive rat and its normotensive control, the Wistar-Kyoto (WKY) rats.[7] BHRs have baseline arterial blood pressure above the normotensive range before they are exposed to chronic stress.[7] BHRs subjected to chronic physical stress or a long-term high-salt diet reliably develop sustained hypertension.[7,8]

Chronic stress activates corticotropin-releasing hormone (CRH)-expressing neurons in the hypothalamus and central nucleus of the amygdala (CeA).[9] The CeA is an important extrahypothalamic region involved in controlling cardiovascular function during psychological stress, fear, and anxiety.[10] Stimulation of the CeA produces blood pressure and heart rate changes similar to those produced by stressful events.[11] Functional magnetic resonance imaging showed that individuals predisposed to hypertension display increased neuronal activity within the amygdala.[12] Also, lesions of the CeA significantly attenuate stress-induced pressor responses in BHRs.[13] The CRH-expressing neurons in the CeA (CeA-CRH neurons) are crucially important for the autonomic responses to chronic stress.[14] However, the role of CeA-CRH neurons in chronic stress-induced hypertension remains largely unknown.

Potassium (K^+) channels are essential for controlling the membrane potential and excitability of neurons.[15] Among many K^+ channel subunits, heteromeric Kv7.2 and Kv7.3, homomeric Kv7.2, or Kv7.5 (encoded by Kcnq2, Kcnq3, and Kcnq5 genes, respectively) constitute the neuronal Kv7/M-channels.[15,16] Compared with homomers, distinct subunits in heteromers may interact efficiently to produce more potent regulation upon the excitability of neurons. For example, co-expression of Kv7.2 and Kv7.3 leads to greater surface expression and larger currents than when either subunit is expressed alone.[17] The M-channel is a non-inactivating voltage-gated K^+ channel that is critically involved in stabilizing the membrane potential to the resting membrane potential because it opens more when the cell membrane depolarizes.[15] Genetic knockdown or acute blockade of Kv7.2/Kv7.3 channels depolarizes neurons and increases their firing activity, whereas the opening Kv7.2/Kv7.3 channels hyperpolarize the cell membrane and inhibit neuronal activity.[18] The important functional role of Kv7 subunits has been highlighted because mutations in Kv7 subunits are associated with benign familial neonatal convulsions, an autosomal dominant neonatal epilepsy.[19,20] Our most recent study revealed that diminished Kv7 channel activity in CeA neurons contributes to elevated sympathetic outflow in spontaneously hypertensive rats.[21] In the present study, we determined the role of Kv7 channels in regulating CeA-CRH neuronal activity in chronic stress-induced sustained hypertension in BHRs.

2. Methods

The experimental protocols and surgical procedures were approved by the Institutional Animal Care and Use Committee of The University of Missouri School of Medicine (#9439) and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The detailed experimental procedures, including anaesthesia and euthanasia, are described in the *online-only Data Supplemental Materials*. The rats were euthanized by decapitation under deep anaesthesia with 5% isoflurane in O_2 to harvest brain tissue for electrophysiological recording and biochemical assays including western blotting and RT–PCR. The rats subjected to immunocytochemical staining were intra-cardiac perfused with 4% paraformaldehyde and 10% sucrose under deep anaesthesia with isoflurane 5% in O_2. The rats used for recording of arterial blood pressure and renal sympathetic nerve activity were decapitated after finishing the experiment when they were still under anaesthesia by urethane and α-chloralose.

2.1 Chronic unpredictable stress

The chronic unpredictable mild stress (CUS) model was executed according to previously reported paradigms with minor modification (see Supplementary material online, *Table S1*).

2.2 Telemetry transmitter implantation and blood pressure measurements

The arterial blood pressure (ABP) of conscious BHRs and WKY rats was continuously monitored by a telemetry system in free-moving rats.

2.3 Identification of CRH neurons in the CeA

The CRH-expressing neurons in the rat CeA were identified by an adeno-associated virus (AAV, serotype 2) viral vector-mediated expression of enhanced green fluorescent protein (eGFP) driven by the full-length of rat CRH promoter. Under anaesthesia induced by inhalation of 2–3% isoflurane, the AAV vector was injected into the CeA.

2.4 Immunofluorescence staining

Immunofluorescence staining was used to determine the spatial distribution of Kv7 channels in the CeA-CRH neurons.

2.5 Chemogenetic approach and viral vector microinjection

Inhibitory hM4Di was specifically expressed in CeA-CRH neurons by bilateral injection of a mixture of two viral vectors including AAV2-CRH-Cre and AAV2-hSyn-hM4Di-DIO-mCherry into the CeA under 2–3% isoflurane anaesthesia. Please refer to detailed information on vectors and microinjection procedures in *Supplemental Materials*.

2.6 Quantitative reverse transcription-polymerase chain reaction (qRT–PCR) and western immunoblotting analysis

Rat brain tissues were harvested through decapitation under isoflurane (5% in O_2 inhalation) anaesthesia and then were used to measure the Kv7 subunit mRNA and protein levels.

2.7 Recording of ABP and renal sympathetic nerve activity (RSNA)

Recording of ABP and RSNA were recorded in rats anaesthetized by a bolus intraperitoneal injection of a mixture of urethane (800 mg/kg) and α-chloralose (60–75 mg/kg), to determine the effect of inhibiting CeA-CRH neurons by the Designer Receptor Exclusively Activated by Designer Drugs (DREADD) approach on ABP, heart rate (HR), and RSNA. The responses of ABP, HR, and RSNA to microinjection of Kv7 blockers into the CeA in unstressed BHRs and CUS BHRs were determined.

2.8 Whole-cell patch-clamp recordings in brain slices were performed

Coronal brain slices were sectioned from the rat brains that were quickly removed under deep anaesthesia induced by 5% isoflurane in O_2. Firing activity and M-currents were recorded from CeA-CRH neurons tagged by eGFP or mCherry in the brain slices.

3. Results

3.1 CUS induces a long-lasting elevation of ABP in BHRs

The baseline mean ABP of WKY rats was significantly lower than that in BHRs [$t_{(11)} = 12.53$, $P < 0.0001$]. CUS treatment significantly elevated ABP in BHRs and WKY rats. The maximum pressor response appeared on Days 10 to 21 after the onset of CUS (*Figure 1A*). Whereas the CUS-induced pressor response lasted for another 21 days in BHRs after cessation of CUS, elevated blood pressure in WKY rats returned to the basal levels 7–10 days after the termination of the CUS procedure (*Figure 1A*). The baseline HR of unstressed BHRs was not significantly different from that of WKY rats (*Figure 1B*). CUS treatment significantly increased HR in BHRs from baseline 268.8 ± 7.7 bpm to 318.2 ± 32.3.3 bpm [$t_{(6)} = 3.760$, $P = 0.0094$]. CUS-induced tachycardia was persistent and remained elevated for at least 3 weeks after termination of CUS (*Figure 1B*). CUS treatment did not significantly alter HR in WKY rats.

3.2 CUS activates CRH neurons

To determine the effect of CUS on CeA-CRH neurons in BHRs, we directly measured the firing activity of CeA-CRH neurons in brain slices. CeA-CRH neurons were identified by a viral-mediated expression of eGFP driven by the rat CRH promoter. We used an immunostaining approach to verify the eGFP-tagged CeA neurons that were CRH positive (*Figure 1C–E*). In conrotatory brain sections, the CeA was a circular-shaped structure with boundaries of 3.7 to 4.6 mm lateral from the midline and 7.5 to 8.5 mm from the surface of the cortex (*Figure 1D*). In addition, the CeA was −2.8 to −3.3 mm anteroposterior from bregma. Images from *Figures 1E*, *2B*, and *5A* were taken from this area. A total of 480 of 491 (97.3 ± 0.62%) eGFP-tagged neurons in the CeA from eight brain sections were positive for CRH immunoreactivity. We performed whole-cell recording on eGFP-expressing neurons visualized within the boundaries of the CeA in brain slice preparation. To minimize the influence of the last stressor, the firing activity of CRH-CeA was recorded on Days 7 and 14 post-CUS procedure. The basal firing activity of eGFP-tagged CeA-CRH neurons did not differ between unstressed BHRs (0.83 ± 0.14 Hz, $n = 6$ neurons from three rats) and CUS-treated BHRs [0.89 ± 0.16 Hz, $n = 7$ neurons from three rats, $t_{(11)} = 0.3434$, $P = 0.7377$], while the baseline firing rate of CeA-CRH neurons in unstressed BHRs was slightly higher than that in WKY rats [0.55 ± 0.2 Hz, $n = 10$ neurons from four rats, $t_{(14)} = 1.027$, $P = 0.3218$, *Figure 1F*]. The firing activities of CeA-CRH neurons from CUS-treated BHRs at Days 7 and 14 after the termination of CUS were significantly higher than those in unstressed BHRs [$F_{(5,33)} = 4.302$, $P = 0.004$, *Figure 1F*]. However, in WKY rats, CUS increased the firing activity of CeA-CRH neurons on Day 7, but not Day 14, after CUS termination. These data suggest that CUS induces a long-lasting increase in the firing activity of CeA-CRH neurons in BHRs but excites transiently CeA-CRH neurons in WKY rats.

