The sodium leak channel NALCN encodes the major background sodium ion conductance in murine anterior pituitary cells

Marziyeh Belal^{1&2*}, Mariusz Mucha¹, Arnaud Monteil³, Paul G Winyard⁴, Robert Pawlak¹, Jamie J. Walker^{5,6&7}, Joel Tabak^{1*}, Mino D C Belle^{1*}.

¹ University of Exeter Medical School, Hatherly Labs, Streatham Campus, Prince of Wales Road, Exeter, Devon, UK.

² Northwestern University, Feinberg School of Medicine, Chicago, IL 60611, United States.

³ IGF, University of Montpellier, CNRS, INSERM, Montpellier, France.

⁴ University of Exeter Medical School, St Luke's Campus, Exeter EX1 2LU

⁵ College of Engineering, Mathematics and Physical Sciences, University of Exeter, Exeter, UK.

⁶ EPSRC Centre for Predictive Modelling in Healthcare, University of Exeter, Exeter, UK.

⁷ Bristol Medical School, Translational Health Sciences, University of Bristol, Bristol, UK.

Abstract

The pituitary gland, the so-called “master gland” produces and secretes a variety of hormones essential for regulating growth and development, metabolic homeostasis, reproduction and the stress response. The interplay between the brain and peripheral feedback signals controls hormone secretion from pituitary cells by regulating the properties of ion channels, and in turn, cell excitability. Endocrine anterior pituitary cells fire spontaneous action potentials to regulate their intracellular calcium level and eventually hormone secretion. However, the molecular identity of the non-selective cationic leak channel involved in maintaining the resting membrane potential at the firing threshold remained unknown. Here, we show that the sodium leak channel NALCN, known to modulate neuronal excitability, also regulates excitability in murine anterior pituitary cells. Using viral transduction combined with electrophysiology and

calcium imaging we show that NALCN encodes the major Na^+ leak conductance which tunes the resting membrane potential close to firing threshold to sustain the intrinsically-regulated firing in endocrine pituitary cells. Genetic interruption of NALCN channel activity, hyperpolarised the membrane potential drastically and stopped the firing activity and consequently abolished the cytosolic calcium oscillations. Moreover, we found that NALCN conductance forms a very small fraction of the total cell conductance yet has a profound impact on modulating pituitary cell excitability. Taken together, our results demonstrate that, NALCN is a crucial regulator of pituitary cell excitability and supports spontaneous firing activity to consequently regulate hormonal secretion. Our results suggest that receptor-mediated and potentially circadian changes in NALCN conductance can powerfully affect pituitary activity and hormone secretion.

Introduction

The electrical activity of cells, in response to external inputs or intrinsically derived stimulation, drives physiological functions that are essential to life, such as breathing, heartbeat, and hormone release (Cui et al, 2016; Protze et al, 2017; Rorsman and Ashcroft, 2018; Bertram et al 2018). This electrical activity relies on depolarising conductances acting on the cellular membrane to maintain the resting membrane potential (RMP) near firing threshold, driving cells to discharge action potentials (APs). In hormone-secreting cells of the pituitary gland, spontaneous electrical activity results in rhythmic Ca^{2+} entry through voltage-gated calcium channels. The subsequent oscillations in cytosolic calcium concentration ($[\text{Ca}^{2+}]_i$) serve a plethora of key physiological purposes, such as triggering hormone secretion, maintaining Ca^{2+} levels within intracellular calcium stores, and regulating gene expression (Mollard & Schlegel, 1996; Kwiecien & Hammond, 1998; Stojilkovic, 2012). Silencing spontaneous firing in pituitary cells immediately abolishes $[\text{Ca}^{2+}]_i$ oscillations and basal hormone secretion (Kucka et al, 2010).

The ability of pituitary cells to produce spontaneous APs is in part due to the depolarised RMP relative to the K^+ equilibrium potential (Fletcher et al, 2018).

70 Replacing extracellular Na⁺ with large impermeable cations such as NMDG⁺
 71 immediately suppresses the cell's ability to become depolarised. Instead,
 72 hyperpolarises the RMP, silences firing activity and abolishes the [Ca²⁺]_i
 73 transients (Simasko, 1994; Sankaranarayan & Simasko, 1996; Kwiecien and
 74 Hammond, 1998; Tsaneva-Atanasova et al, 2007; Kucka et al, 2010, 2012;
 75 Tomic et al, 2011; Liang et al, 2011; Zemkova et al, 2016; Kayano et al, 2019).
 76 This reflects the presence of constitutively-active inward-depolarising currents in
 77 pituitary cells that set the membrane potential close to the firing threshold.
 78 Pharmacological and electrophysiological investigation of this inward leak
 79 current indicate that it is mediated by a TTX-insensitive, voltage-independent
 80 and constitutively active Na⁺-permeable conductance (Fletcher et al, 2018).

81
 82 Whilst the molecular identity of this resting Na⁺ conductance in pituitary cells
 83 remains unknown, the Na⁺ leak channel/nonselective (NALCN) has emerged as
 84 the major background Na⁺-permeable conductance in several neuronal
 85 populations. For example, NALCN is essential for maintaining the spontaneous
 86 generation of action potentials in hippocampal neurons (Lu et al, 2007),
 87 GABAergic and dopaminergic neurons of the midbrain (Lutas et al, 2016;
 88 Philippart and Khaliq, 2018) and neurons of the suprachiasmatic nucleus of the
 89 hypothalamus (Flourakis et al, 2015). In ventral respiratory neurons of the brain
 90 stem, NALCN facilitates rhythmic and CO₂ stimulated breathing, as well as
 91 responsiveness to neuropeptides (Lu et al, 2007; Shi et al, 2016; Yeh et al,
 92 2017).

