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SYMPOSIUM REVIEW

The regulation of neuronal mitochondrial metabolism by calcium

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Abstract Calcium signalling is fundamental to the function of the nervous system, in association with changes in ionic gradients across the membrane. Although restoring ionic gradients is energetically costly, a rise in intracellular Ca^{2+} acts through multiple pathways to increase ATP synthesis, matching energy supply to demand. Increasing cytosolic Ca^{2+} stimulates metabolite transfer across the inner mitochondrial membrane through activation of Ca^{2+}-regulated mitochondrial carriers, whereas an increase in matrix Ca^{2+} stimulates the citric acid cycle and ATP synthase. The aspartate–glutamate exchanger Aralar/AGC1 (Slc25a12), a component of the malate–aspartate shuttle (MAS), is stimulated by modest increases in cytosolic Ca^{2+} and upregulates respiration in cortical neurons by enhancing pyruvate supply into mitochondria. Failure to increase respiration in response to small (carbachol) and moderate (K^+-depolarization) workloads and blunted stimulation of respiration in response to high workloads (veratridine) in Aralar/AGC1 knockout neurons reflect impaired MAS activity and limited mitochondrial pyruvate supply. In response to large workloads (veratridine), acute stimulation of respiration occurs in the absence of MAS through Ca^{2+} influx through the mitochondrial calcium uniporter (MCU) and a rise in matrix $[Ca^{2+}]$. Although the physiological importance of the MCU complex in work-induced stimulation of respiration of CNS neurons is not yet clarified, abnormal mitochondrial Ca^{2+} signalling causes pathology. Indeed, loss of function mutations in MICU1, a regulator of MCU complex, are associated with neuromuscular disease. In patient-derived MICU1 deficient fibroblasts, resting matrix Ca^{2+} is increased and mitochondria fragmented. Thus, the fine tuning of Ca^{2+} signals plays a key role in shaping mitochondrial bioenergetics.

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Irene Llorente-Folch received her PhD from Autonomous University of Madrid in 2013 under the supervision of Prof **Jorgina Satrustegui's** at the Centre for Molecular Biology "Severo Ochoa" CSIC-UAM and Center for Biomedical Network Research on Rare Diseases and was supported by the Grant MITOLAB-CM. Work in Jorgina Satrustegui's lab has focused on calcium signaling to mitochondria in health and disease with emphasis in the roles played by mitochondrial metabolite transporters which are regulated by extramitochondrial calcium. **Michael R. Duchen** studied Physiology and Medicine in Oxford and London. He worked in hospital medicine for a few years before embarking on PhD studies in the Physiology department at UCL, with Tim Biscoe as supervisor and mentor. He has stayed at UCL ever since. He developed an interest in mitochondrial cell biology, and his has focussed on understanding the integration of mitochondrial function and cell signaling events in health and disease.

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Abbreviations AGC, aspartate-glutamate carrier; ANT, adenine nucleotide translocase; CaMC, Ca^{2+}-regulated mitochondrial carrier; DCA, ′dichloroacetic acid; ER, endoplasmic reticulum; KO, knockout; MAS, malate–aspartate shuttle; MCU, mitochondrial calcium uniporter; MCUC, mitochondrial calcium uniporter complex; NCLX, Na^+/Ca^{2+} exchanger; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydroganase; PTP, permeability transition pore; SCaMC/APC, calcium-binding mitochondrial carrier protein.