3.3 CUS-induced sustained hypertension in BHRs was attenuated by chronic suppression of CeA-CRH neurons

Since CUS increased CeA-CRH neuron activity in BHRs, we determined the role of CeA-CRH neurons in CUS-induced sustained hypertension in BHRs by using a chemogenetic approach. We microinjected a mixture of AAV2-hSyn-DIO-hM4Di-mCherry and AAV2-CRH-Cre vectors to express hM4Di in CeA-CRH neurons specifically (*Figure 2A*). The specificity of the AAV2-CRH-Cre vector has been validated independently by others using RNAscope.[22] The expression of hM4Di in CeA-CRH neurons was verified by immunostaining of CRH in mCherry-tagged neurons (*Figure 2B*). Bath application of a DREADD agonist C21 (10 μM) induced a hyperpolarization and significantly decreased the firing rate from 1.4 ±

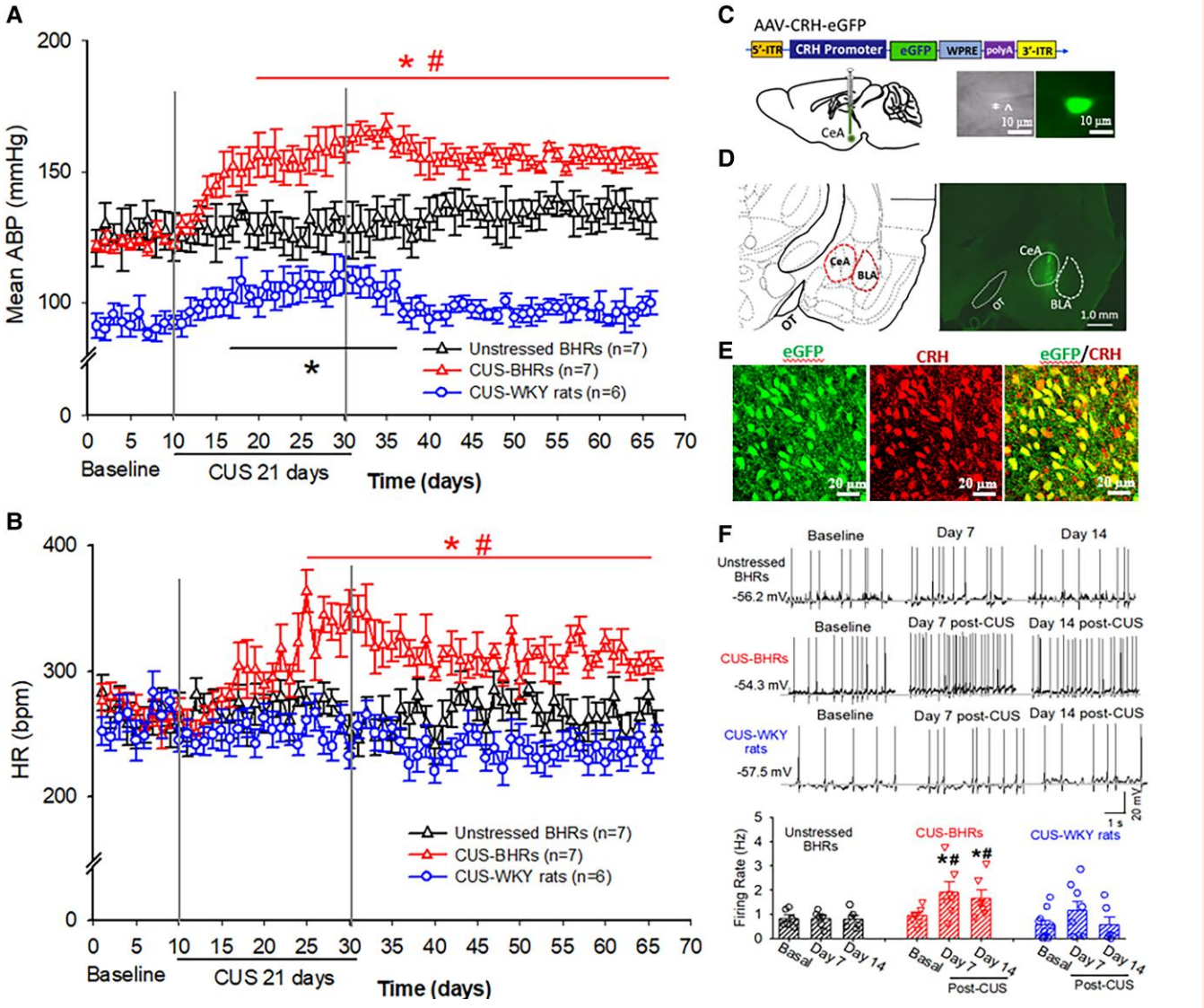


Figure 1 Chronic unpredictable stress (CUS) induced a sustained elevation of blood pressure and a long-lasting hyperactivity of CeA-CRH neurons in BHRs. (*A* and *B*) Mean ABP (*A*) and HR (*B*) were monitored by radiotelemetry before, during, and after CUS in WKY rats ($n = 6$) and BHRs ($n = 7$) and age-matched unstressed BHRs ($n = 7$). Two-way ANOVA with *post hoc* analyses of Tukey multiple comparison tests were used to compare daily mean ABP and HR between experimental groups. (*C*) Schematic diagram depicts constructs of AAV vector containing an eGFP sequence driven by the rat CRH promoter. Inset: An eGFP-tagged CeA neuron indicated by * with a recording electrode indicated by ^ under transmitted light and fluorescent illuminant. (*D*) Diagram and micro-photography show the eGFP expression in the CeA region. (*E*) Immunofluorescence images depict that eGFP-tagged neurons were positive for CRH immunoreactivity. (*F*) Original traces and summary data show that CUS induced a long-lasting increase in the spontaneous firing activity of CeA-CRH neurons in BHRs ($n = 7$ neurons from three rats in groups of Day 7 and Day 14 post-CUS) compared with unstressed BHRs ($n = 6$ neurons from three rats). *$P < 0.05$, compared with the basal values in CUS-BHRs; #$P < 0.05$, compared with respective values in unstressed BHRs; repeated-measures 2-way ANOVA with Dunnett's *post hoc* test. OT, optic tract.

0.2 to 0.4 ± 0.1 Hz [$t_{(5)} = 6.359$, $P = 0.0014$] in six mCherry-expressing neurons recorded from three unstressed BHRs. In contrast, application of C21 (10 μM) did not alter the membrane potential and firing rate in any of six unlabelled neurons [1.5 ± 0.2 Hz before vs. 1.4 ± 0.2 Hz after C21, $t_{(5)} = 0.2000$, $P = 0.8494$, *Figure 2C* and *D*]. These results suggest that CeA-CRH neuron activity was able to be selectively suppressed by the DREADD approach.

Next, we determined if selective inhibition of CeA-CRH neurons mitigates CUS-induced hypertension in BHRs. Blood pressure was monitored by telemetry in conscious BHRs. Two weeks after injection of a mixture of AAV2-DIO-hM4Di and AAV2-CRH-Cre vectors into the CeA, C21 was administered subcutaneously through an implanted osmotic pump at a dose of 1.0 mg/kg of body weight daily for 28 days. CUS procedures were performed during the period of C21 administration. In seven BHRs injected with AAV-hSyn-DIO-hM4Di into the CeA and five BHRs co-injection of AAV-hSyn-DIO-mCherry and AAV-CRH-Cre vectors into the CeA, CUS procedure plus C21 administration elevated mean ABP and HR for at least 3 weeks after cessation of CUS (*Figure 2E* and *F*). However, in eight BHRs injected with AAV-hSyn-DIO-hM4Di and AAV-CRH-Cre vectors, mean ABP and HR were significantly lower during