93
 94 The importance of NALCN as a regulator of electrical responsiveness in
 95 endocrine cells such as pancreatic β cells, is also emerging (Swayne et al
 96 2009). A high level of NALCN mRNA expression in the pituitary gland has been
 97 reported (Swayne et al, 2009). Further, transcriptomic data indicated that every
 98 anterior pituitary cell type significantly expresses NALCN and its known
 99 regulatory subunits (UNC-79, UNC-80, FAM155A) at levels higher than other
 100 known cationic leak channel, such as TRP channels and HCN channels (Paul
 101 Le Tissier, Jacques Drouin and Patrice Mollard, personal communication,
 102 04/2021). Moreover, NALCN has recently been shown to regulate cell
 103 excitability in a pituitary clonal cell line (Impheng et al, 2021). Finally, the
 104 pharmacological profile of NALCN in neurons is similar to that of resting Na⁺

105 conductance in pituitary cells: TTX-insensitive, extracellular Ca^{2+} - and NMDG⁺-
106 sensitive.

107 Together, this strongly suggests that NALCN is the primary contributor to the
108 background Na^{+} conductance and consequently plays a key role in regulating
109 cell excitability in pituitary cells. To test this, we used a lentiviral-mediated
110 knockdown strategy combined with electrophysiology and calcium imaging to
111 evaluate the role of NALCN in regulating the electrical and $[\text{Ca}^{2+}]_i$ activity of
112 murine primary anterior pituitary cells. This multifaceted approach not only
113 allowed us to determine that NALCN is the main contributor to the background
114 Na^{+} -permeable conductance of pituitary cells but also enabled us to estimate
115 the magnitude of this conductance.

116

117 **Results**

118

119 **Primary murine anterior pituitary cells express NALCN channel protein**

120 Previous research revealed the expression of *Nalcn* gene in mouse anterior
121 pituitary gland at the mRNA level (Swayne et al, 2009). Thus, we first confirmed
122 the expression of NALCN protein in primary endocrine pituitary cells. Using
123 fluorescence immunohistochemistry and the use of NALCN antibody directed at
124 the extracellular epitope of NALCN protein, we could detect the presence of
125 NALCN channel in murine endocrine anterior pituitary cells (Figure 1). Here, the
126 majority of the anterior pituitary cells were stained with the NALCN antibody,
127 and no staining was observed when the primary NALCN antibody was omitted
128 (Figure 1H).

129

130 **NALCN regulates spontaneous firing of primary pituitary cells**

131 Given that gadolinium (Gd^{3+}) and flufenamic acid (FFA) block the spontaneous
132 firing of pituitary cells (Kucka et al, 2012) and thus revealing the presence of a
133 functional non-selective cationic conductance (NSCC), it is noteworthy that Gd^{3+}
134 and FFA are not specific blockers of a particular non-selective cationic
135 conductance. Thus, NSCCs other than NALCN may contribute to the effects of
136 FFA and Gd^{3+} on membrane potential. To determine if NALCN is the main
137 NSCC in endocrine anterior pituitary cells, we used a genetic manipulation
138 approach to directly investigate the role of NALCN in the regulation of
139 spontaneous firing in these cells. We set up a lentiviral-mediated knockdown

strategy to decrease NALCN expression level, and then evaluated the resulting changes in electrophysiological properties of pituitary cells. Most untreated control (untreated Ctrl) and scramble control (SCR Ctrl) cells exhibited spontaneous firing activity (Figure 2A,B,D,F), consistent with the previous reports (*reviewed in* Fletcher et al, 2018). In contrast, 90% (28 of 31) of NALCN knockdown (NALCN KD) cells were found to be silent (Figure 2C,D,F), compared to just 17% (5 of 30) in SCR control and 19% (6 of 31) in untreated control cells (Figure 2D,F). In addition, NALCN KD cells exhibited a significantly more hyperpolarised resting membrane potential (RMP) compared to both untreated and SCR control cells (NALCN KD RMP: -63.6 ± 8.5 mV (mean $\pm$ SD), $n=31$ from 12 animals; SCR Ctrl: -45.2 mV $\pm$ 5.8 mV, $n=30$, 12 animals; untreated Ctrl: -47.5 mV $\pm$ 7.8, $n=31$, 12 animals; $p<0.001$, one-way ANOVA with post hoc Bonferroni correction; Figure 2E). There was no significant difference between the SCR control cells and untreated control cells either for the firing frequency (untreated Ctrl: 0.7 ± 0.6 Hz, $n=25$; SCR Ctrl: 0.5 ± 0.4 Hz, $n=25$, $p>0.1$, t -test; Figure 2F) or for the RMP value (untreated Ctrl: -47.6 ± 7.6 mV, $n=31$; SCR Ctrl: -45.2 mV $\pm$ 5.8 mV, $n=30$ $p>0.1$, t -test; Figure 2E). Thus, the viral transduction *per se* did not affect the firing activity or RMP of pituitary cells. In the following sections we will therefore only compare NALCN KD cells with SCR control cells.

160

161 **Small conductance injection restores firing in NALCN KD cells**

162 We next use dynamic-clamp to examine whether the injection of nonselective cationic conductance to silent NALCN KD cells could restore spontaneous firing activity. Indeed, silent NALCN KD cells became active and gained their typical firing activity upon increasing the non-selective cationic conductance (Figure 163 3A). The added conductance required to bring the NALCN KD cells to fire 164 ranged from 0.02 to 0.12 nS (median 0.05 nS, $n=28$, Figure 3B).

168

169 Taken together, this first set of results indicates that NALCN is a key player in 170 pituitary cell excitability, by modulating the RMP and subsequently the firing 171 activity of primary pituitary cells. The amount of NALCN conductance lost by the 172 cells after NALCN KD appears to be on the order of 0.05 nS.

173

174

175

176 **NALCN contributes to the inward Na⁺ leak current in primary pituitary** 177 **cells**

178 To further determine the contribution of the NALCN-mediated inward leak
179 current to the background Na⁺ current of pituitary cells, we compared the inward
180 currents at a holding potential of -80 mV in SCR control and NALCN KD cells to
181 remove any influence from K⁺ channel conductance. The holding current
182 obtained by voltage clamping cells at -80 mV was found to be significantly
183 larger in the SCR control group compared to the NALCN KD group (Figure 4A-
184 C, SCR control (mean ± SD): -0.72 ± 0.2 pA/pF, n=15; NALCN KD: -0.19 ± 0.13
185 pA/pF, n=16; p< 0.001, *t*-test), confirming that the NALCN KD cells were more
186 hyperpolarised. Remarkably, this background Na⁺ conductance was reduced by
187 0.045 nS in SCR control, but by only 0.015 nS in NALCN KD upon the
188 substitution of extracellular Na⁺ with the impermeant cation NMDG⁺. This
189 indicates that the measured inward currents were mediated by a background
190 Na⁺ conductance which was more strongly reduced in NALCN KD cells (Figure
191 4D, SCR control (median ± interquartile): 0.045 ± 0.02 nS, n = 16; NALCN KD:
192 0.015 ± 0.01 nS, n = 15; p < 0.001, Mann Whitney test).

