Large conductance Ca²-activated K (BK) channels promote secretagogue-induced transition from spiking to bursting in murine anterior pituitary corticotrophs

Title: Large conductance Ca^{2+}-activated K^+ channels (BK) promote secretagogue-induced transition from spiking to bursting in murine anterior pituitary corticotrophs

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KEY POINTS

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- Corticotroph cells of the anterior pituitary are electrically excitable and are an integral component of the HPA axis which governs the neuroendocrine response to stress.
- Corticotrophs display predominantly single spike activity under basal conditions that transition to complex bursting behaviours upon stimulation by the hypothalamic secretagogues CRH and AVP, however the underlying mechanisms controlling bursting are unknown.
- In this study, we show that CRH and AVP induce different patterns of corticotroph electrical activity, and we use an electrophysiological approach combined with mathematical modeling to show the ionic mechanisms for these differential effects.
- The data reveals that secretagogue-induced bursting is dependent on large conductance Ca^{2+}-activated K^{+} (BK) channels and is driven primarily by CRH whereas AVP promotes an increase in single-spike frequency through BK-independent pathways involving activation of non-selective cation conductances.
- As corticotroph excitability is differentially regulated by CRH and AVP this may allow corticotrophs to respond appropriately to different stressors.

Word count: 150

ABSTRACT

Anterior pituitary corticotroph cells are a central component of the hypothalamic-pituitary-adrenal (HPA) axis essential for the neuroendocrine response to stress. Corticotrophs are excitable cells that receive input from two hypothalamic secretagogues, corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) to control the release of adrenocorticotrophin hormone (ACTH). Although corticotrophs are spontaneously active and increase in excitability in response to CRH and AVP the patterns of electrical excitability and

underlying ionic conductances are poorly understood. In this study, we have used electrophysiological, pharmacological and genetic approaches coupled with mathematical modeling to investigate whether CRH and AVP promote distinct patterns of electrical excitability and to interrogate the role of large conductance calcium- and voltage-activated (BK) channels in spontaneous and secretagogue-induced activity. We reveal that BK channels do not play a significant role in the generation of spontaneous activity but are critical for the transition to bursting in response to CRH. In contrast, AVP promotes an increase in single spike frequency, a mechanism independent of BK channels but dependent on background non-selective conductances. Co-stimulation with CRH and AVP results in complex patterns of excitability including increases in both single spike frequency and bursting. The ability of corticotroph excitability to be differentially regulated by hypothalamic secretagogues provides a mechanism for differential control of corticotroph excitability in response to different stressors.

Abbreviations: ACTH, adrenocorticotrophic hormone; AVP, arginine vasopressin; BK, large conductance Ca^{2+} - and voltage-activated potassium channel; CRH, corticotrophin-releasing hormone; HPA, hypothalamic-pituitary-adrenal; IK, intermediate conductance Ca^{2+}-activated potassium channel; NMDG, *N*-methyl-D-glucamine; POMC, proopiomelanocortin; PVN, paraventricular nucleus; STREX, stress regulated exon; ZERO, BK channels lacking STREX insert.

INTRODUCTION

Excitable cells, such as neurons and endocrine cells, exhibit diverse patterns of spontaneous electrical activity that can be modified in response to neuropeptide stimulation. These diverse

responses are coordinated by an eclectic array of ion channels and establishing the role of any particular ion channel remains a significant challenge.

Endocrine cells of the anterior pituitary typically display spontaneous activity (Stojilkovic *et al.*, 2010) that is characterized by either single action potentials or bursts of electrical activity, termed "pseudo-plateau bursting", that results in small oscillations of the membrane potential during the active phase of the burst, rather than full spikes (Tsaneva-Atanasova *et al.*, 2007; Stern *et al.*, 2008; Vo *et al.*, 2014). Pseudo-plateau bursting has been proposed to increase intracellular Ca^{2+} to a greater extent than spiking alone which is thought to be important in driving hormone secretion in endocrine cells (Van Goor *et al.*, 2001*c*; Stojilkovic *et al.*, 2005). Previous studies have identified a role for large conductance Ca^{2+}- and voltage-activated potassium (BK) channels in the generation of *spontaneous* pseudo-plateau bursting in anterior pituitary somatotrophs and lactotrophs (Van Goor *et al.*, 2001*a*; Tsaneva-Atanasova *et al.*, 2007; Tabak *et al.*, 2011) whereas gonadotrophs, that express little BK current, show spontaneous single spiking (Van Goor *et al.*, 2001*b*; 2001*c*; Stojilkovic *et al.*, 2010).

However, anterior pituitary corticotrophs, the central hub of the hypothalamic-pituitary-adrenal (HPA) axis which governs the homeostatic response to stress, express BK channels (Shipston & Armstrong, 1996; Shipston *et al.*, 1999; Tsaneva-Atanasova *et al.*, 2007; Brunton *et al.*, 2007; Stern *et al.*, 2008; Liang *et al.*, 2011; Vo *et al.*, 2014) yet display predominantly spontaneous single spike activity but can *transition* to pseudo-plateau bursting when stimulated (Kuryshev *et al.*, 1997; Van Goor *et al.*, 2001*c*; Stojilkovic *et al.*, 2005; Liang *et al.*, 2011). This suggests that under unstimulated conditions BK channels in corticotrophs do not contribute to spontaneous electrical activity but may promote pseudo-plateau bursting in response to the hypothalamic secretagogues corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). CRH and AVP activate distinct G-protein

receptor signaling cascades: the cAMP/PKA and IP3/PKC pathways respectively (Antoni, 1986; King & Baertschi, 1990; Van Goor *et al.*, 2001*a*; Tsaneva-Atanasova *et al.*, 2007; Tabak *et al.*, 2011), both of which have been reported to control BK channel activity and properties in pituitary cells, including corticotrophs (Shipston *et al.*, 1996; Tian *et al.*, 2008; Zhou *et al.*, 2012*b*).

Fast activating BK channels (BK-near), in close proximity to voltage activated calcium channels, have been demonstrated to facilitate pseudo-plateau bursting (Tabak *et al.*, 2011). The rapid activation of the channels that occurs during the upstroke of an action potential limits the spike amplitude and activation of delayed rectifier K^+ channels and thus allows the membrane potential to oscillate, resulting in a burst (Tabak *et al.*, 2011; Vo *et al.*, 2014). BK channels that are located distantly from voltage-gated Ca^{2+} channels (BK-far) are responsible for the termination of a burst (Tsaneva-Atanasova *et al.*, 2007). While the molecular basis for these different populations is not established, importantly functional diversity of BK channel properties in anterior pituitary cells can be conferred by multiple mechanisms including alternative pre-mRNA splicing and post-translational modification of the pore-forming subunit encoded by a single gene, KCNMA1 (for example see Tian *et al.*, 2004; Chen *et al.*, 2005; Tian *et al.*, 2008; Stojilkovic *et al.*, 2010).

In this study, we have developed a mathematical model of corticotrophs and used this in conjunction with pharmacological and genetic approaches to interrogate the role of BK channels in spontaneous and CRH/AVP-evoked electrical activity in native male mouse corticotrophs. We reveal that corticotroph BK channels do not play a significant role in spontaneous electrical activity but are essential for the transition to bursting upon CRH-stimulation. Furthermore, we reveal that AVP, in contrast to CRH, does not promote bursting, rather it increases the frequency of single spikes and is independent of functional BK channels. Thus CRH and AVP engage BK channel-dependent and –independent pathways

respectively and BK channels are essential for the CRH-induced transition to pseudo-plateau bursting in murine corticotrophs.

