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Title: Crosstalk between mitochondria, calcium channels and actin cytoskeleton modulates noradrenergic activity of locus coeruleus neurons.

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Running title: Crosstalk between mitochondria, Ca^{2+} and actin.

Abbreviations: ACSF, artificial cerebrospinal fluid; AP, action potential; $I_{K\text{-}ATP}$, ATP-dependent K^+ channels; CCCP, carbonyl cyanide m-chlorophenylhydrazone (Sigma-Aldrich CAS number 555-60-2, catalogue number C2759); CICR, Ca^{2+}-induced Ca^{2+} release; CNS, central nervous system; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; I_{Cl}, chloride current; $I_{K\text{-}Ca}$, Ca^{2+}-activated K^+ current; K_{Ca}, Ca^{2+}-activated K^+ channel; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; LC, locus coeruleus; Ψm, mitochondrial membrane potential; RRID, Research Resource Identifier (see scicrunch.org); SK, small conductance Ca^{2+}-activated K^+ channels; TTX, tetrodotoxin (Tocris CAS number 4368-28-9, catalogue number 1078)

Abstract

Locus coeruleus (LC) is the name of a group of large sized neurons located at the brain stem, which provide the main source of noradrenaline to the central nervous system, virtually, innervating the whole brain. All noradrenergic signalling provided by this nucleus is dependent on an intrinsic pacemaker process. Our study aims to understand how noradrenergic neurons finely tune their pacemaker processes and regulate their activities. Here we present that mitochondrial perturbation in the LC from mice, inhibits spontaneous firing by a hyperpolarizing response that involves Ca^{2+} entry via L-type Ca^{2+} channels and the actin cytoskeleton. We found that pharmacological perturbation of mitochondria from LC neurons using the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), induced a dominant hyperpolarizing response when electrophysiological approaches were performed. Surprisingly, the CCCP-induced hyperpolarizing response was dependent on L-type Ca^{2+} channel-mediated Ca^{2+} entry, as it was inhibited by: removal of extracellular Ca^{2+}; addition of Cd^{2+}; nifedipine or nicardipine; but not by intracellular dialysis with the Ca^{2+} chelator BAPTA,

the latter indicating that the response was not due to a global change in $[Ca^{2+}]_c$ but does not exclude action at intracellular microdomains. Further to this, incubation of slices with cytochalasin D, an agent that depolymerises the actin cytoskeleton, inhibited the hyperpolarizing response indicating an involvement of the actin cytoskeleton. The data are consistent with the hypothesis that there is crosstalk between mitochondria and L-type Ca^{2+} channels leading to modulation of noradrenergic neuronal activity mediated by the actin cytoskeleton.

Introduction

Noradrenergic activity, i.e. action potential firing and consequently release of noradrenaline, is crucial to brain function. Even though, most of the noradrenaline release in the central nervous system (CNS) comes from a small noradrenergic nucleus called locus coeruleus (LC), its release is fundamental to maintain the brain "awake" and brain conscious activities (Berridge *et al.* 2012, Espana *et al.* 2016, Szabadi 2013). This sole fact, highlights the importance of a tight control of the noradrenergic activity, including the frequency and synchrony of firing (Christie *et al.* 1989, Alvarez *et al.* 2002), patterns of firing (Chu & Bloom 1973, Devilbiss *et al.* 2006), and resultant noradrenaline release, leading to activation of adrenergic receptors throughout the CNS.

Noradrenergic signaling despite having many functional roles such as wakefulness, is also involved in maintaining a degree of CNS "protection" (Feinstein *et al.* 2016). Impairment in the noradrenergic system, including changes in the firing activity, is a key step in developing various neuropathologies (Rommelfanger & Weinshenker 2007, Grudzien *et al.* 2007, Vermeiren & De Deyn 2017).

In a classical view, LC neurons possess an intricate pacemaker activity that allows tight regulation of many physiological processes (de Oliveira *et al.* 2010b, Williams *et al.* 1984). Examples include sleep regulation and vigilance (Roussel *et al.* 1967, Hobson *et al.* 1975), learning and memory (Kety 1972), and attention (Aston-Jones *et al.* 1999, Sara 2009). Key intracellular components underlying LC function include mitochondria. These subserve a crucial role in energy production (Lehninger *et al.* 2008, Bartolak-Suki *et al.* 2017) and Ca^{2+}-dependent signalling pathways via their intracellular Ca^{2+} buffering capacity (Gunter *et al.* 2004, Ishii *et al.* 2006, Gunter *et al.* 2000). They have also been implicated in pathologies such as neuronal death (Randall & Thayer 1992), oxidative stress (Andreyev *et al.* 2005) and certain neurodegenerative diseases (Vanitallie 2008). Some reports have proposed that the multifunctional physiological role of mitochondria allows them to behave as an intracellular "checkpoint" (Henry *et al.* 2002), whereby they closely regulate intracellular signalling pathways and confer resistance to cell death.