Introduction

In this review, we focus on the role played by Ca^{2+} ions in the modulation of cellular respiration, as well as the mechanisms involved. This role for Ca^{2+} is ubiquitous, and most probably can be generalized to all cell types, as will be discussed in the initial part of this review. We then consider in more detail Ca^{2+}-mediated regulation of mitochondrial energy metabolism in neurons as the prototype of the mechanisms involved. Indeed, neurons are responsible for disproportionate oxygen consumption at rest in humans (the brain uses ∼20% of the total oxygen consumed at rest but represents only 2% of body mass; Mink *et al.* 1981). In addition, neurons are critically and almost exclusively dependent on mitochondrial oxidative phosphorylation (OXPHOS) as a major source of ATP and have a limited capacity to upregulate energy supply through glycolysis when OXPHOS is compromised (Herrero-Mendez *et al.* 2009). Mitochondria in these cells represent an exclusive target for Ca^{2+} to guarantee activity-dependent regulation of cellular energy metabolism. Overall, Ca^{2+}-dependent regulation of OXPHOS involves two principal mechanisms: (i) Ca^{2+} entry into mitochondria through the Ca^{2+} uniporter (mitochondrial calcium uniporter; MCU) and (ii) Ca^{2+}-dependent activation of mitochondrial metabolite transporters (Ca^{2+}-regulated mitochondrial carriers), where Ca^{2+} acts on the external surface of the inner mitochondrial membrane. Thus, even though cytosolic and mitochondrial Ca^{2+} signals are usually tightly coupled, they can also have distinct effects on mitochondrial metabolism, ensuring differential regulation in some cases. Because some of the mechanisms employed by Ca^{2+} to modulate respiration have only been described in cells other than neurons, we will refer to other cell types (heart, fibroblasts) throughout the review to address these specific mechanisms, which particularly involve those related to the mitochondrial Ca^{2+} uniporter complex (MCUC) that has only been recently characterized at the molecular level; thus, only a few studies directly address its role in the regulation of OXPHOS.

Cell metabolism and ATP homeostasis

Specialized processes in differentiated cells consume ATP, such as neuronal transmission, muscle contraction, cellular motility and secretion. In addition, energy is required for cellular maintenance and repair to counter the forces of entropy. Events that require the disturbance of ionic gradients across the membrane are also almost invariably associated with Ca^{2+} signals, either through influx across the plasma membrane or by release from internal stores. Restoring ionic gradients by ion pumps in the plasma membrane [Na^+/ Ca^{2+} exchanger (NCLX); Na^+/K^+ ATPase pump; Ca^{2+}/H^+ ATPase exchanger] and within the organelles (Ca^{2+}/H^+ ATPase exchanger) in the endoplasmic reticulum (ER) requires ATP consumption. It has also been known for many years that cells match the rate of ATP production and utilization with little or no measurable change in metabolic intermediates. The maintenance of cellular metabolites during alterations in workload has been termed metabolic homeostasis, and is probably most thoroughly studied in cardiac and skeletal muscle (Balaban 2002; 2006; Glancy *et al.* 2013).

Neurons are also subject to changes in workload. Most of the energy in the brain is consumed by synaptic transmission (Attwell and Laughlin, 2001; Hall *et al.* 2012; Harris *et al.* 2012). In high energy-demanding tissues such as the brain and skeletal and cardiac muscle, the rapid formation of ATP through phosphocreatine and the creatine kinase reaction maintains the distribution of ATP through the cell at almost constant levels and can be important in peak conditions of energy demand (Cerdan *et al.* 1990; Balaban *et al.* 2009). However, overall, the major sustainable source of energy is ATP generated by OXPHOS.

Recent studies have revealed that neuronal activity not only contributes significantly to ATP consumption, but also stimulates ATP synthesis through a Ca^{2+}-dependent increase in OXPHOS (Rangaraju *et al.* 2014). Neuronal activity requires both rapid adaptation of oxidative energy metabolism and a sufficient supply of oxygen and nutrients and, thus, it is very sensitive to altered mitochondrial function (Whittaker *et al.* 2011; Kann *et al.* 2012). Furthermore, mitochondrial fission and redistribution to regions of increased metabolic demand have been observed during sustained impulse activity (Sajic *et al.* 2013), confirming that mitochondrial function is essential for the correct balance of neuronal function in response to an imposed workload.

