Chronic Stress Facilitates Bursting Electrical Activity in Pituitary Corticotrophs

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Running title: Chronic stress facilitates BK-dependent bursting in corticotrophs

KEY POINTS

- Chronic stress (CS) is predicted to modify the electrical excitability of anterior pituitary corticotrophs.
- Electrophysiological recordings from isolated corticotrophs from CS male mice display spontaneous electrical bursting behaviour compared to the tonic spiking behaviour of unstressed corticotrophs.
- The increased spontaneous bursting from CS corticotrophs is BK-dependent and mathematical modelling reveals that the time constant of activation, properties and proportion of BK channels functionally coupled to L-type calcium channels determines the promotion of bursting activity.
- CS (but not unstressed) corticotrophs display CRH-induced bursting even when the majority of BK channels are pharmacologically inhibited, which can be explained by the stochastic behaviour of a small number of BK channels with distinct properties.
- Corticotroph excitability can be finely tuned by the stochastic behaviour of a small number of BK channels dependent on their properties and functional co-localisation with L-type calcium channels to control corticotroph excitability over diverse time domains and physiological challenges.

ABSTRACT

Coordination of an appropriate stress response is dependent upon anterior pituitary corticotroph excitability in response to hypothalamic secretagogues and glucocorticoid negative feedback. A key determinant of corticotroph excitability is large conductance calcium- and voltage- activated (BK) potassium channels that are critical for promoting CRH-induced bursting that enhances ACTH secretion. Previous studies revealed HPA axis hyperexcitability following chronic stress (CS) is partly a function of increased corticotroph output. Thus, we hypothesise that chronic stress promotes corticotroph excitability through a BK-dependent mechanism.

Corticotrophs from CS mice displayed significant increases in spontaneous bursting, that was suppressed by the BK blocker paxilline. Mathematical modelling reveals that the time constant of BK channel activation, plus properties and proportion of BK channels functionally coupled to L-type calcium (Ca^{2+}) channels determines bursting activity. Surprisingly, CS corticotrophs (but not unstressed) display CRH-induced bursting even when the majority of BK channels are inhibited by paxilline; that modelling suggests is a consequence of the stochastic behaviour of a small number of BK channels coupled to L-type Ca^{2+} channels.

Our data reveal that changes in the stochastic behaviour of a small number of BK channels can finely tune corticotroph excitability through stress-induced changes in BK channel properties. Importantly, regulation of BK channel function is highly context dependent allowing dynamic control of corticotroph excitability over a large range of time domains and physiological challenges in health and disease. This is likely to occur in other BK-expressing endocrine cells, with important implications for the physiological processes they regulate and the potential for therapy.

INTRODUCTION

Stress and stress-related disorders represent a significant burden on health and society. Following an acute stress, glucocorticoids mediate the hormonal stress response of the organism and provide negative feedback to limit hypothalamic-pituitary-adrenal (HPA) axis activity to facilitate recovery. However, following chronic stress the HPA axis in both humans and animals is typically characterized by alterations in HPA sensitivity to stressors and glucocorticoid negative feedback. For example, patients with major depression can display HPA hypersensitivity with reduced glucocorticoid feedback and elevated cortisol levels (Nestler *et al.*, 2002) and chronic elevation of glucocorticoids is a major risk factor for human health including predisposition to metabolic syndrome, affective disorders and cardiovascular disease (de Kloet *et al.*, 2005; Walker, 2007; Macfarlane *et al.*, 2008; Quax *et al.*, 2013). In rodents, chronic stress (CS) results in stress hypersensitivity as a result of enhanced corticotrophin-releasing hormone (CRH)-induced adrenocorticotropic hormone (ACTH) release from anterior pituitary corticotrophs measured both *in vivo* and *in vitro* (Young & Akil, 1985; Franco *et al.*, 2016).

Hormone secretion from anterior pituitary cells occurs primarily when the cells are producing bursts of electrical activity. During these bursts, the intracellular Ca^{2+} concentration is elevated to a much higher level and for a much longer duration than when the cell is spiking tonically (Van Goor *et al.*, 2001*b*), and this elevated Ca^{2+} concentration evokes exocytosis of hormone-filled vesicles at a higher rate (Tagliavini *et al.*, 2016). The mechanism for electrical bursting in pituitary corticotrophs, which secrete ACTH when stimulated by CRH, relies on BK-type K^+ channels (Duncan *et al.*, 2015; Fletcher *et al.*, 2017), suggesting it plays a central role in regulating hormone secretion. A similar burst mechanism has been found in other pituitary cell types (Van Goor *et al.*, 2001*a*; Tabak *et al.*, 2011). The key role played by these channels in burst production has been verified by studies in which they were removed pharmacologically or genetically and then BK-type current added back using the dynamic clamp technique (Tabak *et al.*, 2011; Duncan *et al.*, 2015), or in which BK-type current was subtracted using the dynamic clamp, converting the electrical bursting pattern in stimulated corticotrophs to a tonic spiking pattern (Duncan *et al.*, 2016).

We have previously shown that early glucocorticoid feedback (within 2h of glucocorticoid exposure) prevents this BK-channel dependent, CRH-induced bursting and

bursting can be 'rescued' by reintroduction of a fast activating BK current using dynamic clamp (Duncan *et al.*, 2016). We hypothesized that chronic stress may also control corticotroph excitability through regulation of electrical bursting. Indeed, as chronic stress enhances CRH-induced ACTH secretion (Young & Akil, 1985; Franco *et al.*, 2016) we predicted that both spontaneous and CRH-induced electrical bursting may be enhanced. However, whether CS in fact modifies either spontaneous or CRH-evoked excitability has not been experimentally determined and whether CS-induced changes in activity are dependent upon functional BK channels is not known.

In this study, we found that corticotrophs from chronically stressed mice exhibit a high degree of BK-channel dependent bursting activity even under basal (i.e., unstimulated) conditions, in contrast to cells from unstressed animals which exhibit low-frequency tonic spiking in basal conditions. Furthermore, rather surprisingly under conditions in which BK channels are largely blocked pharmacologically we found that CRH could still induce bursting in corticotrophs in chronically stressed mice, in contrast to that observed in unstressed animals.

To further understand these experimental findings, we modified our previous mathematical model of corticotroph electrical excitability (Duncan *et al.*, 2015) to allow us to interrogate both the functional properties of BK channels with distinct properties as well as the stochastic behaviour of BK channel activity. Importantly we find that the time constant for BK channel activation and the proportion of distinct BK channel subtypes functionally coupled to L-type Ca^{2+} channels can explain both the heterogeneity of bursting behaviour and the ability of CRH to promote bursting in chronically stressed corticotrophs when the majority of BK channel activity is blocked pharmacologically. The motivation for this model prediction is prior work showing that BK channel activation must be sufficiently rapid to induce bursting in corticotrophs (Duncan *et al*., 2016) and pituitary cell-line GH4C1 cells (Tabak *et al*., 2011).

Taken together, our data reveal that the electrical activity of corticotrophs can be finely tuned by the stochastic behaviour of a small number of BK channels dependent on their properties and functional co-localisation with L-type Ca^{2+} channels to control corticotroph excitability over diverse physiological conditions.

METHODS

Ethical Approval

Mice expressing green fluorescent protein (GFP) under the proopiomelanocortin (POMC) promoter were used as previously described (Duncan *et al.*, 2015) on a C57/BL6 background. Either litter- or age-matched adult male mice (12 – 14 weeks old) were randomly assigned to each experimental condition. Mice were caged in groups of two to four, under standard laboratory conditions (lights on at 7:00 AM, lights off at 7:00 PM, 21°C, with tap water and chow available *ad libitum*) at the University of Edinburgh. Tissue collection was performed between 9:00 AM and 10:00 AM in accordance with United Kingdom Home Office requirements (PPL P90E1F821) and University of Edinburgh Ethical Review Committee approval (PL24-16).

Reagents

General biochemical reagents used throughout this study were obtained from Sigma-Aldrich and were of analytical-grade quality unless stated otherwise.