METHODS

Animals

Mice lacking the pore-forming exon of the BK channel α-subunit ($BK^{-/-}$ mice; Sausbier *et al.*, 2004) were backcrossed for at least 10 generations with mice expressing GFP under the POMC promoter (Pinto *et al.*, 2004) to generate BK-POMC-GFP mice with a SV129/C57BL6 mixed background. More than 99% of GFP positive cells also stain for ACTH in our assays and thus all GFP positive cells of the anterior pituitary are corticotrophs and lack any functional BK channels in the $BK^{-/-}$ background. The genotype of all animals used was verified for each pituitary isolation and culture generated. The mice show normal gross pituitary morphology and the same number of corticotrophs although pituitary ACTH content, but not POMC mRNA, is suppressed as reported previously for the $BK^{-/-}$ mice (Brunton *et al.*, 2007) compared to their littermate WT controls. Mice were caged in groups of two to four under standard laboratory conditions (lights on at 07:00, lights off at 19:00, 21ºC, with tap water and chow available *ad libitum*). Wild-type (WT) or mice deficient for the BK channel ($BK^{-/-}$) were used from the same litters generated by a cross of mice heterozygous for the BK allele. Male mice, aged 2-5 months, were used for pituitary cell culture with tissue collection performed between 08:30 and 10:00 in accordance with United Kingdom Home Office requirements (PPL 60/4349) and University of Edinburgh ethical review committee.

Pituitary cell culture

Three to four mice were killed by cervical dislocation and the pituitaries removed and cut to remove the intermediate (which contain POMC-expressing melanotrophs) and posterior lobes. The remaining anterior lobe was chopped by hand with a single edged razor blade in two directions (pituitary rotated by 90º). The tissue was digested in a solution of DMEM (Invitrogen) containing 25 mM HEPES, 0.25% trypsin (Worthington) and 10 μg/ml DNAse I for 20 minutes in a 37ºC water bath. The tube was shaken every five minutes to ensure a complete and even digestion. Following digestion, the tissue was allowed to settle to the bottom and the supernatant aspirated. One ml of inhibition solution (DMEM containing 0.5 mg/ml Soybean Trypsin inhibitor, 100 kallikrein units aprotinin (200 x dilution of Sigma stock), 10 μg/ml DNAse I) was added and triturated using a P1000 pipetteman (Gilson) set to 1 ml (~40 times). A further 4 ml of inhibition solution was added and the resulting cell suspension was filtered through a pre-wetted 70 μm nylon mesh (BD Bioscience) and diluted with an equal volume of culture media (DMEM containing 25 mM HEPES, 5 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 0.3% BSA (w/v), 4.2 μg/ml fibronectin and antibiotic/antimycotic (100x dilution of sigma stock)) and spun in a centrifuge at 100 x g for 10 minutes. The supernatant was carefully removed and the cells were gently triturated with 1 ml of culture medium (~40 times). The cell suspension was diluted appropriately with culture media and plated on 12 mm coverslips in a six well plate (four coverslips per well) and incubated at 37ºC in 5% CO_2. Media was changed every two days with an antibiotic/antimycotic free medium (DMEM containing 25 mM HEPES, 5 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 0.3% BSA (w/v) and 4.2 μg/ml fibronectin) and electrophysiological recordings were obtained from cells 24-96 hours post-isolation. Over this culture period, cells displayed a typically simple stellate or ovoid morphology with no significant difference in behavior or response to CRH or AVP between days in culture.

Electrophysiology

Current clamp electrophysiological recordings were obtained from corticotroph cells using the perforated patch mode of the whole-cell patch clamp technique. The pipette solution contained amphotericin B at a concentration of 150 μg/ml and resulted in access resistances typically < 40 MΩ within 10-20 minutes, which allowed stable recordings in excess of 20 minutes. The standard bath (extracellular) solution contained (in mM): 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 (intracellular) solution contained (in mM): 10 NaCl, 30 KCl, 60 K_2SO_4, 1 $MgCl_2$, 10 HEPES, 10 Glucose and 50 Sucrose. The pH was adjusted to 7.2 with KOH, 290 mOsmol/l.

Electrophysiological recordings were performed at room temperature (18-22°C) using Clampex 10.1 (Molecular Devices) with a sampling rate of 10 kHz and filtered at 2 kHz. Patch pipettes were fabricated from borosilicate glass (Garner) using a Model P-97 micropipette puller (Sutter Instruments). Pipette tips were heat polished and had resistances typically between 2-3 MΩ. Cell capacitance of corticotroph cells was ranged from 2-10 pF and compensated series resistance was typically < 20 MΩ. Drugs were applied to cells using a gravity perfusion system with a flow rate of 1-2 ml/min to minimise flow-induced artefacts.

Electrophysiology Analysis

Current clamp recordings were performed using a standard 20 minute protocol unless stated otherwise with analysis performed using Clampfit v10.1 (Molecular Devices). Basal activity was recorded for seven minutes before exposing cells to CRH and/or AVP for three minutes, concluding with a 10 minute washout period. Activity of corticotroph cells was measured in three minute blocks corresponding to basal activity (4-7 min), CRH/AVP-evoked activity (10-13 min) and washout period (17-20 min). Consistent with previous studies, stimulation of

corticotroph cells with CRH and AVP (0.2 nM and 2 nM respectively) resulted in a robust membrane depolarisation (Liang *et al.*, 2011). To account for this, during event analysis the baseline was adjusted relative to the depolarisation so that all events were measured relative to current membrane potential. Measurements were made of membrane potential, event frequency, and mean event duration. An event was defined from the point it reached threshold (Δ 25 mV from baseline) until it fell below a re-arm level (Δ 5 mV). In addition to mean event duration, bursting behaviour was quantified through the calculation of a burstiness factor (BF). This method classifies any event < 100 ms as a spike and events > 100 ms as a burst; a burstiness factor is calculated as the fraction of events that are bursts (Van Goor *et al.*, 2001*b*; 2001*c*; Tabak *et al.*, 2011).

Mathematical Model

The Hodgkin-Huxley formalism is used (Hodgkin & Huxley, 1952) with currents that are present in pituitary corticotrophs. In our model, the potential difference across the plasma membrane varies according to:

$$C_m \frac{dV}{dt} = -\left(I_{Ca} + I_{K-dr} + I_{BK-near} + I_{BK-far} + I_{K-ir} + I_{NS} + I_{noise}\right) \quad (1)$$

where C_m is the membrane capacitance. There are six ionic currents in the model as shown in Figure 1A. I_{Ca} is the high voltage activated dihydropyridine sensitive L-type Ca^{2+} current that is responsible for most Ca^{2+} entry during an action potential. I_{K-dr} is the rapidly activated delayed rectifier K^+ current that is largely responsible for the downstroke of an action potential. The model also contains large-conductance, voltage and Ca^{2+}-activated K^+ channels (BK channels). Some are located near Ca^{2+} channels and respond to Ca^{2+} in microdomains at open Ca^{2+} channels, producing the current $I_{BK-near}$. Others are situated away from Ca^{2+} channels and respond to the mean cytosolic Ca^{2+} concentration, producing the current

$I_{BK-far.}$ BK-near channels represent STREX type channels, while BK-far channels represent ZERO type channels (Shipston *et al.*, 1999; Chen *et al.*, 2005; Zhou *et al.*, 2012) although the spatial distribution of these channel variants, or any of the channels involved in excitability in corticotrophs, is not known. I_{K-ir} is the barium insensitive inward rectifier K^+ current that activates under hyperpolarization. Also, the model has a current produced by non-selective-cation channels, I_{NS}. The effect of system noise is included in the model through the current I_{noise}.