In neurons using glucose as the main metabolic substrate, an increase in workload is necessarily associated

with increased glucose oxidation and augmented oxygen consumption, which is controlled by the mitochondrial proton electrochemical gradient ($\Delta\mu$H) and mainly used for ATP synthesis (Mitchell & Moyle, 1969).

Regulation of OXPHOS in response to work was initially considered to be carried out by a simple feedback of the ATP hydrolysis products ADP and Pi on mitochondrial ATP synthase (Chance & Williams, 1955; Jacobus *et al.* 1982). The classical principles of chemiosmotic coupling dictate that increased ATP production by mitochondria is coupled with increased oxygen consumption by the respiratory chain and an increased substrate supply to mitochondria. However, this is not the only mechanism driving changes in mitochondrial function in response to changes in workload. Indeed, it has become clear that Ca^{2+} regulation of mitochondrial function plays an important role in maintaining ATP homeostasis (McCormack & Denton, 1990; Rizzuto *et al.* 2012)

Calcium signalling and mitochondrial respiration

Ca^{2+} is a versatile and ubiquitous intracellular messenger, acting as a mediator of almost all energy demanding processes in mammalian cells. The capacity of mitochondria to take up large quantities of Ca^{2+} in a membrane potential-dependent manner has been known for decades (Deluca & Engstrom, 1961; Harris, 1977; Nicholls, 1978). Mitochondrial Ca^{2+} accumulation not only serves both as a Ca^{2+} buffering system in the cell, but also as a pathway to modulate the energy metabolism of the cell. Ca^{2+} handling involves a complex dialogue between the mitochondria, the ER, lysosomes, the plasma membrane and the nucleus. Gradients of Ca^{2+} across the membrane reflect a huge free energy and their maintenance represents a significant energetic burden (Glancy & Balaban, 2012; Rueda *et al.* 2014).

It is well known that Ca^{2+}-dependent regulation of OXPHOS is mediated through Ca^{2+} entry into mitochondria through the MCU (Fig. 1). However, the identification of Ca^{2+}-regulated mitochondrial carriers (CaMCs) (del Arco & Satrustegui 1998, del Arco *et al.* 2000) revealed an additional target of cytosolic Ca^{2+} signals in neuronal mitochondria. The critical difference between these pathways is that Ca^{2+}-dependent regulation of OXPHOS through the carriers operates by the action of Ca^{2+} at the outer surface of the inner mitochondrial membrane, rather than in the matrix, and so does not require mitochondrial Ca^{2+} uptake (Fig. 1). $[Ca^{2+}]$cyt-activated increase in ATP production by OXPHOS contributes to metabolic homeostasis (i.e. allows the ATP/ADP and NADH/NAD^+ levels to remain constant) despite an increase in workload, as reported by Glancy & Balaban (2012).

To maintain substrate supply to mitochondria with an elevated respiratory rate, further control mechanisms acting upstream of mitochondria are required. For example, through its association with calmodulin, Ca^{2+} activates phosphorylase kinase, which in turn activates glycogen phosphorylase, initiating glycogen breakdown and so increasing glucose supply. This is not only a general pathway in tissues with significant glycogen stores, such as liver or muscle (Picton *et al.* 1981), but also takes place in astrocytes (Ibrahim *et al.* 1975; Newman *et al.* 2011; Müller *et al.* 2014*a*,*b*) and, to some extent, in neurons (Saez I *et al.* 2014). Thus, Ca^{2+} stimulates both glycogen breakdown and glucose oxidation, increasing ATP supply (McCormack *et al.* 1990; Müller 2014*b*).

The physiological importance of respiratory control by Ca^{2+} in intact neurons and in the CNS *in vivo* is still largely unknown. Rapid Ca^{2+}-dependent changes in oxygen consumption in response to membrane depolarization have been described in cultured Purkinje neurons (Hayakawa *et al.* 2005). However, other studies have found no evidence for a role of cytosolic Ca^{2+} in activity-dependent rises in cerebellar rate of oxygen consumption in the intact brain (Mathiensen *et al.* 2011). The resolution of these questions is confounded by two opposing actions of Ca^{2+} because it not only activates ATP production through the stimulation of OXPHOS, but also increases ATP consumption through the increased energy demand required to recover the ionic resting state.