Mitochondria impact on action potential (AP) production and regulation, by several mechanisms including ion channels, such as ATP-dependent K^+ channels ($I_{K\text{-}ATP}$) (Liu *et al.* 2002, Bergmann & Keller 2004). A decrease in ATP/ADP ratios can activate $I_{K\text{-}ATP}$ channels and cause hyperpolarization (Liu *et al.* 1998). Likewise, other channels such as Ca^{2+}-activated K^+ channels (K_{Ca}) can be modulated by mitochondria (Tano & Gollasch 2014), and recently small conductance Ca^{2+}-activated K^+ channels (SK) were demonstrated to be part of the inner mitochondrial membrane (Dolga *et al.* 2013). K^+ channels modulate several important processes in neurons, such as AP duration and spike frequency adaptation (Storm 1990, Faber & Sah 2003b, Faber & Sah 2003a). Reactive oxygen species, which are primarily produced by mitochondria, can also interact with ion channels and modulate their activity (Ferranti *et al.* 2003). In this regard, it has been reported that impairment of mitochondrial activity in rat LC neurons generates an intracellular Ca^{2+}-dependent hyperpolarization that is driven by both Ca^{2+}-activated K^+ channels and ATP-dependent K^+ channels (Murai *et al.* 1997). We have also demonstrated that the content of oxidants present in LC neurons, is capable of modulating

mitochondrial membrane potential and pacemaker activity in small subpopulations of neurons within the LC nucleus (de Oliveira *et al.* 2012). Thus, there is considerable evidence that mitochondrial and ion channel functions are closely linked in LC neurons and this interaction may be fundamental in regulating noradrenergic activity.

In further exploring mitochondrial-pacemaker relation in LC neurons, we have unveiled a new component in this crosstalk: namely the actin cytoskeleton. Our data indicates interconnection between the actin cytoskeleton and activation of L-type Ca^{2+} channels triggered by agents that are known by modulate the mitochondrial membrane potential (Ψm). These interactions led to a "hyperpolarizing response" that was independent of intracellular Ca^{2+}. This mechanism provides a means for mitochondria to modulate the pacemaker process of LC neurons, connecting mitochondrial function with the firing output, a mechanism previously proposed for heart muscle (Viola et al 1999). In the case of the LC the mechanism could represent an accessory means of contributing to neuronal integration and information processing.

Methods

Preparation of brain slices:

All procedures were approved by The University of Newcastle Animal Care and Ethics Committee (AEC Approval Number A-2009-153), animals were obtained from the breeding facility of the University. Swiss mice (RRID: MGI:2162034; P6-12, both sexes) were used to prepare brain slices containing the LC. Mice were rendered unconscious with ketamine (100 mg/kg i.p. - Sigma-Aldrich CAS Number 1867-66-9, catalogue number BP736) (de Oliveira *et al.* 2010a) and then decapitated. The brain was rapidly removed and immersed in ice-cold "modified sucrose ringer" containing (in mM): 11 glucose, 25 $NaHCO_3$, 235 sucrose, 2.5 KCl, 1 $MgCl_2$, 1 NaH_2PO_4 and 2.5 $CaCl_2$, bubbled with 95% O_2/5% CO_2 (Graham *et al.* 2007). A vibrating

tissue slicer (Leica VT1000S) was used to cut slices (~ 270μm thick) from isolated cerebellum and brain stem. Slices were kept in a recovery chamber (containing ACSF) at room temperature and high oxygen for ~ 2 h before experiments commenced. An upright microscope (Olympus BX50) was used to identify the slice containing the LC. After identification, the slice was transferred to a recording bath and superfused with artificial cerebrospinal fluid (ACSF) containing (in mM): 11 Glucose, 25 $NaHCO_3$, 120 NaCl, 2.5 KCl, 1 NaH_2PO_4, 2.5 $CaCl_2$ and 1 $MgCl_2$, constantly bubbled with 95% O_2/5% CO_2 (pH 7.4). LC neurons were visualized using infrared video microscopy with differential interference contrast optics and identified according to their large size and location near the ventrolateral border of the fourth ventricle. When recordings were obtained from mechanically isolated LC neurons it was noted in the text. Most experiments presented were undertaken in neurons within slices.