Chronic stress paradigm and cell culture

We used an established 14-day repeated homotypic restraint stress paradigm as a simple, highly reproducible and tractable model of chronic stress producing profound changes at the transcriptomic, cell physiology, systems and behavioural level in C57/Bl6 mice, including induction of anxiety and depression-like behaviour (Kim & Han, 2006). Mice were randomly assigned to one of two experimental groups: 1) control, non-stressed (Control); and 2) restraint stress for 30 minutes per day, for 14 days (Chronic Stress). For the restraint group, mice were placed individually in clear plastic restraint tubes (CH Technologies (USA) Inc., Westwood, NJ) of an internal diameter of 31 mm with a variable pusher, adjusted based on animal size. On day 15, mice were sacrificed and pituitary glands removed. Anterior pituitary cells were acutely isolated by trypsin digestion as previously described (Duncan *et al.*, 2015). Each cell preparation used anterior pituitary glands collected from three animals. Cells were cultured on 12 mm coverslips (Warner Instruments) in serum-free media (DMEM containing 25 mM HEPES, 5 µg/mL insulin, 50 µg/mL transferrin, 30 nM sodium selenite, 0.3% BSA [wt/vol], 4.2 µg/mL fibronectin, and antibiotic/antimycotic [100x dilution of Sigma stock]) and incubated at 37°C in 5% CO_2. Serum-free media (lacking antibiotic/antimycotic) was changed

every 2 days and electrophysiological recordings were obtained from cells 24–96 hours post-isolation.

Electrophysiology

Electrophysiological recordings were obtained from GFP-identified corticotroph cells using the perforated patch mode of the whole-cell patch clamp technique. Amphotericin B was used at a concentration of 150 µg/mL in pipette solution, which resulted in access resistances typically less than 40 MΩ within 10–20 minutes and allowed stable recordings in excess of 40 minutes. The standard bath solution (extracellular) contained the following (in millimoles): 140 NaCl, 5 KCl, 2 $CaCl_2$, 0.1 $MgCl_2$, 10 HEPES, and 10 glucose. The pH was adjusted to 7.4 with NaOH, 300 mOsmol/L. The standard pipette solution (intracellular) contained the following (in millimoles): 10 NaCl, 30 KCl, 60 K_2SO_4, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 50 sucrose. The pH was adjusted to 7.3 with KOH, 290 mOsmol/L. Recordings were performed at room temperature (18°C–22°C) to facilitate stable recordings of more than 30 minutes required for these assays and obtained using Clampex 10.3 (Molecular Devices) with a sampling rate of 10 kHz and filtered at 2 kHz. Patch pipettes were fabricated from borosilicate glass (King Precision Glass, Inc) using a model P-97 micropipette puller (Sutter Instruments). Pipette tips were heat polished and had resistances typically between 2 and 3 MΩ. Compensated series resistance was typically less than 20 MΩ and capacitance of corticotrophs ranged from 2 to 10 pF. A gravity driven perfusion system was used to apply drugs to the cells with a flow rate of 1–2 mL/min to minimize flow-induced artifacts.

Dynamic Clamp

Dynamic clamp experiments were performed using a separate digital acquisition card and computer running the software QuB (Milescu *et al.*, 2008). In the current clamp mode of the patch amplifier (Axopatch 200B; Molecular Devices), membrane potential V was used to compute the current going through the BK channels, $I_{BK} = g_{BK} f (V_K - V)$, with f obtained by integrating

$$\tau_{BK} \frac{df}{dt} = f_\infty(V) - f \tag{1}$$

in real time using the forward Euler method (Milescu *et al.*, 2008), with an average time step of 21 µsec (maximum ≤100 µsec), and the steady-state BK channel activation given by

$$f_{\infty}(V) = \left[1 + exp\left(\frac{(v_f - V)}{s_f}\right)\right]^{-1} \quad (2)$$

The calculated BK current was injected back into the cell through the same digital acquisition card. Typical parameter values were as follows: g_{BK} = 0.5-4 nS; τ_{BK} = 5 ms; s_f = 2 mV.$v_f$values of -5 mV and -20 mV were used to model ZERO and STREX channels respectively.

Electrophysiological Analysis

Current clamp recordings were analysed as previously described (Duncan *et al.*, 2015, 2016) using Clampfit v.10.3 (Molecular Devices). CRH-evoked activity was measured immediately after 3 minutes of CRH stimulation (0.2 nM). In addition to the mean event duration, bursting behaviour was quantified through the calculation of a burst factor. This method classifies any event <100 ms as a spike and events >100 ms as a burst; a burst factor is calculated as the proportion of events that are bursts (Duncan *et al.*, 2015, 2016). Data are presented as mean ± SD, where n represents the number of cells, from pituitary preparations each using three animals. Statistical analysis of electrophysiological parameters was performed using R 4.0.5. Data consisting of two groups was analysed using a Welsh Two Sample t-test or Paired t-test as appropriate. Mixed-effects linear models were created using the *nlme:lme* package, and used to compare membrane potential, event frequency and event duration between the two experimental groups; group (control or CS), treatment (base or CRH), and their interaction were used as fixed effects and the cell ID was used as a random effect. Event frequency and event duration were log-transformed to meet the assumption of normality for the model's residuals (for the frequency) and to correct for heteroscedasticity (for the duration); all other models' assumptions were met. A generalised linear mixed-effect model was created using the *lme4:glmer* function, and used to compare burstiness factor, to account for the factor that this is bound between 0 and 1. Post-hoc comparisons were performed using Tukey's test when main effects or interactions were found to be significant.

The Mathematical Model

We modified a previously published mathematical model of corticotroph electrical activity to include two BK channel splice variants, STREX and ZERO (Xie & McCobb, 1998; Chen *et al.*, 2005; Duncan *et al.*, 2015), and to implement a stochastic treatment of BK channels. There are three ordinary differential equations for the membrane potential (*V*), the activation

variable for delayed-rectifier K^+ channels (*n*), and for the bulk free cytosolic Ca^{2+} concentration (*c*). The differential equation for membrane potential is:

$$C_m \frac{dV}{dt} = -(I_{Kdr} + I_{Kir} + I_{Ca} + I_{NS} + I_L + I_{IK} + I_{BK}) \quad (3)$$

where C_m is the membrane capacitance and *t* is time in ms. There are seven ionic currents in the voltage equation: a delayed rectifier K^+ current (I_{Kdr}), an inward rectifier K^+ current (I_{Kir}), an L-type Ca^{2+} current (I_{Ca}), a non-selective-cation current (I_{NS}), a leak current (I_L), an intermediate conductance K^+ current (I_{IK}), and big conductance K^+ current (I_{BK}). Mathematical expressions for the currents are:

$$I_{Kdr} = g_{Kdr} n (V - V_K) \quad (4)$$

$$I_{Kir} = g_{Kir} r_\infty(V)(V - V_K) \quad (5)$$

$$I_{Ca} = g_{Ca} m_\infty(V)(V - V_{Ca}) \quad (6)$$

$$I_{NS} = g_{NS}(V - V_{NS}) \quad (7)$$

$$I_L = g_L(V - V_L) \quad (8)$$

$$I_{IK} = g_{IK} i_\infty(c)(V - V_K) \ . \quad (9)$$

The factors g_x are maximum conductance parameters, and V_x are Nernst potentials. The spontaneous activity of control corticotrophs typically consists of low-frequency spiking with no evidence of periodicity. This is a clear indicator of stochastic behavior. To produce stochastic spontaneous spiking in the model we introduce background noise in several conductances that are responsible for triggering action potentials. In particular, g_{Ca}, g_{NS}, and g_{Kir} were varied at each time step by randomly selecting a value from a normal distribution about a mean given in Table 1, with variances $\sigma_{Ca} = 0.1$, $\sigma_{NS} = 0.02$, and $\sigma_{Kir} = 0.2$ nS. The amplitudes and variances were chosen so that the rate of spontaneous spiking in the control model cell would be similar to that of the actual control cell. The activation variable for the delayed rectifier changes over time according to

$$\frac{dn}{dt} = \frac{n_\infty(V) - n}{\tau_n} \quad (10)$$

where τ_n is a time constant and $n_\infty(V)$ is the equilibrium function for the variable. This, and all other equilibrium functions of *V*, have the form:

$$x_\infty(V) = \frac{1}{1 + \exp(\frac{v_x - V}{s_x})} \quad (11)$$

Activation variables for all other currents vary on a faster time scale, and are assumed to be at quasi-equilibrium with V or c. The IK current is gated by Ca^{2+}, and not voltage, and its activation function is:

$$i_\infty(c) = \frac{c^2}{c^2+k_{IK}^2} \tag{12}$$

The bulk free intracellular Ca^{2+} concentration varies according to the differential equation:

$$\frac{dc}{dt} = -f_c(\alpha I_{Ca} + k_c c) \tag{13}$$

where f_c is the fraction of cytosolic Ca^{2+} that is free (not bound to buffers), α converts current to concentration, and k_c is the Ca^{2+} pump rate.