Mitochondrial Ca^{2+} uptake and its impact on mitochondrial function

The inner mitochondrial membrane maintains a high membrane potential ($\Delta\Psi$) (~150–180 mV negative to the cytosol) and Ca^{2+} uptake, an electrogenic process, induces a small transient mitochondrial depolarization (Vitorica & Satrustegui, 1985; Duchen, 1992), which correlates with the rising phase of $[Ca^{2+}]$cyt, reflecting the Ca^{2+} current across the inner mitochondrial membrane. Because, in most cells, the mitochondrial matrix represents a small proportion of the total cell volume, and there is a strong electrochemical potential favouring the accumulation of Ca^{2+} into mitochondria, the movement of relatively few Ca^{2+} ions promotes large concentration changes in the matrix, with obvious signalling potential. Mechanisms of Ca^{2+} buffering in the matrix are poorly understood. Ca^{2+} uptake is facilitated by locating mitochondria close to microdomains of high $[Ca^{2+}]$cyt, allowing for its operation even in the absence of global $[Ca^{2+}]$cyt signals (Rizzuto *et al.* 1998; Contreras *et al.* 2010).

Ca^{2+} entry into mitochondria requires the recently identified MCU (Baughman *et al.* 2011; de Stefani *et al.* 2011), which forms part of a large complex, the MCUC (Rizzuto *et al.* 2012; Marchi & Pinton, 2014; Pendin *et al.*

2014), whose components are still not fully resolved. The complex includes MCUb, a dominant negative component of the oligomeric channel (Raffaello *et al.* 2013), the Ca^{2+} sensitivity modulators MICU1 and MICU2 (Perocchi *et al.* 2010; Mallilankaraman *et al.* 2012*b*; Bai *et al.* 2013; Plovanich *et al.* 2013; Patron *et al.* 2014), MCU regulator 1 (Mallilankaraman *et al.* 2012*a*; but see Paupe *et al.* 2015) and essential MCU regulator (Sancak *et al.* 2013). Although the topology of MCU was initially a matter of debate (Drago *et al.* 2011), its N- and C-terminal domains probably span into the mitochondrial matrix and its nine amino acid linker (i.e. the DIME domain) between the two transmembrane domains faces the intermembrane space (Baughman *et al.* 2011; Martell *et al.* 2012). The existence of only two putative transmembrane domains strongly suggests that an active and functional uniporter channel could only be formed by oligomers of MCU.

On the other hand, a K^+/H^+ antiporter (Froschauer *et al.* 2005; Nowikovsky *et al.* 2012; De Marchi *et al.* 2014; Doonan *et al.* 2014; Nowikovsky & Bernardi, 2014; Nowikovsky *et al.* 2004) has also been proposed to mediate Ca^{2+}/H^+ exchange; first, as a Ca^{2+} efflux pathway (Jiang *et al.* 2009; 2013; Tsai *et al.* 2014) and, more recently, as a Ca^{2+} influx pathway alternative to the MCU (Doonan *et al.* 2014). Furthermore, other routes of Ca^{2+} entry into mitochondria, such as a rapid mode of uptake (Gunter & Sheu, 2009; Ryu *et al.* 2010), might be responsible for residual Ca^{2+} uptake in mitochondria from MCU deficient cells (Pan *et al.* 2013) and this requires further investigation (Bondarenko *et al.* 2013; 2014).