The non-selective-cation current has constant conductance in our model $I_{NS}(V) = g_{NS}(V - V_{NS})$. The voltage-dependent currents with dynamic conductances (g) are as follows:

$$I_{Ca}(V) = g_{Ca} m_\infty(V)(V - V_{Ca}) \tag{2}$$

$$I_{K-dr}(V) = g_K n(V - V_K) \tag{3}$$

$$I_{K-ir}(V) = g_{K-ir} r_\infty(V)(V - V_K) \tag{4}$$

$$I_{BK-far}(V, c) = g_{BK-far} bk_f (V - V_K) \tag{5}$$

$$I_{BK-near}(V, c_{dom}) = g_{BK-near} bk_n (V - V_K) \tag{6}$$

We assume that the Ca^{2+} channels and inward rectifying K^+ channels activate instantaneously. The gating variable for the activation of delayed rectifier K^+ current (n) has first order kinetics and changes with time according to

$$\frac{dn}{dt} = \frac{n_\infty(V) - n}{\tau_n}. \tag{7}$$

The steady-state functions are

$$x_\infty(V) = \frac{1}{1 + e^{\left(\frac{v_x - V}{s_x}\right)}}, \qquad x = n, m, r. \tag{8}$$

For the near and far populations of BK channels we use the model of (Tsaneva-Atanasova *et al.*, 2007) for pituitary somatotrophs. The gating variables are

$$\frac{dbk_n}{dt} = \frac{bk_{n_\infty}(V, c_{DOM}) - bk_n}{\tau_{bk_n}} \tag{9}$$

$$\frac{dbk_f}{dt} = \frac{bk_{f_\infty}(V, c) - bk_f}{\tau_{bk_f}} \tag{10}$$

where Ca_{DOM} is the free Ca^{2+} concentration in a microdomain and c is the mean free cytosolic Ca^{2+} concentration. The equilibrium functions are:

$$bk_{n_\infty}(V, c_{DOM}) = \frac{1}{1 + \exp\frac{-\left(V - V_{bk-near}(c_{DOM})\right)}{k_{bk}}}, \tag{11}$$

$$bk_{f_\infty}(V, c) = \frac{1}{1 + \exp\frac{-\left(V - V_{bk-far}(c)\right)}{k_{bk}}}. \tag{12}$$

where

$$V_{BK-near}(c_{DOM}) = V_{BK_0} - k_{s\square ift} \ln \frac{c_{DOM}}{k_{Ca_{BK-near}}}, \tag{13}$$

$$V_{BK-far}(c) = V_{BK_0} - k_{s\square ift} \ln \frac{c}{k_{Ca_{BK-far}}} \tag{14}$$

and domain Ca^{2+} is modeled as proportional to the Ca^{2+}-current:

$$c_{DOM} = -AI_{Ca}(V).$$

The equation for the mean intracellular Ca^{2+} concentration is

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

where f is the fraction of free Ca^{2+} in the cytosol, α converts current to concentration and k_c is the Ca^{2+} pump rate.

The current due to channel noise is $I_{noise} = \sigma_N \omega$ where σ_N is noise amplitude and ω is a Wiener variable. The model is implemented in the XPPAUT software program (Ermentrout, 2002) using the Euler method (dt=0.1 ms), and the computer code is available for free download from http://www.math.fsu.edu/~bertram/software/pituitary.

Parameters of the model are listed in Table 1 and the steady-state activation functions for the currents are shown in Figure 1B & C.

Statistics

The data were expressed as mean ± SEM (standard error of the mean), n = number of independent experiments. Statistical analysis was performed as appropriate by student's T-test and ANOVA analysis with Bonferroni-Holm *post hoc* test (Microsoft Excel). Significant differences between groups were defined at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

RESULTS

CRH/AVP increases frequency and bursting in corticotroph cells

In corticotrophs isolated from POMC-GFP male mice spontaneous activity was observed in more than 90% of cells recorded under current-clamp using the perforated patch-clamp approach, as previously observed in female corticotrophs (Liang *et al.*, 2011) with predominantly large amplitude single-spike action potentials (Figure 2A & B). Very occasional longer duration bursts of activity were observed, together with single spike action

potentials, in less than 6% of all corticotrophs analysed under basal conditions. Corticotrophs had a resting membrane potential of -53.7 ± 1.5 mV, displayed spontaneous action potentials at a frequency of 0.34 ± 0.14 Hz and had a mean cell capacitance of 4.43 ± 0.94 pF (Figure 2D & E). The mean event duration of 69 ± 26 ms and a burstiness factor (BF) of 0.18 ± 0.10 are both consistent with predominantly single-spike action potential behaviour (Figure 2F & G) in the unstimulated state. Replacement of external sodium ions with the large organic cation *N*-methyl-D-glucamine ($NMDG^+$) resulted in a significant ($p = 0.00059$) hyperpolarisation of 22.7 ± 2.8 mV ($n = 5$) within one minute, accompanied by a cessation of spontaneous activity which was fully reversible following washout. These data are consistent with that previously observed in female mouse corticotrophs and that a background sodium conductance is important for setting the resting membrane potential of corticotrophs, as in other pituitary cells (Tomic *et al.*, 2011; Liang *et al.*, 2011).

In vivo, corticotrophs are exposed to pulses of both CRH and AVP released from the hypothalamus in response to stress, resulting in the release of ACTH. Thus corticotrophs were stimulated with CRH and AVP (0.2 and 2 nM respectively) at concentrations chosen to be physiologically relevant and within peak concentrations in portal circulation in response to stress (Gibbs & Vale, 1982; Sheward & Fink, 1991). A three minute exposure to CRH/AVP resulted in a robust depolarisation and an increase in firing frequency (Figure 2A) with a transition from predominantly single-spike action potentials (Figure 2B) to complex bursting patterns (Figure 2C) that included both single spike and 'pseudo-plateau burst' behaviours. Following CRH/AVP exposure, there was a significant ($p = 0.0031$) membrane depolarisation to -47.4 ± 0.8 mV (Figure 2D) and a significant ($p = 0.0080$) increase in event frequency to 0.86 ± 0.18 Hz (Figure 2E). This was accompanied by a significant ($p = 0.016$) increase in mean event duration (564 ± 160 ms) following CRH/AVP stimulation (Figure 2F) as well as a significant ($p = 0.00014$) increase in the burstiness factor to 0.79 ± 0.06 (Figure

2G), indicating a switch to predominantly pseudo-plateau bursting following CRH/AVP exposure. After 10 min washout of CRH/AVP, membrane potential returned to -52.5 ± 1.3 mV which was not significantly different to baseline (Figure 2D). However, corticotroph activity remained elevated compared to pre-stimulation with event frequency elevated at 0.88 ± 0.2 Hz (Figure 2E). Although event duration and the burstiness factor were elevated compared to pre-stimulation levels, they were suppressed compared to levels 3 min following CRH/AVP application (Figure 2F & G). These data reveal that exposure of corticotrophs to physiological levels of the hypothalamic secretagogues CRH and AVP result in a complex patterns of excitability that include increases in event frequency and a transition to pseudo-plateau bursting.

Modeling of the CRH/AVP effect in pituitary corticotrophs

To examine whether our mathematical model of the corticotroph can recapitulate the key features of both spontaneous (basal) activity and the subsequent depolarisation and transition to bursting following CRH and AVP stimulation we changed ionic current parameters in the model that correspond to currents implicated in the control of corticotroph excitability. The model consists of six ionic currents (Figure 1A & B): L-type Ca^{2+} current (I_{Ca}), delayed rectifier K^+ current ($I_{K\text{-}dr}$), inward rectifier K^+ current ($I_{K\text{-}ir}$,) BK-near ($I_{BK\text{-}near}$) and BK-far ($I_{BK\text{-}far}$) currents and non-selective current (I_{NS}) with a simple geometry as the spatial organization of ion channels and Ca^{2+} pools in corticotrophs is very poorly characterised. See methods for full description.

L-type Ca^{2+} channels are critical for CRH/AVP stimulated ACTH secretion and CRH has been reported to increase L-type Ca^{2+} current in a variety of corticotroph models (Mollard *et al.*, 1987; Guérineau *et al.*, 1991; Kuryshev *et al.*, 1995). CRH and AVP have been reported to activate non-selective cation channels (Takano *et al.*, 1996; Mani *et al.*, 2009) in

corticotrophs or other cells and a non-selective Na^+-dependent conductance is critical for determining the resting membrane potential (Liang *et al.*, 2011). Although CRH has been reported to regulate inward rectifier K^+ current $I_{K\text{-}ir}$ in rat corticotrophs (Kuryshev *et al.*, 1997) the barium sensitive $I_{K\text{-}ir}$ has no functional effect in mouse corticotrophs (Liang *et al.*, 2011). Distinct BK channel splice variants, including the stress regulated exon (STREX) and ZERO (lacking STREX insert) splice variants of the channel are expressed in the anterior pituitary and murine corticotrophs (Shipston & Armstrong, 1996; Shipston *et al.*, 1999; Brunton *et al.*, 2007; Liang *et al.*, 2011). The model incorporates two distinct BK channel populations: BK-near and BK-far. While the molecular identity of these populations is not prescribed, the characteristics of these populations are similar to those of the STREX and ZERO variants, respectively. For example, BK-near (STREX) have a significantly left shifted apparent voltage for half maximal activation compared to BK-far (ZERO) channels (Xie & McCobb, 1998; Chen *et al.*, 2005) (Figure 1C). Moreover, activation of the cAMP/PKA pathway shifts the properties of STREX channels to channels with ZERO-like properties including a significant right shift in the half maximal voltage of activation and faster activation kinetics (Tian *et al.*, 2001*a*; 2004; Chen *et al.*, 2005; Zhou *et al.*, 2012*a*).