We used a stochastic description of the BK channels. This was motivated primarily by the small number of BK channels estimated to be present in pituitary cells, and the relatively large effect of the opening of a single BK channel due to its large single-channel conductance (Richards *et al.*, 2020). In excitable cells, BK channels are often functionally colocalised with voltage-dependent Ca^{2+} channels either through assembly of the two channel types in macromolecular assemblies, or as a result of overlapping calcium nanodomains from multiple voltage-dependent Ca^{2+} channels close to a BK channel (Roberts *et al.*, 1990; Marrion & Tavalin, 1998; Berkefeld *et al.*, 2006; Prakriya & Lingle, 2000; Montefusco *et al.*, 2017). Although the precise molecular arrangement in corticotrophs, as for any other anterior pituitary cell type, is not known bursting behaviour requires functional co-localisation (Duncan *et al.*, 2015; Tabak *et al.*, 2011; Montefusco *et al.*, 2017). In our modelling, we therefore partition the BK channels into those that are colocalised with Ca^{2+} channels and those that are not. Altogether, then, we considered four populations of BK channels (Fig. 1A): STREX-type BK channels that are near Ca^{2+} channels, and thus activate quickly (denoted as s_n); ZERO-type BK channels that are near Ca^{2+} channels (z_n); STREX-type BK channels that are far from Ca^{2+} channels and thus activate much more slowly (s_f); ZERO-type BK channels that are far from Ca^{2+} channels (z_f. The STREX splice variant is known to have an activation function that is left-shifted relative to the ZERO variant (Xie & McCobb, 1998; Tian *et al.*, 2004; Chen *et al.*, 2005), and in our model the half-activation voltage of a STREX channel is $v_s = -20$ mV, while that for a ZERO channel is $v_z = -5$ mV (Fig. 1B).

Each BK channel can be in either a closed state or an open state, and is treated as a Markov process with closed-to-open rate δ and open-to-closed rate γ. For the BK channels that are colocalised with Ca^{2+} channels (BK-near channels):

$$\delta_{sn} = \frac{s_\infty(V)}{\tau_{BK-n}} \quad , \quad \delta_{zn} = \frac{z_\infty(V)}{\tau_{BK-n}} \tag{14}$$

and

$$\gamma_{sn} = \frac{1-s_\infty(V)}{\tau_{BK-n}} \quad , \quad \gamma_{zn} = \frac{1-z_\infty(V)}{\tau_{BK-n}} \quad . \tag{15}$$

The time constants for STREX and ZERO channels colocalised with L-type Ca^{2+} channels (τ_{BK-n}) are assumed to be the same. For the BK channels that are far from Ca^{2+} channels the close-to-open rate is much smaller due to the much lower Ca^{2+} concentration, so the time constant for channel opening (τ_{CO}) is much larger than for colocalised channels. The open-to-closed time constant is much smaller, since this is independent of Ca^{2+} concentration. Hence, for the non-colocalised BK channels (BK-far channels):

$$\delta_{sf} = \frac{s_\infty(V)}{\tau_{CO}} \quad , \quad \delta_{zf} = \frac{z_\infty(V)}{\tau_{CO}} \tag{16}$$

and

$$\gamma_{sf} = \frac{1-s_\infty(V)}{\tau_{BK-f}} \quad , \quad \gamma_{zf} = \frac{1-z_\infty(V)}{\tau_{BK-f}} \quad . \tag{17}$$

The total number of ZERO channels is constant, and denoted as N_z. Similarly, the total number of STREX channels is constant, N_s. Letting β_z and β_s denote the fraction of ZERO and STREX channels colocalised with Ca^{2+} channels, the number of channels in each of the four populations is $N_{z_n} = \beta_z N_z$, $N_{z_f} = (1-\beta_z)N_z$, $N_{s_n} = \beta_s N_s$, and $N_{s_f} = (1-\beta_s)N_s$. The number of channels of type *x* that are open at any point in time is denoted as N_x^o. The BK current is then:

$$I_{BK} = g_{BK}\left(N_{z_n}^o + N_{z_f}^o + N_{s_n}^o + N_{s_f}^o\right)(V - V_K) \tag{18}$$

where g_{BK} is the single-channel conductance of a BK channel.

The drug paxilline is an effective and specific BK channel antagonist. However, the inhibition of BK channels by paxilline is inversely related to channel open probability with paxilline largely blocking the closed state (Zhou & Lingle, 2014; Zhou *et al.*, 2020). Thus, it is unlikely that all BK channels colocalised with Ca^{2+} channels are blocked by paxilline even at high concentrations. In our simulations, we assume that when paxilline is present, approximately 90% of the BK channels are blocked at each point in time. The random selection of blocked channels was biased so that closed channels are 10 times more likely to be targeted than open BK channels. Thus, at each time step, approximately 10% of the

population of BK channels is chosen to be in an unblocked state. These unblocked channels are much more likely to be in the open state than the closed state, but at that (or subsequent) times steps an unblocked channel can transition to the closed state.

The activation time constant for BK channels that are colocalised with Ca^{2+} channels (τ_{BK-n}) is critical in determining whether a model cell spikes are bursts. Smaller values of the time constant facilitate bursting (Duncan *et al*., 2016). In model simulations, we assumed that both stimulation with CRH and chronic stress impact this parameter. Under basal conditions we used τ_{BK-n} in the range of 17-26 ms for unstressed cells, and 2-26 ms for stressed cells. Under CRH-stimulated conditions we used τ_{BK-n} in the range of 2-8 ms for unstressed cells and 2-17 ms for stressed cells. All ranges were sampled in increments of 3 ms.

To simulate the application of CRH, which acts on BK channels through protein kinase A (Tian *et al.*, 2001, 2004), we increased the conductance of the Ca^{2+} current from $g_{Ca} = 1.85$ nS to 2.1 nS, increased the number of ZERO channels from $N_z = 5$ to 20 to reflect activation of this BK isoform, and decreased the number of STREX channels from $N_s = 10$ to 5 to reflect inhibition of this BK isoform.

To simulate corticotrophs from chronically stressed animals we increased the fraction of ZERO-type BK channels that are colocalised with Ca^{2+} channels from $\beta_z = 0.2$ to 0.8.

To construct the box plots and histograms of Fig. 9 and Fig. 10 we uniformly sampled three parameters: β_s, β_z, and τ_{BK-n}. The β_s parameter was sampled from 0.1 to 0.5 in increments of 0.1. The β_z parameter was sampled from 0.1 to 0.5 in increments of 0.1 for unstressed cells and 0.5 to 0.9 in increments of 0.1 for stressed cells. The τ_{BK-n} parameter was sampled according to the ranges discussed above, in increments of 3 ms. For each sample, a simulation was performed with the model cell for a duration of 50 s and the burst factor determined. This was done for 1000 samples for each class of cell (unstressed and basal, unstressed and stimulated, stressed and basal, stressed and stimulated).

All parameter values are given in Table 1. The computer simulations were performed using the Euler method in MATLAB with time step $\Delta t = 0.05$ ms. Computer codes can be downloaded from www.math.fsu.edu/~bertram/software/pituitary.