Preparation of acutely dissociated LC neurons:

Brain slices were cut using the protocol described in "Preparation of brain slices" and allowed to recover for 1-2h. Slices were then placed in the ACSF-containing recording chamber and neurons were isolated using a custom made vibrating device according to an adapted protocol (Akaike & Moorhouse 2003). This instrument was used to vibrate a fine glass electrode with a sealed tip just above the tissue surface, generally providing ~15 healthy LC neurons. After isolation, neurons were left for 15 min to settle and attach to the glass on the bottom of the recording chamber. Electrophysiological properties of isolated LC neurons were not obviously distinguishable from those of LC neurons in slices as previously reported (de Oliveira *et al.* 2011). Both, presented similar electrophysiological characteristics including pacemaker currents and spontaneous firing. Input resistance (IR) and capacitance for isolated neurons were 322±32 MΩ and 37.2±1.8 pF respectively (n=5). IR was similar to that of 298±12 MΩ recorded from mechanically isolated LC neurons and higher than the IR previously reported for LC neurons in slices (e.g. 250±9.5 MΩ (de Oliveira et al. 2010a); see also (de Oliveira et al. 2011,

de Oliveira et al. 2010b, Williams et al. 1984). The finding of higher IR values for isolated LC neurons most likely resulted from reduction of the dendritic arbor by the isolation procedure.

Electrophysiology:

Axopatch-1C amplifier was used to perform recordings in whole cell voltage clamp or current clamp recording modes. Electrodes had resistances ranging 1.8 – 2.5 MΩ. Data were sampled at 100 kHz and low-pass filtered at 5 kHz. Neurons were considered to be adequately voltage clamped when no 'breakthrough' spikes were observed during voltage ramp application (de Oliveira et al. 2010b). Our exclusion criteria consisted in analyse the response of each neuron on current clamp, i.e. neurons that presented irregular firing, and/or lower input resistance and/or were unusual hard to make a proper seal with the patch pipette were excluded from the experiments.

Solutions and pharmacology:

To minimise perfusion-associated wash out during whole cell voltage clamp experiments, we used a protocol previously described (de Oliveira et al. 2010b). Briefly, 10s after intracellular access was gained the treatment perfusion was started most commonly by using a rapid change (> 90% change over ~ 2 s) local-perfusion system (Graham *et al.* 2006) and in some cases by standard bath perfusion (cases noted in the text). The rapid change local-perfusion system was used to avoid unspecific effects produced by drug application in slices. It consisted of a very thin silica tube attached to a net of silicon tubes connected to a small container where drugs (working solutions) are stored. The tip of the silica tube was placed just above the brain slice into the recording chamber. When the local perfusion system was turned on, it produced a stream of solution delivering treatments in a small area in front of the silica tube, allowing only neurons inside this small area receive the desired treatment in a very fast

fashion. The suction system of the recording chamber responsible to pump ACSF off, also promptly pumped off the drugs delivered by the local perfusion system, allowing only neurons in front of the silica tube have constant contact with the drug applied and not the whole slice. Depending on the location of the neuron being assessed into the LC nucleus, the silica tube of the fast change local-perfusion system was relocated to cover the area where the neuron was located. Recordings were generally made from 2-3 cells in each slice with some 30 minutes ACSF washout between drug applications. The standard internal pipette solution contained (in mM): 8 NaCl, 135 K methyl sulphate, 10 HEPES, 2 Mg_2ATP, 0.3 Na_3GTP, 0.1 EGTA, pH: 7.3. In some experiments 10 mM BAPTA (Sigma-Aldrich CAS number 85233-19-8, catalogue number A4926) was added to the standard internal solution (cases noted in the text). An additional procedure, which was aimed at depolymerising the actin cytoskeleton, involved incubating slices for 1-2 h with 1 μM cytochalasin D (Sigma-Aldrich CAS number 22144-77-0, catalogue number C8273). The slice was then transferred to the recording chamber that was perfused with ACSF. Whole cell recordings commenced and 1 μM CCCP (carbonyl cyanide m-chlorophenylhydrazone), (Sigma-Aldrich CAS number 555-60-2, catalogue number C2759) was applied using the standard experimental protocol. $CdCl_2$, BAPTA and Tetrodotoxin ((TTX) - Tocris CAS number 4368-28-9, catalogue number 1078) were made up as stock solutions in water with the others all made up with DMSO, the final concentration applied containing ≤ 0.1% DMSO. The experiment presented in Figure 10 used exclusively the internal pipette solution containing (in mM): 1 $MgCl_2$, 130 CsCl, 10 EGTA, 10 HEPES, 0.2 GTP, 2 ATP, pH: 7.3. All slice experiments were carried out at 33 ± 2 °C.

***Acquisition and analysis**:*

Data were acquired using Axograph 4.8 software ((RRID:SCR_014284) ITC-16 interface, and a Mac G4 computer), and analysed using Axograph X 1.1.0 software. A -5 mV pulse delivered shortly after achieving the whole cell-recording mode was used to measure input resistance, cell

capacitance and series resistance by the software. JPCalc (Barry 1994) was used for junction potentials corrections: -8.5 mV and -10 mV for the junction potentials between ACSF in the bath and K methyl sulphate or 10mM BAPTA internal pipette solutions, respectively. Recordings were filtered at 1 kHz using Axograph X software prior to analysis. "n" presented for each experiment represents number of cells/neurons accessed. At least three individual animals were used to prepare brain slices containing the LC for each experiment. No randomization method or blinding were performed in the present work.