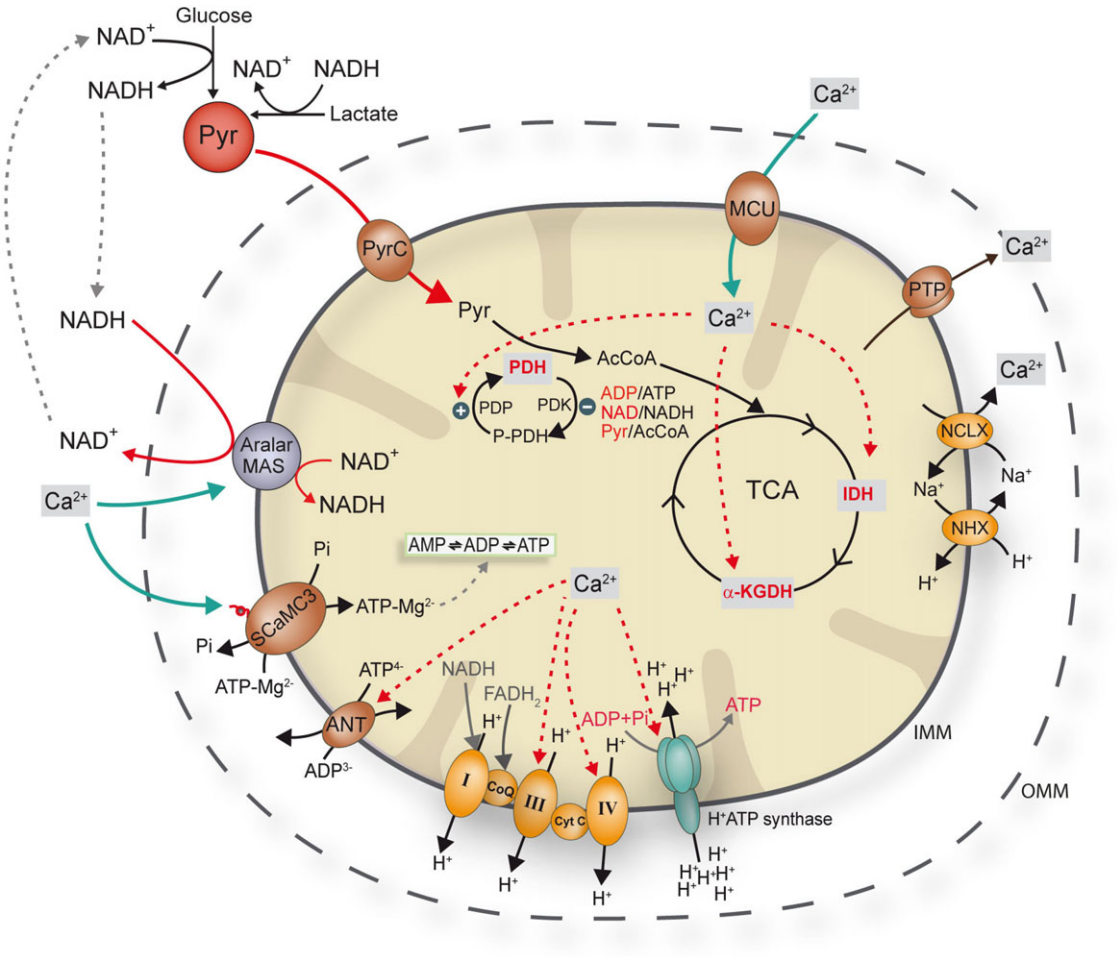

Figure 1. Schematic representation of Ca^{2+}regulation of mitochondrial respiration
Tricarboxylic acid cycle enzymes are highly sensitive to changes in $[Ca^{2+}]$, which presumably binds directly to isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase (α-KGDH), whereas PDH is activated by the Ca^{2+}-sensitive pyruvate dehydrogenase phosphatase. Complex IV and complex III may also be regulated by intra-mitochondrial Ca^{2+}. Matrix Ca^{2+} may also regulate OXPHOS through an effect on the ANT and on the F1Fo-ATP synthase. Extramitochondrial Ca^{2+} activates Aralar/AGC1-MAS activity and SCaMC-3. P-PDH, phosphorilated pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase; Pyr, pyruvate; AcCoA, acetyl coenzyme A; TCA: tricarboxylic acid cycle; NHX, Na^+/H^+ exchanger;

Efflux pathways are also essential for equilibrating mitochondrial and cytosolic Ca^{2+} (Takeuchi *et al.* 2015). The major pathway is a Na^+/Ca^{2+} exchanger that is distinct from the exchanger at the plasma membrane and was recently characterized as NCLX (Palty *et al.* 2010), whereas the Ca^{2+}/H^+ exchanger remains elusive (Fig. 1).