193

194 These findings indicate that NALCN contributes most of the inward leak
195 conductance in pituitary cells. Moreover, we obtained a rough estimate of the
196 contribution of NALCN channels to the background Na⁺ conductance by noting
197 that the difference in background Na⁺ conductance between the SCR control
198 and NALCN KD group is 0.045 – 0.015 = 0.03 nS. This is probably an
199 underestimate since the NALCN KD may not have removed all the channels,
200 but it implies that a change in background Na⁺ conductance of just 0.03 nS can
201 have a profound effect on the electrical activity of endocrine anterior pituitary
202 cells. This is in agreement with the observation that adding 0.05 nS of inward
203 leak conductance in NALCN KD cells restore electrical activity (Figure 3B).

204

205 **NALCN is required for spontaneous intracellular Ca²⁺ oscillations in** 206 **primary pituitary cells**

207 Previous studies have shown that the pattern of spontaneous firing determines
208 the amplitude and duration of intracellular Ca²⁺ oscillations in anterior endocrine
209 pituitary cells (Stojilkovic et al, 2005, Stojilkovic et al, 2012). Thus, we next

210 tested whether NALCN KD affects spontaneous intracellular Ca^{2+} transients in
 211 these cells. Consistent with a previous report (Tomić et al, 2011), approximately
 212 57 % (51 of 90) of SCR control pituitary cells exhibited spontaneous intracellular
 213 Ca^{2+} transients (Figure 5A). In contrast, only 11 % (4 of 36) of NALCN KD cells
 214 displayed low-amplitude intracellular Ca^{2+} transients, the remaining 89 % were
 215 quiescent and did not generate any intracellular Ca^{2+} oscillations. (Figure 5B).
 216 To quantitatively compare the size of the intracellular Ca^{2+} fluctuations between
 217 the SCR control and NALCN KD cells, the standard deviation of the Ca^{2+} trace
 218 for each individual cell was calculated over 600 seconds. Comparing the
 219 standard deviation between the two groups revealed a statistically-significant
 220 difference (Figure 5C, SCR control: median=0.04, n=90; NALCN KD:
 221 median=0.015, n=36; $p < 0.001$, Mann Whitney test). The final peak in each trace
 222 in Figure 5A,B represents the extracellular K^+ (15 mM) induced depolarisation
 223 and consequently a rise in $[\text{Ca}^{2+}]_i$, which was used as a control for the viability
 224 of cells. This indicated that NALCN plays a role in regulating intracellular Ca^{2+}
 225 oscillations of primary pituitary cells.

226

227 **NALCN mediates a low extracellular Ca^{2+} -induced depolarisation in** 228 **primary pituitary cells**

229 In primary anterior pituitary cells, the lowering or removal of extracellular Ca^{2+}
 230 causes membrane depolarisation and silencing of spontaneous firing activity but
 231 with no effect on $[\text{Ca}^{2+}]_i$ (Stojilkovic, 2006; Sankaranarayanan and Simasko,
 232 1996; Kwiecien and Hammond, 1998; Tsaneva-Atanasova et al, 2007). The
 233 removal of extracellular Ca^{2+} in hippocampal neurons activates slow and
 234 sustained inward leak currents through the NSCC (Chu et al, 2003). It is
 235 reported that the removal of extracellular Ca^{2+} activates the UNC80-NALCN
 236 complex, increasing the NALCN current and thus regulating excitability in
 237 neurons (Lu et al, 2010). Therefore, we next explored the possibility that
 238 NALCN could be responsible for the low extracellular Ca^{2+} -induced
 239 depolarisation in pituitary cells. To test this hypothesis, the inward leak current
 240 was measured at a holding potential of -80 mV before and after reducing
 241 extracellular Ca^{2+} from 2 mM to 0.1 mM.

242

243 Consistent with previous studies in neurons, removal of extracellular Ca^{2+}
 244 significantly increased the inward leak current in SCR control pituitary cells over

a time-course of 20 to 200 seconds (median $\pm$ interquartile: -0.8 ± 0.6 pA/pF to -1.5 ± 0.5 pA/pF, $n = 15$, $p < 0.001$, Kruskal-Wallis, Figure 6A,C). Subsequent replacement of extracellular Na^+ with NMDG $^+$ reduced the holding inward leak current (-1.5 ± 0.5 pA/pF to -0.3 ± 0.2 pA/pF, $n = 15$, $p < 0.001$, Kruskal-Wallis, Figure 6A,C), indicating that the extracellular Ca^{2+} -induced depolarisation is Na^+ mediated. In contrast, lowering the extracellular Ca^{2+} in NALCN KD pituitary cells results in a small rise in the inward leak current (-0.37 ± 0.35 pA/pF to -0.5 ± 0.25 pA/pF, $n = 13$, $p < 0.05$, Figure 6B,C). The substitution of extracellular Na^+ with NMDG $^+$ in NALCN KD cells still reduced the inward leak current but only marginally by comparison (-0.5 ± 0.25 pA/pF to -0.2 ± 0.25 pA/pF, $n = 13$, $p < 0.01$, Figure 6C), which reinforces the fact that NALCN is involved. This current could result from residual NALCN still expressed in the NALCN KD cells (since knocking down NALCN may not necessarily result in a 100% knock out) or contribution from other NSCCs, or from the seal conductance.

260

The overall rise in the inward leak current after reducing extracellular Ca^{2+} in control pituitary cells was significantly higher than that in NALCN KD pituitary cells (median $\pm$ interquartile in SCR control: -0.6 ± 0.6 pA/pF, $n = 13$, NALCN KD: -0.13 ± 0.1 pA/pF, $n = 13$, $p < 0.001$, Mann Whitney test, Figure 6D). Our results show that, similarly to what has been observed in neurons, NALCN current is reduced by extracellular Ca^{2+} and is involved in the low Ca^{2+} -induced depolarisation observed in pituitary cells.