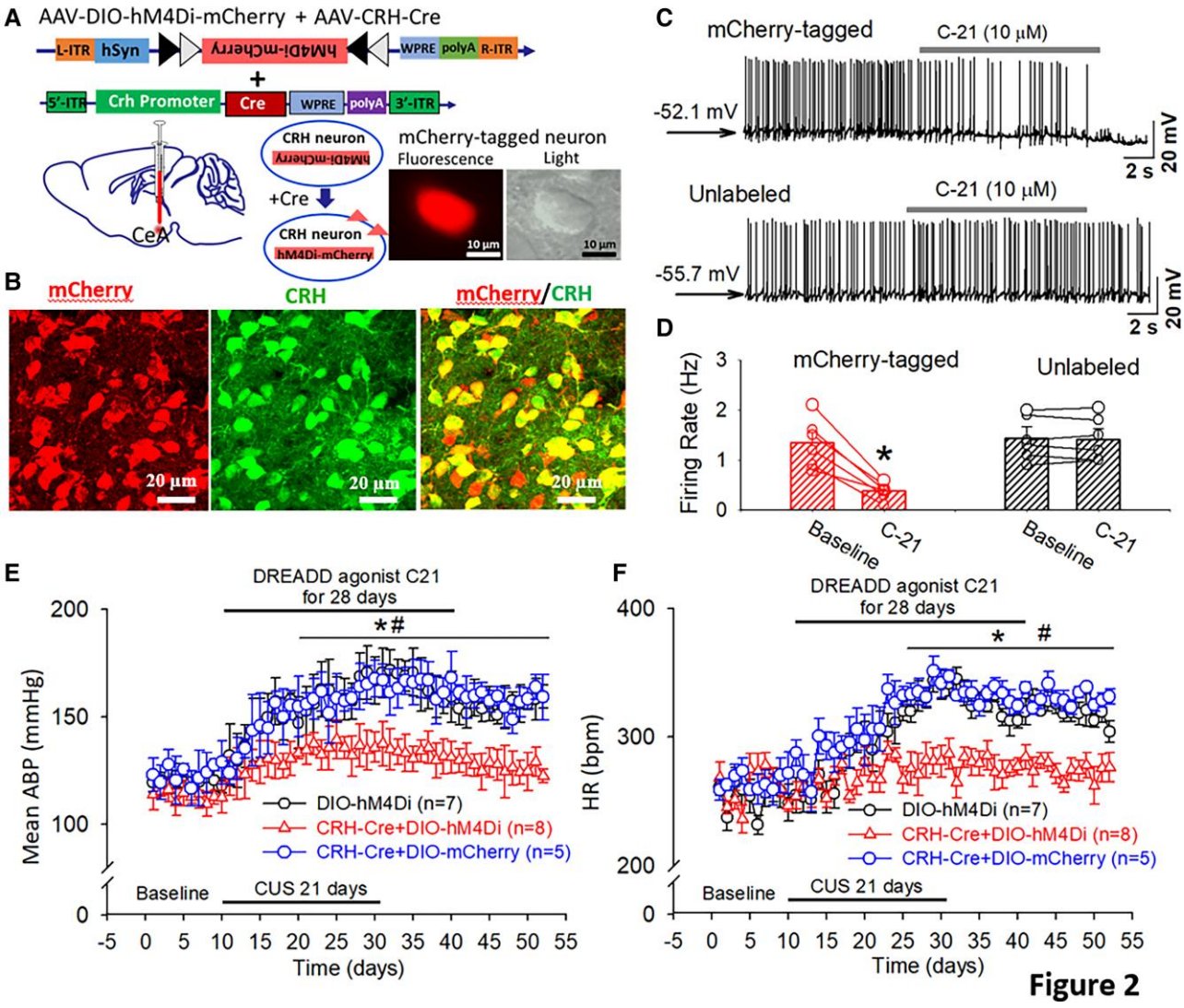


Figure 2 Chemogenetic inhibition of CeA-CRH neurons effectively alleviated CUS-induced sustained hypertension in BHRs. (*A*) Schematic diagram shows the structures of viral vectors, injection of the viral vectors, and expression of hM4Di on CeA-CRH neurons. Inset images show a mCherry-tagged CeA neuron viewed under transmitted light and fluorescent illuminant. (*B*) Immunofluorescence images show the mCherry-tagged CeA neurons were positive for CRH immunoreactivity. (*C* and *D*) Representative raw tracings (*C*) and summary data (*D*) depict that bath application of 10 μM C21 selectively decreased the firing activity of mCherry-tagged CRH neurons ($n = 6$ neurons from three unstressed BHRs) but did not alter the firing activity of unlabelled neurons ($n = 6$ neurons from three BHRs). Repeated-measures ANOVA with Dunnett's *post hoc* test was used to compare the firing activity between groups. (*E* and *F*) CUS elevated mean ABP (*E*) and HR (*F*) in BHRs injected with AAV-DIO-hM4Di viral vector ($n = 7$) or co-injection of AAV-hSyn-DIO-mCherry and AAV-CRH-Cre into the CeA vectors. Administration of C21 (1.0 mg/kg/day for 28 days) through implanted osmotic pump significantly mitigated CUS-induced pressor response and tachycardia in BHRs injected with AAV-CRH-Cre and AAV-DIO-hM4Di viral vectors ($n = 8$). Two-way ANOVA with *post hoc* analyses of Tukey multiple comparison tests were used to compare daily mean ABP and HR between experimental groups. *$P < 0.05$, compared with baseline values; #$P < 0.05$, compared with BHRs injected with AAV-DIO-hM4Di.

CUS plus C21 administration and after the termination of the CUS (*Figure 2E* and *F*). In order to determine if suppression of CeA-CRH neurons alters baseline ABP and HR, C21 (1.0 mg/kg of body weight daily for 28 days) was administered through the implanted osmotic pump in six unstressed BHRs injected with AAV-DIO-hM4Di and AAV-CRH-Cre vectors. C21 administration had no significant effect on ABP and HR (see Supplementary material online, *Figure S1*). In addition, C21 administration did not change ABP in BHRs subjected to sham surgery ($n = 6$, Supplementary material online, *Figure S1*). These data suggest that increased CeA-CRH neuronal activity is required for CUS-induced hypertension in BHRs.

3.4 Selectively suppressing CeA-CRH neurons decreased sympathetic vasomotor tone in CUS-induced hypertension in BHRs

Heightened sympathetic vasomotor tone plays a critical role in neurogenic hypertension.[23] Since selective inhibition of CeA-CRH neurons decreased

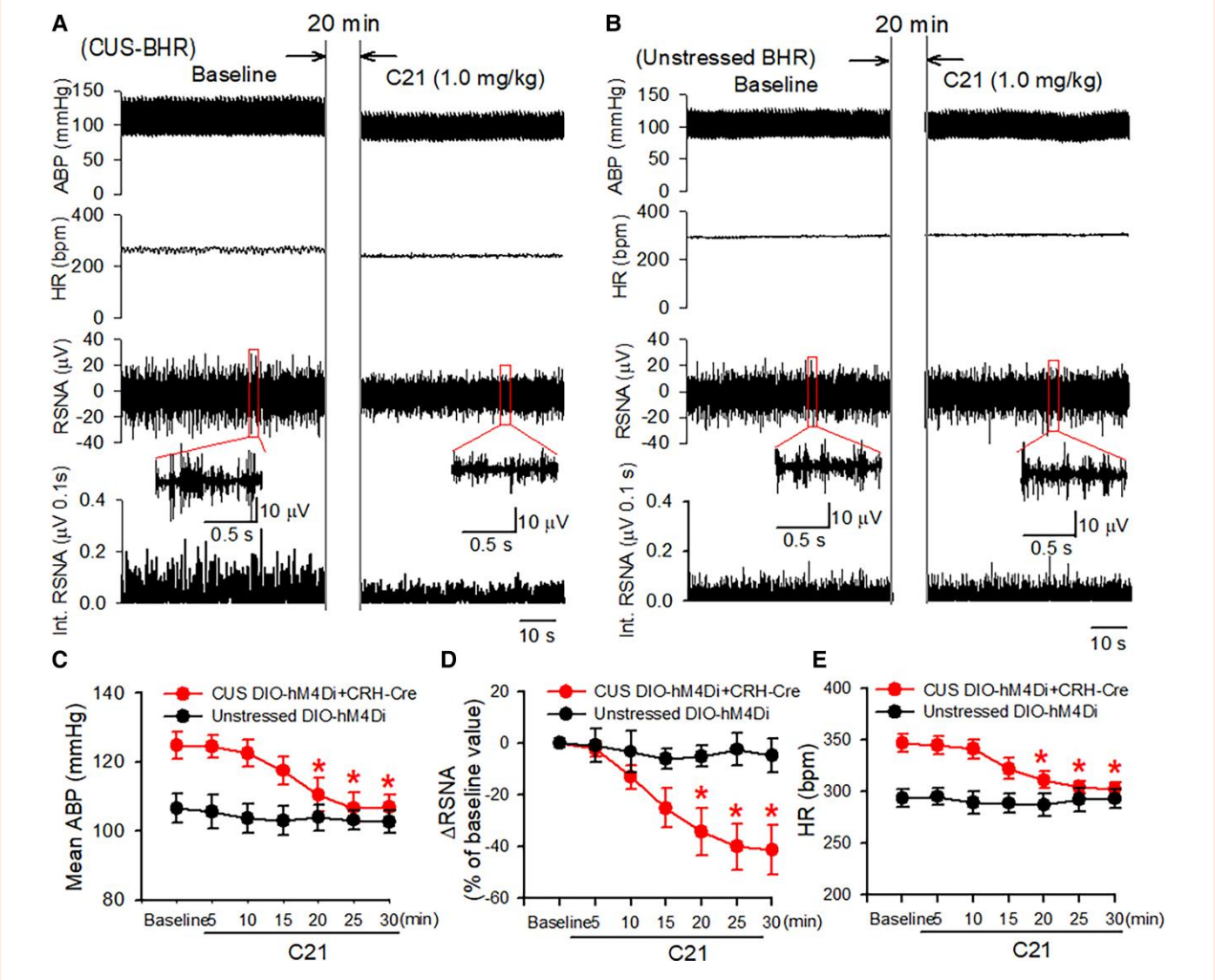


Figure 3 Chemogenetic inhibition of CeA-CRH neurons effectively decreased sympathetic outflow and ABP in CUS-treated BHRs. (*A* and *B*) Raw recording traces show that intravenous bolus injection of C21 (1.0 mg/kg) decreased ABP, RSNA, and HR in CUS-treated BHRs injected with AAV-CRH-Cre and AAV-DIO-hM4Di into the CeA (*A*), while C21 did not alter ABP, RSNA, and HR in BHRs injected with AAV-DIO-hM4Di into the CeA (*B*). Recording of ABP, RSNA, and HR in CUS-BHRs were performed 14 days after termination of CUS treatment. (*C*, *D*, and *E*) Summary data show changes in mean ABP (*C*), RSNA (*D*), and HR (*E*) in response to C21 (i.v. 1.0 mg/kg) in six CUS-BHRs injected with AAV-CRH-Cre and AAV-DIO-hM4Di and BHRs injected with only AAV-DIO-hM4Di ($n = 6$). *$P < 0.05$, compared with the respective baseline. Repeated-measures ANOVA with Dunnett's *post hoc* test.

CUS-induced hypertension, we speculate that inhibition of CeA-CRH neurons would reduce sympathetic outflow in CUS-treated BHRs. To test this hypothesis, we injected AAV-hSyn-DIO-hM4Di and AAV-CRH-Cre vectors into the CeA of six BHRs for selectively expressing hM4Di in CeA-CRH neurons. After 2-week recovery from the surgery, these BHRs were exposed to a 21-day CUS treatment. Two weeks after termination of CUS procedure, ABP, renal sympathetic nerve activity (RSNA), and HR were recorded in anaesthetized CUS-treated BHRs. The basal ABP, RSNA, and HR were significantly higher in CUS-treated BHRs ($n = 6$) than unstressed BHRs ($n = 6$) (*Figure 3* and Supplementary material online, *Figure S3*). Administration of C21 (1.0 mg/kg, i.v.) significantly decreased mean ABP, RSNA, and HR in these six BHRs (*Figure 3*). However, C21 administration did not alter mean ABP, RSNA, and HR in six BHRs injected with AAV-hSyn-DIO hM4Di vector only. These data suggest that heightened CeA-CRH neuronal activity mediates CUS-induced increases in sympathetic vasomotor tone in BHRs.

