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Regulation of corticotropin-releasing hormone neuronal network activity by noradrenergic stress signals

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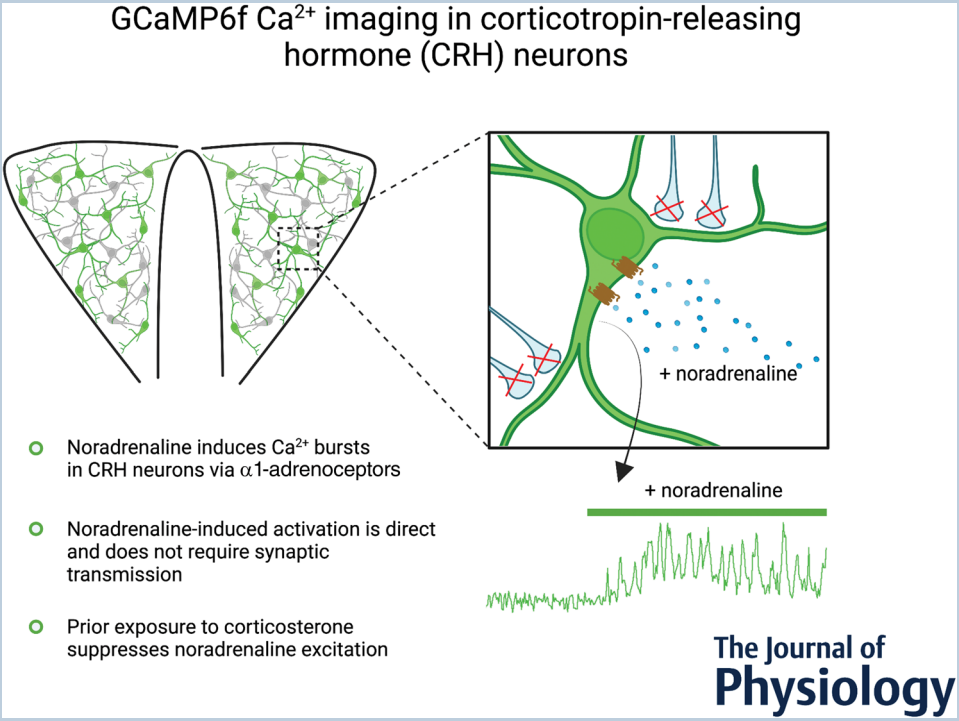

Abstract Noradrenaline is a neurotransmitter released in response to homeostatic challenge and activates the hypothalamic–pituitary–adrenal axis via stimulation of corticotropin-releasing hormone (CRH) neurons. Here we investigated the mechanism through which noradrenaline regulates activity within the CRH neuronal network. Using a combination of *in vitro* GCaMP6f Ca^{2+} imaging and electrophysiology, we show that noradrenaline induces a robust increase in excitability in a proportion of CRH neurons with many neurons displaying a bursting mode of activity. Noradrenaline-induced activation required α_1-adrenoceptors and L-type voltage-gated

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Ca^{2+} channels, but not GABA/glutamate synaptic transmission or sodium action potentials. Exposure of mice to elevated corticosterone levels was able to suppress noradrenaline-induced activation. These results provide further insight into the mechanisms by which noradrenaline regulates CRH neural network activity and hence stress responses.

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Abstract figure legend Corticotropin-releasing hormone (CRH) neurons control the hypothalamic–pituitary–adrenal axis. One neurotransmitter that is important for the activation of CRH neurons in response to stress is noradrenaline. Using GCaMP6f Ca^{2+} imaging, we investigated how noradrenaline regulates the activity of CRH neurons. Here we show that noradrenaline induces a robust increase in activity in a proportion of CRH neurons with many neurons displaying a bursting mode of activity. Noradrenaline-induced activation required α_1-adrenoceptors and L-type voltage-gated Ca^{2+} channels, but not GABA/glutamate synaptic transmission. Exposure of mice to the stress hormone corticosterone was able to suppress noradrenaline-induced activation.

Key points

- GCaMP6f Ca^{2+} imaging and on-cell patch-clamp recordings reveal that corticotropin-releasing hormone neurons are activated by noradrenaline with many neurons displaying a bursting mode of activity.
- Noradrenaline-induced activation requires α_1-adrenoceptors.
- Noradrenaline-induced Ca^{2+} elevations persist after blocking $GABA_A$, AMPA, NMDA receptors and voltage-gated Na^+ channels.
- Noradrenaline-induced Ca^{2+} elevations require L-type voltage-gated Ca^{2+} channels.
- Corticosterone suppresses noradrenaline-induced excitation.

Introduction

The hypothalamic–pituitary–adrenal (HPA) axis is controlled by a population of hypothalamic corticotropin-releasing hormone (CRH) neurons located in the paraventricular nucleus (PVN) of the hypothalamus. Stress relevant information is relayed from multiple brain areas to these neurons in order to control their patterns of spiking activity (Bains et al., 2015; Focke & Iremonger, 2020; Kondoh et al., 2016). Increases in CRH neuron activity in turn control both the expression of stress associated behaviours and activation of the HPA axis (Daviu & Bains, 2021; Kim & Iremonger, 2019).

One neurotransmitter that is important for the activation of CRH neurons in response to stress is noradrenaline (Cole & Sawchenko, 2002; Flak et al., 2014; Helmreich et al., 2001; Itoi et al., 1994; Plotsky, 1987). Noradrenergic inputs to the CRH population predominantly originate from the A1 cell group in the ventral lateral medulla and the A2 cell group in the nucleus of the solitary tract (Cunningham & Sawchenko, 1988; Sawchenko & Swanson, 1982). Lesioning of these noradrenergic inputs or local blockade of noradrenergic receptors within the PVN inhibits the secretion of corticosterone following stress (Flak et al., 2014; Ritter et al., 2003). Previous work suggests that CRH neurons express α_1-adrenoceptors (Chen et al., 2019; Cummings & Seybold, 1988; Day et al., 1999) indicating that noradrenaline may directly excite these neurons.

Despite this, recent work suggests that noradrenaline activation of CRH neurons is dependent on an indirect glutamate pathway instead (Chen et al., 2019). Specifically it was shown that activation of α_1-adrenoceptors on CRH neurons triggered release of a retrograde transmitter which excited astrocytes to release ATP. Astrocytic ATP then triggered spiking in glutamate interneurons which innervate CRH neurons leading to enhanced glutamate release and subsequent CRH neuron excitation (Chen et al., 2019). We speculated that in addition to this mechanism, noradrenaline may also excite CRH neurons directly via α_1-adrenoreceptor mechanisms as has been shown in hypothalamic magnocellular neurons (Randle et al., 1986; Yamashita et al., 1987) and non-identified parvocellular neurons (Munier et al., 2022). A second question that remains unaddressed is how noradrenaline regulates the pattern of CRH neuron activity at both the single cell and the network level. To address these questions, we performed Ca^{2+} imaging in brain slices where CRH neurons were targeted with the genetically encoded Ca^{2+} sensor GCaMP6f.