Thus in our model we first simulated the application of CRH/AVP through changes to three ionic currents: the L-type Ca^{2+} current (I_{Ca}), the non-selective cation current (I_{NS}), and the BK-near current ($I_{BK\text{-}near}$). Figure 3A & B shows the model corticotroph cell exhibiting spontaneous spiking over the first 100 seconds as seen experimentally. The slow spiking is due to the noise that pushes the voltage randomly above spike threshold with the resting membrane potential set close to threshold by g_{NS}. The application of CRH/AVP is simulated by increasing the conductances g_{NS} and g_{Ca} (Table 1). Also, the BK-near channel time constant τ_{bk_n} is decreased along with $k_{Ca_{BK-near}}$ (Table 1), producing a faster activation of this current and shifting its activation curve rightward to overlap that of the BK-far activation

curve (Figure 1C, blue curve becomes like red curve). After these changes to the model parameters, the low frequency spiking changes to high-frequency bursting (Figure 3A & C). Moreover, the spike amplitude decreases substantially (by ~20 mV) and the base membrane potential increases (by ~20 mV), as seen experimentally. The effects of drug washout is simulated by slowly changing parameters back to their original values (Figure 3A).

CRH and AVP differentially regulate corticotroph excitability in vitro

CRH and AVP act synergistically to increase ACTH secretion from corticotroph cells and activate distinct G-protein coupled receptor activated intracellular signaling cascades: the cAMP/PKA and IP3/PKC pathways, respectively. Stimulation of corticotrophs with combined secretagogues CRH/AVP increased both firing frequency and bursting behaviour. Previous studies have reported that CRH and AVP are individually able to regulate electrical excitability in corticotrophs (e.g see Mollard *et al.*, 1987; Guérineau *et al.*, 1991; Lee & Tse, 1997; Tse & Lee, 1998). We thus asked whether CRH and AVP at physiologically relevant concentrations reported in the portal circulation (Gibbs & Vale, 1982; Sheward & Fink, 1991), promote distinct, or similar, patterns of electrical activity when applied alone in an attempt to dissect key conductances that may be the targets of these neuropeptides.

Individually, both CRH (Figure 4A & B) and AVP (Figure 4C & D) were still able to produce an increase in corticotroph excitability. However, the patterns of stimulated excitability were distinct. Treatment of corticotrophs with 0.2 nM CRH resulted in a significant ($p = 0.0057$) depolarisation from -53.0 ± 2.4 mV to -43.0 ± 3.8 mV. Firing frequency increased significantly ($p = 0.043$) from 0.13 ± 0.07 Hz to 1.45 ± 0.68 Hz (Figure 4E). Following 10 min washout, event frequency had significantly declined to 0.40 ± 0.22 Hz in contrast to the sustained elevation of excitability observed in cells treated with combined CRH/AVP (0.88 ± 0.24 Hz). A transition from spiking to bursting was observed in all tested

cells and mean event duration increased significantly ($p = 0.021$) from 63 ± 49 ms to 378 ± 267 ms following CRH stimulation (Figure 4F). In contrast, cells treated with 2 nM AVP alone failed to produce a significant depolarisation, but there was a significant ($p = 0.033$) increase in firing frequency from 0.32 ± 0.17 Hz to 1.77 ± 0.42 Hz (Figure 4E). Firing frequency remained elevated (1.33 ± 0.60 Hz) following 10 min washout suggesting that AVP promotes a sustained increase in activity. Interestingly, AVP alone failed to induce a transition from spiking to bursting in any cell examined. Mean event duration was 55 ± 46 ms under basal conditions and did not significantly increase (105 ± 78 ms) following AVP exposure (Figure 4F). AVP has been reported to stimulate intracellular Ca^{2+} release, although most reports utilize supraphysiological AVP levels an order of magnitude greater than used here, that has been reported to activate Ca^{2+}-activated potassium currents that produce brief hyperpolarization in rat corticotrophs (Corcuff *et al*., 1993). We never see an AVP-induced hyperpolarization under our recording conditions suggesting that either AVP (2nM) does not significantly promote intracellular Ca^{2+} release in our system or this calcium elevation is not efficiently coupled to activation of Ca^{2+}-activated potassium channels.

These data suggest that CRH can drive a transition to pseudo-plateau bursting while AVP promotes a sustained increase in single-spike frequency but does not support a transition to bursting. This predicts that CRH and AVP differentially control conductances important for increased electrical excitability in corticotrophs and that conductances regulated by the cAMP/PKA pathway are likely responsible for the transition to bursting.

Distinct conductances differentially regulate spike frequency and bursting in the corticotroph model

The experimental findings we observed after the addition of CRH only or AVP only were further investigated in our model. In the experiments, adding only CRH (without AVP)

changes the basal single-spike activity to bursting (Figure 4A & B). Since CRH may act on several conductances, we asked which conductances were necessary for conversion to bursting. We found that increasing either the non-selective-cation current conductance g_{NS} or Ca^{2+} current conductance g_{Ca} increases spike frequency but does not produce bursting. However, making the BK-near channels similar to BK-far by reducing τ_{bk_n} of the BK-near channel from 20 ms to 4 ms and right-shifting its activation curve ($k_{Ca_{BK-near}}$ from 18 to 6 μM) is sufficient to convert spiking to bursting without the need to make any other changes (Figure 5A & B). A small additional increase of the L-type conductance did not prevent that transition to bursting, but increased burst frequency and amplitude.

The data in Figure 4C & D show that application of AVP alone greatly increases the spike frequency but does not convert spiking to bursting. We found that increasing g_{NS} alone (from 0.1 to 0.2 nS) is sufficient to generate the effect seen in experiments. That is, an increase in firing frequency and a slight depolarisation of the membrane potential without a transition to bursting (Figure 5C). In accordance with a role for g_{NS} in AVP action, step current injection alone only stimulates an increase in firing frequency, not a transition to bursting (for example a 1 pA current injection results in a 2.41 ± 0.31 (n = 4) fold increase in action potential frequency). Moreover, inhibition of the non-selective sodium conductance results in membrane hyperpolarization and prevents CRH/AVP induced depolarisation and increase in excitability (Liang *et al.*, 2011). Changes in g_{Ca} alone increases spike frequency and amplitude, while changes in τ_{bk_n} and $k_{Ca_{BK-near}}$ convert spiking to bursting. Hence the model suggests that changes in BK-near cause the CRH-induced transition to bursting, and an increase in the non-selective cation conductance causes the increased spike frequency induced by AVP.

In the model, BK channels have almost no effect on the spontaneous low-frequency spiking activity. Figure 6A shows spontaneous activity in the absence of BK conductance. When application of CRH and AVP together is simulated the spike frequency greatly increases and the model cell is depolarised, but there is no bursting (Figure 6B). However, when even a small fraction (15%) of BK conductance remains there is a mixture of bursting and fast spiking (Figure 6C).

CRH/AVP-evoked bursting activity is suppressed by pharmacological inhibition of BK channels

The model predictions of Figure 6 were tested in corticotrophs exposed to the selective BK channel inhibitor paxilline (1 μM). To investigate the role of BK channels in regulating basal activity, corticotrophs were exposed to paxilline for three minutes. In 3/3 cells tested, paxilline had no effects on spontaneous corticotroph activity for all parameters measured. These results are not surprising as the BK channel has been suggested to promote bursting behaviour which is uncommon under basal conditions.