Statistics:

GraphPad Prism 4.02 (RRID:SCR_002798) was used to prepare graphics, assess normality and perform statistic tests when applicable. Shapiro-Wilk normality test was used to assess normality and Wilcoxon signed rank test was used to perform statistic. No outlier test was conducted in the present work. Electrophysiological recordings are presented as representative experiments from different neurons (n stated on each experiment).

Results

Effect of CCCP on voltage-dependent currents and spontaneous LC firing

The effect of mitochondrial perturbation on spontaneous firing in LC neurons was investigated by rapid application (Methods) of the mitochondrial protonophore CCCP (Fig. 1). Application of 1 μM CCCP initially produced an increase in firing rate that peaked ~ 60 s after CCCP application (Fig. 1A). This was followed by a large hyperpolarization, which peaked after ~ 180 s and completely abolished spontaneous firing (n=8), AP discharge could be re-established by injection of depolarizing current (Fig. 1A, black bar). Voltage clamp recordings made at -53mV and -78mV holding potentials confirmed the effect shown in figure 1A, with

application of 1 μM CCCP initially inducing a small inward current (evident at a holding potential of -78 mV) followed by a large outward current that peaked ~ 5 min after CCCP application (Fig. 1B; n=5 both). The effect of Antimycin A (Sigma-Aldrich CAS number 1397-94-0, catalogue number A8674), a mitochondrial complex III blocker, was also analysed. 1 μM Antimycin A produced a hyperpolarization that closely resembled that produced by 1μM CCCP (Fig. 2; n=3). Antimycin A experiment was irreversible; after the first neuron be accessed all other neurons in the same slice were excluded due our exclusion criteria (see methods). This fact yielded an n of 3 for this particular experiment.

The voltage dependence of the CCCP-induced currents was investigated using two voltage clamp protocols (Fig. 3). The first involved applying depolarizing ramps (40 mV/s) to LC neurons in the presence of 1 μM CCCP from a holding potential of -58.5 mV. When voltage ramps were applied 60 s and 180 s after CCCP application two effects were observed on the evoked currents: initially, the neurons exhibited an "inward inflection" at hyperpolarized membrane potentials, then with increasing depolarization an "outward inflection" appeared (Fig. 3A, B; n=8). The differential current, calculated by subtracting the current recorded in ACSF from that in 1 μM CCCP (i.e. test minus control), showed the induced leakage current reversed at -82 ± 0.7 mV (Fig. 3C, n=8). A second voltage clamp protocol, involved applying depolarizing pulses from a holding potential of -88 mV in the presence of 1 μM TTX (tetrodotoxin). Application of depolarizing pulses from a holding potential of -88 mV revealed that the CCCP-induced "leakage" current was "non-inactivating", at least for the duration of the applied pulses, as shown by the representative differential recordings (i.e. test minus control) in figure 3D (recorded after 180 s in 1μM CCCP; n=9). I-V plots constructed from the pulse-evoked currents (Fig. 3E, n=9) exhibited a similar relationship as those elicited by depolarizing ramps, with the differential currents exhibiting a reversal potential of -83 ±1.2 mV (Fig. 3F, n=9). This reversal potential suggests the CCCP-induced leakage current is either carried by K^+ or Cl^- ions.

Effect of CCCP on isolated LC cells

Isolated LC neurons where no glial cells were present, while not showing the initial depolarizing response to application of CCCP (1 μM), exhibited a large slow hyperpolarizing response that inhibited spontaneous AP discharge (Fig. 4; n=5). The response was similar but faster than that observed in slices. As reported on the methods section, electrophysiological properties from isolated and LC neurons presented in slices were not obviously different (de Oliveira et al. 2011).

Dependency of the CCCP-induced hyperpolarization on L-type Ca^{2+}channels

It has been reported that the hyperpolarization induced by the protonophore FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) in rat LC neurons is due to activation of K-ATP sensitive channels (Murai et al., 1997), a result obtained using slow bath drug application. We also found that the CCCP-induced hyperpolarization was inhibited by 10 μM glibenclamide (K^+-ATP channel blocker) when using the same methodological approach (i.e. slow bath drug application; please see methods about differences from rapid change local-perfusion systems - data not show).

However, given the previously reported CCCP-induced increase in $[Ca^{2+}]_c$ (de Oliveira et al. 2012), it remains possible that Ca^{2+}-dependent K^+ channels (K_{Ca}) could also subserve a role to produce hyperpolarization in LC neurons. In order to test this, we examined the dependency of the CCCP-induced hyperpolarization on external and cytosolic $[Ca^{2+}]$. The hyperpolarization induced by 1 μM CCCP was abolished when the slice was superfused with Ca^{2+}-free (500 μM EGTA) ACSF solution (Fig. 5; n=5). To examine whether CCCP action was dependent on bulk $[Ca^{2+}]_c$ within LC neurons experiments were undertaken using a rapid Ca^{2+} chelator (BAPTA) dialysed into LC neurons. The CCCP-induced hyperpolarization was not affected by the presence of 10 mM BAPTA in the recording pipette (Fig. 6A; n=13). Additionally, voltage clamp

experiments showed that intracellular addition of BAPTA did not block the CCCP-induced outward current (Fig. 6B; n=8).