3.5 CUS decreases Kv7 channel expression in the CeA and reduces M-currents in CeA-CRH neurons

The Kv7 channel carries non-inactivating M currents that crucially regulate neuronal excitability.[15,16,24] To determine if CUS alters Kv7 channels, we measured protein and mRNA levels of Kv7.2, Kv7.3, and Kv7.5 subunits in the CeA in unstressed BHRs and CUS-treated BHRs. The tissue samples for western blot and PCR analysis were taken from unstressed BHRs and CUS-treated BHRs 10–14 days after the termination of CUS procedure.

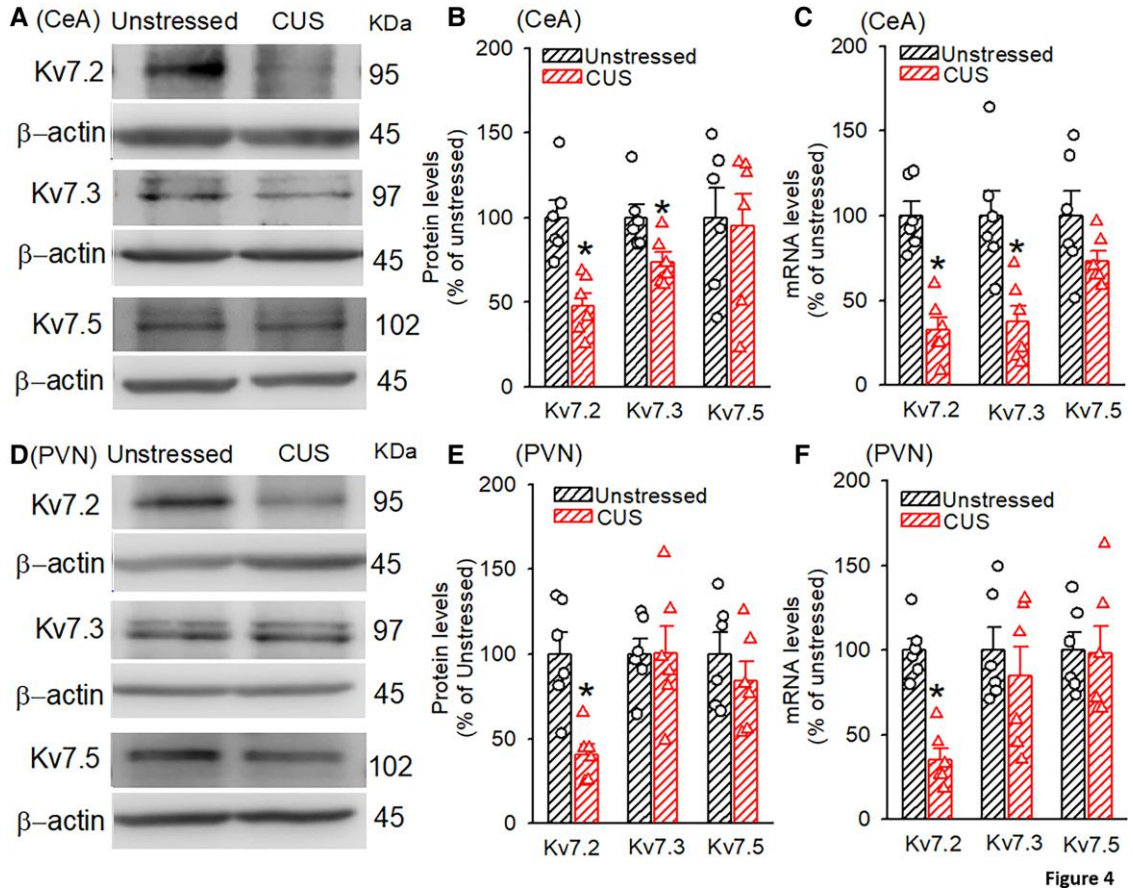


Figure 4 CUS decreased protein and mRNA expression levels of Kv7.2, Kv7.3, and Kv7.5. (*A*, *B*, and *C*) Original gel images (*A*), quantification of immunoblot band density (*B*), and summary data of mRNA levels (*C*) show the total protein and mRNA levels of Kv7.2, Kv7.3, and Kv7.5 in the CeA in unstressed and CUS-treated BHRs ($n = 6$ samples in each group). (*D*, *E*, and *F*) Original gel images (*D*), quantification of band density (*E*), and summary data of mRNA levels (*F*) show the protein and mRNA levels ($n = 6$ samples in each group) of Kv7.2, Kv7.3, and Kv7.5 in the PVN in unstressed and CUS-treated BHRs. In these protein assays, each sample consists of respective tissues from one rat. *$P < 0.05$, compared with values in unstressed BHRs (unpaired Student's *t*-test). PVN, paraventricular nucleus.

Compared with unstressed BHRs, Kv7.2 and Kv7.3 subunit protein levels were significantly decreased in the CeA in CUS-treated BHRs [Kv7.2: $t_{(10)} = 4.202$, $P = 0.0018$; Kv7.3: $t_{(10)} = 2.712$, $P = 0.0219$, $n = 6$ samples for each group, *Figure 4A* and *B*]. Also, Kv7.2 and Kv7.3 mRNA levels in the CeA were significantly lower in CUS-treated BHRs than in unstressed BHRs [Kv7.2: $t_{(10)} = 4.525$, $P = 0.0040$; Kv7.3: $t_{(10)} = 3.537$, $P = 0.0054$, $n = 6$ samples for each group, *Figure 4C*]. Kv7.5 subunit protein and mRNA expression levels did not differ between CUS-treated BHRs and unstressed BHRs. To determine if CUS-induced decreases in Kv7 channel protein and mRNA levels is limited to the CeA, we also measured Kv7 channels protein and mRNA levels in the paraventricular nucleus (PVN). The Kv7.2 protein and mRNA expression levels were significantly reduced in the PVN tissue in CUS BHRs compared with unstressed BHRs [protein: $t_{(10)} = 4.16$, $P = 0.0019$; mRNA: $t_{(10)} = 6.727$, $P < 0.0001$, $n = 6$ samples for each group, *Figure 4D to F*]. However, protein and mRNA levels of Kv7.3 and Kv7.5 subunits did not differ significantly between unstressed BHRs and CUS-treated BHRs.

We next determined if CUS alters M-currents, which are carried by Kv7 channels, in CeA-CRH neurons. We first determined the distribution of Kv7.2, Kv7.3, and Kv7.5 subunits in CeA-CRH neurons using immunofluorescence staining in brain slice containing the eGFP-tagged CeA-CRH neurons (*Figure 5A*). Then, M-currents were recorded in eGFP-tagged CeA-CRH neurons in brain slices by using whole-cell patch-clamp techniques. A selective M-channel opener retigabine (10 μM) and a blocker XE-991 (10 μM) were used to determine the total and basal M-currents, respectively. The basal M-currents were defined as XE-991-sensitive currents without retigabine. The total M-currents were referred to as XE-991-sensitive currents in the presence of retigabine. Compared with unstressed BHRs ($n = 7$ neurons from four BHRs), both basal and total M-currents were significantly reduced in CeA-CRH neurons in CUS-treated BHRs at Day 7 post-CUS [total M-currents: $F_{(2,21)} = 20.43$, $P < 0.0001$; basal M-currents: $F_{(2,21)} = 62.12$, $P < 0.0001$, $n = 9$ neurons from four BHRs at Day 7 post-CUS and $n = 8$ neurons from four BHRs at Day 14 post-CUS, *Figure 5B* and *C*].

3.6 Diminished Kv7 channel activity contributed to CUS-induced hyperactivity of CeA-CRH neurons in BHRs

To determine whether diminished Kv7 channel activity was involved in the hyperactivity of CeA-CRH neuron in CUS-treated BHRs, we tested the effect of the M channel blocker XE-991 on the firing activity of CeA-CRH neurons. The basal firing rates of CeA-CRH neurons in CUS-treated