To investigate the role of BK channels in the CRH/AVP response, corticotroph cells were treated with paxilline (1 μM) for four minutes prior to CRH/AVP exposure. Paxilline remained present throughout the remainder of the recording. In comparison to control cells, paxilline treated cells showed predominantly single-spike action potentials under basal conditions (Figure 7A). In contrast, there was a significant reduction in CRH/AVP-evoked bursting behaviour (Figure 7B). Under basal conditions, paxilline-treated cells had a resting membrane potential of -54.2 ± 2.8 mV and a spontaneous event frequency of 0.27 ± 0.20 Hz (n = 7). Paxilline treated cells also had a mean event duration of 148 ± 39 ms and a burstiness factor of 0.23 ± 0.13. The basal properties of paxilline treated cells were not significantly different to untreated cells for all parameters measured.

Following CRH/AVP exposure, there was a significant ($p = 0.0068$) depolarisation from -54.2 ± 2.8 mV to -46.3 ± 2.9 mV in paxilline-treated cells which was no different from controls. Event frequency significantly ($p = 0.044$) increased to 0.69 ± 0.16 Hz (Figure 7C) which was not significantly different to untreated cells (0.86 ± 0.18 Hz). A transition to bursting was observed in only 4/8 cells after CRH/AVP stimulation, compared to 7/7 of control cells. CRH/AVP was unable to produce a significant increase in mean event duration (178 ± 58 ms) in paxilline-treated cells (Figure 7D), which was significantly ($p = 0.021$) reduced compared to untreated cells (564 ± 160 ms). The burstiness factor following CRH/AVP stimulation was 0.43 ± 0.15 which was significantly ($p = 0.018$) reduced compared to untreated cells (0.79 ± 0.06).

BK-knockout mice show reduced bursting compared to wild-types

Pharmacological blockade of BK channels resulted in a reduction in CRH/AVP-evoked bursting activity, as predicted by the model. To further investigate the role of BK channels in promoting bursting, cells isolated from BK channel knockout mice ($BK^{-/-}$) were exposed to CRH/AVP for three minutes following the same 20 minute protocol. The mean cell capacitance of $BK^{-/-}$ cells was 4.16 ± 0.40 pF ($n = 6$) which was not significantly different from wild-type cells (4.89 ± 0.29 pF).

Current clamp recordings revealed 4/6 $BK^{-/-}$ cells displayed spontaneous activity with 6/6 showing an increase in activity following CRH/AVP stimulation (Figure 8A & B). In comparison to paxilline-treated cells, corticotrophs isolated from $BK^{-/-}$ mice showed no difference in basal membrane potential (-53.7 ± 2.3 mV), frequency (0.40 ± 0.19 Hz), mean event duration (153 ± 106 ms) or burstiness factor (0.15 ± 0.11) compared to wild-type cells ($n = 6$). CRH/AVP exposure resulted in a significant ($p = 0.00074$) depolarisation to -45.1 ± 2.4 mV which was not significantly different to wild-type. CRH/AVP was able to induce a

significant ($p = 0.046$) increase in firing frequency to 2.30 ± 0.79 Hz (Figure 8C). Interestingly, peak firing frequency was significantly ($p = 0.040$) elevated compared with controls (0.86 ± 0.18 Hz) representing a 2.7-fold increase in CRH/AVP-evoked activity. Following CRH/AVP stimulation, bursting activity was observed in 3/6 cells. Basal mean event duration was 153 ± 106 ms which did not significantly increase (305 ± 127 ms) following CRH/AVP (Figure 8D).

These pharmacological and genetic data combined show that CRH/AVP is still able to produce a robust increase in electrical activity in $BK^{-/-}$ cells. However, the increase in activity was largely associated with an increase in firing frequency rather than a transition to bursting supporting a key role for BK channels in the CRH/AVP –induced transition to bursting.

DISCUSSION

In this paper we have exploited electrophysiological, pharmacological and genetic approaches in conjunction with mathematical modelling of anterior pituitary corticotroph cells to examine the mechanisms controlling secretagogue-induced electrical excitability. Importantly, our data reveal that the hypothalamic neuropeptides CRH and AVP stimulate distinct patterns of electrical excitability. CRH promotes bursting behaviour that is dependent upon functional BK channels whereas AVP promotes an increase in firing frequency of single action potentials through BK-independent pathways likely involving activation of non-selective conductances. Thus, as distinct stressors can stimulate differential release of CRH and AVP and corticotroph excitability is differentially controlled by these two hypothalamic secretagogues, the pattern of corticotroph excitability may be distinct for different stressors.

In this study we examined corticotrophs from male mice expressing GFP under control of the POMC promoter to aid visual identification. In agreement with previous studies in female

mice (Liang *et al.*, 2011) > 90% of male corticotrophs displayed spontaneous activity with predominantly single action potentials that varied widely in frequency (0.01 to 1 Hz) in unstimulated conditions using the perforated patch-clamp recording approach. In < 6% of corticotrophs we observed very occasional longer bursts of activity alongside single spikes. Whether these infrequent longer events in the basal state reflects stochastic channel behaviour or reflect the activity of endogenous signaling pathways that control ion channel function, as has been reported for the cAMP-signaling pathways in other pituitary cells (Kucka *et al*., 2013), remains to be determined. However, the predominant phenotype of unstimulated murine corticotrophs was single spike activity of variable frequency that did not differ over the 1-4 days in culture. A previous study in mouse corticotrophs reported that corticotrophs are not spontaneously active (Lee *et al.*, 2011), however this employed conventional whole cell recording that results in intracellular dialysis, a procedure that abolishes spontaneous activity in murine corticotrophs (Liang *et al.*, 2011). The modeling, in conjunction with electrophysiological analysis, revealed that the resting membrane potential was largely determined by the non-selective sodium conductance with single spike activity resulting from stochastic fluctuations that bring the voltage above spike threshold. In accordance with other anterior pituitary cell types that display single spike activity under basal conditions, spontaneous corticotroph activity was unaffected by pharmacological or genetic inhibition of the BK channel (Van Goor *et al.*, 2001*b*; 2001*c*).

Stimulation of corticotrophs with physiological concentrations of CRH/AVP resulted in a robust depolarisation and increase in event frequency coupled with a transition from predominantly single-spike action potentials to complex bursting patterns. The modeling analysis revealed that the transition to the complex bursting pattern following combined CRH/AVP could be explained primarily by changes in three conductances: the L-type Ca^{2+} conductance, non-selective cation conductance and the properties of the BK-near

conductance. Separately CRH and AVP are able to drive an increase in electrical activity of the corticotroph but differentially modulate the pattern of electrical activity. These distinct patterns result from the differential contribution of these three conductances in CRH and AVP-stimulated excitability. Corticotrophs stimulated with CRH alone promoted a transition to bursting, an effect dependent upon changes that make the BK-near channels like the BK-far channels, i.e., a right shifted activation curve and faster activation. Thus regulation of BK-near alone may control bursting, although regulation of g_{Ca} and g_{NS} are required for the significant depolarisation of resting membrane potential and increase in the frequency of spikes and bursts. Indeed, when BK channels are blocked pharmacologically, or deleted genetically, stimulation promotes an increase in single spike activity. In contrast, AVP stimulated an increase in single spike frequency without a transition to bursting, an effect dependent upon g_{NS} in the model. Intriguingly, AVP alone (or CRH/AVP) resulted in an increase in event frequency which remains elevated at the end of the washout period whereas with CRH alone activity declines during washout. This suggests that AVP prolongs the duration of the response to hypothalamic secretagogues and supports the hypothesis that CRH mediates a rapid increase in ACTH secretion whereas AVP acts to elicit a plateau phase (Lee & Tse, 1997; Tse & Lee, 1998). Clearly the ability of CRH and AVP to promote distinct patterns of electrical excitability, and consequently distinct patterns of Ca^{2+} dynamics in corticotrophs, is likely to be important for both short-term control of ACTH secretion as well as longer-term control of gene transcription and other Ca^{2+}-regulated mechanisms in corticotrophs. Important in this regard will be a greater understanding of the spatial distribution of the ion channels controlling excitability, intracellular Ca^{2+} stores and their relationship to secretory vesicle localisation and release. Cytological and ultrastructural analysis of corticotrophs *in situ* in rat has revealed heterogeneity in corticotroph morphologies including 'stellate' and ovoid cell types (e.g., see Childs, 1987; Yoshimura &

Nogami, 1981). The proportion of these morphological distinct corticotrophs differs between species (e.g., human corticotrophs tend to be ovoid or round) as well as during development and in response to environmental challenge. Based on electron microscopy analysis ACTH containing granules are typically localised toward the cell periphery and in a proportion of corticotrophs accumulate in extended processes. More recent analysis of the three dimensional architecture of murine corticotrophs from POMC-GFP expressing mice also shown heterogeneity in corticotroph morphology as well as extensive anatomical networks *in situ* (Budry et al., 2011). Whether such morphologically distinct corticotrophs display distinct properties, regulation or function is not known. However, we saw no difference in behavior or responses in the simple stellate and ovoid cells analysed in our short-term cultures.