These findings are contradictory, as on the one hand they indicate a role for Ca^{2+} entry yet on the other they indicate that an increase in $[Ca^{2+}]_c$ within the LC neuron is not fundamental to the hyperpolarizing response. Therefore, we examined the properties of the Ca^{2+} channels involved. The hyperpolarizing response induced by 1 μM CCCP was abolished by the addition of 300 μM $CdCl_2$, in both current (Fig. 7A; n=6) and voltage clamp (Fig. 7B; n=5). More specifically, application of nifedipine ((10 μM) Sigma-Aldrich CAS number 21829-25-4, catalogue number N7634), an L-type Ca^{2+} channel blocker, markedly inhibited the hyperpolarization (Fig. 8A; n=5) and reduced the outward current (Fig. 8B; n=5). Application of nicardipine ((5 μM) Sigma-Aldrich CAS number 54527-84-3, catalogue number N7510), another L-type Ca^{2+}channel antagonist also markedly inhibited the hyperpolarization (data not show). Together these data indicate a fundamental role of L-type Ca^{2+} channels in the neuronal response to mitochondrial inhibition.

Ca^{2+} currents are involved in transmitter release that can alter the pacemaker process of spontaneous neurons. Our results demonstrated that it is not the case for our experimental model, were spontaneous postsynaptic currents (i.e. inward spikes in the voltage clamp recording) were still present when CCCP was co-treated with either one or other blocker (Figs. 8B or 7B). If nifedipine or Cd^{2+} had a major impact on neurotransmitter release these spontaneous events should have been markedly altered, which was not the case.

Involvement of the actin cytoskeleton on the CCCP-induced hyperpolarization

Another link between inhibition of mitochondria and the hyperpolarizing response was provided by experiments aimed at depolymerising the actin cytoskeleton. The actin cytoskeleton is known to mediate cross talk between mitochondria and L-type Ca^{2+} channels in

cardiac myocytes (Viola & Hool 2010). We investigated this interaction in LC neurons by pre-treating slices with the actin cytoskeleton depolymerising agent, cytochalasin D (1μM). Following this treatment, CCCP (1μM) no longer induced hyperpolarization and resultant cessation of AP activity (Fig. 9; n=9). This result demonstrates an involvement of the actin cytoskeleton in linkage between mitochondria and L-type Ca^{2+} channels in modulation of LC neurons and hence noradrenergic signalling. Cytochalasin D (1μM) treatment had a minimal impact in spontaneous postsynaptic currents as demonstrated by Figure 10B, suggesting that cytochalasin treatment did not impair neurotransmitter release in our experimental model. We have also tested if cytochalasin treatment impaired activation of postsynaptic receptors. For this, we tested the response of glycine post synaptic receptors before and in the presence of cytochalasin D. As shown in Figure 10A, the response to glycine application in LC neurons was not obviously altered.

Discussion

Pacemaking activity in generation of APs is a crucial function in many CNS neurons (Alberts 2008, Bean 2007). This activity is tightly controlled and consumes considerable energy that is largely provided by mitochondria via ATP production (Attwell & Laughlin 2001, Byrne & Roberts 2004). Mitochondria also regulate Ca^{2+} buffering and free radical generation (Werth & Thayer 1994, Mitchell 1976, Cadenas & Davies 2000) with dysfunction in mitochondrial metabolism leading to both controlled and uncontrolled cell death (Gross *et al.* 1999, Martinou & Green 2001, Xu *et al.* 2006). In the present study, we investigated a mitochondrial mechanism, which inhibits AP discharge in the LC, this providing the main noradrenergic innervation of the brain. The mitochondrial mechanism could impact noradrenergic signalling in two ways: first, by modulating the intrinsic LC pacemaker, thus helping to produce diverse firing patterns; and second, by reducing the ATP demand from the pacemaker process itself, thus preserving LC neurons from an imminent energy shortage.