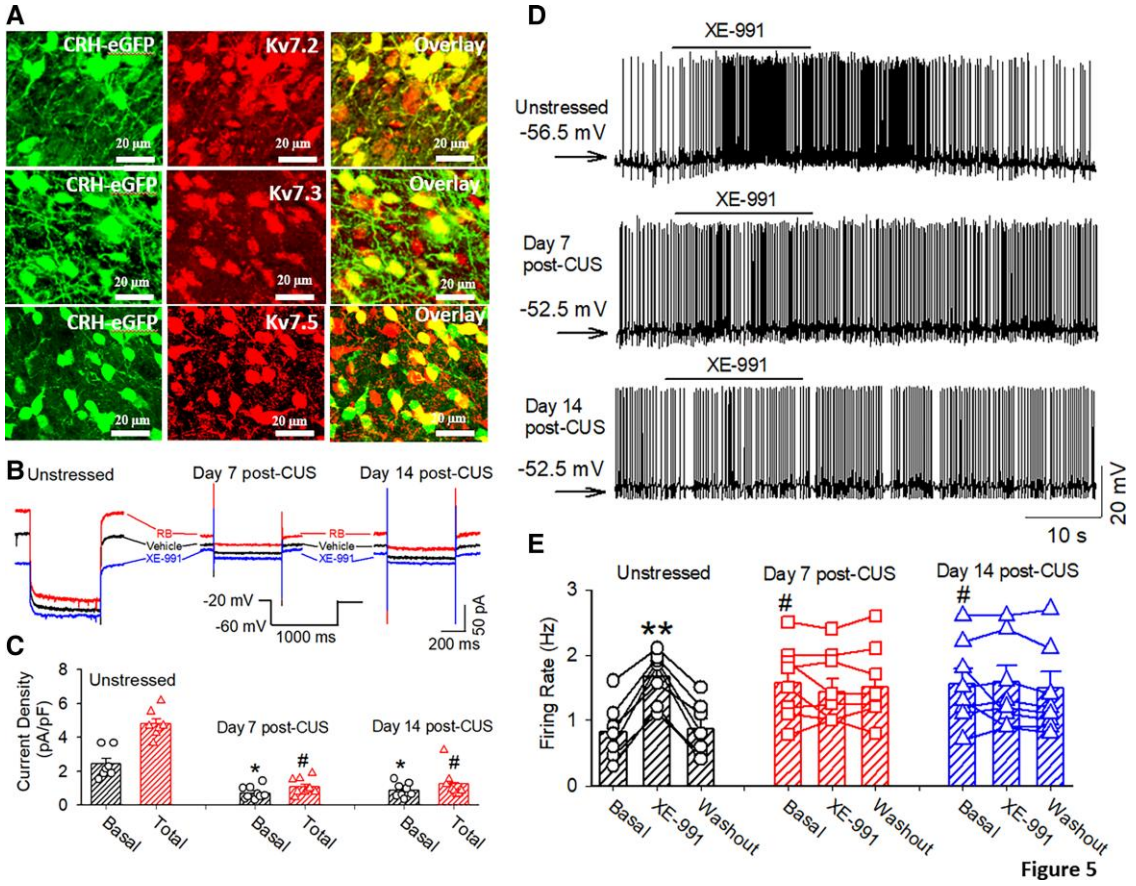


Figure 5 CUS decreases M-currents in CeA-CRH neurons and the excitatory effect of Kv7 channel blocker. (*A*) Immunofluorescence images show the distribution of Kv7.2, Kv7.3, and Kv7.5 immunoreactivities on CeA-CRH neurons. (*B*) Raw traces of voltage-clamp recording show M-currents in CeA-CRH neurons before and after applying an M-channel blocker XE-991 (10 μM) and an M-channel opener retigabine (10 μM) in unstressed BHRs and CUS-treated BHRs at Day 7 and Day 14 after the termination of CUS. (*C*) Summary data shows that calculated basal and total M-currents were significantly diminished in CeA-CRH neurons in CUS-treated BHRs after the termination of CUS ($n = 9$ neurons in four CUS-treated BHRs at Day 7 post-CUS and $n = 8$ neurons in four CUS-treated BHRs at Day 14 post-CUS) compared with unstressed BHRs ($n = 7$ neurons in four BHRs). *$P < 0.05$, compared with basal M current density in unstressed BHRs; #$P < 0.05$, compared with total M current density in unstressed BHRs. (*D* and *E*) Raw tracings (*D*) and summary data (*E*) show the effect of Kv7 channel blocker XE-991 (10 μM) on the spontaneous firing activity of CeA-CRH neurons in unstressed BHRs ($n = 7$ neurons from four BHRs), CUS-treated BHRs at Days 7 and 14 post-CUS ($n = 8$ neurons from four BHRs in each group). *$P < 0.05$, compared with the baseline values in the group; #$P < 0.05$, compared with the corresponding values in unstressed BHRs. Repeated-measures ANOVA with Dunnett's *post hoc* test. RB, retigabine.

BHRs post-CUS 7 days and 14 days were significantly higher than those in unstressed BHRs [$F_{(2,20)} = 4.561$, $P = 0.0233$, $n = 7$ neurons from four unstressed BHRs, $n = 8$ neurons from four CUS-treated BHRs on Day 7 post-CUS, and $n = 8$ neurons from four BHRs at Day14 post-CUS, *Figure 5D* and *E*]. Bath application of XE-991 (10 μM) significantly increased the spontaneous firing rate from 0.82 ± 0.2 to 1.7 ± 0.1 Hz in these seven CeA-CRH neurons in unstressed BHRs [$F_{(2,18)} = 9.267$, $P = 0.0017$] and depolarized these neurons from -57.5 ± 1.2 to -50.6 ± 1.5 mV [$F_{(2,18)} = 6.773$, $P = 0.0064$]. However, bath application of XE-991 (10 μM) did not significantly alter the spontaneous firing rate of CeA-CRH neurons in CUS BHRs on Days 7 and 14 post-CUS [7 days: 1.6 ± 0.2 vs. 1.4 ± 0.2, $F_{(2,21)} = 0.06852$, $P = 0.9340$, $n = 8$ from four BHRs; 14 days: 1.5 ± 0.2 vs. 1.6 ± 0.2, $F_{(2,21)} = 0.1111$, $P = 0.8954$, $n = 8$ from four BHRs, *Figure 5D* and *E*].

We also assessed the excitability of eGFP-tagged CeA-CRH neurons by measuring their action potentials in response to depolarizing currents (0–70 pA). Action potentials were elicited by applying depolarizing currents at a duration of 1 s with 5 s intervals to CeA-CRH neurons in unstressed and CUS-treated BHRs. Compared with unstressed BHRs, the injected currents elicited significantly more APs in CUS-treated BHR at Days 7 and 14 post-CUS (see Supplementary material online, *Figure S4*). Furthermore, bath application of XE-991 (10 μM) increased the number of APs at each depolarizing current level in unstressed BHRs but did not alter the elicited action potentials in CUS-treated BHRs (see Supplementary material online, *Figure S4*).

In addition, we determined the role of Kv7 channels in the CeA in controlling sympathetic outflow in unstressed and CUS-treated BHRs. Bilateral microinjection of the specific Kv7 blocker XE-991 (6.7 nmol in 50 nl aCSF into each side),[21,25] into the CeA significantly increased mean ABP, RSNA, and HR in unstressed BHRs [mean ABP: $P < 0.0001$, $F_{(2,18)} = 53.06$; RSNA: $P = 0.0002$, $F_{(2,18)} = 143.2$; HR: $P = 0.0189$, $F_{(2,18)} = 10.03$, $n = 7$; *Figure 6*]. On the other hand, in CUS-treated BHRs, microinjection of the same dose of XE-991 into the CeA failed to alter mean ABP, RSNA, and HR (*Figure 6B–F*). These data suggest that chronic stress impairs the ability of Kv7 channels in the CeA in restraining sympathetic outflow in BHRs.

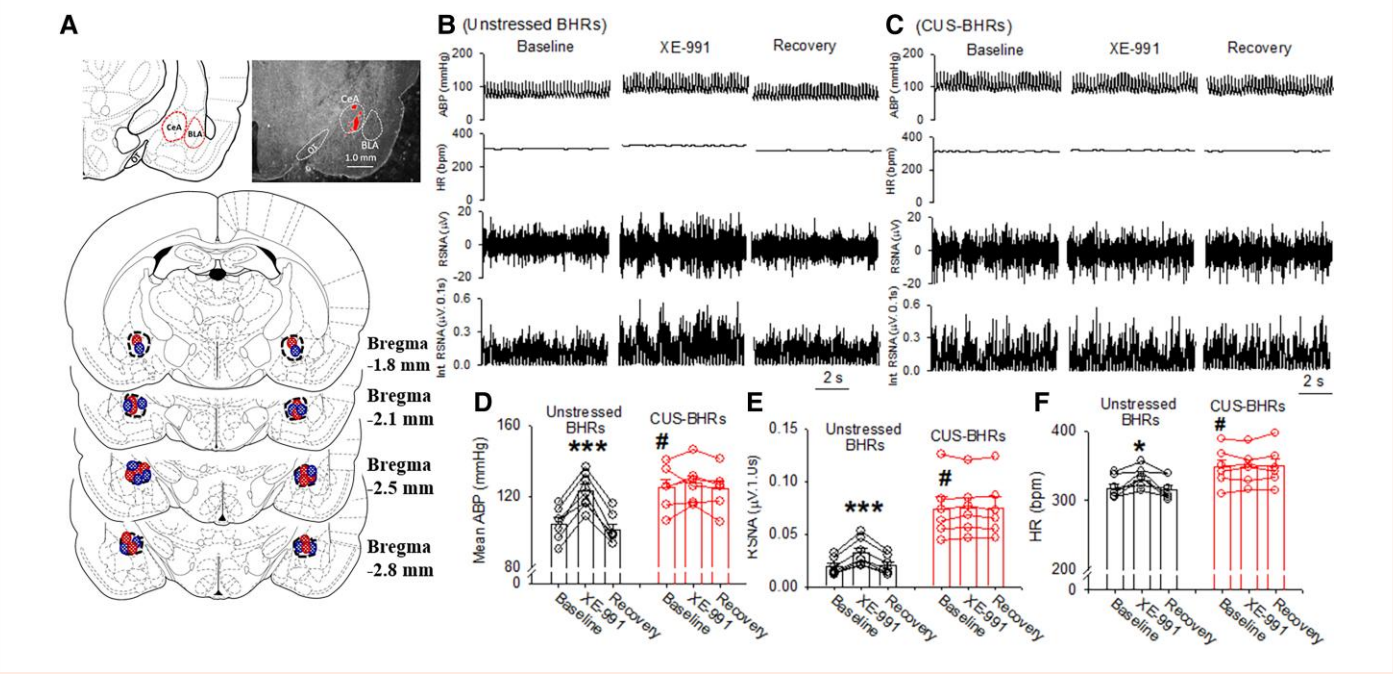


Figure 6 Blocking Kv7 channels in the CeA increased sympathetic output in unstressed BHRs but not in CUS-treated BHRs. (*A*) Diagram and representative microphotography (upper panel) and schematic drawings (lower panels) depict the microinjection sites of XE-991 in the CeA in unstressed BHRs and CUS-treated BHRs. (*B* and *C*) Raw recording traces show the effect of microinjection of XE-991 into the CeA on ABP, RSNA, and HR in unstressed BHRs (*B*) and CUS-treated BHRs (*C*). (*D*, *E*, and *F*) Summary data show changes in mean ABP (*D*), RSNA (*E*), and HR (*F*) in response to microinjection of XE-991 into the CeA in unstressed BHRs ($n = 7$) and CUS-treated BHRs ($n = 6$). *$P < 0.05$, ***$P < 0.0001$, compared with the respective baseline values; #$P < 0.05$, compared with basal values in unstressed BHRs. Repeated-measures ANOVA with Dunnett's *post hoc* test. 3V, third ventricle.