Parameter	Value	Parameter	Value
C_m	7pf	v_s	-20 mV
g_{Kdr}	6.5nS	v_z	-5mV
g_{Kir}	0.93nS	v_n	-5mV
g_{Ca}	$1.85-2.1$nS	v_m	-20mV
g_{NS}	0.12nS	v_{Kir}	-50mV
g_L	0.2nS	s_n	10mV
g_{IK}	0.5nS	s_m	12mV
g_{BK}	0.2nS	s_s	2mV
V_{Ca}	60mV	s_z	2mV
V_K	-70mV	s_{Kir}	-1 mV
V_{NS}	-20mV	α	0.0015μM/fC
V_L	-50mV	f_c	0.005
τ_n	30ms	k_c	0.12 μM
τ_{BK-n}	varied	N_z	$5-20$
τ_{BK-f}	5ms	N_s	$5-10$
τ_{CO}	1000 ms	β_s	varied
k_{IK}	0.4μM	β_z	varied

RESULTS

Chronic stress alters electrical activity of corticotrophs cells

As previously described (Duncan *et al.*, 2015; Fletcher *et al.*, 2017), corticotroph cells display primarily single-spike action potentials under basal conditions, and the majority of cells (>90%) transition to pseudo-plateau bursting behaviour following CRH stimulation (Fig. 2A and B). In this study, current-clamp recordings were obtained from corticotrophs isolated from mice subjected to 14 days of restraint stress. In contrast to controls, chronic stress corticotrophs displayed two distinct phenotypes (Fig. 2). A subset of cells (10/15 cells), designated "stressed bursters", display some spontaneous bursting activity and increased bursting with CRH stimulation (Fig. 2C and D). A smaller proportion of cells (5/15 cells), designated "stressed spikers", displayed no bursting under basal conditions and show purely an increase in single-spike frequency following CRH stimulation (Fig 2E and F).

Resting membrane potential of CS cells (n = 15 cells from 3 preparations) was -51.7 ± 7.0 mV and CRH caused a depolarisation to -47.9 ± 4.2 mV (Fig. 3A). In comparison, control cells (n = 10 cells from 5 preparations) had a basal and CRH-stimulated membrane potential of -52.5 ± 5.0 mV and -50.3 ± 6.3 mV respectively. There was no significant effect of CRH (P = 0.105) or chronic stress (P = 0.434), nor an interaction between the two (P = 0.597), suggesting that changes seen in CS cells occur independently of resting membrane potential. In addition, the properties of single action potentials were not different between control (n = 8) and CS cells (n = 9) which displayed spontaneous spiking behavior. There was no significant difference (P = 0.820) in mean spike duration between control (22 ± 7 ms) and CS cells (23 ± 13 ms), and no significant difference (P = 0.316) in mean spike amplitude between control and CS cells (67 ± 16 mV and 58 ± 16 mV respectively). Taken together, this suggests that the major effect of CS is to regulate the mechanism(s) that control bursting behaviour.

Basal and CRH-stimulated event frequency of control cells was 0.77 ± 0.78 Hz and 1.01 ± 0.68 Hz respectively (Fig. 3B). Basal event frequency of CS cells was 0.65 ± 0.42 Hz which increased to 1.72 ± 1.38 Hz following CRH stimulation. There was no significant effect of CRH (P = 0.0740) or chronic stress (P = 0.439), nor an interaction between the two (P = 0.960). However, CS cells displayed a wider range of CRH-evoked event frequency (from 0.33 Hz to 4.53 Hz) compared to controls (from 0.51 Hz to 2.81 Hz). This much greater variability reflects the two subpopulations of CS cells; the "stressed bursters" displayed lower event frequencies,

while “stressed spikers”, which did not transition to bursting, displayed higher event frequencies.

As previously demonstrated, stimulation of control cells with CRH results in the almost exclusive transition to bursting behaviour (Fig. 3C and D). Under basal conditions, mean event duration and burst factor of control cells was 60 ± 48 ms and 0.18 ± 0.21 respectively, corresponding to predominantly single-spike activity. Following CRH stimulation, event duration increased significantly ($P < 0.0001$) to 470 ± 267 ms (Fig. 3C) and burst factor increased significantly ($P = 0.0032$) to 0.78 ± 0.25 (Fig. 3D). In contrast, CS cells showed a mix of spiking and bursting activity under basal conditions. Basal event duration of CS cells was 381 ± 469 ms and was significantly increased compared to control cells ($P = 0.046$). Basal burst factor of CS cells was 0.46 ± 0.44, which was not significantly different to controls ($P = 0.063$). Following CRH stimulation, CS cells showed a mixture of spikers and bursters. This corresponded to event duration ranging from 15 ms to 2582 ms (mean 629 ± 764 ms) and burst factor ranging from 0 to 1 (mean 0.53 ± 0.44). Importantly, while the mean CRH-induced event duration and burst factor was not significantly different to controls ($P = 0.380$ and $P = 0.107$ respectively), the variation in both event duration and burst factor was much higher in corticotrophs from CS animals.

Changes in electrical activity in CS cells do not result from changes in corticotroph cell size or calcium handling

Recent modelling data has predicted that larger pituitary cells have a greater tendency to display bursting behavior (Richards *et al.*, 2020). To determine whether CS results in an increase in corticotroph cell size that may lead to increased basal bursting we measured cell capacitance during electrophysiological experiments. The mean capacitance of CS cells, which is proportional to cell surface area, was 5.10 ± 1.80 pF, which was not significantly different ($P = 0.598$) to control cells (4.78 ± 1.03 pF). In addition, there was no correlation between cell capacitance and basal event duration in all cells recorded ($R^2 = 0.007$). We therefore conclude that the electrical properties of CS cells are not due to changes in cell size.

Bursting in corticotroph cells is dependent on BK channels which are largely driven by calcium influx though L-type calcium channels (Duncan *et al*., 2015, 2016). Previous modelling of BK channel regulation by colocalised voltage gated calcium channels in some endocrine cells suggest that an increase in number of L-type Ca^{2+} channels colocalised with BK channels

can promote bursting as a result of overlapping calcium nanodomains (Prakriya & Lingle, 2000; Montefusco *et al*., 2017). To address whether changes in calcium handling might underly the changes in CS corticotroph behaviour we took two approaches. Firstly, using dynamic clamp in unstressed (control) corticotrophs, we increased the L-type calcium current alone. This resulted in an increase in single-spike frequency but did not induce bursting *per se*, an effect recapitulated in our mathematical model (Fig. 4A). Addition of 0.5 nS g_{Ca} (v_f = -5 mV, s_f = 12 mV, τ = 0.1 ms) to control cells (n = 4 cells from 3 preparations) resulted in an increase in event frequency 0.86 ± 0.43 Hz to 1.49 ± 0.74 Hz (P = 0.0301) but had no significant effect (P = 0.871) on event duration (event duration was 15 ± 5 ms after addition of g_{Ca}, compared to basal event duration of 14 ± 4 ms). Secondly, as an increase of g_{Ca} in dynamic clamp does not recapitulate the biological effects of increasing intracellular free calcium in cells we used our mathematical model of corticotroph excitability to probe the effect of enhancing intracellular free calcium by reducing calcium buffering capacity (Fig. 4B). In the corticotroph model, increasing the fraction of intracellular calcium that is free resulted in an increase in spike frequency but did not induce bursting. Taken together, while we cannot exclude that changes in calcium handling might contribute towards enhanced basal bursting in CS cells, an increase in calcium current or calcium buffering capacity *alone* is not sufficient to increase spontaneous bursting in CS corticotrophs.

Mathematical Modelling Suggests a Mechanism for Bursting in Basal Conditions in Stressed Cells

In the mathematical model (see Methods), the cell produces a low-frequency spiking pattern with occasional bursts under basal conditions (Fig. 5A). CRH-stimulated cells were simulated by making changes to the Ca^{2+} channel conductance, and the activation time constant for BK-near channels as well as the number of ZERO and STREX type channels, as described in Methods and figure captions. With these changes, the model cell primarily exhibits bursting behaviour (Fig. 5B), as is typically observed in the actual cells when stimulated with CRH (Figs. 2 and 3). Key elements for this change in behaviour from spiking to bursting is an increased co-localisation of BK channels near Ca^{2+} channels, and a decrease in the BK channel activation time constant for colocalised channels. We have previously shown that increasing fast-activating BK conductance facilitates bursting in corticotrophs (Duncan *et al.*, 2015, 2016).