We have called "hyperpolarizing response" the mechanism elicited by CCCP. This mechanism can be explained as follows: The electrophysiological data presented in Fig. 3 demonstrated that at 60s, 180s or longer application times of CCCP, there is a large outward current along the entirely pacemaker region of neurons present in slices. Based in our results, this current grows larger as CCCP is applied for longer times (i.e. hyperpolarizing current at 180s is larger than 60s). This outward current was very similar independent of the depolarizing protocol (ramps or pulses) appearing on top of the voltage-induced ones, suggesting the presence of an extra outward current instead of modulation of voltage-dependent currents by CCCP treatment. As demonstrated previously (de Oliveira et al. 2010b), the pacemaker process of mice LC neurons is dependent on a pool of depolarizing and hyperpolarizing membrane currents present at the interspike interval. Any addition of a new current on this pool (depolarizing or hyperpolarizing) would result in a new outcome, consequently modulating the pacemaker process of LC neurons. Fig. 3 demonstrated the presence of "an extra" hyperpolarizing/outward current induced by CCCP. By itself, initially it is sufficient to abolish the pacemaker process of LC neurons without any hyperpolarization once all depolarizing membrane currents present at the pacemaker region are "neutralized" by this extra current. As the CCCP treatment grows stronger with longer application times (Fig. 3), the hyperpolarizing current becomes larger than all depolarizing membrane currents, producing the massive hyperpolarization observed here.

Mitochondrial disruption has been shown to initiate outward currents in several types of neuron (Inoue *et al.* 1991, Murai et al. 1997, Pisani *et al.* 2006, Song *et al.* 2010). We also observed an outward current (or hyperpolarization) in mouse LC neurons when mitochondria were disrupted by the protonophore CCCP or the complex III inhibitor Antimycin A (Figs 1-3). CCCP induced-hyperpolarizing response was also present in isolated LC neurons (Fig. 4), demostrating this phenomenon does not require glial cells to develop. It is important to note though, that the initial depolarization (Fig. 1A and B) induced by CCCP treatment was absent in isolated LC neurons, suggesting that glial cells may be involved in this initial event. We have

previously demonstrated that CCCP treatment generates relatively small changes in Ψm (de Oliveira et al. 2012), suggesting that the hyperpolarizing response observed here may also occur during normal physiological processes, given that the range of Ψm fluctuation in living neurons is within the levels previously demonstrated by our group (Buckman & Reynolds 2001, de Oliveira et al. 2012).

The mechanism by which mitochondria activate the hyperpolarizing response is intriguing, as the response was inhibited by removal of extracellular Ca^{2+} and activation of Ca^{2+} entry particularly through L-type Ca^{2+} channels, but not by global cytosolic Ca^{2+} as confirmed using a fast Ca^{2+} chelator (Figs 5-8). This suggests that Ca^{2+} entry through channels such as the L-type Ca^{2+} channel acts in microdomains to produce their effect (O'Connell *et al.* 2004, Marques-da-Silva & Gutierrez-Merino 2014). It is supported by the fact that L-type Ca^{2+} channels have sites in their molecular structure capable of form very tight microdomains with different intracellular molecules. This tight binding is necessary to activate specific Ca^{2+}-depended pathways as a bulk increase in $[Ca^{2+}]$ by itself is a "promiscuous" initial transduction signal. Ca^{2+} is able to diffuse around 7nm in a 10mM BAPTA environment before chelation (Parekh 2008), suggesting that tight plasmalemmal microdomains may not be affected by intracellular BAPTA as high Ca^{2+} concentrations are able form very close to the opening of the pore, impacting on the efficiency of BAPTA. This is sufficient to activate Ca^{2+}-dependent molecules bound tightly to the channel and transduce Ca^{2+} signaling further. The finding that depolymerisation of the cytoskeleton (Fig. 9) adds further intrigue, and may be fundamental to maintaining the structural integrity of the microdomains ensuring linkage between L-type Ca^{2+} channels and the mitochondria. Notably, a linkage between L-type Ca^{2+} channels and the mitochondria has been demonstrated in isolated cardiac cells, where polymers of F-actin link L-type Ca^{2+} channels to mitochondria producing a feedback between the activities of these two components (Viola *et al.* 2009, Viola & Hool 2010). It was also demonstrated that Ca^{2+} channels are involved with neuroinflammation in vulnerable regions, i.e. *Substantia Nigra pars compacta* and LC (Duda *et al.* 2016), and it is also known that L-type Ca^{2+} channels can modulate free radical content in neuronal populations (Pachauri *et al.*

2013). The actin cytoskeleton has also been demonstrated to modulate Ψm in other cellular models, where oxidative damage of biomolecules and reactive oxygen species content were modulated by the degree of polymerization of the actin cytoskeleton (de Oliveira *et al.* 2007). This cytoskeletal component, has also been associated with many different physiological situations involving mitochondria that potentially progress to the neuroinflammation, oxidative stress and/or aging (Bartolak-Suki et al. 2017, D'Ambrosi *et al.* 2014). Thus, it seems likely that L-type Ca^{2+} channels are linked to mitochondrial function and could modulate the content of free radicals into a cell. This itself is likely to modulate cellular function, such as a pacemaking as previously demonstrated for LC neurons (de Oliveira et al. 2012). It is important to note, cytochalasin D pre treatment performed here had a minimal impact on spontaneous and glycine-induce postsynaptic currents (Figure 10), suggesting no modulation of neurotransmitters release.