4. Discussion

Chronic stress often induces a short-term elevation of blood pressure in normotensive animals while causing long-lasting hypertension in animals susceptible to high blood pressure.[26–28] Chronic unpredictable stress increases sympathetic nerve activity and blood pressure in normotensive rats.[27,29,30] In this study, we found that CUS induced long-lasting hypertension in BHRs, whereas the CUS-induced blood pressure elevation in WKY rats quickly returned to baseline levels after the termination of CUS. The CUS-induced sustained hypertension in BHRs likely results from the interaction between inherent predispositions and environmental stimuli.[7,30,31] Furthermore, we found that CUS-induced hypertension in BHRs is tightly associated with a significantly increased excitability of CeA-CRH neurons for a long-lasting period after the termination of CUS. Selective suppression of CeA-CRH neurons using a chemogenetic approach is sufficient to alleviate sustained hypertension and heightened sympathetic outflow in CUS-treated BHRs.

CRH is a ubiquitous neuropeptide synthesized by CRH expressing neurons in the CeA, bed nucleus of the stria terminalis (BNST), and PVN.[32] CRH plays an important role in regulating the hormonal, autonomic, and behavioural responses to stress.[33] The CeA is an essential brain region that mediates the stress response.[34] The CeA-CRH neurons project to the locus coeruleus (LC) to modulate the neuronal activity,[35] anxiety-like behaviour, and hippocampus-dependent memory.[36] Furthermore, CeA-CRH neurons are crucial in chronic stress-induced autonomic responses.[14] In this study, the CeA-CRH neurons were targeted by an AAV-mediated expression of eGFP or hM4Di guided by the rat CRH promoter. The AAV-CRH construct has been validated using RNAscope by others.[22] In addition, CeA-CRH neurons in CRH-Cre rat[37] showed a similar density and distribution pattern as the CeA-CRH neurons identified by the AAV-CRH constructs in this study. The virus-driven expression of eGFP was primarily located within the CeA, with a limited spread to the basal medial amygdala, whereas few eGFP expressions were noted in the other surrounding regions. Thus, it is unlikely that the expression outside of CeA weakens the conclusion that the observed effects were specific to the area of the CeA in response to the manipulations. The CeA-CRH neurons project to many brain regions including the lateral parabrachial nucleus, LC, and nucleus tractus solitarius, which are involved in regulating blood pressure.[37] Activation of CRH neurons leads to sequential CRH release and synthesis.[38] We did not assess the effects of CRH produced by CeA-CRH neurons within the CeA or other brain regions on blood pressure directly in this study. It is likely that CHR in the CeA activates CeA neurons that connect to the rostral ventrolateral medulla (RVLM) or the PVN to increase blood pressure and sympathetic outflow. Because our present study was focused on the role of CeA-CRH neurons in stress-induced hypertension, we did not use Gq DREADDs to stimulate CeA-CRH neurons. Nevertheless, we have shown that stimulation of CeA neurons by blocking M-channels increases blood pressure and sympathetic outflow.[21] Thus, we expect that stimulation of CeA-CRH neurons using approaches such as stimulatory DREADDs increases blood pressure in BHRs. We plan to perform such experiments in the future. We found that CUS induced a long-lasting increase in excitability of CeA-CRH neurons, and a long-lasting elevation of ABP in BHRs. On the other hand, CUS induced a transient increase in excitability of CeA-CRH neurons and elevation of ABP in WKY rats. Although the baseline firing activities of CeA-CRH neurons were not significantly different between WKY rats and BHRs, the baseline blood pressure in BHRs is significantly higher than WKY rats, suggesting that CeA-CRH neurons play a minimal role in controlling the baseline blood pressure in unstressed condition. This notion is supported by findings showing that specific inhibition of CeA-CRH neurons by DREADD does not change baseline blood pressure and sympathetic activity in BHRs and WKY rats.

The most salient finding of this study is that suppression of CeA-CRH neurons significantly alleviates CUS-induced sustained hypertension and elevated sympathetic outflow in BHRs. We used an AAV viral vector-mediated CRH Cre recombinase expression to selectively express an inhibitory DREADD, hM4Di, on CeA-CRH neurons. Using the viral vector-mediated expression of rat CRH Cre-dependent expression of DREADD allowed us to target rat CRH neurons and manipulate their activity remotely *in vivo*. Furthermore, stereotaxic microinjection of viral vectors limited the Cre-guided expression of hM4Di in the CeA. The efficacy of the viral vector-mediated transfection of hM4Di in CeA-CRH neurons was very high since the vast majority of the CeA-CRH neurons expressed hM4Di. In BHRs with hM4Di expression in the CeA-CRH neurons, C21 significantly attenuated CUS-induced sustained elevation of ABP and increased RSNA in BHRs. Clozapine N-oxide (CNO) may activate DREADDs receptor through its metabolic clozapine,[39] producing confounding effects by acting on non-DREADD targets.[40] Thus, we used C21, a potent and selective cholinergic muscarinic receptor agonist for DREADD activation in our *in vivo* studies.[41] In addition, we found that C21 at the doses used in this study did not alter blood pressure and sympathetic nerve activity in either conscious rats or anaesthetized rats without hM4Di expression in CeA-CRH neurons.

We found that suppression of CRH neurons using DREADD decreased the elevated blood pressure and RSNA in CUS-induced sustained hypertension, indicating that the increase in blood pressure is closely associated with increases sympathetic outflow in the animal model.[42,43] Since CUS induces a long-lasting elevation of arterial blood pressure during and post-CUS procedure, it is likely that increases in blood pressure during and post-CUS were associated with elevated sympathetic outflow. However, it is not clear if increases in RSNA precede elevation of blood pressure during the CUS. Because the DREADD-induced suppression of CeA-CRH neurons covered 21 days of CUS and 7 days after stress (sustained phase), we were able to determine the role of CeA-CRH neurons in the development (during CUS) and maintenance of CUS-induced sustained hypertension in BHRs. CeA-CRH neurons likely play an important role in developing CUS-induced sustained elevation of ABP and sympathoexcitation in BHRs.

Angiotensin II and AT1 and AT2 receptors play an important role in mediating stress-induced pressor responses. For instance, AT1a receptor-expressing neurons in the subfornical organ and RVLM critically mediate pressure response during stress.[44,45] Although AT1a receptors are expressed on a subpopulation of CRH-expressing neurons in the CeA and PVN,[46] genetical ablation of AT1a receptors in CRH-expressing neurons had no effect on blood pressure and heart rate.[46] There are few AT2 receptor-expressing neurons in the basolateral amygdala and lateral division of the CeA,[47] suggesting that AT2 receptors play a minor role in regulating blood pressure during stress. It remains unclear whether CUS alters expression of AT1R and AT2R in the CeA-CRH neurons, and these alterations contribute to CUS-induced hypertension in BHRs.

Different mechanisms might be involved in the development and sustained phases of CUS-induced hypertension in BHRs. In this study, we examined Kv7 channel-mediated CRH neuronal activity to determine the role of Kv7 in sustained hypertension induced by chronic stress. Neuronal Kv7 channels, including Kv7.2, Kv7.3, and Kv7.5 are the principal molecular components of the slow voltage-gated and non-inactivating M-currents, which stabilize membrane potentials to the resting membrane potential and critically regulate neuronal excitability.[15] Our immunofluorescence staining revealed that Kv7.2, Kv7.3, and Kv7.5 subunits were expressed on CeA-CRH neurons. The protein and mRNA levels of Kv7.2 and Kv7.3 in the CeA were significantly decreased 2 weeks after termination of CUS compared with unstressed BHRs. Consistently, CUS profoundly decreased the M-currents recorded from identified CeA-CRH neurons in BHRs. We specifically determined the role of Kv7 in CUS-induced sustained hypertension in BHRs, thus, we did not measure the Kv7 expression levels in WKY rats because this study and others found that these rats did not show persistent hypertension after CUS.[30] We also measured the Kv7 protein and mRNA levels in the PVN, another brain region involved in regulating neuroendocrine and cardiovascular function,[23,48] in CUS-treated BHRs. CUS decreased Kv7.2 protein and mRNA levels in the PVN tissue but did not alter Kv7.3 and Kv7.5 levels. Although the CeA receives projections from PVN neurons containing oxytocin or vasopressin,[49,50] CRH neurons in the PVN do not directly connect to CeA-CRH neurons. It is not clear whether these oxytocinergic or vasopressinergic projections from the PVN to CeA affect the CeA-CRH neurons during the CUS. On the other hand, the CeA does not innervate the PVN directly[51] but heavily innervates brain regions such as the BNST. In turn, projections from the BNST innervate the PVN. It has been shown that the CeA-BNST-PVN pathway is involved in emotional stress.[52] This pathway is potentially involved in regulating blood pressure and sympathetic outflow in CUS-induced hypertension, as well as the release of CRH from the PVN to the pituitary gland where it is the primary secretagogue of ACTH activating the HPA axis during stress.[53] Chronic stress reduces the expression of glutamate AMPA receptors in the frontal cortex through suppression of DNA methyltransferase 3A activity.[54] Furthermore, Kv7.2 mRNA levels are regulated by an epigenetic modification that involves lysine dimethyltransferases G9a.[55] Thus, further studies are needed to define the epigenetic mechanisms involved in the chronic stress-induced reduction of Kv7 channel expression in the CeA.