Using this Ca^{2+} imaging approach, we report that bath-applied noradrenaline (10 μM) robustly activates a subset of CRH neurons. Excitatory responses to noradrenaline were heterogeneous and often included bursting. Noradrenaline-induced Ca^{2+} elevations were shown to be mediated via α_1-adrenoceptors. Importantly, noradrenaline-induced Ca^{2+} elevations did not require voltage-gated sodium channels or synaptic transmission. Consistent with previous studies which have shown that corticosterone can suppress the intrinsic excitability of CRH neurons (Bittar et al., 2019), we found that corticosterone also suppressed noradrenaline-induced activation of CRH neurons. Together, these data demonstrate that noradrenaline can directly activate CRH neurons and that this activation can be suppressed by exposure to corticosterone.

Methods

Ethical approval

All experiments were approved by the University of Otago Animal Ethics committee (AUP67-16, AUP18-79) in accordance with the New Zealand Animal Welfare Act.

Animals

All experiments were carried out in male and female hemizygous Crh-IRES-cre;Ai95D (GCaMP6f) mice. Experiments with chronic corticosterone treatment were only carried out in male mice. Both Crh-IRES-Cre (Taniguchi et al., 2011) (stock no. 012704) and Ai95D (Madisen et al., 2015)(stock no. 024105) were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were aged 2–4 months and were housed under 12:12 h lighting schedule with *ad libitum* access to food and water.

Brain slice preparation

Mice were euthanised by cervical dislocation and the brain was immediately removed. The brain was then placed in ice-cold cutting solution which contained (in mM): 87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 0.5 $CaCl_2$, 6 $MgCl_2$, 25 D-glucose and 75 sucrose, bubbled with 95% O_2–5% CO_2. The brain was then sliced on a vibratome (Leica Biosystems (Wetzlar, Germany) VT1000S or VT1200S) into 200 μm thick coronal sections. Brain slices containing the PVN were then incubated in a tissue bath at 30°C containing artificial cerebrospinal fluid (ACSF; mM: 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.5 $MgCl_2$ and 10 glucose) bubbled with 95% O_2–5% CO_2.

GCaMP6f Ca^{2+} imaging

Brain slices were transferred to a tissue bath on a fixed-stage Olympus (Tokyo, Japan) BX51WL upright fluorescence microscope and constantly perfused with 95% O_2–5% CO_2 ACSF at 30°C. Differential interference contrast microscopy was first used to locate the PVN with a ×40 water immersion objective (NA = 0.8). To image GCaMP6f fluorescence, we used a xenon arc lamp (Lambda DG-4, Sutter Instrument Co., Novato, CA, USA) with a green fluorescent protein (GFP) filter cube allowing for transmission of excitation light in the 470–490 nm range. Emitted light was passed through a dichroic mirror and band pass emission filter (500–520 nm) and was focused onto a CCD camera (Hamamatsu Photonics (Hamamatsu, Japan) ORCA-ER). Once CRH neurons expressing GCaMP6f were localised and focused on, movies were recorded using MicroManager (ImageJ, NIH, Bethesda, MD, USA). Images were acquired at a frame rate of 2 Hz, with an exposure time of 75 ms per frame and 4 × 4 pixel binning.

Simultaneous electrophysiology and confocal Ca^{2+} imaging

Slices were transferred to a tissue bath on a fixed-stage upright confocal microscope (Olympus FV1000). Slices were perfused with recording ACSF at a temperature of 30°C. Loose seal on-cell recordings were performed with borosilicate glass pipettes filled with ACSF (resistance 3–6 MΩ). Recordings were performed in voltage clamp with the holding current kept at 0 pA. Imaging was performed with an Olympus FV1000 confocal microscope fitted with a ×40, 0.8 NA objective lens. GCaMP was excited with a 488 nm argon laser (Melles Griot (Carlsbad, CA, USA)). Emitted light was detected by a photomultiplier tube after passing through a bandpass filter (505–605 nm).

For experiments shown in Fig. 1*D* and *E* and Fig. 3, noradrenaline (10 μM) was bath-applied at the start of the experiment in order to excite CRH neurons. Once a CRH neuron displaying activity was localised, the recording electrode was then lowered into the bath and a loose seal (approximately 7–20 MΩ) recording was obtained from that neuron. Simultaneous, Ca^{2+} imaging movies were recorded using Olympus FV10-ASW 4.1 software. For all recordings, frame scans were performed with the confocal aperture wide open, on 3–3.5× zoomed regions, at approximately 2 Hz. In 16 neurons, we performed simultaneous Ca^{2+} imaging and on-cell recordings before and after the bath-application of tetrodotoxin (TTX; 1 μM). For five of the recorded neurons, only 10 μM noradrenaline was in the bath prior to bath-application of TTX. In the remainder of recordings, 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX; 10 μM),

DL-2-amino-5-phosphonopentanoic acid (DL-AP5; 40 μM), picrotoxin (50 μM) and noradrenaline (10 μM) were in the bath prior to application of TTX.

Electrophysiological recordings were amplified 10x with a Multiclamp 700B amplifier (Molecular Devices, San Jose, CA, USA), filtered at 1 kHz, digitised using a Digidata 1440A (Molecular Devices) and recorded using Clampex software (Molecular Devices).

Drug application

The following drugs were dissolved in recording ACSF before being bath-applied: noradrenaline (10 μM, Sigma-Aldrich, St Louis, MO, USA), yohimbine (10 μM, Sigma-Aldrich), CNQX (10 μM, Sigma-Aldrich), DL-AP5 (40 μM, Tocris Bioscience, Bristol, UK), picrotoxin (50 μM, Sigma-Aldrich), tetrodotoxin (TTX, 1 μM, Alomone Labs, Jerusalem, Israel), $CdCl_2$ (100 μM, Sigma-Aldrich), nifedipine (100 μM, Sigma-Aldrich). Prazosin (10 μM, Sigma-Aldrich) was puff-applied onto the brain slice directly due to issues with its sticking in the perfusion lines.

Corticosterone treatment

To elevate corticosterone levels, mice were given corticosterone dissolved in the drinking water. This non-invasive method has previously been used to chronically elevate plasma corticosterone levels in mice (Bittar et al., 2019; Gourley et al., 2013; Karatsoreos et al., 2010). Stock solutions of corticosterone (4-pregnene-11β,21-diol-3,20-dione, Sigma-Aldrich) were first prepared in ethanol. Stock solutions were then dissolved in tap water to a final concentration of 25 μg/ml corticosterone and 0.25% ethanol. Vehicle treated mice (control) received only 0.25% ethanol in tap water. Both groups received modified drinking water for 14 days. Corticosterone drinking water was replaced every 4–5 days.