Noradrenergic signalling, as primarily mediated by LC neurons, is involved in many brain functions including attention and alertness (Aston-Jones et al. 1999), memory and learning process (Kety 1972) and participation in stress disorders (Van Bockstaele & Valentino 2013). Noradrenaline signalling produced by activity of noradrenergic neurons acts on different adrenoreceptors. For instance, β receptors activation has been demonstrated to suppress the key pro inflammatory promoter nuclear factor kappa B (NFκB) (Ishii *et al.* 2015). Reduce neuroinflammation acting in diverse situations such as glial-mediated inflammation (Schlachetzki *et al.* 2010), cytokine and type 2 nitric oxide synthase expression in astrocytes and microglia (Galea & Feinstein 1999) were also demonstrated. These activities are dependent on the intrinsic pacemaker process and degree of synchronicity of LC neurons that ultimately decides when and where noradrenaline should be released. It is known that mitochondria are directly involved with inflammation as well as $[Ca^{2+}]_c$ deregulation (Bartolak-Suki et al. 2017, Georgieva *et al.* 2017). Here we present a third player; the actin cytoskeleton which appears to participate in the crosstalk between these two cellular components. This mechanism could contribute to fine tuning LC pacemaking and hence noradrenergic signalling across the cortex.

We hypothesize, clearly extrapolating the findings demonstrated here, if the mechanism involving the crosslink between mitochondria-actin cytoskeleton -L-type Ca^{2+} channels fails, then all noradrenergic signalling coming from LC neurons could be lost and all the neuroprotective/anti-inflammatory action of noradrenaline could be lost together. Even though many neurological diseases such as Parkinson and Alzheimer are preceded by a loss of LC neurons (Feinstein et al. 2016), whether this has a dominant role at the initial events of these diseases remains speculative once inflammation is still not fully understood.

In conclusion, we have demonstrated that changes in mitochondrial function produces a response capable of modulate the pacemaker activity of noradrenergic neurons from LC. This is achieved by a combination of L-type Ca^{2+} channels, external Ca^{2+} entry and a new player in this crosstalk named actin cytoskeleton. All these components combined produced a hyperpolarizing response as result. Our findings open up new areas for research in understanding the consequences of metabolic challenge on brain function and noradrenergic signalling.

--Human subjects --

Involves human subjects:

If yes: Informed consent & ethics approval achieved:

=> if yes, please ensure that the info "Informed consent was achieved for all subjects, and the experiments were approved by the local ethics committee." is included in the Methods.

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=> if Yes, insert "All experiments were conducted in compliance with the ARRIVE guidelines." unless it is a Review or Editorial

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Legends

Figure 1: Effect of CCCP treatment on spontaneous LC firing and voltage-dependent currents. ***A,*** Representative current clamp recording showing application of 1 μM CCCP in ACSF abolished spontaneous action potential (AP) discharge and hyperpolarized the membrane potentialin an LC neuron. AP discharge could be reinstated by depolarization (horizontal bar) and partially reversed after CCCP washout (n=8). Lower records show 60s and 180s of 1 μM CCCP treatment on an expanded time scale. ***B,*** Representative voltage clamp recordings at holding potentials of -53 and -78 mV showing that 1 μM CCCP induced a large outward current (n=5 both holding potentials). All data obtained from LC neurons present in slices; "n" presented for each experiment represents number of cells/neurons accessed from at least three different mice.

Figure 2: Effect of the mitochondrial complex III blocker Antimycin A in LC neurons. Application of 1 μM Antimycin A caused hyperpolarization and inhibited AP firing. Lower records show 60s and 240s of 1 μM Antimycin A treatment on an expanded time scale (n=3). Data obtained from LC neurons present in slices; "n" presented for each experiment represents number of cells/neurons accessed from at least three different mice.

Figure 3: Voltage dependence of the CCCP-induced currents on LC neurons. ***A***, Representative recording of the currents evoked by depolarizing ramps during application of 1 μM CCCP in ACSF (test solution), showing the current evoked in ACSF (control solution; light grey line) and 1 μM CCCP 60s (dashed grey line) and 180s (solid black line) after rapid application of this test

solution. ***B***, I-V plot for the currents presented in A. ***C***, Mean differential currents (i.e. CCCP test – control) with records obtained 60s (light grey line) and 180s (black line) after rapid application of CCCP (n=8 for **A**, **B** and **C**). ***D,*** Representative depolarizing pulse-evoked differential currents obtained by taking the currents in test solution (i.e. 1 μM CCCP + 1 μM TTX ACSF) and subtracting the corresponding currents in control (i.e. 1 μM TTX ACSF). Figure shows representative recording for 180s of 1 μM CCCP + 1 μM TTX ACSF application. ***E,*** Averaged I-V plot showing the currents evoked by pulses for control (light grey line) and the test CCCP solution 60s (dashed grey line) and 180s (solid black line) after its application. ***F,*** Mean differential I-V plots (i.e. test minus control) 60s (light grey dashed line) and 180s (black dashed line) after application of the CCCP solution (n=9 for **D**, **E** and **F)**. "n" presented for each experiment represents number of cells/neurons accessed from at least three different mice. **C** and **F** Graphs show mean ± SEM.