268

269 Discussion

270

271 NALCN regulates the excitability of murine anterior pituitary cells

All endocrine anterior pituitary cell types produce intrinsically-regulated firing. This firing is crucial for maintaining their normal physiology such as basal hormone secretion, gene expression and Ca^{2+} stores level (Kwiecien and Hammond, 1998; Stojilkovic et al, 2010; Fletcher et al, 2018). However, the molecular identity of the major non-selective cationic conductance (NSCC) that enables spontaneous firing in pituitary cells is not fully determined (Fletcher et al, 2018). Pharmacological studies have revealed that the NSCC is unequivocally Na^+ -dependent and TTX-resistant (Simasko, 1994;

280 Sankaranarayanan and Simasko, 1996; Kucka et al, 2010; Liang et al, 2011;
281 Tomic et al, 2011; Zemkova et al, 2016). Some studies have suggested that
282 transient receptor cation channels (TRPC), as well as hyperpolarisation-
283 activated cyclic nucleotide-gated channel (HCN) may contribute to the NSCC
284 described in pituitary cells and related cell lines (Tomic et al, 2011; Kucka et al,
285 2012; Kayano et al, 2019, Kretschmannova et al, 2012). However, these
286 deductions were based on RNA expression profile as well as on the use of non-
287 selective pharmacological compounds. In addition, no genetic manipulations
288 (e.g. modification of the ion channel expression level) were performed to
289 demonstrate their contribution to NSCC. A recent study, however, implicates
290 NALCN as a major contributor to the NSCC in the GH3 pituitary cell line
291 (Impheng et al, 2021).

292

293 To determine whether this finding extends to native pituitary cells, we applied
294 genetic manipulation to knockdown NALCN channel, in combination with
295 electrophysiology and calcium imaging to investigate its role in regulating cell
296 excitability and $[Ca^{2+}]_i$ oscillations in cultured murine anterior pituitary cells. We
297 found that 1) NALCN channel encodes a major Na^+ leak conductance to sustain
298 the spontaneous firing activity in endocrine pituitary cells, 2) NALCN is the main
299 contributor to the depolarising NSCC in pituitary cells, without which the cells
300 remain silent and hyperpolarised. 3) NALCN is crucial for maintaining
301 spontaneous intracellular Ca^{2+} transients in these cells. 4) As in neurons,
302 NALCN activity is sensitive to the extracellular Ca^{2+} level in pituitary cells.
303 Taken together, these results support a critical role for NALCN in both
304 maintaining the spontaneous firing and the subsequent intracellular Ca^{2+}
305 transients in primary pituitary cells.

306

307 We found that NALCN KD pituitary cells are significantly hyperpolarised (by
308 about 15 mV) compared to control pituitary cells. Moreover, 90% of NALCN KD
309 pituitary cells were entirely silent and did not generate any action potentials.
310 Interestingly, restoring the non-selective cationic conductance in NALCN KD
311 cells using dynamic clamp made these cells generate action potentials in the
312 normal frequency range for pituitary cells. However, discontinuing NALCN-like
313 conductance injection (or dynamic-clamp mimic of NALCN conductance)
314 immediately silenced the cells, retuning the membrane potential to the

hyperpolarised state. On the other hand, in the majority of silent control pituitary cells, the removal of the NALCN-like conductance did not return them to silent state but made them sustain the normal firing (data not shown).

A small NALCN conductance sustains a large depolarising drive

The comparison between background Na^+ inward current in control cells and NALCN KD cells showed that the amount of NALCN conductance knocked down in our experiments was about 0.03 nS. This is consistent with the median 0.05 nS background Na^+ conductance required to activate silent NALCN KD cells. This dynamic clamp-based estimate does not rely on measurements of small noisy currents (a few pA) in voltage clamp, and therefore provides an estimate that is not affected by poor signal-to-noise ratio. The profound effect on electrical activity of such a small conductance results from the high input resistance of pituitary cells, on the order of 5 G Ω . Assuming a reversal potential around 0 mV for this conductance, at a membrane potential of -60 mV the current through a 0.05 nS NALCN conductance is 3 pA. If the membrane resistance is 5 G Ω , the depolarisation due to the current is therefore 15 mV. Again, this is consistent with our finding that NALCN KD cells were hyperpolarised by about 15 mV.

We have used the same knockdown approach as Impheng et al (2021), who have reported an estimated NALCN conductance of about 30 pS/pF in GH3 cells. This represents a conductance about three times larger than our estimate in native pituitary cells (10 pS/pF, given our average pituitary cell capacitance of 5 pF). We used a slightly higher extracellular concentration of Mg^{2+} than Impheng et al, 2021 (1 mM vs 0.8 mM). Given that divalent cations block NALCN (Chua et al 2020), this may explain part of the difference between our reported conductance values. It is also possible that GH3 cells simply express higher levels of NALCN. A limitation of this work is that we have not distinguished the different pituitary cell types we have recorded. Different cell types may express different average levels of NALCN. If such heterogeneity exists, then our estimate of NALCN conductance is an aggregate of the average NALCN conductance expressed by each cell-type we have sampled.

349 There is no available estimate for NALCN single-channel conductance, and we
350 are probably under-estimating NALCN conductance in pituitary cells with a
351 knockdown-based approach, since there may still be some NALCN channels
352 expressed in the KD cells. Nevertheless, our estimate of 0.05 nS implies that
353 cells have few active NALCN channels on their membrane. This would explain
354 why our recordings of the non-specific cation current (Figure 4A, 6A), and
355 others' (Liang et al 2011) are very noisy. Transient block by divalent cations,
356 and possibly random channel gating, since NALCN has weak voltage sensitivity
357 (Bouasse et al, 2019, Chua et al 2020), would result in relatively large current
358 fluctuations if the number of channels is low.