Our central prediction for the effects of chronic stress on corticotroph electrical properties from the model is that it increases the fraction of ZERO-type BK channels that are functionally colocalised with Ca^{2+} channels as well as the range of the activation time constant for BK-near channels (see Methods). That is, we assume that chronic stress increases β_z to 0.8 and decreases the activation time constant for the colocalised channels to $\tau_{BK-n} = 2$ ms and above for both CRH-stimulated and unstimulated cells. As a result, the model cell often produces bursts in basal conditions (Fig. 5C), as observed experimentally (Figs. 2 and 3). When the application of CRH is simulated, the model cell remains in a bursting state with an increased event frequency (Fig. 5D), again as in experiments (Figs. 2 and 3).

Paxilline eliminates CRH-induced bursting in control cells, but not in all CS cells

We have previously demonstrated that BK channels play an important role in CRH-induced bursting in corticotroph cells, and that pharmacological blockade of BK channels with paxilline, a specific inhibitor of all BK channels irrespective of their molecular composition, prevents transition to bursting (Duncan *et al.*, 2015). Additionally, we have shown that inhibition of bursting by glucocorticoids can be rescued by addition of BK current using dynamic clamp (Duncan *et al.*, 2016). To determine whether the increase in both basal and CRH-evoked bursting behaviour seen in a subset of CS corticotrophs (10/15 cells) was BK dependent, cells were treated with the specific BK channel inhibitor paxilline (1 µM). When we applied paxilline to unstressed cells (n = 5 cells from 3 preparations), it had no significant effect on spontaneous activity (Fig. 6A and B) and inhibited bursting when CRH was applied in the presence of paxilline (Fig. 6C) as previously reported (Duncan *et al.*, 2015). Paxilline was applied to CS corticotrophs displaying spontaneous bursting (n = 3 cells from 2 preparations). Blockade of BK channels in "stressed bursters" caused a rapid cessation of basal bursting behaviour (Fig. 6D and E). This was reflected in a decrease in event duration from 162 ± 93 ms to 34 ± 29 ms (P = 0.0467). However, bursting recommenced in 3/3 cells following stimulation with 0.2 nM CRH (Fig. 6F) with event duration significantly increasing (P = 0.0287) to 192 ± 49 ms. Thus, the combination of CS and stimulation with CRH was effective at overcoming the inhibitory effect of paxilline on bursting behaviour. Is it possible that the few BK channels left unblocked by paxilline would be sufficient to generate bursting when the cell is both stressed and stimulated, but not either alone? To address this question we employed mathematical modelling, as described next.

Mathematical Modelling Can Explain Bursting in the Presence of Paxilline

One of the unexpected findings from corticotrophs of CS animals was the prevalence of bursting electrical activity even when the BK channel antagonist paxilline was present. However, this only occurred in CRH-stimulated cells (Fig. 6). To understand why, we simulated the application of paxilline in the mathematical model by assuming that the drug blocks approximately 90% of the BK channels (see Methods for details). In basal conditions, two channels remain unblocked and in CRH conditions three are unblocked. Although this channel number is small, the very large conductance (100-300 pS) of a single open BK channel (Lee & Cui, 2010; Latorre *et al.*, 2017) and the very small size (diameter of 7-15 μm) of corticotrophs (Duncan *et al.*, 2015; Fletcher *et al.*, 2017) makes the stochastic opening of just a few channels a significant event. In our simulations, we found that paxilline had little effect under basal conditions (Fig. 7A), where the cell spikes with low frequency with or without paxilline. In the CRH-stimulated model cells, the BK channel antagonist eliminated bursting (Fig. 7B), as found experimentally (Fig. 6C). In simulations of CS cells, paxilline eliminated bursting under basal conditions (Fig. 7C, compare with Fig. 6E). However, when the model cell was stimulated with CRH a mixed pattern of spiking and bursting was produced (Fig. 7D), similar to what is seen experimentally (Fig. 6F). Thus, bursting is possible in the model, even when all but three of the BK channels are blocked by paxilline.

To determine why paxilline blocks CRH-induced bursting in model unstressed cells, but not in model CS cells, we looked at the timing of BK channel openings during a spike or burst of spikes for both model cell types. Figure 8A and B shows the timing of BK-near channel openings during a burst in an unstressed (Fig. 8A) and CS (Fig. 8B) model cell without any BK channel blockage. Both ZERO-type channel openings (depicted with purple circles) and STREX-type channel opening (depicted with orange circles) occur throughout the burst when the cell is depolarized. This is shown as raster plots in the lower panels of Fig. 8A and B. The number of open ZERO-type BK-near channels is in purple, while the number of open STREX-type BK-near channels is in orange. Because a higher fraction of ZERO-type channels are colocalised with Ca^{2+} channels in the model CS cell, there are more instances of multiple ZERO-type channel openings. BK channels not colocalised with Ca^{2+} channels open later than the BK-near channels, and thus do not contribute to burst generation.

In simulations in which paxilline is present, the situation is quite different. For an unstressed CRH-stimulated cell, there are very few channel openings near the peak of a spike,

and the resulting BK current is too small to promote bursting (Fig. 8C). In the case of the CS cell, there are a sufficient number of ZERO-type channel openings to convert a single spike into a burst (Fig. 8D). This occurs because 80% of the ZERO-type channels are assumed to be colocalised with Ca^{2+} channels (in contrast to the 20% of STREX-type channels), so even if 90% of all BK channels are blocked by paxilline, there are enough BK-near channels to provide the hyperpolarizing current necessary to convert a spike into a burst.

In summary, the model suggests that the reason bursting in the presence of paxilline occurs in the CS cell and not the unstressed cell is that the total BK current near the peak of an action potential is greater in the CS cell (Fig. 8D) than in the unstressed cell (Fig. 8C). It has been shown previously in mathematical studies with experimental verification employing the dynamic clamp technique that the presence of fast-activating BK current is decisive in converting a single spike into a burst of spikes (Duncan *et al*., 2016; Tabak *et al*., 2011; Vo *et al*., 2014). We add to that by showing, in Fig. 8, that it is the BK channel openings near the peak of the action potential that are key for burst production.

Dynamic Clamp Supports a Key Model Prediction about the Role for STREX Channels in Lengthening Bursts

A key prediction from our modelling data is that the fraction of the two BK channel types with different properties, ZERO and STREX, functionally coupled with L-type Ca^{2+} channels is important for the transition to bursting as well as *the duration* of bursting. Sufficient BK-ZERO activity is sufficient to produce bursting, whereas the additional presence of STREX would produce a longer duration of bursting. Indeed, in model cells in basal conditions, introduction of ZERO alone can induce bursting behaviour (Fig. 9B). However, addition of STREX-type BK conductance results in bursting events of longer duration, even though the total added BK conductance is smaller (Fig. 9C). This could be recapitulated in dynamic clamp experiments in unstressed corticotrophs (n = 7 cells from 3 preparations) exposed to paxilline to block endogenous BK channel activity. Under basal conditions these cells transitioned from spiking (Fig. 9D) to primarily bursting upon introduction of BK-ZERO (Fig. 9E). When both ZERO-type and STREX-type currents were added to the same cell, the burst duration was increased even though the total added BK conductance was smaller (Fig. 9F). Addition of $g_{BK-ZERO}$ increased event duration from 42 ± 21 ms to 185 ± 106 ms (P = 0.0893). A combination of both $g_{BK-ZERO}$ and $g_{BK-STREX}$ significantly (P = 0.0334) further

increased event duration to 363 ± 215 ms, compared to $g_{BK-ZERO}$ alone. These data support synergistic roles for the two types of BK channels in shaping the electrical behaviour of the corticotrophs.

Heterogeneity in Spontaneous Activity Can Be Explained By Heterogeneity in BK Channel Activation Time Constant and Location

A peculiar finding from the data in Fig. 3D is that for both unstimulated and stimulated CS cells the burst factors covered almost the entire interval from 0 to 1. A closer examination of the data indicated that some cells from CS animals spiked tonically with almost no bursting events (the "stressed spikers"), while others were at the other extreme, with about 80-85% of events consisting of bursts whether stimulated or not (the "stressed bursters"). In contrast, the burst factor was always small in the unstressed cells under basal conditions, and only increased when stimulated with CRH. We wished to understand the mechanism for this great heterogeneity in activity of the stressed cells. To this end, we performed computer simulations in which the values of the BK-channel co-localisation parameters (β_s and β_z) and the BK-near activation time constant (τ_{BK-n}) were randomly sampled as described in Methods. The parameter space is thus 3-dimensional, with each sample reflecting a particular choice of β_s, β_z, and τ_{BK-n}. The model was then run for a duration of 50 s with this choice of parameters and the burst factor computed. For each condition (unstressed basal, unstressed stimulated, CS basal, CS stimulated) 1000 samples were generated. We asked what ranges of τ_{BK-n} values were sufficient to qualitatively match the burst factor distributions in the data of Fig. 3D.