How may CRH and AVP control conductances required for stimulation of distinct patterns of electrical activity? CRH and AVP operate through two distinct intracellular signalling cascades activated via distinct GPCRs (Antoni, 1986; King & Baertschi, 1990; Stojilkovic *et al.*, 2010). CRH exerts its actions exclusively through CRHR1 in corticotroph cells resulting in an increase in cytosolic cAMP with subsequent activation of downstream effectors, predominantly PKA. In contrast AVP acts through V1B receptors, and results in the cleavage of PIP_2 to IP_3 and DAG, the latter leading to the activation of PKC. Voltage dependent Ca^{2+} influx via L-type Ca^{2+} channels is essential for CRH and AVP-evoked ACTH secretion. CRH, via PKA has been shown in a number of models to stimulate Ca^{2+} influx by activating L-type Ca^{2+} channels, most likely via PKA phosphorylation of the channel (Kuryshev *et al.*, 1996). Both CRH and AVP have been reported to activate non-selective cation conductances (Takano *et al.*, 1996; Mani *et al.*, 2009), thus providing potential pathways for driving cellular depolarisation. Although the molecular identity of these non-selective conductances has not been defined, work in other pituitary cell types suggest that it may, in part, be

mediated via transient receptor potential (TRP)–like conductances that are also regulated by the cAMP/PKA pathway (Tomic *et al.*, 2011).

Our data reveals that BK channels play an important role in CRH-stimulated generation of bursting behaviour in corticotroph cells. Previous studies have identified a correlation between the level of BK expression in anterior pituitary cell types and the incidence of spontaneous bursting activity (Van Goor *et al.*, 2001*b*; 2001*a*). Furthermore, previous studies have suggested that there exist two populations of BK channels involved in the generation of bursting (Tsaneva-Atanasova *et al.*, 2007). BK channels located in close proximity to voltage-gated Ca^{2+} channels (BK-near) are important in the generation of a burst whereas BK channels located distantly from Ca^{2+} channels (BK-far) are required for burst termination. Our model predicts that CRH promotes bursting by decreasing the time constant of BK-near and shifting the activation curve so that it becomes like BK-far.

In vivo, global genetic deletion of BK channels results in mice with a blunted HPA axis response to acute stress, although the phenotype is likely manifest through changes at multiple levels of the HPA axis (Brunton *et al.*, 2007). BK channels are expressed in several corticotroph models including murine corticotrophs where at least two splice variants, STREX and ZERO, are predominantly expressed (Shipston & Armstrong, 1996; Shipston *et al.*, 1999; Brunton *et al.*, 2007; Liang *et al.*, 2011). CRH can inhibit total BK conductance in corticotrophs (Shipston *et al.*, 1996; Tian *et al.*, 2008) and in expression systems PKA dependent protein phosphorylation of either the STREX or ZERO variant results in channel inhibition or activation, respectively (Tian *et al.*, 2001*b*; 2004; Chen *et al.*, 2005). More importantly, the properties of the STREX and ZERO variants correspond to the key features of BK-near and BK-far in the model, respectively, and a key effect of PKA phosphorylation of STREX is to convert it to a phenotype more closely corresponding to ZERO. Thus, a potential mechanism for CRH induced bursting may involve PKA-dependent

phosphorylation of STREX variant channels that may underlie the BK-near conductance while also potentially enhancing BK-far. Although PKC can also regulate BK channels it can only regulate STREX variant channels under defined conditions, including channels that have already been phosphorylated by PKA (Zhou *et al.*, 2012), suggesting that combined CRH/AVP may have an additional effect on BK-near. Furthermore, as glucocorticoids have been shown to prevent PKA-mediated regulation of BK channels (Shipston *et al.*, 1996; Tian *et al.*, 1998) it will be of interest to examine whether glucocorticoid feedback may control CRH-induced bursting in native mouse corticotrophs. Clearly, alternative explanations for the molecular composition of these BK conductances are plausible and warrant future investigation.

Corticotroph cells treated with paxilline or isolated from $BK^{-/-}$ mice showed no difference in spontaneous activity compared with controls, which suggests that BK channels are not responsible for regulating resting membrane potential or for the generation of spontaneous activity in corticotrophs. The model suggests that CRH/AVP promotes bursting primarily through BK channels and based on these observations one would predict that in the absence of BK, corticotrophs excitability driven by CRH/AVP would result mostly in an increase in single-spike frequency. Following CRH/AVP stimulation, both paxilline-treated and $BK^{-/-}$ cells showed an increase in event frequency but failed to significantly transition to bursting. In both cases bursting was not completely abolished, which suggests that although BK channels greatly facilitate bursting, they are not absolutely necessary. Indeed, this has been predicted in a prior modeling study of pituitary cells (Teka *et al.*, 2011). Furthermore, bursting was more prevalent in $BK^{-/-}$ cells, compared to WT cells acutely exposed to the BK inhibitor paxilline, which could be the result of compensation through changes in expression of other ionic conductances. Previous studies have revealed that intermediate conductance Ca^{2+}-activated potassium channels (IK) may control bursting activity in female corticotroph

cells (Liang *et al.*, 2011), although the IK inhibitor TRAM-34 has no effect in male corticotrophs. This suggests that male and female corticotrophs display sexually dimorphic mechanisms to control bursting.

In conclusion, we reveal that CRH and AVP regulate distinct patterns of electrical excitability in corticotrophs. Importantly, the CRH-induced transition to bursting is dependent upon functional BK channels whereas AVP promotes an increase in spike frequency alone that is independent of BK channel function. The ability of these neuropeptides to engage distinct modes of electrical excitability is likely to have important functional consequences for the regulation of corticotrophs in response to different stressors.

ADDITIONAL INFORMATION

Competing Interests

None of the authors have any competing interests to disclose.

Author Contributions

PJD, SS, JT, PR, RB & MJS designed the studies; PJD, SS & JT performed research; PJD, SS, JT, RB & MJS analysed data; PJD, SS, JT, RB & MJS wrote the paper. All authors have seen and approved the final version of the manuscript.

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Figure 1: A) Schematic diagram of the ionic currents in the pituitary corticotroph model. **B)** Steady-state activation functions for the K^+ (black), Ca-L (green) and K-ir (magenta) channels and **C)** for the BK-far (red) and BK-near (blue) channels.