359

360 **NALCN activity is modulated by extracellular Ca^{2+} in pituitary cells**

361 Under both physiological and pathological circumstances extracellular Ca^{2+}
362 ($[\text{Ca}^{2+}]_e$) can drop markedly in various brain regions (Ren, 2011) and serum
363 (Ferry et al, 1997). This variation of serum Ca^{2+} appears to regulate
364 adrenocorticotrophic hormone (ACTH) secretion from pituitary corticotrophs.
365 Indeed, variations in serum Ca^{2+} within physiological range is associated with a
366 drastic change in ACTH secretion (Isaac et al, 1984; Fuleihan et al, 1996). At
367 the cellular level, the reduction in $[\text{Ca}^{2+}]_e$ results in membrane depolarisation of
368 cultured rat lactotrophs and somatotrophs (Sankaranarayanan and Simasko,
369 1996; Tsaneva-Atanasova et al, 2007). Conversely, an increase in $[\text{Ca}^{2+}]_e$, but
370 also in $[\text{Mg}^{2+}]_e$, to 10 mM with a concomitant blockade of calcium channel
371 resulted in a hyperpolarisation of the membrane potential of cultured rat pituitary
372 somatotrophs (Tsaneva-Atanasova et al, 2007). It has been speculated that the
373 underlying mechanism of low $[\text{Ca}^{2+}]_e$ – induced membrane depolarisation could
374 involve either the inhibition of Ca^{2+} -activated potassium channels and/or the
375 increase of a Na^+ background conductance (Sankaranarayanan and Simasko,
376 1996; Tsaneva-Atanasova et al, 2007).

377

378 NALCN was found to be sensitive to blockade by $[\text{Ca}^{2+}]_e$ in neurons and GH3
379 cells (Lu et al, 2010; Bouasse et al, 2019; Impheng et al, 2021). Our results
380 show that in mice anterior pituitary cells, NALCN channels are also sensitive to
381 $[\text{Ca}^{2+}]_e$ blockade, and mediate the low $[\text{Ca}^{2+}]_e$ -induced membrane
382 depolarisation. Two different mechanisms have been proposed to underlie
383 NALCN blockade by $[\text{Ca}^{2+}]_e$. First, this blockade could occur through the Ca^{2+} -

sensing receptor (CaSR) *via* a G_q-protein dependent pathway that ultimately induces NALCN phosphorylation by protein kinase C (Lu et al, 2010; Lee et al, 2019). Alternatively, this blockade could occur through direct binding of Ca²⁺ within the NALCN pore (Chua et al, 2020). It remains to determine which mechanism is involved in the NALCN modulation by [Ca²⁺]_e in primary pituitary cells. Of note, Zemkova and colleagues reported that lowering [Ca²⁺]_e had no effect on the membrane potential of corticotrophs (Zemkova et al, 2016) even though Letissier and colleagues found these cells express the NALCN-coding mRNA (personal communication). Possibly, the short amount of given time (20 seconds) during which the [Ca²⁺]_e was removed may not be long enough to allow the membrane potential to depolarise (Zemkova et al, 2016). In our experiments, the inward leak current in the majority of primary pituitary cells started to increase within 20 seconds to about a minute after removal of [Ca²⁺]_e and continued to increase within the next few minutes until it reached a steady state. This timeline suggests that a transduction cascade instead of a direct pore effect could be involved.

400

401 **NALCN modulation by neurohormones: a key player in pituitary cell** 402 **response to hypothalamic signals?**

403 In addition to Ca²⁺ sensing, NALCN can be negatively and positively regulated
404 by G-protein dependent and independent signalling pathways (Lu et al, 2009;
405 Swayne et al, 2009; Philippart & Khaliq, 2018). We have seen above that a
406 small NALCN conductance has a powerful effect on pituitary cell electrical
407 excitability. Together, this suggest that NALCN modulation by hypothalamic
408 signals would therefore provide a powerful way to control pituitary activity and
409 regulate pituitary hormones secretion. Several studies have suggested that a
410 NSCC is essential for facilitating pituitary cell response to hypothalamic signals.
411 For instance, several groups have shown that hyperpolarising the cells by
412 removing extracellular Na⁺ supresses both growth hormone releasing hormone
413 (GHRH)-induced Ca²⁺ influx (Lussier et al, 1991; Naumov et al, 1994) and
414 GHRH-induced growth hormone secretion from rat pituitary *in vitro* (Kato et al,
415 1988). In the same study, it was found that TTX had no effect on either GHRH-
416 induced GH secretion or GHRH-induced Ca²⁺ influx (Kato et al, 1988).
417 Interestingly, it was shown that in the absence of extracellular Na⁺, changing the
418 resting membrane potential by increasing the extracellular K⁺ to 15 or 30 mM

completely rescued the GHRH-induced GH secretion (Kato et al, 1988). Removal of extracellular Na^+ also substantially delayed the stimulatory response to corticotrophin-releasing hormone (CRH) by approximately 13 minutes in murine corticotroph cells (Liang et al, 2011). Consistent with these observations, Tomić et al, (2011) found that after removal of bath Na^+ , stimulating adenylyl cyclase with forskolin (adenylyl cyclase and protein kinase A activator) does not rescue spontaneous $[\text{Ca}^{2+}]_i$ transients in pituitary cells. These experiments suggest a critical role of NALCN in the response of pituitary cells to hypothalamic neurohormones. However, since these experiments essentially removed the depolarising component of NALCN, they cannot be used to assess whether NALCN is activated directly by neurohormones. In other words, the experiments cannot distinguish whether NALCN acts permissively to enable cells to respond to neurohormones or is directly involved in mediating the effects of the hormones. Thus, it could be of value to explore the impact of hypothalamic neurohormones on NALCN channel in each anterior pituitary cell type to identify the key effector ion channels involved in the regulation of pituitary hormone secretion.

The approach based on NALCN knockdown and electrophysiology developed here outlines a way to make this distinction. For example, NALCN depolarising current can be reintroduced using dynamic clamp in NALCN KD cells. In this way, while the native NALCN channel is prevented to directly mediate the action of neurohormones, its permissive action on membrane potential will be conserved. Alterations of cellular response to neurohormones under these circumstances imply that NALCN participates directly in receptor-mediated response to neurohormones.