Data analysis

Image analysis was performed with Clampfit (Molecular Devices), Olympus Fluoview 1000 and ImageJ software. Regions of interest were drawn around fluorescent CRH neuron soma and average fluorescence intensity was measured for each imaging frame. Changes in fluorescence ($\Delta F/F$) were calculated by $(F - F_0)/F_0$ where F is the fluorescence and F_0 is the baseline fluorescence. To detect discrete Ca^{2+} events, we used the 'detect peak' and prominence functions in MATLAB (The MathWorks Inc., Natick, MA, USA). The threshold at which a change in fluorescence was detected as an event was set by taking the fifth highest peak during the 60 s baseline period and adding 1.5 standard deviations (SD) calculated from this 60 s baseline period. For each cell, we recorded the number of Ca^{2+} events, the average amplitude and frequency of the events. Cells were considered inactive if their maximum prominence during noradrenaline application was less than the baseline mean fluorescence plus 3 SD of the baseline (mean F_o + 4SD > MaxProminance = Inactive).

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). All reported values are the means ± SD. Comparisons between groups were carried out using either an unpaired Student's t-test or one-way ANOVA with Tukey's multiple comparisons tests. $P < 0.05$ was considered statistically significant.

Results

Noradrenaline-induced activation of CRH neurons

To determine how noradrenaline regulates the activity of CRH neurons in the PVN, we performed GCaMP6f Ca^{2+} imaging in brain slices. In the absence of external stimulation, CRH neurons were generally not spontaneously active. Bath application of 10 μM noradrenaline induced Ca^{2+} transients in a subset of CRH neurons in each imaging field. While individual responses to noradrenaline were heterogeneous, typical responses consisted of an initial large increase in Ca^{2+} followed by a lower level of sustained activity often with recurrent bursts (Fig. 1*A*). We found no difference in the number of CRH neurons activated by noradrenaline between male and female mice ($P = 0.306$, data not shown).

We next performed an experiment where noradrenaline was applied three times. Each application was 180 s in duration and separated by approximately 12 min. Many CRH neurons showed the same type of neuronal activity when repeatedly exposed to noradrenaline (Fig. 1*B*). When the area under the curve was analysed for each noradrenaline application, we found no significant difference between the three $\Delta F/F$ responses ($F_{(2,48)} = 0.3342$, $P = 0.718$, data not shown). These data demonstrate that CRH neurons can be repetitively activated by noradrenaline.

We next sought to determine the relationship between Ca^{2+} elevations and electrical spiking. To do this, we bath-applied noradrenaline, performed Ca^{2+} imaging and then targeted active cells for loose-seal on-cell patch-clamp recordings (Fig. 1*C*–*E*). We found that increases in spiking activity were time-locked with increases in GCaMP6f fluorescence. In five neurons that exhibited clear bursting activity, we correlated the total cumulative fluorescence during each burst of action potentials with the number of action potentials in each burst (Fig. 1*F* and *G*). This analysis confirmed that

there was a significant correlation between integrated fluorescence during each burst and spike number (Fig. 1*G*).

Noradrenaline acts through the α_1-adrenoceptor to activate CRH neurons

Previous literature has shown noradrenaline can excite CRH neurons via α_1-adrenoceptors and inhibit CRH neurons via α_2-adrenoceptors (Chen et al., 2019). Consistent with this, we found that blocking α_1-adrenoceptors with prazosin (10 μM) led to a significant decrease in noradrenaline-induced activation of CRH neurons measured with Ca^{2+} imaging (Fig. 2*B*). In slices that had α_1-adrenoceptors blocked with prazosin, the number of cells active per slice was significantly lower compared to slices without prazosin (control, 7.53 ± 3.22 cells active per slice, $n = 17$; prazosin, 1.17 ± 0.75 cells active per slice, $n = 6$; one-way ANOVA, $F_{(4,50)} = 14.89$, $P < 0.0001$; control *vs.* prazosin *post hoc* test $P = 0.0007$; Fig. 2*F*). The few cells that were still activated by noradrenaline showed significantly lower numbers of Ca^{2+} events per neuron (one-way ANOVA $F_{(4,50)} = 10.67$, $P < 0.0001$; control *vs.* prazosin *post hoc* test $P = 0.0001$, Fig. 2*G*) and reduced mean event amplitude (one-way ANOVA $F_{(4,49)} = 2.997$, $P = 0.027$; control *vs.* prazosin *post hoc* test $P = 0.022$, Fig. 2*H*).

To investigate the role of α_2-adrenoceptors, we applied yohimbine (10 μM) prior to noradrenaline. Blockade of α_2-adrenoceptors with yohimbine caused a significant increase in noradrenaline-induced activation of CRH neurons (13.09 ± 3.86 cells active per slice, $n = 11$, $P = 0.0002$ compared to control, Fig. 2*C* and *F*). While the number of Ca^{2+} events was slightly higher in the presence of yohimbine, this was not statistically significant (control,