Figure 4: Response to CCCP in isolated LC neurons. Representative current clamp recording showing that application of 1μM CCCP abolished AP discharge and induced a lasting hyperpolarization. Lower records show 60s and 180s of 1 μM CCCP treatment on an expanded time scale and the inset shows a freshly isolated LC neuron obtained by mechanical dissociation (see Methods, n=5). "n" presented for each experiment represents number of cells/neurons accessed from at least three different mice.

Figure 5: The CCCP-induced hyperpolarization is inhibited in Ca^{2+} free ACSF. Representative current clamp recordings showing that the CCCP-induced hyperpolarization was not present when LC neurons were bath perfused with 500 μM EGTA Ca^{2+}-free ACSF. 1 μM CCCP was aplied normally through local fast perfusion. Lower records show 60s and 180s of 1 μM CCCP treatment on an expanded time scale (n=5). Data obtained from LC neurons present in slices;

"n" presented for each experiment represents number of cells/neurons accessed from at least three different mice.

Figure 6: Intracellular dialysis with BAPTA does not inhibit the CCCP-induced hyperpolarization and outward current. ***A*,** Representative current clamp recording showing that the CCCP-induced hyperpolarization and resultant cessation of spontaneous firing was evoked in LC neurons dialysed with internal pipette solution containing 10 mM BAPTA. Action potential discharge could be reinstated by depolarization (horizontal bar). Lower records show 60s and 180s of 1 μM CCCP treatment in LC neurons dialysed with internal pipette solution containing 10 mM BAPTA on an expanded time scale (n=13). ***B*,** Representative voltage clamp recordings at holding potential of -53 mV showing that the outward current induced by 1 μM CCCP remained present when neurons were dialysed with internal pipette solution containing 10 mM BAPTA (n=8). Data obtained from LC neurons present in slices; "n" presented for each experiment represents number of cells/neurons accessed from at least three different mice.

Figure 7: The CCCP-induced hyperpolarization is inhibited by a non-selective blocker of Ca^{2+} channels. ***A*,** Representative current clamp recording showing that the CCCP-induced hyperpolarization was not evoked when $CdCl_2$ (0.3 mM) was included in the ACSF solution. Lower records show 60s and 180s of 1 μM CCCP treatment on an expanded time scale when $CdCl_2$ (0.3 mM) was included in the ACSF solution (n=6). ***B*,** Representative voltage clamp recordings at holding potentials of -53 mV showing that the outward current induced by 1 μM CCCP was not present when $CdCl_2$ (0.3 mM) was included in the ACSF solution (n=5). Data obtained from LC neurons present in slices; "n" presented for each experiment represents number of cells/neurons accessed from at least three different mice.

Figure 8: The CCCP-induced hyperpolarization is dependent on L-type Ca^{2+} channel activity. ***A,*** Representative current clamp recording showing the calcium channel blocker, nifedipine (10μM), markedly inhibited the CCCP-induced hyperpolarization. Lower records show 60s and 180s of 1 μM CCCP + 10μM nifedipine co-treatment on an expanded time scale (n=5). ***B,*** Representative voltage clamp recording (holding potential -53 mV) demonstrating the effect of 10μM nifedipine co-treatment on the CCCP-induced outward current (n=5). Data obtained from LC neurons present in slices; "n" presented for each experiment represents number of cells/neurons accessed from at least three different mice.

Figure 9: Effect of pre-treatment with Cytochalasin D on the CCCP-induced hyperpolarization. Representative current clamp recording showing that pre-treatment with 1μM cytochalasin D, a potent inhibitor of actin polymerization, prevented the hyperpolarization induced by 1μM CCCP (n=9). Lower records show 60s and 180s of 1 μM CCCP in LC neurons pre-treated with 1μM cytochalasin D on an expanded time scale. Data obtained from LC neurons present in slices; "n" presented for each experiment represents number of cells/neurons accessed from at least three different mice.

Figure 10: Effect of pre treatment with Cytochalasin D glycine-induced and spontaneous currents. ***A,*** Representative voltage clamp recording showing that glycinergic currents persist after treatment with cytochalasin D. The normally inhibitory currents were inward due a high Cl- internal solution (see methods; n=4 control, and n=6 glycine). ***B,*** Expanded record segments from the experiment shown in A in ACSF control (upper records) and in ACSF after 2 h pre-exposure to 1μM cytochalasin D. Such spontaneous activity was observed in all cytochalasin D treated LC neurons (n=6). Data obtained from LC neurons present in slices; "n" presented for each experiment represents number of cells/neurons accessed from at least three different mice.