A number of enzymes in the mitochondrial matrix are regulated by Ca^{2+}. In particular, the citric acid cycle dehydrogenases are extremely sensitive to Ca^{2+}, which presumably binds directly to isocitrate dehydrogenase and α-ketoglutarate dehydrogenase, whereas pyruvate dehydroganase (PDH) is activated by Ca^{2+}-sensitive phosphatase activity (Balaban, 2009; Denton, 2009) (Fig. 1). Ca^{2+} also modulates F1-FoATPase activity, promoting ATP generation at a given driving force, thus increasing the velocity of ATP production through a post-translational modification whose specific mechanism remains elusive (Balaban *et al.* 2009; Glancy & Balaban, 2012). Direct Ca^{2+}-dependent activation of F1-ATP synthase by S100A1 has also been implicated in the heart (Borries *et al.* 2007). The fact that respiration rate increases with workload may also imply Ca^{2+} activation of some of the respiratory complexes (Balaban *et al.* 2009; Glancy & Balaban 2012) (Fig. 1). Ca^{2+} may also regulate OXPHOS through effects on adenine nucleotide translocase (ANT), which mediates the electrogenic exchange of ADP3- with ATP4- between the cytosol and the mitochondrial matrix without modifying the net content of adenine nucleotides (Klingenberg, 2008) (Fig. 1). A rise in intramitochondrial $[Ca^{2+}]$ diminishes ANT activity (Moreno-Sanchez, 1983), lowering the matrix ADP content, which decreases F1-FoATPase activity.

Signalling by extramitochondrial calcium

Aspartate-glutamate carriers (AGCs) and calcium-binding mitochondrial carrier proteins (SCaMCs/APCs). Aspartate-glutamate carriers and ATP-Mg/Pi transporters (SCaMCs/APCs) (del Arco & Satrústegui, 1998; Palmieri *et al.* 2001; del Arco & Satrústegui, 2004; Fiermonte *et al.* 2004; Satrústegui *et al.* 2007) are the two classes of mitochondrial carriers activated by extramitochondrial Ca^{2+} (Pardo *et al.* 2006; Contreras *et al.* 2007; Traba *et al.* 2008; Traba *et al.* 2012).

AGCs are components of the malate–aspartate shuttle (MAS) and, under physiological conditions of polarized mitochondria, comprise the main site of regulation of the shuttle (Satrustegui *et al.* 2007). AGCs are activated by modest increases of extramitochondrial $[Ca^{2+}]$ at concentrations not far from the resting state. For example, Aralar/AGC1, the isoform prevailing in the brain, has an $S_{0.5}$ of 324 nM Ca^{2+} (Palmieri *et al.* 2001; Pardo *et al.* 2006; Contreras *et al.* 2007; Satrustegui *et al.* 2007). Aralar/AGC1 activation results in the transfer of cytosolic reducing equivalents into the mitochondrial matrix, increasing substrate supply to mitochondria. Moreover, the MAS oxidizes cytosolic NADH, enhancing pyruvate production from lactate and glucose (Safer *et al.* 1971). Extramitochondrial Ca^{2+} activation of Aralar/AGC1-MAS activity results in the net transfer of reducing equivalents (NADH) from the cytosol to mitochondria, which increases the state 3 respiration rate when using glutamate plus malate as substrates in the presence of a physiological cytosolic free-Ca^{2+} concentration (Gellerich *et al.* 2012, 2013), as well as promotion of Ca^{2+}-dependent pyruvate oxidation in the mitochondria (Gellerich *et al.* 2012; 2013) (Fig. 1). Interestingly, the mitochondrial pyruvate carrier, recently characterized at the molecular level (Bricker *et al.* 2012; Herzig *et al.* 2013), has a low affinity for pyruvate (K_m of 0.6 mM in rat liver, Paradies *et al.* 1983) and so may limit pyruvate oxidation (Schell & Rutter, 2013). This may be important with respect to the role of Aralar/AGC1-MAS activity in increasing pyruvate concentration, favouring its transport into the mitochondrial matrix.