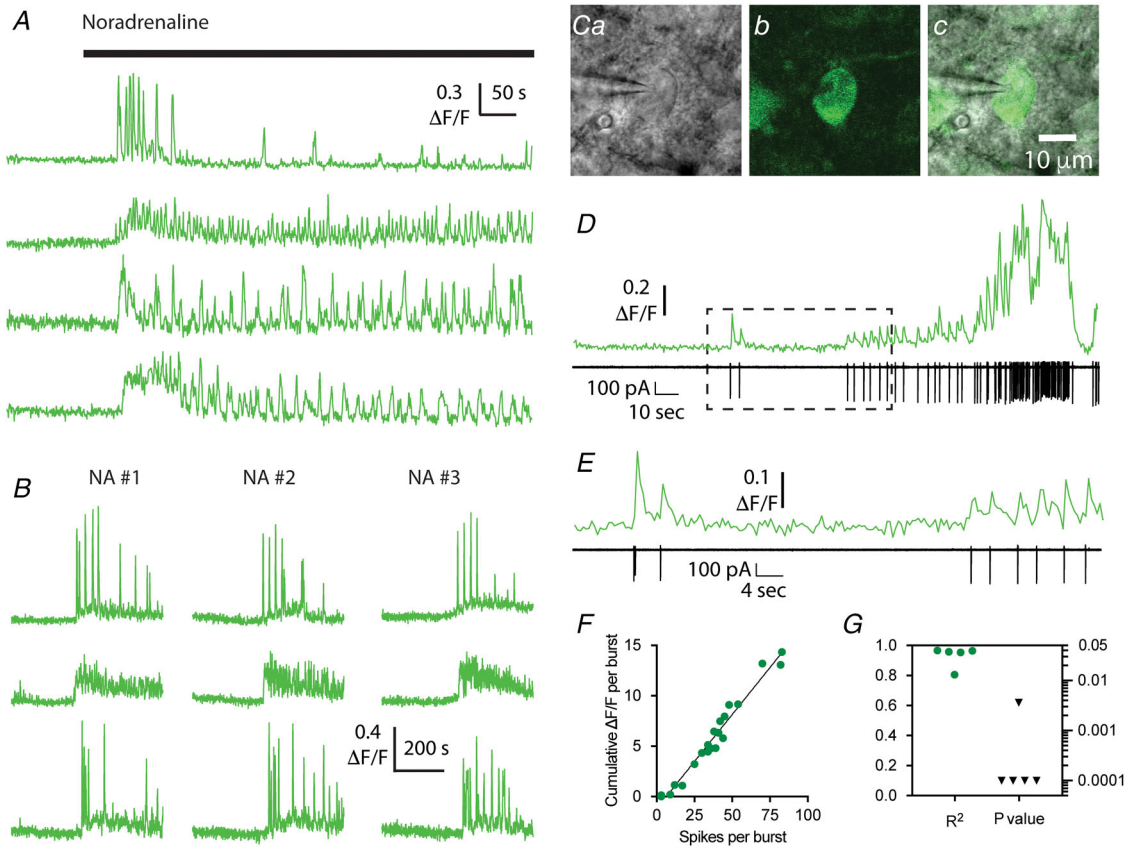

Figure 1. Noradrenaline induces heterogeneous patterns of excitation in CRH neurons
A, four examples of GCaMP6f calcium responses from different CRH neurons in response to bath-application of noradrenaline. *B*, repeated bath-applications of noradrenaline (NA) to the same neuron evoke responses with similar kinetics. *C*, images showing on-cell patch clamp recording from a CRH neuron expressing GCaMP6f in an acute brain slice of the PVN. *a*, transmitted light image; *b*, confocal image of GCaMP6f fluorescence; *c*, merged image. *D*, simultaneous electrical (black) and GCaMP6f fluorescence (green) traces from the soma of a CRH neuron. Traces illustrate that spontaneous bursts of spikes coincide with increases in GCaMP6f fluorescence. Region inside dashed box expanded in *E*. *F*, relationship between total cumulative fluorescence and spike count per burst from a single representative neuron. *G*, correlation r^2 and *P*-values of total cumulative fluorescence and spike count in response to burst activity. [Colour figure can be viewed at wileyonlinelibrary.com]

24.20 ± 11.47 events per cell *vs.* yohimbine, 31.97 ± 6.87 events per cell; $P = 0.103$). There was also no significant effect on mean event amplitude (control, 0.28 ± 0.13 $\Delta F/F$ *vs.* yohimbine, 0.24 ± 0.09 $\Delta F/F$, $P = 0.635$).

Together these findings suggest that noradrenaline-induced excitation is mediated by α_1 receptors and that α_2 receptors act to restrain noradrenaline-induced excitation.

Noradrenaline induced excitation does not require glutamate or GABA synaptic inputs

To determine the role that fast synaptic transmission plays in noradrenaline-induced activation of CRH neurons, we blocked AMPA and NMDA receptors with CNQX (10 μM) and DL-AP5 (40 μM) and then washed in noradrenaline. In the presence of these glutamate receptor antagonists, noradrenaline activated 9.25 ± 3.50 cells per slice ($n = 8$, Fig. 2*D* and *F*), which was not significantly different from control ($P = 0.698$). In the presence of the $GABA_A$ receptor antagonist picrotoxin (50 μM), noradrenaline also activated a similar number of CRH neurons compared to control (picrotoxin, 8.54 ± 2.57 cells active per slice, $P = 0.902$ compared to control). Blocking glutamate or GABA synaptic transmission had no effect on number of Ca^{2+} events per cell (CNQX/DL-AP5, $P > 0.999$; picrotoxin, $P = 0.916$) or event amplitude (CNQX/DL-AP5, $P = 0.976$; picrotoxin, $P = 0.212$).

To investigate this phenomenon further, we performed simultaneous on-cell patch clamp recordings with Ca^{2+} imaging. We bath-applied noradrenaline in the presence of CNQX (10 μM), DL-AP5 (40 μM) and picrotoxin (50 μM) and then targeted active cells for on-cell recording. In 11 out of 11 recorded CRH neurons, noradrenaline-induced Ca^{2+} elevations coincided with bursts of electrical spiking even when fast glutamate and GABA synaptic transmission was blocked (Fig. 3). We then bath-applied TTX (1 μM) to block sodium spikes. TTX inhibited sodium spiking recorded electrophysiologically in all neurons. However, in 9 out of 11 neurons, Ca^{2+} bursts continued after sodium spiking had been inhibited with TTX (Fig. 3*A*–*C*). In 2 of the 11 recorded neurons, TTX inhibited both electrical spikes