Because Kv7 channels critically regulate the membrane potential and neuronal excitability,[16] we assessed if impaired Kv7 channel activity is involved in the hyperactivity of CeA-CRH neurons induced by CUS. We found that the selective Kv7 channel blocker XE-991 profoundly increased the spontaneous firing activity of labelled CeA-CRH neurons in brain slices from unstressed BHRs. However, spontaneous firing activity of CeA-CRH neurons in CUS-treated BHRs was not altered by the XE-991 application. Furthermore, in unstressed BHRs, XE-991 increased the number of action potentials induced by depolarizing currents applied to the CeA-CRH neurons but did not alter current injection-induced action potentials in CUS-treated BHRs. These findings support the notion that impaired Kv7 channel activity contributes to chronic stress-induced hyperactivity of CeA-CRH neurons in BHRs. In addition, we found that blocking Kv7 channels in the CeA increased RSNA and ABP in unstressed BHRs but did not significantly alter RSNA and ABP in BHRs subjected to CUS. These data suggest that chronic stress impairs the function of Kv7 channels in the CeA and thereby, in restraining sympathetic outflow in BHRs.

There are several limitations of the current study. For example, this study focuses on examining the effect of CUS on blood pressure and sympathetic outflow in BHRs. Thus, we did not assess the effects of CUS paradigm on body and organ weights, corticosterone levels, and depression and anxiety-like behaviours, which were assessed in a previous study.[56,57] Furthermore, it is not clear if CUS differentially affect locomotion in WKY rats and BHRs, which will be determined in future studies. It has been shown that hypertension leads to damage of multiple organs due to high blood pressure and elevated sympathetic outflow.[58,59] However, this study was focused on the mechanism of increased central sympathetic outflow in stressed-induced hypertension, we did not assess the damage of target organs in CUS-induced hypertension in BHRs and will explore this in our future studies.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' Contributions

Z.-F.S., H.-L.P., and D.-P.L. designed the study. Z.-F.S., H.Z., P.Z., J.G.P., X.-L.K., and D.-P.L. performed the experiments and data analysis. H.-L.P., A.K.J., H.Z., Z.L., and D.-P.L. interpreted the findings. Z.-F.S., Z.L., E.T.Y., and D.-P.L. wrote the first draft of the manuscript. H.-L.P., E.T.Y., H.-M.C., X.-L.K., Z.L., H.Z., and A.K.J. revised and commented on the manuscript.

Conflict of interest: none declared.

Funding

This study was supported by the National Heart, Lung, and Blood Institute grants HL142133 and HL139523, and HL159157 to D.-P. L.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

References

1. Singh M, Singh AK, Pandey P, Chandra S, Singh KA, Gambhir IS. Molecular genetics of essential hypertension. *Clin Exp Hypertens* 2016;**38**:268–277.
2. Friedman R, Iwai J. Genetic predisposition and stress-induced hypertension. *Science* 1976; **193**:161–162.
3. Matthews KA, Katholi CR, McCreath H, Whooley MA, Williams DR, Zhu S, Markovitz JH. Blood pressure reactivity to psychological stress predicts hypertension in the CARDIA study. *Circulation* 2004;**110**:74–78.
4. Kanayama N, Tsujimura R, She L, Maehara K, Terao T. Cold-induced stress stimulates the sympathetic nervous system, causing hypertension and proteinuria in rats. *J Hypertens* 1997;**15**:383–389.
5. Grotto I, Grossman E, Huerta M, Sharabi Y. Prevalence of prehypertension and associated cardiovascular risk profiles among young Israeli adults. *Hypertension* 2006;**48**:254–259.
6. Wang R, Lu X, Hu Y, You T. Prevalence of prehypertension and associated risk factors among health check-up population in Guangzhou, China. *Int J Clin Exp Med* 2015;**8**: 16424–16433.
7. Lawler JE, Cox RH, Sanders BJ, Mitchell VP. The borderline hypertensive rat: a model for studying the mechanisms of environmentally induced hypertension. *Health Psychol* 1988;**7**: 137–147.
8. Hunt RA, Tucker DC. Developmental sensitivity to high dietary sodium chloride in borderline hypertensive rats. *Hypertension* 1993;**22**:542–550.
9. Butler RK, Oliver EM, Sharko AC, Parilla-Carrero J, Kaigler KF, Fadel JR, Wilson MA. Activation of corticotropin releasing factor-containing neurons in the rat central amygdala and bed nucleus of the stria terminalis following exposure to two different anxiogenic stressors. *Behav Brain Res* 2016;**304**:92–101.
10. Hsu DT, Chen FL, Takahashi LK, Kalin NH. Rapid stress-induced elevations in corticotropin-releasing hormone mRNA in rat central amygdala nucleus and hypothalamic paraventricular nucleus: an in situ hybridization analysis. *Brain Res* 1998;**788**:305–310.
11. Gelsema AJ, McKitrick DJ, Calaresu FR. Cardiovascular responses to chemical and electrical stimulation of amygdala in rats. *Am J Physiol* 1987;**253**:R712–R718.
12. Gianaros PJ, Sheu LK, Matthews KA, Jennings JR, Manuck SB, Hariri AR. Individual differences in stressor-evoked blood pressure reactivity vary with activation, volume, and functional connectivity of the amygdala. *J Neurosci* 2008;**28**:990–999.
13. Sanders BJ, Wirtz-Nole C, DeFord SM, Erling BF. Central amygdaloid lesions attenuate cardiovascular responses to acute stress in rats with borderline hypertension. *Physiol Behav* 1994;**56**:709–713.
14. Gray TS. Amygdaloid CRF pathways: role in autonomic, neuroendocrine, and behavioral responses to stress. *Ann N Y Acad Sci* 1993;**697**:53–60.
15. Brown DA, Passmore GM. Neural KCNQ (Kv7) channels. *Br J Pharmacol* 2009;**156**: 1185–1195.
16. Delmas P, Brown DA. Pathways modulating neural KCNQ/M (Kv7) potassium channels. *Nat Rev Neurosci* 2005;**6**:850–862.
17. Stewart AP, Gomez-Posada JC, McGeorge J, Rouhani MJ, Villarroel A, Murrell-Lagnado RD, Edwardson JM. The Kv7.2/Kv7.3 heterotetramer assembles with a random subunit arrangement. *J Biol Chem* 2012;**287**:11870–11877.
18. Mucha M, Ooi L, Linley JE, Mordaka P, Dalle C, Robertson B, Gamper N, Wood IC. Transcriptional control of *KCNQ* channel genes and the regulation of neuronal excitability. *J Neurosci* 2010;**30**:13235–13245.
19. Biervert C, Steinlein OK. Structural and mutational analysis of KCNQ2, the major gene locus for benign familial neonatal convulsions. *Hum Genet* 1999;**104**:234–240.
20. Singh NA, Charlier C, Stauffer D, DuPont BR, Leach RJ, Melis R, Ronen GM, Bjerre I, Quattlebaum T, Murphy JV, McHarg ML, Gagnon D, Rosales TO, Peiffer A, Anderson VE, Leppert M. A novel potassium channel gene, KCNQ2, is mutated in an inherited epilepsy of newborns. *Nat Genet* 1998;**18**:25–29.
21. Sheng ZF, Zhang H, Zheng P, Chen S, Gu Z, Zhou JJ, Phaup JG, Chang HM, Yeh ETH, Pan HL, Li DP. Impaired Kv7 channel activity in the central amygdala contributes to elevated sympathetic outflow in hypertension. *Cardiovasc Res* 2021;**118**:585–596.
22. Engelke DS, Zhang XO, O'Malley JJ, Fernandez-Leon JA, Li S, Kirouac GJ, Beierlein M, Do-Monte FH. A hypothalamic-thalamostriatal circuit that controls approach-avoidance conflict in rats. *Nat Commun* 2021;**12**:2517.
23. Zhou JJ, Ma HJ, Shao JY, Pan HL, Li DP. Impaired hypothalamic regulation of sympathetic outflow in primary hypertension. *Neurosci Bull* 2019;**35**:124–132.
24. Peters HC, Hu H, Pongs O, Storm JF, Isbrandt D. Conditional transgenic suppression of M channels in mouse brain reveals functions in neuronal excitability, resonance and behavior. *Nat Neurosci* 2005;**8**:51–60.
25. Bian G, Liu J, Guo Y, Yang Y, Li L, Qiao H, Li W, Xu T, Zhang Q. Kv7.2 subunit-containing M-type potassium channels in the lateral habenula are involved in the regulation of working memory in Parkinsonian rats. *Neuropharmacology* 2020;**168**:108012.
26. Esler M, Eikelis N, Schlaich M, Lambert G, Alvarenga M, Dawood T, Kaye D, Barton D, Pier C, Guo L, Brenchley C, Jennings G, Lambert E. Chronic mental stress is a cause of essential hypertension: presence of biological markers of stress. *Clin Exp Pharmacol Physiol* 2008;**35**: 498–502.
27. Komnenov D, Quaal H, Rossi NF. V_{1a} and V_{1b} vasopressin receptors within the paraventricular nucleus contribute to hypertension in male rats exposed to chronic mild unpredictable stress. *Am J Physiol Regul Integr Comp Physiol* 2021;**320**:R213–R225.
28. Lawler JE, Barker GF, Hubbard JW, Schaub RG. Effects of stress on blood pressure and cardiac pathology in rats with borderline hypertension. *Hypertension* 1981;**3**:496–505.
29. Schaeuble D, Packard AEB, McKlveen JM, Morano R, Fourman S, Smith BL, Scheimann JR, Packard BA, Wilson SP, James J, Hui DY, Ulrich-Lai YM, Herman JP, Myers B. Prefrontal cortex regulates chronic stress-induced cardiovascular susceptibility. *J Am Heart Assoc* 2019;**8**: e014451.
30. Zhou JJ, Shao JY, Chen SR, Li DP, Pan HL. α2δ-1–Dependent NMDA receptor activity in the hypothalamus is an effector of genetic-environment interactions that drive persistent hypertension. *J Neurosci* 2021;**41**:6551–6563.
31. Sanders BJ, Lawler JE. The borderline hypertensive rat (BHR) as a model for environmentally-induced hypertension: a review and update. *Neurosci Biobehav Rev* 1992; **16**:207–217.
32. Bloom FE, Battenberg EL, Rivier J, Vale W. Corticotropin releasing factor (CRF): immunoreactive neurones and fibers in rat hypothalamus. *Regul Pept* 1982;**4**:43–48.
33. Koob GF. Corticotropin-releasing factor, norepinephrine, and stress. *Biol Psychiatry* 1999;**46**: 1167–1180.
34. Adhikari A. Distributed circuits underlying anxiety. *Front Behav Neurosci* 2014;**8**:112.
35. Van Bockstaele EJ, Peoples J, Valentino RJ. Anatomic basis for differential regulation of the rostrolateral peri-locus coeruleus region by limbic afferents. *Biol Psychiatry* 1999;**46**: 1352–1363.
36. Paretkar T, Dimitrov E. The central amygdala corticotropin-releasing hormone (CRH) neurons modulation of anxiety-like behavior and hippocampus-dependent memory in mice. *Neuroscience* 2018;**390**:187–197.
37. Pomrenze MB, Millan EZ, Hopf FW, Keiflin R, Maiya R, Blasio A, Dadgar J, Kharazia V, De Guglielmo G, Crawford E, Janak PH, George O, Rice KC, Messing RO. A transgenic rat for investigating the anatomy and function of corticotrophin releasing factor circuits. *Front Neurosci* 2015;**9**:487.
38. Aguilera G, Liu Y. The molecular physiology of CRH neurons. *Front Neuroendocrinol* 2012;**33**: 67–84.
39. Gomez JL, Bonaventura J, Lesniak W, Mathews WB, Sysa-Shah P, Rodriguez LA, Ellis RJ, Richie CT, Harvey BK, Dannals RF, Pomper MG, Bonci A, Michaelides M. Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. *Science* 2017;**357**: 503–507.
40. MacLaren DA, Browne RW, Shaw JK, Krishnan Radhakrishnan S, Khare P, Espana RA, Clark SD. Clozapine N-oxide administration produces behavioral effects in Long–Evans rats: implications for designing DREADD experiments. *eNeuro* 2016;**3**:ENEURO.0219–16.2016.
41. Thompson KJ, Khajehali E, Bradley SJ, Navarrete JS, Huang XP, Slocum S, Jin J, Liu J, Xiong Y, Olsen RHJ, Diberto JF, Boyt KM, Pina MM, Pati D, Molloy C, Bundgaard C, Sexton PM, Kash TL, Krashes MJ, Christopoulos A, Roth BL, Tobin AB. DREADD agonist 21 is an effective agonist for muscarinic-based DREADDs *in vitro* and *in vivo*. *ACS Pharmacol Transl Sci* 2018; **1**:61–72.
42. Johnson AK, Xue B. Central nervous system neuroplasticity and the sensitization of hypertension. *Nat Rev Nephrol* 2018;**14**:750–766.
43. Seravalle G, Grassi G. Sympathetic nervous system and hypertension: new evidences. *Auton Neurosci* 2022;**238**:102954.
44. Chen D, Jancovski N, Bassi JK, Nguyen-Huu TP, Choong YT, Palma-Rigo K, Davern PJ, Gurley SB, Thomas WG, Head GA, Allen AM. Angiotensin type 1A receptors in C1 neurons of the rostral ventrolateral medulla modulate the pressor response to aversive stress. *J Neurosci* 2012;**32**:2051–2061.
45. Krause EG, de Kloet AD, Scott KA, Flak JN, Jones K, Smeltzer MD, Ulrich-Lai YM, Woods SC, Wilson SP, Reagan LP, Herman JP, Sakai RR. Blood-borne angiotensin II acts in the brain to influence behavioral and endocrine responses to psychogenic stress. *J Neurosci* 2011;**31**: 15009–15015.
46. Hurt RC, Garrett JC, Keifer OP Jr, Linares A, Couling L, Speth RC, Ressler KJ, Marvar PJ. Angiotensin type 1a receptors on corticotropin-releasing factor neurons contribute to the expression of conditioned fear. *Genes Brain Behav* 2015;**14**:526–533.
47. Yu Z, Swiercz AP, Moshfegh CM, Hopkins L, Wiaderkiewicz J, Speth RC, Park J, Marvar PJ. Angiotensin II type 2 receptor-expressing neurons in the central amygdala influence fear-related behavior. *Biol Psychiatry* 2019;**86**:899–909.
48. Zhou JJ, Gao Y, Zhang X, Kosten TA, Li DP. Enhanced hypothalamic NMDA receptor activity contributes to hyperactivity of HPA axis in chronic stress in male rats. *Endocrinology* 2018; **159**:1537–1546.
49. Ferretti V, Maltese F, Contarini G, Nigro M, Bonavia A, Huang H, Gigliucci V, Morelli G, Scheggia D, Manago F, Castellani G, Lefevre A, Cancedda L, Chini B, Grinevich V, Papaleo F. Oxytocin signaling in the central amygdala modulates emotion discrimination in mice. *Curr Biol* 2019;**29**:1938–1953.e6.