Figure 10A shows a dot-plot of the burst factors in the 3-dimensional parameter space for model CS cells that are in basal conditions. The size and color of the dots indicates the size of the burst factor (small blue dots indicate cells that primarily spike and thus have small burst factor, while large red dots indicate cells that primarily burst and thus have large burst factor). As is clear from this plot, the main parameter for determining whether a cell is spiking or bursting is τ_{BK-n}: the burst factor is larger for smaller values of this time constant.

Figure 10B shows box plots of the burst factor for the four classes of model cells that resulted from uniformly sampling the parameter space, noting that the parameter ranges that define the four classes of cells differ (see Methods). For the control model cells in basal conditions, a range of τ_{BK-n} values from 17-26 ms produced electrical patterns with low burst

factors (median=0.14) and low variation (interquartile range, IQR=0.08). For the control CRH-stimulated model cells, a range of τ_{BK-n} values from 2-8 ms produced electrical patterns with much higher burst factors (median=0.85) and greater variation (IQR=0.33). For the CS basal model cells with a range of τ_{BK-n} values from 2-26 ms the burst factors were typically larger (median=0.28) and showed much greater variation (IQR=0.47) than the comparable unstressed model cells. Finally, for the CS and stimulated model cells with a range of τ_{BK-n} values from 2-17 ms the burst factors were similarly large (median=0.58) and large variation (IQR=0.61). Thus, with these ranges of τ_{BK-n} values for the four classes of model cells, the burst factor box plots share features of the data. Most importantly, the simulations display the similar greater burst factor heterogeneity in the chronically stressed cells compared to the unstressed cells.

The top panels of Fig. 11 show histograms of the burst factor for the four populations of model cells (the same populations used to make Fig. 10B). For control model cells in basal conditions there is a unimodal distribution (Fig. 11A, blue), with mean (blue dashed line) of approximately 0.1. However, for CS model cells in basal conditions the distribution of burst factors is much more spread out and apparently multimodal (Fig. 11A, red). There is a cluster of cells that primarily spike (left), a cluster that primarily bursts (right), and a third cluster that exhibits a mix of bursting and spiking (middle). With this multimodal distribution, the mean (red dashed line) provides little or no useful information about the distribution itself. For the control model cells that are CRH-stimulated, all have time courses where the majority of events are bursts (Fig. 11B, blue), consistent with the role of CRH as a stimulating factor for corticotrophs. For the CS and CRH-stimulated model cells, there again appears to be a multimodal distribution. One mode consists of cells that primarily spike (left), another that contains cells that primarily burst (right), and one mode consists of cells with a roughly even mix of spiking and bursting (middle).

The model burst factor distributions are compared with experimental histograms using the experimental data in the bottom row of Fig. 11. These histograms (n = 10 cells from 5 preparations for control groups, and n = 15 cells from 3 preparations for CS groups) display some similarity to the model histograms, even though the sample number is much lower. In particular, the distribution of burst factors is unimodal for control cells in basal conditions, with most cells having burst factor below 0.3 (Fig. 11C, blue). In contrast, the burst factors for CS cells in basal conditions has an apparent bimodal distribution, with a primarily-spiking

subpopulation and a primarily-bursting subpopulation (Fig. 11C, red). Again, for a multimodal distribution like this the mean value (red dashed line) provides little or no useful information. The control cells in the presence of CRH are almost entirely distributed with burst factor of 0.5 or greater (Fig. 11D, blue), very much as in the model histogram. The CS CRH-stimulated cells appear to have a multimodal distribution (Fig. 11D, red), as in the model simulations, with subpopulations of spikers (the "stressed spikers"), subpopulations of bursters (the "stressed bursters") and two cells with roughly equal fractions of spikes and bursts. Remarkably, the mean values of the experimental distributions (dashed lines in Fig. 11C, D) are similar to those of the model cell subpopulations (dashed lines in Fig. 11A, B).

DISCUSSION

Anterior pituitary corticotrophs have to be able to respond with an appropriate output in the face of very different physiological challenges, for example in response to an acute or chronic stress. A key determinant of output is the ability to control the pattern of electrical excitability between single spike activity and bursting behaviour (Duncan *et al.*, 2015, 2016; Fletcher *et al.*, 2017). Here we show that in mouse male corticotrophs the distinct properties, stochastic activity and functional localisation of large conductance calcium- and voltage- activated potassium (BK) channels with L-type Ca^{2+} channels allows fine tuning of corticotroph excitability in the face of short- and long-term physiological challenges.

Chronic stress promotes BK-channel dependent spontaneous bursting in corticotrophs

By integrating patch clamp electrophysiological analysis of native murine corticotrophs with mathematical modelling and dynamic clamp we reveal that isolated corticotrophs from CS male mice display spontaneous electrical bursting behaviour compared to the tonic spiking behaviour of corticotrophs from unstressed mice. This enhanced spontaneous bursting behaviour is dependent on functional BK channels as it can be blocked by the specific BK channel inhibitor paxilline. Importantly, CS corticotrophs display a greater heterogeneity in spontaneous spiking and bursting behaviour that is dependent on three main factors: i) the properties of different populations of BK channels in corticotrophs, that is the fraction of each population functionally coupled to Ca^{2+} influx through L-type Ca^{2+} channels; and , ii) the time constant for BK channel activation for BK channels close to L-type Ca^{2+} channels. Importantly, these properties determine the timing of BK channel activity during a typical spike and hence the effect of the channel population on the ability to transition to and sustain bursting behaviour. Mathematical modelling and dynamic clamp experiments reveal that the properties of the distinct populations of BK channels correlate with the two major BK channel splice variants expressed in corticotrophs: ZERO and STREX and their relative coupling to voltage dependent Ca^{2+} influx. Moreover, the distinct regulation of these two variants by the cAMP-dependent protein kinase A signalling pathway (Tian *et al.*, 2001, 2004) that is controlled by the major secretagogue CRH provides a mechanism for the transition to bursting behaviour in the presence of CRH.

Importantly, mathematical modelling revealed that the spontaneous bursting observed in corticotrophs from CS mice results from a larger proportion of BK channels being

open at the peak of a typical spike than in corticotrophs from unstressed mice. This is a consequence of a greater proportion of the ZERO variant channels being open at the peak of an action potential in CS corticotrophs as a result of their properties. Modelling predicts that a greater proportion are functionally coupled to Ca^{2+} influx through L-type Ca^{2+} channels and is critically dependent upon the time constant for BK channel activation being sufficiently small.

While our dynamic clamp analysis supports this prediction, definitive analysis of CS-induced changes in activation time constant of native BK channels in metabolically intact corticotrophs remains a significant challenge. We cannot easily discriminate BK currents resulting from a potentially very small number of BK channels colocalised to Ca^{2+} channels from those further away from Ca^{2+} channels in whole cell recordings. Definitive analysis would require, for example, single channel patch clamp recordings in which we can simultaneously monitor both BK and Ca^{2+} currents in the same patch.

How CS might increase the fraction of BK channels coupled to Ca^{2+} channels also remains to be determined, however, a number of different mechanisms may play a role. The expression of BK channels and alterative splicing of the ZERO and STREX variants can be dynamically regulated including by circulating glucocorticoid hormones, intrinsic cellular excitability as well as a diverse array of other signalling pathways (Xie & McCobb, 1998; Xie & Black, 2001; Lai & McCobb, 2006; Chatterjee *et al.*, 2009). In addition, increased functional coupling to Ca^{2+} channels and/or changes in activation time constant may result from a wide variety of mechanisms including through postranslational modification of BK channel pore forming subunits themselves, assembly with regulatory and other accessory subunits and/or changes in components of BK channel macromolecular signalling complexes that are likely important for localisation with Ca^{2+} channels (Lee & Cui, 2010; Berkefeld *et al.*, 2010; Contreras *et al.*, 2013; Shipston & Tian, 2016; Montefusco *et al.*, 2017; Latorre *et al.*, 2017). Clearly, understanding the diversity of mechanisms by which this predicted enhanced functional coupling occurs in CS warrants further investigation.