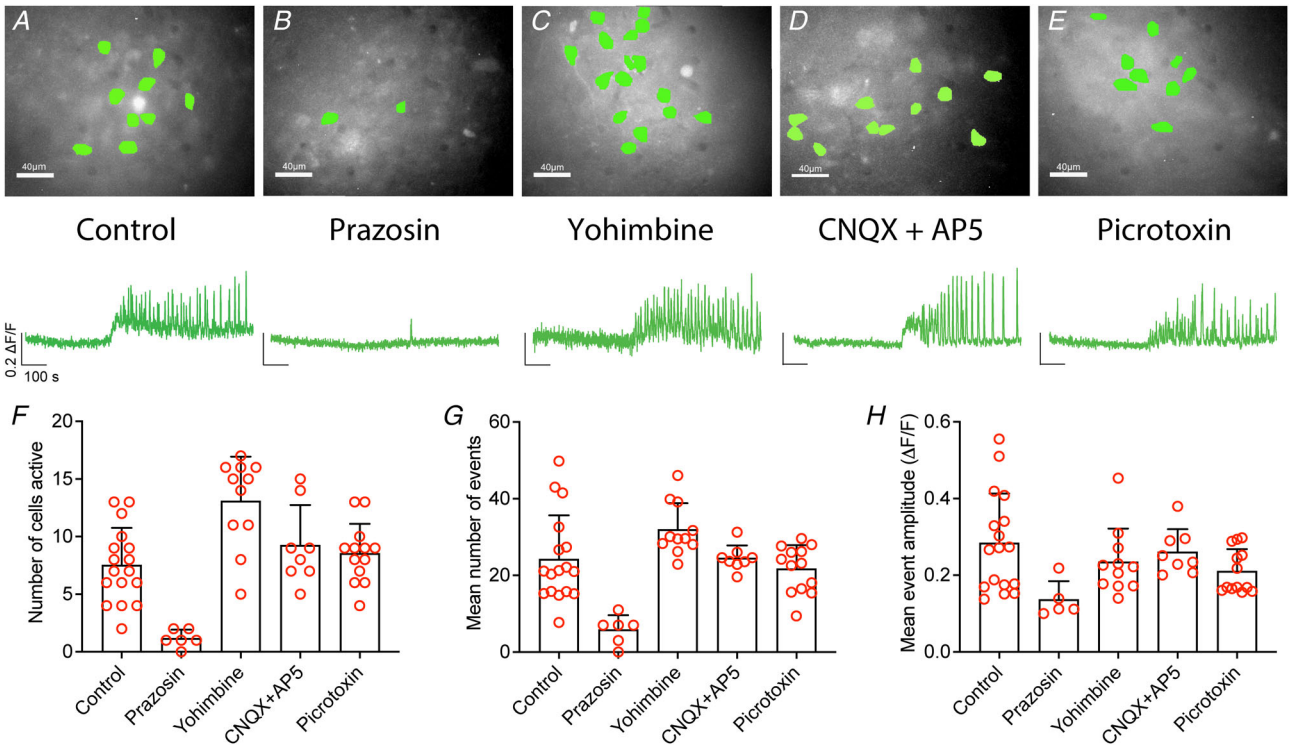

Figure 2. Noradrenaline-induced Ca^{2+} events in CRH neurons require α_1-adrenoceptors
A–*E*, top, representative images showing the number of neurons in each imaging region that were activated by noradrenaline. Activated cells are highlighted green. Each image is from a different brain slice pre-treated with no drugs (control) (*A*), prazosin (*B*), yohimbine (*C*), CNQX and DL-AP5 (*D*) or picrotoxin (*E*). Bottom, representative GCaMP6f fluorescence traces of noradrenaline responses in control, prazosin, yohimbine, CNQX and DL-AP5, or picrotoxin. *F*, summary graph showing number of neurons activated by noradrenaline per brain slice for each drug treatment condition. *G*, summary graph showing number of events detected (mean per active neuron per slice) in response to noradrenaline for each drug treatment condition. *H*, summary graph showing the amplitude of events (mean per active neuron per slice) in response to noradrenaline for each drug treatment condition. [Colour figure can be viewed at wileyonlinelibrary.com]

and Ca^{2+} bursts (Fig. 4*D*). We then repeated these experiments in the absence of glutamate and GABA receptor blockers. In all five recorded CRH neurons, bursts of electrical spikes coincided with elevations in somatic Ca^{2+}. In all five CRH neurons, bath-application of TTX inhibited sodium spiking activity recorded electrophysiologically, but did not inhibit on-going Ca^{2+} bursts.

Together, these data show that fast glutamate and GABA synaptic transmission is not required for noradrenaline-induced Ca^{2+} bursts. Secondly, these experiments show that in the presence of glutamate and GABA receptor antagonists, Ca^{2+} and electrical spike bursts occur together. Finally, and most surprisingly, noradrenaline-induced Ca^{2+} elevations can persist when sodium action potentials are blocked.

Effect of blocking sodium action potentials on noradrenaline-induced Ca^{2+} events

To further investigate the effect of TTX on noradrenaline-induced Ca^{2+} responses, we switched back to Ca^{2+} imaging without on-cell recordings and applied noradrenaline in the continued presence of TTX (Fig. 4*A*). We found that prior inhibition of sodium action potentials with TTX had no impact on the number of CRH neurons activated by noradrenaline (TTX + noradrenaline, 6.67 ± 1.63 cells active per slice, $n = 6$; one-way ANOVA $F_{(4,46)} = 11.33$, $P < 0.0001$; control *vs.* TTX *post hoc* test $P = 0.963$, Fig. 4*A* and *E*). The mean number of Ca^{2+} events per cell (one-way ANOVA $F_{(4,46)} = 3.801$, $P = 0.0094$; control *vs.* TTX *post hoc* test $P > 0.999$, Fig. 4*F*) and event amplitude were also not significantly different from control (one-way ANOVA $F_{(4,44)} = 0.756$, $P = 0.560$; control *vs.* TTX *post hoc* test $P = 0.990$, Fig. 4*G*). Next we bath-applied CNQX, DL-AP5, picrotoxin and TTX together before and during bath-application of noradrenaline (Fig. 4*B*). In the presence of combined synaptic and sodium channel blockers, noradrenaline still activated 6.93 ± 3.45 cells per slice, which was not different from TTX alone ($P = 0.999$). There was also no difference in mean number of Ca^{2+} events ($P = 0.985$) or mean Ca^{2+} event amplitudes compared to TTX alone ($P > 0.999$). Together these results confirm that noradrenaline-induced Ca^{2+} bursts do not require sodium spikes or glutamate/GABA synaptic input.

Noradrenaline-induced Ca^{2+} bursts are underpinned by opening of voltage-gated calcium channels

To test the hypothesis that noradrenaline acts directly on the CRH neurons to drive opening of voltage-gated calcium channels (VGCCs), we performed experiments in which glutamatergic and GABAergic synaptic inputs, action potential firing and VGCCs were blocked with the combination of CNQX, DL-AP5, picrotoxin, TTX as well