The SCaMCs (del Arco & Satrustegui, 2004) or APCs (Fiermonte *et al.* 2004) are adenine nucleotide carriers that perform the electroneutral exchange of ATP-Mg^{2-} or $HADP^-$ with HPO_4^{2-} between the cytosol and the mitochondrial matrix (Joyal & Aprille 1992; Nosek & Aprille 1992; Fiermonte *et al.* 2004). The SCaMCs are activated by extramitochondrial Ca^{2+} with an $S_{0.5}$ of activation within the range of the MCU complex of ~3–4 μM for the brain and liver isoform SCaMC-3/Slc25a23 (Amigo *et al.* 2013) and of ~12.7 μM for the tumor cell isoform SCaMC-1/Slc25a24 (Traba *et al.* 2012). The direction and magnitude of the transport depend on the relative concentrations of ATP-Mg^{2-} or $ADPH^-$ and Pi. The Mg^{2+} and H^+ associated with ATP and ADP are essential for transport through the carrier. Although its main substrate is magnesium-bound ATP, the carrier can also exchange free ADP and, to a lesser extent, free AMP (Asimakis & Aprille 1980; Fiermonte *et al.* 2004). Thus, SCaMC activity regulates the total adenine nucleotide pool in the mitochondrial matrix; the sum of ATP+ADP+AMP (Fig. 1).

By changing the matrix adenine nucleotide content, the ATP-Mg^{2-}/Pi carriers play an important role in the regulation of the mitochondrial metabolic pathways that have adenine nucleotide-dependent enzymes, including pyruvate carboxylase (gluconeogenesis), carbamyl phosphate synthetase (urea cycle), protein synthesis and the import of nuclear encoded proteins into mitochondria (Aprille, 1993; Satrustegui *et al.* 2007). The mitochondrial adenine nucleotide content increases in adult liver mitochondria upon glucagon treatment as a result of the activity of the liver ATP-Mg^{2-}/Pi carrier SCaMC-3 (Aprille, 1988, 1993, Amigo *et al.* 2013).

The SCaMCs appear to be important for mitochondrial maturation after birth. Indeed, the mitochondrial adenine nucleotide pool increases several-fold in newborn rat liver mitochondria within 3 h after birth, coinciding with the maturation of mitochondrial respiration (Sutton & Pollak, 1978; Valcarce *et al.* 1988).

Calcium regulation of mitochondrial respiration in intact neurons: basal state

The contribution of Ca^{2+} signalling to respiration in intact neurons may be inferred from the effects of the disruption of genes involved in the process. In neuronal cultures under basal conditions and in the presence of physiological glucose concentrations, the rate of respiration of cerebrocortical neurons is ∼30% of maximal uncoupled respiration and is driven by a continuous ATP demand (74% is coupled; Llorente-Folch *et al.* 2013). Spontaneous Ca^{2+} signals in embryonic neurons in culture decrease in frequency upon maturation (Gu & Spitzer, 1995). Distinct aspects of neuronal differentiation encoded by the frequency of spontaneous intracellular Ca^{2+} transients (Gu & Spitzer, 1995) remain unknown. A major role for intracellular Ca^{2+} under these conditions is improbable because basal respiratory activity is not influenced by the presence or absence of extracellular Ca^{2+} (Llorente-Folch *et al.* 2013) or by the presence or absence of SCaMC-3 (Llorente-Folch *et al.* 2013). In addition, although the influence MCU in respiration of cortical neuronal cultures is unknown, total body basal oxygen consumption in MCU knockout (KO) mice on an outbred genetic background was the same as that of control mice (Pan *et al.* 2013), suggesting that the lack of MCU does not cause major respiratory defects. However, a compensatory effect on basal respiration in MCU KO neurons in this model cannot be ruled out because an MCU KO strain on the C57BL/6 genetic background was embryonic lethal (Murphy *et al.* 2014).