Perspectives

Our data demonstrates that, as in neurons, NALCN is a crucial player in pituitary cell excitability by conducting a Ca^{2+} -sensitive background Na^+ conductance that regulates membrane potential. Our data thus suggests that NALCN plays a key role in regulating hormone secretion from pituitary cells. Our approach of combining NALCN knockdown and dynamic clamp can now be used to determine whether NALCN directly participates in mediating pituitary responses to hypothalamic signals. The development of *Nalcn* knockout mouse

454 in a specific population of anterior pituitary cells is now required to examine the
455 impact of NALCN on the different pituitary hormones.

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460

461 **Materials and Methods**

462

463 **Primary cell culture**

464 Murine endocrine anterior pituitary cell cultures were prepared between 8:00
465 and 11:00 AM from wild-type C57BL/6J mice as required. Mice were kept in
466 groups of two to four under standard circumstances at the University of Exeter
467 (UK) animal unit: Lights on at 6:00 AM, lights off at 6:00 PM at 21°C, tap water
468 and food were available *ad libitum*. The adult mice aged between two to six
469 months were selected randomly regardless of their sex. All the animal work was
470 carried out according to the standards of the Home Office (England) and the
471 University of Exeter. Three or four mice were culled via cervical dislocation and
472 then were decapitated in accordance with Schedule 1 procedures. After
473 removing the brain, the pituitary gland was removed from the sella turcica (bony
474 cavity) and placed in a 100x21 mm culture dish (Thermo Fisher Scientific, UK)
475 containing 150 µL 4°C DMEM (Dulbecco's modified Eagle's medium with high
476 glucose and 25mM HEPES from Sigma Aldrich (Merck), UK) located on ice.
477 Under a dissection microscope, the intermediate and posterior lobes were
478 removed using a scalpel blade (size 10), and the anterior lobes were chopped
479 to small pieces manually. Subsequently, the chopped tissues were transferred
480 into a 50 mL falcon tube containing 2.5 mL DMEM supplemented with 207
481 TAME Units/mL trypsin and 36 Kunitz Units/mL DNase I (both from Sigma
482 Aldrich, UK), and then incubated in 37°C water bath for 10 minutes. Every 5
483 minutes, the tube was swirled to disperse the tissue pieces evenly to achieve a
484 thorough digestion. After 10 minutes, the suspension was gently triturated 20-30
485 times using a 1 mL pipette tip. At the end of the digestion step, an inhibition
486 solution containing 5 mL DMEM supplemented with 0.25 mg/mL Lima soybean
487 trypsin inhibitor, 100 kallikrein unit aprotinin and 36 Kunitz Units/ml DNase I
488 (Sigma Aldrich, UK) was added to the digestion solution, and the cell

suspension was left for a few minutes to inactivate the trypsin enzyme activity. The resulting suspension was finally filtered through a cell strainer with 70 μ m nylon mesh (Merck) and was centrifuged at 100xg for 10 minutes. The pellet was resuspended in 500-600 μ L DMEM solution and then 60 μ L was plated on each 15 mm diameter round coverslip (Thermo Fisher Scientific, UK) in a 12-well plate. After 20 minutes, once the cells were securely attached to the bottom of coverslips, 1 mL of growth medium (DMEM + 2.5% FBS + 0.1 % Fibronectin + 1% antibiotic-penstrep, from Sigma Aldrich, UK) was added to each well and then incubated at 37°C in a 5% CO₂ incubator. The culture medium was replaced with antibiotic-free growth-medium 6 hours later. The growth medium was refreshed every two days. 5 μ L concentrated suspension of lentivirus was added to the medium in each well of a 12-well plate. Fresh growth medium was substituted 24 hours after transduction. Green fluorescence cells were usually observable 2-3 days after transduction and selected for electrophysiological recording. Each batch of primary pituitary cell culture was utilized for up to 5 days after transduction for electrophysiological recordings and calcium imaging.

505

506 **Lentivirus**

A microRNA-adapted shRNA based on miR-30 for specific NALCN silencing cloned in the lentiviral pGIPZ plasmid and targeting the 5'-GCAACAGACTGTGGCAATT-3' region of the rat NALCN-encoding RNA was obtained from a commercial source (Dharmacon #V2LMM_90196). A non-silencing scramble control was used in our experiments (Dharmacon #RHS4346). HpaI/BamHI are the restriction sites in pGIPZ plasmid between which the NALCN silencing shRNA was inserted.

514

515 **Electrophysiological recording**

Electrophysiological recordings from pituitary cells were performed at room temperature using single-cell amphotericin-perforated patch-clamp technique. The recordings were obtained using an Axopatch 700B amplifier and Clampex 10.1 (Molecular Devices) with a sampling rate of 10 kHz and filtered at 2 kHz (lower pass). Patch pipettes were fabricated from borosilicate glass with filament (outer diameter: 1.50 mm and inner diameter: 0.86 mm, Warner Instrument-multi channel system distributor) and pulled using a micropipette puller (Sutter Instruments, model P-97). Pipette tips were then fire polished and

523

524 had a resistance ranging from 4 to 6 MΩ. Once a high resistance seal was
525 formed (>10 GΩ), usually within 10 minutes of patching, the access resistance
526 (or series resistance) would reduce to less than 50 MΩ, and then recording
527 started. If the seal resistance was less than 10 GΩ, the cell was discarded. In
528 current clamp mode, series resistance was compensated by Bridge-Balance
529 and was usually less than 40 MΩ. Junction potential was not corrected. In
530 voltage clamp mode, compensated series resistance (60%) was normally less
531 than 50 MΩ and the capacitance of recorded pituitary cells ranged between 4
532 and 6 pF (electronic compensation was done *via* whole-cell mode of multiclamp
533 700B). During recording, the cells were constantly perfused using a gravity-
534 driven perfusion system, with a flow rate of 0.5 mL/min with extracellular
535 solution containing (in millimolar) 138 NaCl, 5 KCl, 10 alpha-D-glucose, 25
536 HEPES, 0.7 Na₂HPO₄, 1 MgCl₂ and 2 CaCl₂. The pH was adjusted to 7.4 with
537 NaOH, and the osmolality was 305 mOsmol/L. Patch pipettes were filled with an
538 intracellular solution containing (in millimolar) 10 NaCl, 100 K-Gluconate, 50
539 KCl, 10 HEPES, and 1 MgCl₂. The pH was adjusted to 7.2 with KOH, 295
540 mOsmol/L. The osmolality of the solutions was maintained by adding an inert
541 ingredient sucrose. 5 µl Amphotericin-B of a stock solution (20 mg/mL in
542 dimethyl sulfoxide) was added to 1 mL of pipette solution to achieve a final
543 concentration of 50 µg/ml. Other concentrations such as 10 µl Amphotericin-B
544 were also tried, however 5 µl resulted in more durability of the recording (all
545 salts and Amphotericin-B were purchased from Sigma Aldrich).