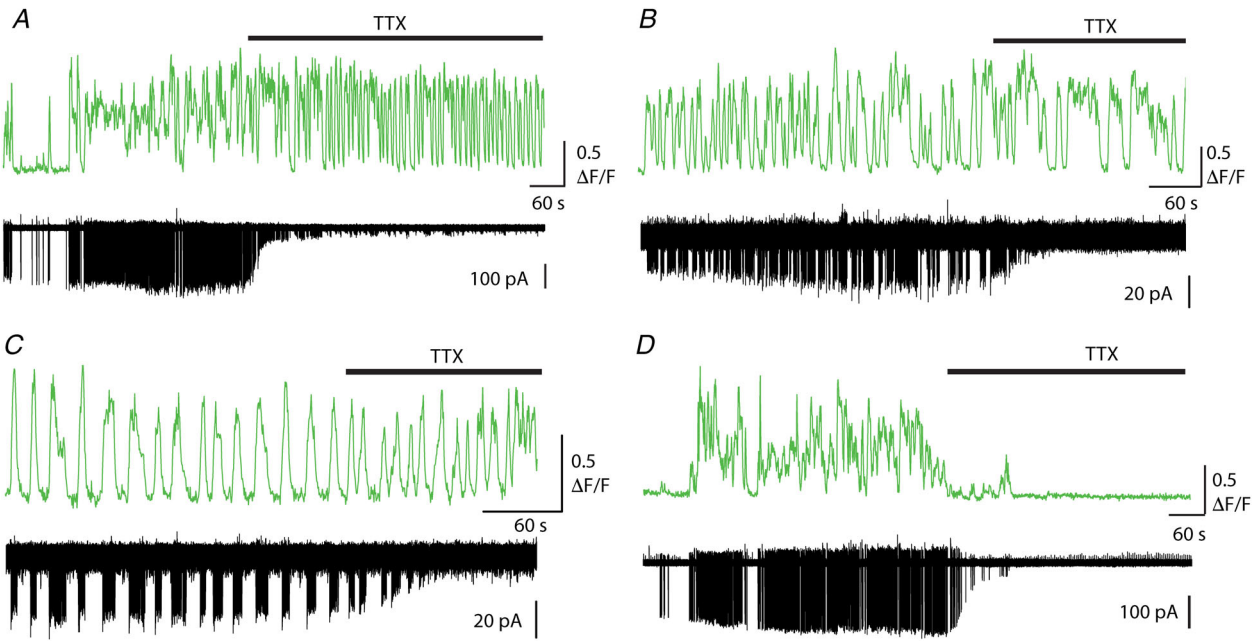

Figure 3. Noradrenaline-induced Ca^{2+} events do not require sodium action potentials
Simultaneous electrical (black) and GCaMP6f fluorescence (green) traces from the soma of a CRH neuron recorded in the continued presence of CNQX, DL-AP5, picrotoxin and noradrenaline. *A–C*, example recordings where application of TTX (black bar) leads to cessation of spiking activity measured electrophysiologically; however, Ca^{2+} events persist. *D*, example recording where application of TTX inhibits both electrical spiking and Ca^{2+} events. [Colour figure can be viewed at wileyonlinelibrary.com]

as the non-specific VGCC antagonist $CdCl_2$ (100 μM). In the presence of this drug cocktail, noradrenaline-induced excitation was significantly reduced (synaptic blockers + TTX + $CdCl_2$, 1.25 ± 0.50 cells active per slice ($n = 4$) *vs.* synaptic blockers + TTX, $P = 0.006$, Fig. 4*C* and *E*). The few cells that were active in the all blockers + $CdCl_2$ condition did not have a significantly different number of mean events per cell ($P = 0.176$, Fig. 4*F*) or mean amplitude of events compared to the synaptic blockers + TTX condition ($P = 0.838$, Fig. 4*G*). The fact that some Ca^{2+} events remained in the presence of $CdCl_2$ suggests that at the concentration used, $CdCl_2$ may not be blocking all VGCCs.

We speculated that L-type Ca^{2+} channels may be responsible for the noradrenaline-induced Ca^{2+} bursts. To test this, we repeated the previous experiment with the same synaptic and sodium channel blockers but with the specific L-type VGCC antagonist nifedipine (100 μM). Similar to the results with $CdCl_2$, the number of cells active per slice in response to noradrenaline was significantly reduced in the presence of nifedipine (synaptic blockers + TTX + nifedipine, 1.50 ± 0.97 cells active per slice, $n = 10$, $P = 0.0002$ compared to synaptic blockers + TTX, Fig. 4*D* and *E*). The few cells that were active in the all blockers + nifedipine condition did not show significantly different numbers of events per cell ($P = 0.174$) or amplitude of events ($P = 0.917$) compared to the synaptic blockers + TTX condition. Overall, these results indicate that VGCCs play an important role in noradrenaline-induced Ca^{2+} events in CRH neurons.

Corticosterone suppresses noradrenaline-induced activation of CRH neurons

We have previously shown that chronic elevations in corticosterone leads to a suppression of intrinsic excita-

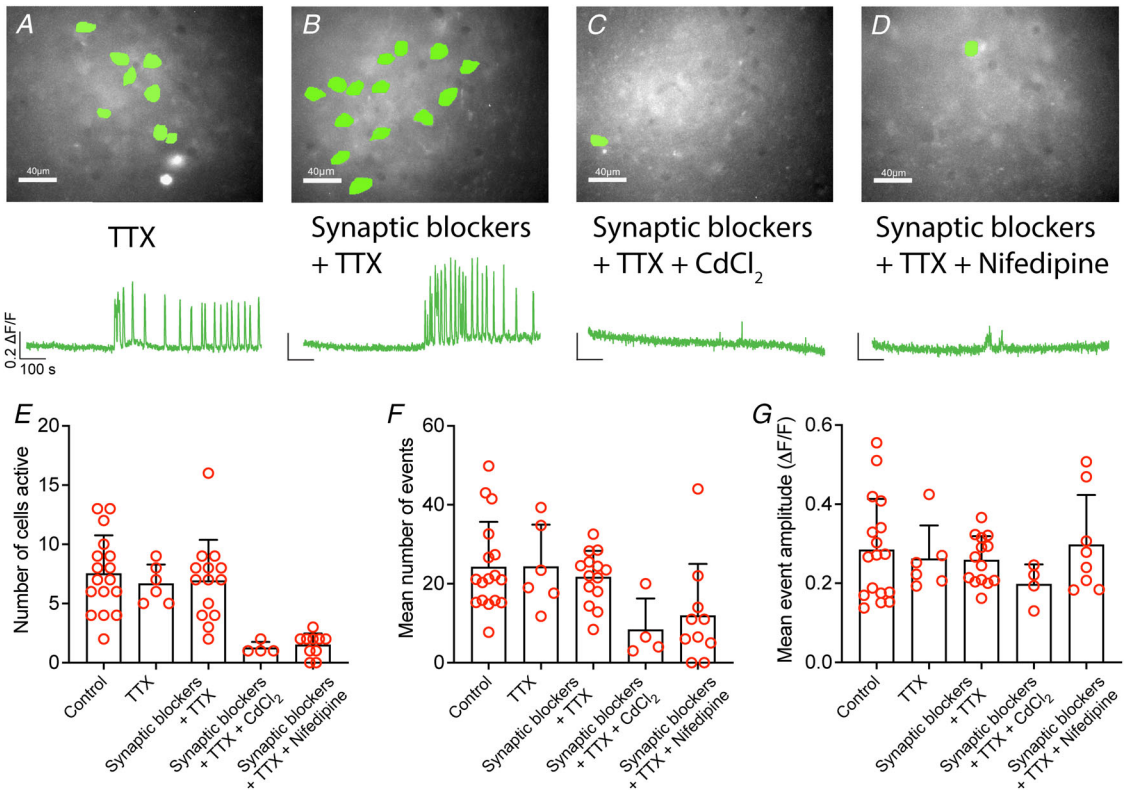

Figure 4. Noradrenaline-induced Ca^{2+} events require voltage-gated Ca^{2+} channels
A–D, top, representative images showing the number of neurons that were activated by noradrenaline. Activated cells are highlighted in green. Each image is from a different brain slice pre-treated with one of the following: TTX (*A*), glutamate/$GABA_A$ receptor antagonists + TTX (synaptic blockers + TTX) (*B*), synaptic blockers + TTX + voltage-gated Ca^{2+} channel antagonist $CdCl_2$ (*C*), synaptic blockers + TTX + L-type Ca^{2+} channel antagonist nifedipine (*D*). Bottom, representative GCaMP6f fluorescence traces of noradrenaline responses in TTX, synaptic blockers + TTX, synaptic blockers + TTX + $CdCl_2$ and synaptic blockers + TTX + nifedipine. *E*, summary graph showing number of neurons activated by noradrenaline per brain slice for each drug treatment condition. *F*, summary graph showing number of events detected (mean per active neuron per slice) in response to noradrenaline for each drug treatment condition. *G*, summary graph showing the average amplitude of events (mean per active neuron per slice) in response to noradrenaline for each drug treatment condition. [Colour figure can be viewed at wileyonlinelibrary.com]