Stochastic behaviour of BK channels allows CRH- and BK channel- dependent bursting

An initially surprising observation in our electrophysiological assays was that in CS corticotrophs, in contrast to corticotrophs from unstressed mice, CRH could still significantly promote bursting in the presence of the BK channel specific inhibitor paxilline used at

saturating concentrations to largely inhibit macroscopic currents (IC_{50} is in 10 nanomolar range (Zhou & Lingle, 2014; Zhou *et al.*, 2020)). While the simplest explanation would be that CRH engages BK-independent mechanisms in CS, but not unstressed corticotrophs, two properties of BK channels and their blockade by paxilline prompted us to explore an alternative hypothesis that the stochastic behaviour of a small number of BK channels is sufficient to allow bursting. Firstly, BK channels have a large conductance (in physiological potassium gradients > 100 pS, a single channel conductance that is 1 to 2 orders of magnitude larger than most potassium channels (Lee & Cui, 2010; Latorre *et al.*, 2017)). Thus, the opening of a small number or very low activity of a larger population of BK channels can have significant physiological effects, especially in small cells such as corticotrophs. Secondly, the inhibitory effect of paxilline is inversely related to the BK channel open probability (Po) (Zhou & Lingle, 2014; Zhou *et al.*, 2020) with paxilline inhibiting BK channels by an almost exclusive closed channel block mechanism. Thus, even in the continued presence of paxilline, BK channels with very high Po can remain largely unblocked by paxilline, conditions that are likely the case for BK channels during the peak of an action potential that are functionally coupled to voltage gated Ca^{2+} influx (Zhou & Lingle, 2014) and in corticotrophs where resting membrane potential is also typically depolarized (~ -45 mV) compared to neurons. Because of our stochastic implementation of BK channel kinetics in the model, we were in a position to explore this possibility. Indeed, we found that with only a few ZERO-type BK channels colocalised with Ca^{2+} channels it was possible to rescue bursting in CRH-stimulated CS cells. The ability of CRH to induce significant bursting in CS corticotrophs in the presence of paxilline would thus also fit with our observations that CS results in a greater proportion of BK channels being functionally coupled to voltage gated Ca^{2+} influx creating conditions where BK channel blockade by paxilline is less efficient.

Implications for corticotroph and endocrine physiology

Our findings, that the properties of different populations of BK channels including their time constant for activation and functional coupling to L-type Ca^{2+} channels in corticotrophs that can be modified by physiological challenge, has important implications for both corticotroph physiology and endocrine excitability more generally. In corticotrophs, our studies show that the properties and function of BK channels can be finely tuned by: acute (seconds to minutes) hypothalamic input from CRH to promote the transition from spiking to

bursting; short-term (tens of minutes – hours) by glucocorticoid negative feedback that prevents CRH-induced bursting (Duncan *et al.*, 2015, 2016); and, longer-term control (days/weeks), for example in response to a major physiological challenge such as CS. While the precise mechanisms underlying the changes in BK channel function over these very wide time domains are largely unknown, the ability to differentially and dynamically change BK channel properties and regulation in response to acute and chronic challenge provides a mechanism to fine tune corticotroph output in response to changing physiological demands. In the case of corticotrophs, acute exposure to the hypothalamic secretagogue CRH engages BK channels to promote the transition from single cell spiking that is blocked by early glucocorticoid feedback (Duncan *et al.*, 2015, 2016), whereas in CS, as shown here, the increased spontaneous bursting activity is dependent upon BK channels being functional under non-stimulated conditions. One of the important features of the regulation of excitability in CS is that overall the mean changes in several parameters of excitability are not significantly different but the variability across the population is much higher in corticotrophs from CS animals. In fact, the burst factors of CS cells spans the full range of values resulting in a population of cells that are primarily spikers and a population of cells that are primarily bursters (Fig. 10). With such distributions, mean values provide little or no useful information. The large variation across the population in CS may allow a much wider dynamic range to be achieved by the entire corticotroph population for example, by allowing appropriate responses to acute stress during CS and as seen in other contexts and pituitary cell types (Snyder *et al.*, 1977; Billiard, 1996; Tomaiuolo *et al.*, 2010; Romanò *et al.*, 2017).

BK channels have also been shown to be important in controlling bursting in other pituitary and endocrine cells (Van Goor *et al.*, 2001*a*; Tabak *et al.*, 2011). While a functional role for BK channels has largely been ascribed to cells that express large BK currents, our work emphasizes the need to interrogate the functional role of BK channels in a physiologically context dependent manner. For example, low level of BK channel expression may correlate with the lack of a role for BK channels in spontaneous bursting activity in some pituitary cell types (Van Goor *et al.*, 2001*c*, 2001*a*). However, because of the potential impact of the stochastic behaviour of a few large conductance BK channels, in particular in small cells with high input resistance, the role of BK channels may be very context dependent as we see for corticotrophs in response to acute CRH exposure or chronic stress. Thus, the modification of the properties and functional localisation of a small number of BK channels with voltage gated

Ca^{2+} influx by different physiological challenges may have a profound effect on the control of cellular excitability more broadly in pituitary and other endocrine cell types.

Data availability statement

Data supporting the results presented in this manuscript are included in the manuscript figures. Computer codes for model are available at www.math.fsu.edu/~bertram/software/pituitary

Competing Interest

All authors declare they have no competing interests/conflicts of interest in this work.

Author contributions

PJD, MF & NR contributed to the acquisition, analysis and interpretation of data for the work. PJD, MF, NR, PLeT, RB & MJS contributed to the conception, design, interpretation and drafting of the work and revised it critically for important intellectual content. All authors have approved the final version of the manuscript; 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; and all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Figure Legends

Abstract figure legend: Coordination of an appropriate stress response is dependent upon anterior pituitary corticotroph excitability in response to hypothalamic secretagogues and glucocorticoid negative feedback. A mathematical model of corticotroph cells (middle) was generated to predict how changes in ion channel properties may explain modulation of corticotroph excitability by chronic stress (CS). A key determinant of corticotroph excitability is large conductance calcium- and voltage- activated (BK) potassium channels that are critical for promoting CRH-induced bursting that enhances ACTH secretion. Bursting behaviour is modulated by the properties of two BK channel splice variants, ZERO (BKz) and STREX (BKs), as well as their proximity to L-type Ca^{2+} channels (near or far). Electrophysiological data was generated from corticotrophs isolated from POMC-GFP mice (top). Mice subjected to a two-week CS paradigm (14 days of restraint stress; 30 minutes per day) showed an increase in basal pseudo-plateau bursting behaviour. Experimental observations were used to refine the mathematical model and predict how varying the properties of BK ZERO and STREX populations could determine bursting behaviour (right). The model predicted that a combination of ZERO and STREX conductances was more efficient at enhancing bursting behaviour compared to ZERO alone (bottom). The dynamic clamp technique (left) was used to test the model prediction (left). Artificial ZERO and STREX currents were injected into native corticotroph which was shown to enhance bursting behaviour. By characterising changes in excitability in CS corticotrophs using complementary experimental and modelling techniques, we aim to identify potential targets for therapeutic intervention.

Figure 1: (A) Illustration of the four populations of BK channels used in the model, and the effect that CRH has on channel activation and deactivation. The red arc indicates co-localisation of BK and L-type Ca^{2+} channels. (B) Activation curves for BK channels of STREX (purple) and ZERO (orange) types.

Figure 2: Representative perforated patch clamp electrical recordings from corticotrophs from unstressed and chronically stressed (CS) male mice. (A) A cell from an unstressed mouse under basal conditions exhibiting tonic spiking. (B) The same cell in the presence of CRH. (C) A cell from a stressed mouse under basal conditions exhibiting bursting. (D) The same cell with application of CRH. Because the cell in panels C & D bursts whether stimulated or not

stimulated it is referred to as a “stressed burster”. (E) A different cell from a stressed mouse exhibiting spiking. (F) The same cell continues to spike, but at a higher rate, in the presence of CRH. Because the cell in panels E & F always spikes, it is referred to as a “stressed spiker”.