546

547 **Dynamic clamp**

548 With dynamic clamp we can effectively change the biophysical property of ion
549 channels (such as activation, inactivation, gating kinetics and conductances) as
550 we choose and investigate how these manipulations affect the pattern of
551 electrical activity in real time. In other words, we can establish the contribution
552 of a type of ion channel to regulating various aspects of electrical activity
553 (Milescu et al, 2008).

554

555 In this study, a second computer and an analogue-to-digital acquisition card
556 (DAQ) were installed to run the dynamic clamp module in the software QuB
557 (Milescu et al, 2008). In the current clamp mode of the Axopatch 700B amplifier,
558 the membrane potential (V_m) of a patched cell was recorded in real time and

559 passed to the computer running QuB as an input for a mathematical expression
 560 of non-selective cationic leak channels : $I_{NS}=g_{NS} (V_m - E_{NS})$ which defines the
 561 corresponding current (I_{NS}) going through them. The sodium leak conductance
 562 (g_{NS}) was changed manually. The calculated I_{NS} was then injected back to the
 563 cell via the same DAQ and then the membrane voltage response was recorded.
 564 Thus, the injected NALCN-like current is varied dynamically, unlike conventional
 565 current clamp in which the injected current is constant across all time points.
 566 The reversal potential (E_{NS}) for this channel was considered zero since this
 567 channel is permeable to different monovalent cations (e.g. K^+ and Na^+) but
 568 primarily to Na^+ (Lu et al, 2007; Chua et al, 2020).

569

570 **Measurement of cytosolic calcium in single pituitary cells**

571 The coverslips with pituitary cells were bathed in the extracellular solution
 572 (containing same ingredients as described in the above electrophysiology
 573 section) with 2 μ M fura-2 AM (Thermo Fischer Scientific-# F1221) for 45 minutes
 574 at 37°C. The cells were then rinsed three times with the extracellular solution
 575 using a 2 mL Pasteur pipette. Following this, the coverslips were mounted onto
 576 the recording chamber (volume ~ 0.2 mL) on the stage of an inverted
 577 microscope (Nikon eclipse Ti). Cells were constantly perfused with the
 578 extracellular solution at room temperature using a gravity-driven perfusion
 579 system. Cells were excited every 1 second with alternating 340-nm and 380-nm
 580 light beams (20 millisecond exposure time) originating from a Lambda DG-4
 581 wavelength switcher (Sutter Instrument Company). Light intensity was reduced
 582 by 50% before hitting the cells using an appropriate filter. The intensity of
 583 emitted light was measured at 520 nm and images were acquired by a
 584 Hamamatsu digital camera C1344 set to 4 × 4 binning. Hardware control was
 585 achieved by TI Workbench software developed by T. Inoue (As used by Tabak
 586 et al, 2010). Using this software, regions of interest (ROI) were selected around
 587 the cells that were not overlapping with other cells and a single background ROI
 588 was selected in an empty space. Pixel values within each region of interest
 589 were averaged for both 340 and 380 excitation wavelengths and then
 590 subtracted from the background. Following this, a ratio (r) was computed
 591 according to the formula:

$$592 \quad r = (ROI_{340} - ROI_{background340}) / (ROI_{380} - ROI_{background380})$$

593 Trace analysis was performed in MATLAB.

594

595 **Immunohistochemistry**

596 Pituitary glands were extracted and fixed overnight in 4% PFA in PBS. The
 597 following day, 70 µm thick sections were cut using a vibratome (Campden
 598 Instruments, UK), placed on poly-L-Lysine coated microscope slides (VWR) and
 599 left to dry. Next, the sections were blocked with 10% FBS in PBST (PBS
 600 containing 0.01% Triton X-100) for one hour at room temperature. Primary
 601 rabbit anti-NALCN antibody at 1:500 dilution (Alomone Labs, Israel, #ASC-022)
 602 in 10% FBS/PBST was applied to the sections and incubated in humid chamber
 603 at 4 C° degrees overnight. The next day, sections were washed 3 times for 15
 604 min with PBST. Then, the secondary 488-Alexa-Fluor conjugated antibody (at
 605 1:1000, Molecular Probes) was applied in 10% FBS/PBST solution for 1 hour at
 606 room temperature, followed by a series of three 15 min washes with PBST. The
 607 nucleic acids present in the pituitary gland cells were visualised with TOTO-3
 608 (1:2000, Thermo Fisher Scientific). Sections were incubated with TOTO-3 for 15
 609 min on an agitated surface, then washed three times (each time 10 min) and
 610 subsequently mounted with Fluorsave medium (Calbiochem). The controls were
 611 the sections where primary antibody was omitted. Images were obtained using
 612 a Zeiss LSM 5 Exciter confocal microscope run by Zen software.

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614 **Statistics**

615 The type of statistical test used in each case is specified in the result using
 616 MATLAB.

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802 Figure 1

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821 Figure 1. The NALCN ion channel is expressed in mouse pituitary gland.

822 Immunofluorescence staining revealed the presence of NALCN protein in
823 mouse pituitary gland (n=3). NALCN is shown in green. TOTO-3 (shown in
824 magenta) was used to visualise cellular nucleic acids. **A)** Transverse section of
825 a pituitary gland under 10X magnification excited with 488 nm light to detect
826 NALCN immunofluorescence. **B)** NALCN visualisation in the area defined by
827 dashed square in A. **C)** Toto-labelled nucleic acid in the same area. **D)** Merged
828 image from B and C. B, C and D are under 20X magnification. **E-G)** are under
829 higher digital magnification using confocal microscopy. **H)** Negative control
830 tissue for which the primary anti NALCN antibody was omitted. The scale bars
831 indicate 500 μ m in panel A, and 20 μ m in panels D and G.