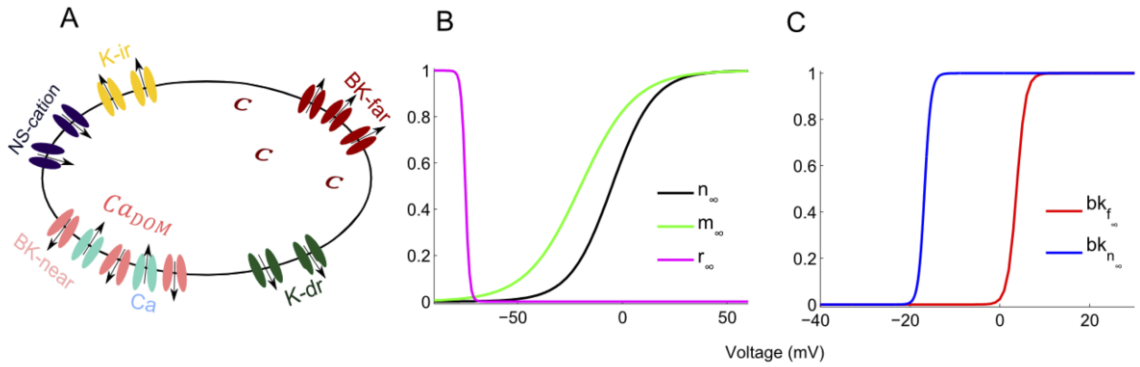

Figure 2: A) Representative current clamp recording of a corticotroph cell exposed to 0.2 nM CRH and 2 nM AVP for three minutes. Under basal conditions **B) c**orticotroph cells display predominantly single-spike action potentials which **C)** transition to complex bursting patterns following CRH/AVP exposure. Grey shading indicates membrane potential between -50 mV and +10 mV. Summary bar graphs illustrating that stimulation with CRH/AVP results in **D)** a membrane depolarisation coupled with **E)** an increase in event frequency, **F)** event duration and **G)** burstiness factor (BF). Data are means ± SEM, (n = 7/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t-test compared to base values.

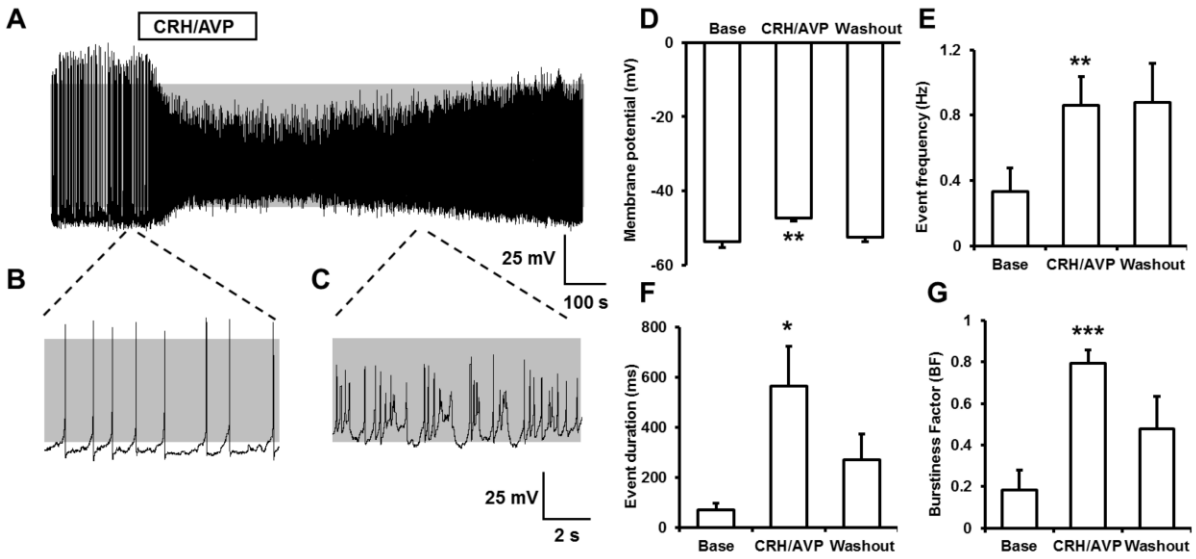

Figure 3: Adding CRH/AVP transforms spontaneous spiking to bursting in the corticotroph model. The CRH/AVP effect is modeled as an increase in g_{Ca} from 1.8 nS to 2.2 nS, an increase in g_{NS} from 0.1 nS to 0.2 nS, a decrease in τ_{bk_n} from 20 ms to 4 ms and a decrease in $k_{Ca_{BK-near}}$ from 18 μM to 6 μM.

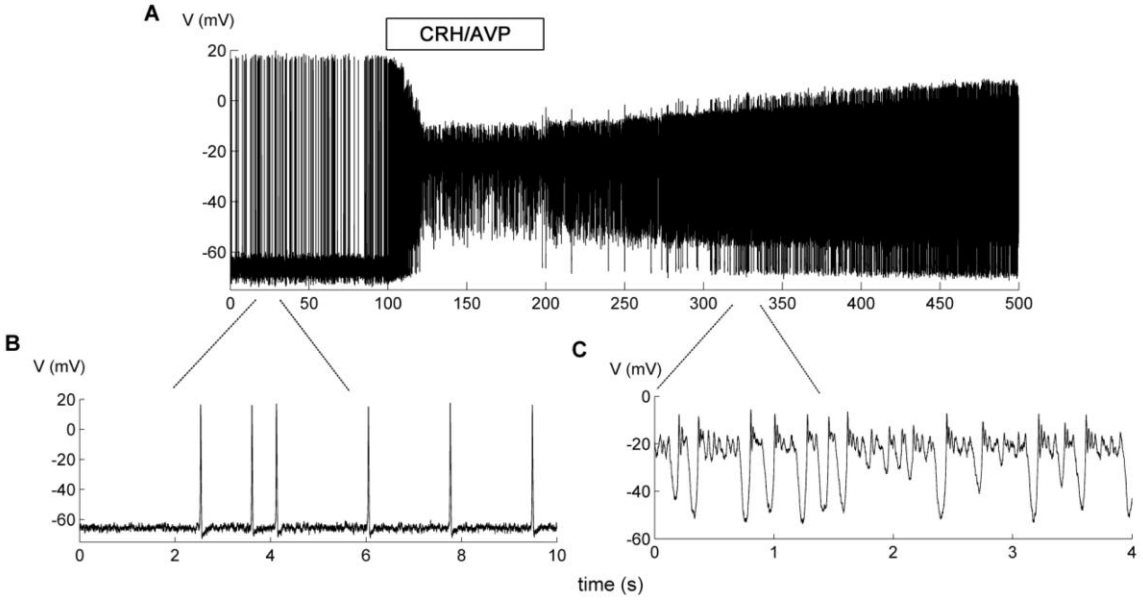

Figure 4: Representative traces of corticotroph cells before **A)** and following **B)** 0.2nM CRH alone or before **C)** and after **D)** exposure to 2 nM AVP for 3 min. Summary bar graphs reveal that **E)** individually CRH and AVP can both induce an increase in event frequency but **F)** only CRH is able to produce an increase in event duration which corresponds to a transition to bursting behaviour. Data are means ± SEM, (n > 3/group). * $p < 0.05$, ** $p < 0.01$, **E)** ANOVA and **F)** Mann-Whitney U test compared to base values.

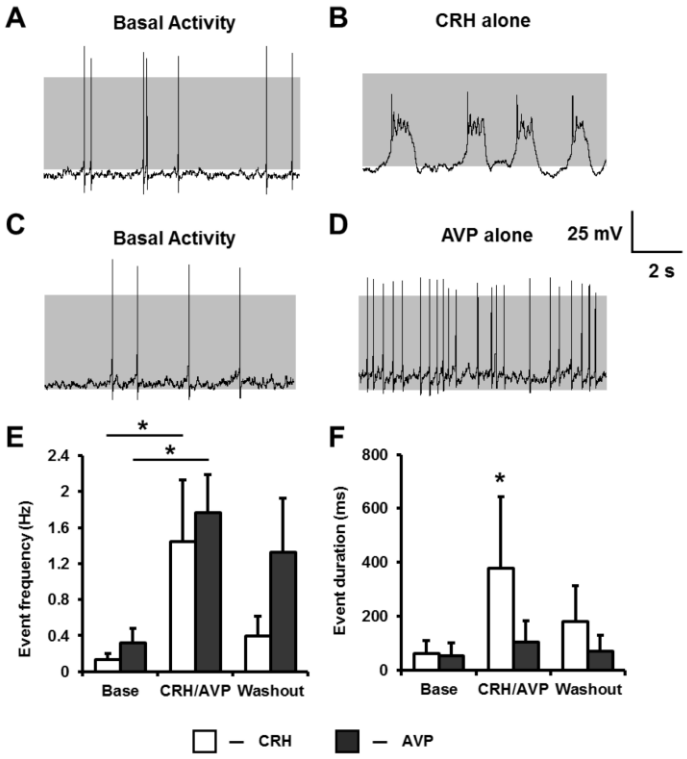

Figure 5: Simulations of CRH and AVP alone. **A)** Basal spiking activity. **B)** CRH alone transforms spiking to high frequency bursting due to changes in the currents I_{Ca} (g_{Ca} is increased from 1.8 nS to 2.2 nS) and $I_{BK\text{-}near}$ (τ_{bk_n} is decreased from 20 ms to 4 ms and $k_{Ca_{BK-near}}$ is decreased from 18 μM to 6 μM). **C)** AVP alone modulates I_{NS} (g_{NS} is increased from 0.1 nS to 0.2 nS) which leads to fast spiking.