bility in CRH neuron as assessed by electrical spiking responses to injected current steps (Bittar et al., 2019). Given these previous data, we hypothesised that chronic elevations in corticosterone would also suppress CRH neuron responses to noradrenaline measured with Ca^{2+} imaging. In control treated mice, bath-application of 10 μM noradrenaline activated on average 8.22 $\pm$ 3.96 neurons per brain slice ($n = 9$). However, in brain slices from animals treated with corticosterone, application of noradrenaline resulted in the activation of significantly fewer CRH neurons (2.80 $\pm$ 1.69 neurons per brain slice, $n = 15$, $P = 0.0001$, Fig. 5). In those neurons which were excited in the corticosterone treated group, the number of events per cell and event amplitude were not significantly different from values in the control group (events per cell: $P = 0.063$; event amplitude: $P = 0.568$). In the same experiments, noradrenaline was washed out and we then bath-applied 20 mM of KCl to robustly activate all live CRH neurons in the brain slice. In control treated mice, this led to the activation of 27.11 $\pm$ 9.31 neurons per slice ($n = 9$). The number of neurons activated in response to KCl in the corticosterone treated group was 25.20 $\pm$ 7.87 ($n = 15$) and was not significantly different from the control group ($P = 0.596$). This experiment also allowed us to determine the percentage of CRH neurons in our imaging field that were responsive to noradrenaline across the groups. In the control group, noradrenaline activated 32.2 $\pm$ 18.0% of CRH neurons, which was significantly higher than the 10.8 $\pm$ 5.9% of neurons activated in the corticosterone treated group ($P = 0.0003$). These findings confirm that corticosterone treatment can also suppress noradrenaline-induced activation of CRH neurons.

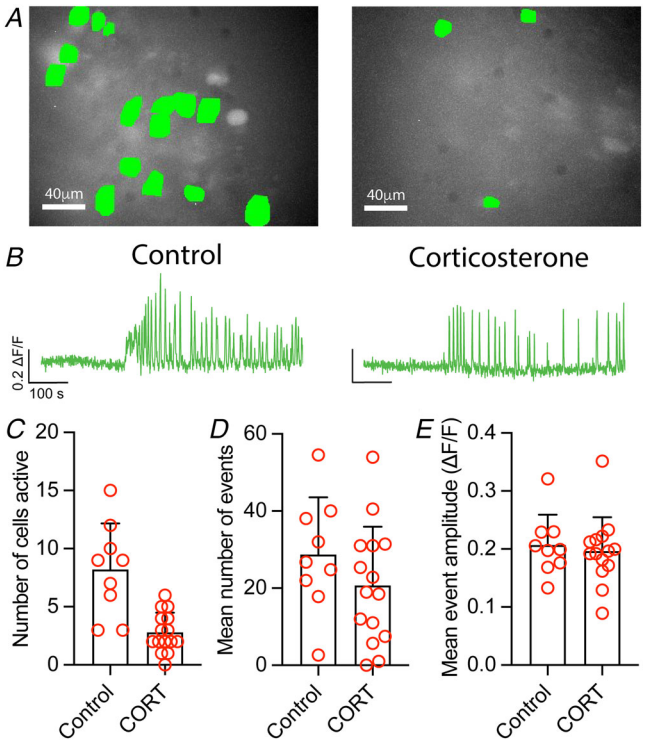

Figure 5. Noradrenaline-induced Ca^{2+} events are suppressed by chronic corticosterone
A, representative images showing the number of neurons that were activated by noradrenaline in a brain slice from a control mouse (left) or a mouse treated with corticosterone in the drinking water for 2 weeks (right). Activated cells are highlighted green. *B*, representative GCaMP6f fluorescence traces of noradrenaline responses from a control mouse or a mouse treated with corticosterone. *C*, summary graph showing number of neurons activated by noradrenaline per brain slice for control and corticosterone conditions. *D* and *E*, summary graphs showing number of events (mean per active cell per brain slice) and amplitude of events (mean per active cell per brain slice). There was no significant difference between groups for either number of events ($P = 0.220$) or event amplitude ($P = 0.673$). [Colour figure can be viewed at wileyonlinelibrary.com]

Discussion

Brainstem noradrenergic neurons are activated in response to stress (Dayas et al., 2001). A subset of these neurons innervate the PVN where they synapse onto CRH neurons (Liposits et al., 1986). Here we show that noradrenaline drives repetitive Ca^{2+} elevations in a subset of CRH neurons within the PVN. This excitation required the activation of α_1-adrenoceptors but did not require GABA/glutamate synaptic transmission or even sodium action potentials. Instead, noradrenaline-induced activation could be blocked following antagonism of L-type VGCCs. Noradrenaline-induced activation was also found to be suppressed in animals chronically treated with corticosterone. Together, these data show that noradrenaline can directly activate CRH neurons and that this excitation is subject to corticosteroid negative feedback.

While there is strong evidence that noradrenergic terminals make direct appositions with CRH neurons (Fuzesi et al., 2007; Flak et al., 2009; Johnson et al., 2018), previous work has suggested that noradrenergic excitation is mediated indirectly via regulation of afferent synaptic input (Chen et al., 2019; Daftary et al., 1998; Han et al., 2002). The study by Chen et al. (2019) showed that activation of α_1-adrenoceptors on CRH neurons triggered release of a retrograde transmitter which excited astrocytes to release ATP. Astrocytic ATP then triggered spiking in glutamate interneurons which innervate CRH neurons leading to enhanced glutamate release and subsequent CRH neuron excitation. In this current study, we found that noradrenaline-induced excitation was independent of GABA or glutamate synaptic transmission and also did not require sodium action potentials. While these finding appear at odds with the results of Chen et al., it is possible that two parallel forms of noradrenaline-induced excitation exist: one that is direct as shown in the current study, and another that is indirect,

as shown by Chen et al. It could even be possible that the noradrenaline-induced Ca^{2+} bursts observed in this current study may be important for driving the retrograde neurotransmitter release reported in Chen et al.