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837 Figure 2

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850 Figure 2. Knock-down of NALCN silences electrical activity of primary

851 **murine pituitary cells.** Representative traces of spontaneous firing activity

852 from **A)** an untreated and **B)** a scramble control (SCR Ctrl) pituitary cell. **C)**

853 Representative trace of electrical activity in a NALCN knockdown (NALCN KD)

854 pituitary cell. **D)** Percentages of active and silent cells in untreated control, SCR

855 Ctrl and NALCN KD pituitary cells. **E)** Resting membrane potential of untreated

856 control, SCR Ctrl and NALCN KD pituitary cells (data presented as mean $\pm$ SD,

857 untreated control: -47.6 ± 7.6 mV, $n=31$ vs SCR control: -45 mV ± 5.8 mV,

858 $n=30$, $p>0.1$; NALCN KD: -63.6 ± 8.5 mV, $n=31$ vs SCR Ctrl and untreated

859 control: $p < 0.001$, one-way ANOVA with Bonferroni correction), the box

860 represents the standard error, the whiskers represent standard deviation (SD).

861 **F)** Distribution of firing frequency between untreated Ctrl, SCR Ctrl and NALCN

862 KD cells over a course of 600 seconds (data presented as mean $\pm$ SD, NALCN

863 KD: 0 Hz, $n= 31$ vs SCR Ctrl: 0.5 ± 0.4 Hz, $n= 25$ and vs untreated Ctrl: $0.7 \pm$

864 0.6 Hz, $n= 25$, $p < 0.001$; SCR Ctrl vs untreated Ctrl: $p > 0.1$, one-way ANOVA

865 with Bonferroni correction). Only three out of 31 NALCN KD cells exhibited firing

866 activity, which occurred at a lower frequency (0.1 ± 0.02 Hz) relative to the

untreated and SCR controls. In each graph, triple asterisks (***) represents $p < 0.001$.

Figure 3

Figure 3. Addition of nonselective cationic conductance restores firing activity in silent NALCN KD pituitary cells. A) Two representative voltage traces of silent NALCN Knock-down (NALCN KD) pituitary cells. Firing activity was restored once the nonselective cationic conductance (g) was increased by a very minute amount, such as 0.02 (right panel, the cell started silent and then discharges APs mid-way) or 0.04 nS (left panel). The cells immediately returned to a silent and hyperpolarised state after removal of the added conductance. **B)** The distribution of added conductance (g) values required for restoring the firing activity in NALCN KD pituitary cells. Median: 0.05 nS, n=28.

Figure 4

Figure 4. NALCN contributes most of the inward Na^+ leak conductance in endocrine anterior pituitary cells. A) Representative trace of an inward Na^+ leak current ($V_h = -80 \text{ mV}$) in a SCR Ctrl pituitary cell as revealed by the substitution of extracellular Na^+ with NMDG $^+$. **B)** Representative trace of an inward leak current in a NALCN KD pituitary cell as revealed by the substitution of extracellular Na^+ with NMDG $^+$. **C)** The density of the inward Na^+ leak current

in NALCN KD cells was significantly reduced compared to SCR Ctrl (data presented as mean $\pm$ SD, SCR Ctrl: -0.72 ± 0.2 pA/pF (-5.2 ± 1.9 pA), $n=15$; NALCN KD: -0.19 ± 0.13 pA/pF (-1.5 ± 0.9 pA), $n=16$; $p < 0.001$, t -test). **D)** The background Na^+ conductance was more strongly reduced by Na^+ removal in control cells than in NALCN KD cells (data represented as Median $\pm$ interquartile, SCR control: 0.045 ± 0.02 nS, $n = 16$; NALCN KD: 0.015 ± 0.01 nS, $n = 15$; $p < 0.001$, Mann Whitney test).

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Figure 5

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Figure 5. NALCN KD impacts on intracellular Ca^{2+} transients.

A) Representative trace of intracellular Ca^{2+} oscillations in an SCR control pituitary cell. The final peak in each graph represents the extracellular K^+ (15 mM)-induced rise in $[\text{Ca}^{2+}]_i$, which was used to check cell viability. **B)** A representative trace of $[\text{Ca}^{2+}]_i$ in a NALCN KD pituitary cell. **C)** The standard deviation of each $[\text{Ca}^{2+}]_i$ trace was calculated and statistical analysis revealed a significant alteration of $[\text{Ca}^{2+}]_i$ oscillations between the two groups (SCR Ctrl: median= 0.04, $n = 90$ from 4 mice; NALCN KD: median= 0.015, $n = 36$ from 4 mice; $p < 0.001$, Mann Whitney test). Box: inter-quartile, Whiskers: range, excluding outliers, Central line: median.

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Figure 6

Figure 6. NALCN is sensitive to the changes in extracellular Ca^{2+} level. A) Representative trace of inward leak current in a SCR control pituitary cell. Reducing extracellular Ca^{2+} significantly increased the inward leak current over a time-course of 20 to 200 seconds (b). Subsequent replacement of extracellular Na^{+} with NMDG $^{+}$ strongly reduced the holding inward leak current (c). **B)** Representative trace of the inward leak current in a NALCN KD pituitary cell; lowering the extracellular Ca^{2+} resulted in a small rise in the inward leak

1007 current (b, applied for a longer time to rule out any further change in current).
1008 Subsequent replacement of extracellular Na^+ with NMDG^+ reduced this inward
1009 current (c). **C)** Normalised inward leak current in 2mM Ca^{2+} (a), 0.1 mM Ca^{2+}
1010 (b), and 0.1 mM Ca^{2+} + NMDG^+ (c) in SCR control (left) and NALCN KD cells
1011 (right). **D)** The overall rise in inward leak current after reducing extracellular
1012 Ca^{2+} in SCR control cells (left), compared to NALCN KD cells (right). Median $\pm$
1013 interquartile in SCR Control: -0.6 ± 0.6 pA/pF, $n = 13$; NALCN KD: -0.13 ± 0.1
1014 pA/pF, $n = 13$, $p < 0.001$, Mann Whitney test. Box: inter-quartile, Whiskers:
1015 range excluding outliers, Central line: median.
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