Figure 3: Quantification of electrical features in corticotrophs from unstressed (Control) and chronically stressed (CS) mice in basal conditions or stimulated by CRH. (A) There was no effect of CS on basal or CRH-induced membrane potential (P = 0.434). (B) Basal and CRH-evoked event frequency is not significantly altered by chronic stress (P = 0.439), but CS cells show a wider range of CRH-induced event frequency. CS results in a higher instance of spontaneous bursting activity which is represented by an in increase in event duration (C) and burst factor (D). Basal event frequency was significantly (P = 0.046) increased in CS cells but not burst factor (P = 0.063). CRH significantly increased event duration and burst factor in control cells (P < 0.0001 and P = 0.00320). In contrast, CS cells showed a mixture of bursting and spiking following CRH-stimulation, resulting in a wide range of event duration and burst factors. CRH-induced event duration and burst factor was not significantly different to controls (P = 0.380 and P = 0.107 respectively). There are n = 10 cells from 5 preparations in each control group and n = 15 cells from 3 preparations in each chronically stressed group.

Figure 4: Effect of changes in Ca^{2+} dynamics. (A) In control unstressed corticotrophs increasing g_{Ca} (g_{Ca}= 0.5 nS, v_f = -5 mV, τ = 0.1 ms) using dynamic clamp results in an increase in spontaneous spike frequency but does not induce spontaneous bursting. (B) In the model, a decrease in Ca^{2+} buffering in the control unstressed model cell (an increase in the parameter f_c from 0.005 to 0.05), resulting in elevated intracellular free Ca^{2+}, increases spontaneous spike frequency but does not induce spontaneous bursting. In all cases, $g_{NS} = 0.125$ nS and $\tau_{BK-n} = 15$ ms.

Figure 5: Model simulations of control and stressed cells. (A) Under basal conditions, an unstressed model cell typically exhibit low-frequency spiking. (B) When application of CRH is simulated, an unstressed model cell shows a high degree of bursting. (C) Under basal conditions, a stressed model cell often bursts, unlike the case in unstressed cells. (D) When stimulated with CRH, a stressed model cell again often bursts. For these simulations: control

cell values $\beta_z = 0.2$, $\tau_{BK-n} = 5$ ms; stressed cell values $\beta_z = 0.8$, $\tau_{BK-n} = 5$ ms; basal cell values $g_{Ca} = 1.85$nS, N_s = 10, $N_z = 5$; stimulated cell values $g_{Ca} = 2.1$nS, N_s = 5, $N_z = 20$.

Figure 6: Paxilline (1 μM) has differential effects in stressed and unstressed cells. (A) An example of an unstressed corticotroph under basal conditions (n = 5 cells from 3 preparations). (B) The same cell with application of paxilline. The BK channel antagonist has no apparent effect on the spiking behaviour. (C) The same cell in the presence of both paxilline and CRH. Although the event frequency is greater, bursting is not produced. (D) A mix of spiking and bursting in a stressed cell (n = 3 cells from 1 preparation). (E) Paxilline eliminates bursting in the stressed cell. (F) Subsequent application of CRH rescues bursting in the stressed cell.

Figure 7: Model simulations of control and stressed cells in the presence of paxilline. (A) An unstressed model cell exhibits low-frequency spiking in basal conditions. (B) Even with CRH present, a control model cell exhibits spiking and no bursting, with an increased event frequency. (C) A stressed model cell spikes in basal conditions. (D) With CRH stimulation, a stressed model cell exhibits bursting mixed with spiking. For these simulations: control cell values $\beta_z = 0.2$, $\tau_{BK-n} = 5$ ms; stressed cell values $\beta_z = 0.8$, $\tau_{BK-n} = 5$ ms; basal cell values $g_{Ca} = 1.85$nS, N_s = 10, $N_z = 5$; CRH-stimulated cell values $g_{Ca} = 2.1$nS, N_s = 5, $N_z = 20$. Paxilline is simulated by blocking approximately 90% of the BK channels.

Figure 8: Model simulations of CRH-stimulated cells show the timing of BK-near channel openings of STREX and ZERO types. (A) In a model control cell, there are BK-near channels openings of each type throughout the burst. The top figure shows the timing of channel openings superimposed on the burst, while the lower figure shows a raster plot. ZERO-type channel openings are in purple, STREX-type openings are in orange. (B) In a model stressed cell there are more multiple channel openings of ZERO-type channels, but openings still occur throughout the burst. (C) In the presence of paxilline, a model control cell produces action potentials, but not bursts. The few BK-near channel openings occur near the action potential peak, and are of ZERO type. (D) In the presence of paxilline, a model stressed cells bursts, with BK-near channel openings near the peaks of the spikes and are primarily of ZERO type. For these simulations: control cell values $\beta_z = 0.2$, $\tau_{BK-n} = 5$ ms; stressed cell values $\beta_z = 0.8$,

$\tau_{BK-n} = 5$ ms; for all cases $g_{Ca} = 2.1$nS, N_s = 5, $N_z = 20$. Paxilline is simulated by blocking approximately 90% of the BK channels.

Figure 9: Model simulations and dynamic clamp experiments show a role for ZERO and STREX current in promoting bursting. (A) In the presence of paxilline, a model cell displays spiking behaviour. For these simulations $\beta_z = 0.2$, $\tau_{BK-n} = 5$ ms, $g_{Ca} = 1.85$nS, N_s = 10, $N_z = 5$. (B) The addition of ZERO-type current ($g_{BK-z} = 3.8$nS, $v_f = -5$ mV, $\tau_{BK} = 5$ms) results in a transition to bursting behaviour. (C) When a smaller ZERO-type BK conductance ($g_{BK-z} =$ 2nS, $v_f = -5$ mV, $\tau_{BK} = 5$ms) is supplemented with a STREX-type conductance ($g_{BK-s} =$ 1.35nS, $v_f = -20$ mV, $\tau_{BK} = 5$ms), the burst duration is increased, although the total added BK conductance ($g_{BK-z} + g_{BK-s} = 3.35$nS versus 3.8 nS) is smaller. Dynamic clamp was used to mimic conditions in model simulations. (D) In the presence of paxilline, unstressed corticotrophs cells (n = 7 cells from 3 preparations) displayed spiking activity. (E) The addition of a ZERO-type conductance ($g_{BK-ZERO}$= 4 nS, v_f = -5 mV, τ_{BK}= 5 ms) resulted in short duration bursting activity. (F) A combination of ZERO-type ($g_{BK-ZERO}$= 2 nS, v_f = -5 mV, τ_{BK}= 5 ms) and STREX-type ($g_{BK-STREX}$= 1 nS, v_f = -20 mV, τ_{BK}= 5 ms) conductance resulted in longer burst events, even though the total added conductance is smaller.

Figure 10: (A) Distribution of burst factors in stressed model cells in basal conditions over a 3-dimensional parameter space. The size and color of each dot indicate the burst factor. Parameter values that give small blue dots are cells that are primarily spiking, while those with large red dots are primarily bursting. (B) Burst factor quantification calculated from populations of 1000 model cells with β_s, β_z, and τ_{BK-n} determined through random sampling as described in Methods and the text.

Figure 11: Histograms of burst factors from model cells and experiments. (A) Model control cells have a unimodal distribution in basal conditions, but stressed cells have a multimodal distribution. (B) Model control and CRH-stimulated cells have burst factor greater than 0.5. Stressed CRH-stimulated cells have a multimodal burst factor distribution. (C) Burst factor histograms of the data from Fig. 3D. Like in the model, the distribution is unimodal for control cells in basal conditions, but multimodal for stressed cells in basal conditions. (D) Experimental burst factor distributions of CRH-stimulated cells again recapitulate features of

the model distributions, with most control cells having burst factor greater than 0.5 and stressed cells exhibiting a multimodal burst factor distribution. There are n = 10 cells from 5 preparations in each control group and n = 15 cells from 3 preparations in each chronically stressed group.

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