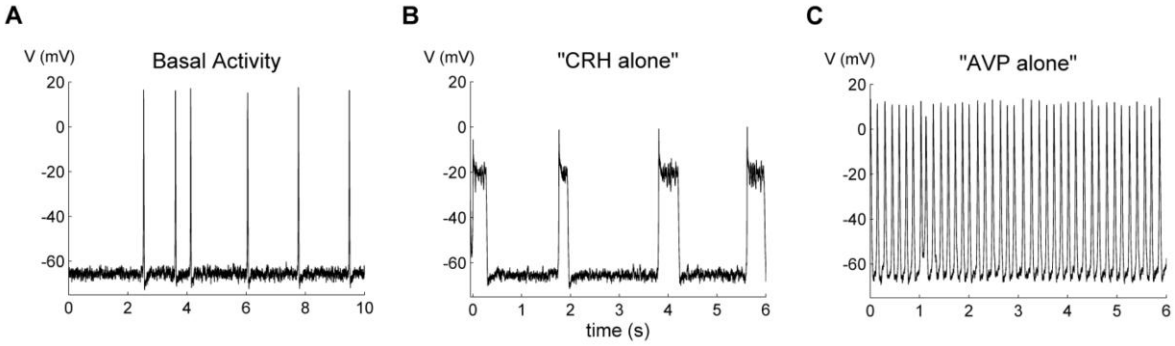

Figure 6: Simulations with little or no BK conductance. **A)** Basal spiking activity in the absence of BK conductance. **B)** CRH/AVP increases spike frequency without bursting when there is no BK conductance. **C)** When some BK conductance remains, CRH/AVP elicits some bursting mixed with fast spiking.

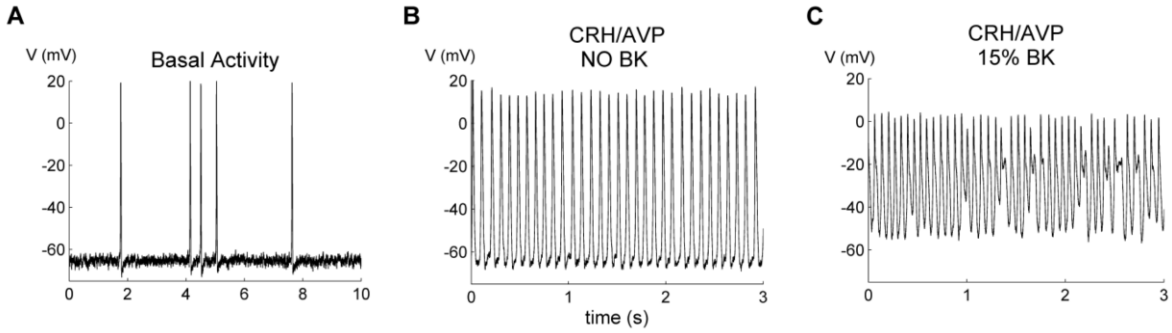

Figure 7: A) Representative traces of corticotrophs pretreated with 1 μM paxilline which **B)** reduces CRH/AVP-evoked bursting behaviour. **C)** Paxilline has no effect on the ability for CRH/AVP to increase event frequency but **D)** significantly reduces event duration. Data are means ± SEM, (n = 7/group). * $p < 0.05$, ** $p < 0.01$ ANOVA compared to respective base values.

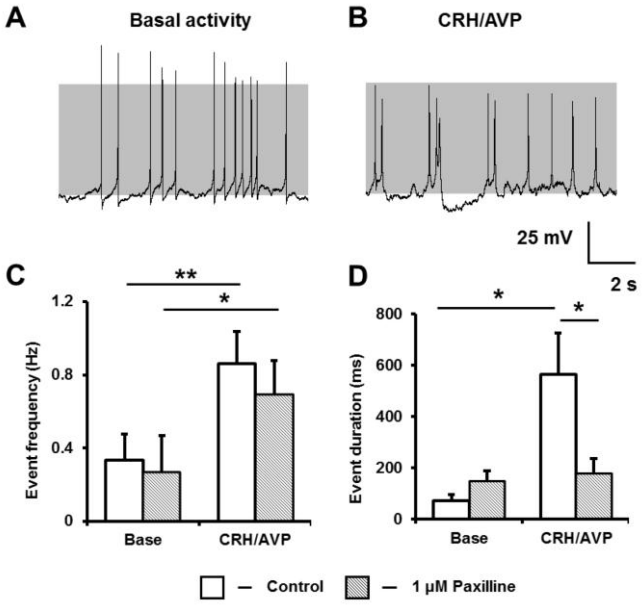

Figure 8: A) Representative traces of corticotrophs isolated from $BK^{-/-}$ mice which **B)** also results in a reduction in bursting following CRH/AVP stimulation. **C)** $BK^{-/-}$ cells show a higher event frequency following CRH/AVP compared to wild-type cells but **D)** show a decrease in event duration. Data are means ± SEM, (n > 6/group). * $p < 0.05$, ** $p < 0.01$, ANOVA compared to respective base values.

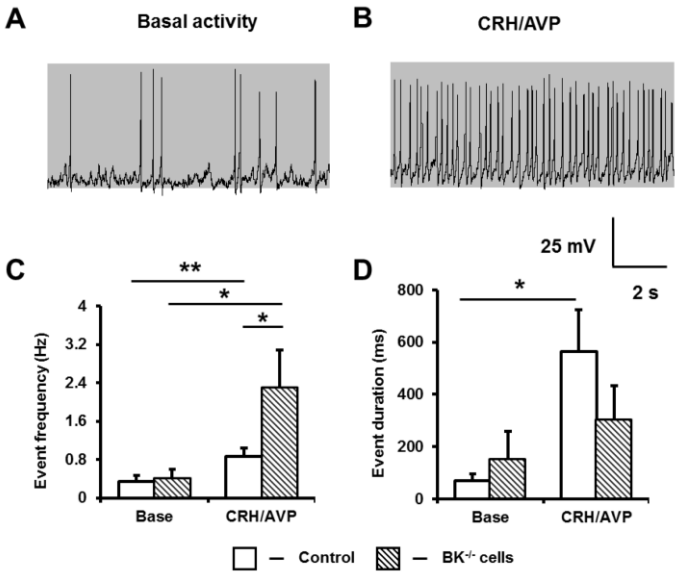

Table 1: Parameter values for mathematical model of corticotroph excitability

Parameter	Value	Parameter	Value
g_{Ca}	1.8 nS (basal), 2.2 nS (CRH)	$k_{Ca_{BK-near}}$	18 μM (basal), 6 μM (CRH)
g_{NS}	0.1 nS (basal), 0.2 nS (AVP)	$k_{Ca_{BK-far}}$	6 μM
g_{K}	8.2 nS	k_{bk}	3 mV
g_{K-ir}	1 nS	sm	10
$g_{BK-near}$	2 nS	sn	10
g_{BK-far}	1 nS	sr	-1
V_{Ca}	60 mV	V_{BK_0}	0.1 mV
V_{NS}	-10 mV	k_{shift}	18
V_{K}	-75 mV	A	0.15
v_r	-60 mV	k_c	0.12 μM
v_m	-20 mV	f	0.01
v_n	-5 mV	α	0.0015
τ_{bk_n}	20 ms (basal), 4 ms (CRH)	σ_N	5 pA
τ_{bk_f}	4 ms	C_m	6 pF
$\boldsymbol{\tau_n}$	40 ms		