Despite the points noted above, there are also several differences between our current study and past work. These differences may influence whether direct or indirect effects of noradrenaline are more or less likely to be observed. Firstly, while a range of noradrenaline concentrations have been used in the past, 100 μM noradrenaline has been the most commonly used concentration (Chen et al., 2019; Daftary et al., 1998; Han et al., 2002). In contrast, all of our experiments utilised a lower concentration of 10 μM. Secondly, previous work has primarily used whole-cell recordings to measure changes in excitability. It is well known that whole-cell recordings can disrupt the intracellular milieu, which can impact signalling pathways and ion channel currents (Kato et al., 1993; Nakajima et al., 1992; Velumian et al., 1997; Zhang et al., 1994). The Ca^{2+} imaging approach used in this current study avoids this issue. It is also important to consider that as CRH neurons commonly have an input resistance >1 GΩ (Bittar et al., 2019; Power & Iremonger, 2021), small membrane currents may produce large depolarisations. Therefore, it is also possible that noradrenaline-induced inward currents have not been observed previously simply due to their small magnitude. One final point to consider is the possibility that noradrenaline-induced direct membrane depolarisation only occurs in a subset of CRH neurons. Previous work sampled from CRH neurons in a non-biased manner. However, in this current study, our analysis was limited to only the CRH neurons which showed Ca^{2+} responses to noradrenaline (i.e. those which were activated by noradrenaline). Based on comparing the number of CRH neurons responding to 10 μM noradrenaline versus the number responding to KCl, we estimate that the fraction of CRH neurons activated by noradrenaline in our study was approximately 32% of the population. Combined with the observation of heterogeneous Ca^{2+} activity patterns in response to noradrenaline, our data clearly show that different CRH neurons in the PVN process noradrenergic signals differently.

Our data support the concept that many CRH neurons are directly depolarised by noradrenaline. Data from hypothalamic magnocellular neurons has shown that activation of the α_1-adrenoceptor leads to depolarisation which drives a bursting pattern of activity (Randle et al., 1986; Yamashita et al., 1987). Randle et al. (1986) performed intracellular electrophysiological recordings from magnocellular neurons and showed that noradrenaline or α_1-adrenoceptor agonists depolarised these neurons by reducing a K^+ leak current. This reduction in K^+ conductance by α_1-adrenoceptor activation is not only seen in magnocellular neurons but has also been demonstrated in several other cell types (Kamimura et al., 2000; Larkman & Kelly, 1992; McCormick, 1992; Pan et al., 1994). While not specifically tested in this current study, this mechanism fits with our current data. Specifically, α_1-adrenoceptor inhibition of a resting K^+ conductance would depolarise the neuron leading to the opening of L-type VGCCs and Ca^{2+} influx. These resulting Ca^{2+} events persisted in the presence of TTX demonstrating that they do not require sodium action potentials. While Ca^{2+} spikes have not been previously reported in CRH neurons, many other neurons are known to exhibit regenerative Ca^{2+} events, especially in dendritic regions (Stuart & Spruston, 2015). As well as being important for synaptic plasticity (Williams et al., 2007), these events have been shown to be important for dendritic transmitter release (Ludwig et al., 2016; Regehr et al., 2009;). As noted above, this idea fits with previous data showing that noradrenaline can promote dendritic peptide release from CRH neurons (Chen et al., 2019).

The patterns of neural activity induced by noradrenaline were heterogeneous, ranging from bursting to a sustained Ca^{2+} elevation. Bursting is commonly seen in neuroendocrine neurons, including magnocellular neurons (Brown et al., 2013) and GnRH neurons (Constantin et al., 2021), and has recently been recorded *in vivo* in CRH neurons (Ichiyama et al., 2022). Bursting is thought to be important in driving efficient exocytosis of large dense core vesicles from nerve terminals (Dreifuss et al., 1971; Dyball et al., 1988; Harris et al., 1969; Jackson et al., 1991). Research in both magnocellular neurons and GnRH neurons has demonstrated that burst firing is determined by intrinsic membrane properties and is regulated by Ca^{2+} influx. In magnocellular vasopressin neurons, bursting requires Ca^{2+}-dependent afterdepolarising potentials (Andrew & Dudek, 1983; Bourque, 1986; Brown, 2004; Li & Hatton, 1997). In GnRH neurons, burst patterning is regulated by Ca^{2+}-activated K^+ channels (Liu & Herbison, 2008; Lee et al., 2010). Interestingly, in GnRH neurons, bursting observed *in vitro* also does not require GABA or glutamate synaptic transmission (Lee et al., 2012).

In this study, we also report that noradrenaline-induced excitation of CRH neurons was suppressed following chronic exposure to corticosterone. This is consistent with our previous work showing that either acute stress hormone exposure (Kim et al., 2019) or 14-day treatment with corticosterone in the drinking water (Bittar et al., 2019) suppresses intrinsic excitability in CRH neurons. However, it is also possible that chronic corticosterone exposure reduced expression levels of α_1-adrenoceptors. Indeed, previous work has shown expression of α_{1b}-adrenoceptor mRNA in CRH neurons is inversely proportional to corticosterone levels (Day et al., 1999).

One limitation of our study is that noradrenaline was bath-applied. While this approach allows for the effects of noradrenaline to be studied in a simple and reproducible manner, CRH neurons exposed to bath-applied noradrenaline will not experience the same profile and kinetics of noradrenaline compared to that released endogenously from nerve terminals. One way to address this issue would be to induce noradrenaline release within the brain slice with optogenetic stimulation, as has been performed in previous studies (Chen et al., 2019; Inoue et al., 2013). In future work, it would be interesting to investigate if the effects of noradrenaline on CRH neuron excitability differ between bath-application and optogenetic approaches.

Overall, this work provides several new insights into how noradrenergic signals regulate CRH neuron network activity. We show that exposure to noradrenaline can drive many CRH neurons into a bursting mode of activity and this excitation does not require indirect innervation by local GABA or glutamate circuits. Most surprisingly, repetitive Ca^{2+} bursts also do not require sodium action potentials. Finally, we show that chronic corticosterone elevations can suppress noradrenaline-induced excitation, consistent with previous demonstrations of corticosteroid negative feedback (Kim & Iremonger, 2019). Together, these findings provide insight into how noradrenergic signals may regulate CRH neural network activity in response to stress.

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Additional information

Data availability statement

All data supporting the findings of this study are available upon reasonable request.

Competing interests

None.

Author contributions

Conception and design of the research: J.M.G., A.S., K.J.I. Data collection: J.M.G., A.S. Data analysis: J.M.G., A.S., S.Z., J.S.K., K.J.I. Manuscript preparation: J.M.G., A.S., S.Z., J.S.K., K.J.I. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

CRH, hypothalamus, noradrenaline, stress

Supporting information

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