50. Hernandez VS, Hernandez OR, Perez de la Mora M, Gomora MJ, Fuxe K, Eiden LE, Zhang L. Hypothalamic vasopressinergic projections innervate central amygdala GABAergic neurons: implications for anxiety and stress coping. *Front Neural Circuits* 2016;**10**:92.
51. Sawchenko PE, Imaki T, Potter E, Kovacs K, Imaki J, Vale W. The functional neuroanatomy of corticotropin-releasing factor. *Ciba Found Symp* 1993;**172**:5–21; discussion 21-29.
52. Kubota N, Amemiya S, Yanagita S, Kita I. Neural pathways from the central nucleus of the amygdala to the paraventricular nucleus of the hypothalamus are involved in induction of yawning behavior due to emotional stress in rats. *Behav Brain Res* 2023;**436**:114091.
53. Choi DC, Furay AR, Evanson NK, Ulrich-Lai YM, Nguyen MM, Ostrander MM, Herman JP. The role of the posterior medial bed nucleus of the stria terminalis in modulating hypothalamic–pituitary–adrenocortical axis responsiveness to acute and chronic stress. *Psychoneuroendocrinology* 2008;**33**:659–669.
54. Wei J, Cheng J, Waddell NJ, Wang ZJ, Pang X, Cao Q, Liu A, Chitaman JM, Abreu K, Jasrotia RS, Duffney LJ, Zhang J, Dietz DM, Feng J, Yan Z. DNA methyltransferase 3A is involved in the sustained effects of chronic stress on synaptic functions and behaviors. *Cereb Cortex* 2020;**31**:1998–2012.
55. Laumet G, Garriga J, Chen SR, Zhang Y, Li DP, Smith TM, Dong Y, Jelinek J, Cesaroni M, Issa JP, Pan HL. G9a is essential for epigenetic silencing of K(+) channel genes in acute-to-chronic pain transition. *Nat Neurosci* 2015;**18**:1746–1755.
56. Marin MT, Cruz FC, Planeta CS. Chronic restraint or variable stresses differently affect the behavior, corticosterone secretion and body weight in rats. *Physiol Behav* 2007;**90**:29–35.
57. Gao Y, Zhou JJ, Zhu Y, Kosten T, Li DP. Chronic unpredictable mild stress induces loss of GABA inhibition in corticotrophin-releasing hormone-expressing neurons through NKCC1 upregulation. *Neuroendocrinology* 2017;**104**:194–208.
58. Grassi G, Seravalle G, Dell'Oro R, Mancia G. Sympathetic mechanisms, organ damage, and antihypertensive treatment. *Curr Hypertens Rep* 2011;**13**:303–308.
59. Mancia G, Seravalle G, Grassi G. Sympathetic nervous factors, pressure variability and organ damage in arterial hypertension. *Ann Ital Med Int* 1997;**12**:217–222.

Translational perspective

We found that hyperactivity of corticotropin-releasing hormone **(**CRH) neurons in the central nucleus of the amygdala (CeA), likely due to diminished Kv7 channel activity, plays a major role in the development of chronic stress-induced hypertension. Our study suggests that CRH neurons in the brain may be targeted for treating chronic stress-induced hypertension. Thus, increasing Kv7 channel activity or overexpressing Kv7 channels in the CeA may reduce stress-induced hypertension. Further studies are needed to delineate how chronic stress diminishes Kv7 channel activity in the brain.