Basal respiration in neurons diminished by ∼46% in the absence of Aralar/AGC1 (Llorente-Folch *et al.* 2013), although this may be a consequence of a lack of MAS itself, which decreases pyruvate supply to mitochondria, rather than a lack of Ca^{2+} signalling through Aralar/AGC1. Indeed, Aralar/AGC1 is not absolutely dependent on Ca^{2+}; in Ca^{2+}-free media, MAS activity is attenuated by ∼70% (Pardo *et al.* 2006; Contreras *et al.* 2007) and this may be sufficient to maintain basal respiration in neurons in culture. It must be noted that these considerations apply to the basal state in cultured neurons, and not necessarily to brain neurons in which baseline activity is energetically much higher (Raichle & Mintun, 2006). In addition to a low basal respiratory rate, Aralar/AGC1 KO neurons have also a limited maximal uncoupled respiratory rate, which may be rescued by exogenous pyruvate (Llorente-Folch *et al.* 2013).

Calcium regulation of mitochondrial respiration in intact neurons: response to workloads

To characterize the role of Ca^{2+} in the regulation of energy metabolism, the double role of Ca^{2+} in regulating OXPHOS has to be considered: (i) as an inducer of workload (i.e. as an inducer of ATP utilization to restore Ca^{2+} levels) and (ii) as a regulator of mitochondrial transporters or dehydrogenases (i.e. as a signal molecule). To dissect these aspects, we applied different stimuli to produce different workloads as a result of an increase in cytosolic Na^+ and/or Ca^{2+}. We imposed a high workload using veratridine, a moderate workload with depolarization using isosmotic high K^+, and a small workload using carbachol, which mobilizes Ca^{2+} from ER. With each of these stimuli, the impact of the Ca^{2+} signals, on the mitochondrial population also differed. Those produced in response to veratridine and K^+ stimulation increase matrix Ca^{2+}, whereas that induced by carbachol primarily exerts an action at the external face of the inner mitochondrial membrane. Experiments were conducted in the presence or absence of 2 mM Ca^{2+} or in cells loaded with BAPTA-AM, a rapid Ca^{2+} chelator, which allows for a Ca^{2+}-induced workload at the same tine as preventing Ca^{2+} signalling (Llorente-Folch *et al.* 2013).

An increase in ATP demand to restore the ionic resting state after a stimulus will in turn stimulate OXPHOS, which might also be regulated by Ca^{2+} itself. In all cases analysed, the oxygen consumption rate (OCR) was severely reduced in the absence of Ca^{2+}. In particular, the absence of Ca^{2+} during veratridine stimulation abolishes Ca^{2+}-regulatory mechanism because the veratridine-induced workload is mainly driven by the massive entry of Na^+ to the cytosol. Moreover, the fall in cytosolic ATP in Ca^{2+}-free media was even more pronounced than that in the presence of Ca^{2+}, which is attributable to the absence of Ca^{2+}-mediated stimulation of mitochondrial respiration. Consequently, these experiments clearly demonstrated the role of Ca^{2+} as a regulatory signal to stimulate OXPHOS (Llorente-Folch *et al.* 2013).

For every Ca^{2+} ion that enters the mitochondria, $3H^+$ must enter the matrix to remove it (assuming NCLX stoichiometry $3Na^+$:$1Ca^{2+}$ and $1Na^+$:$1H^+$ for NHE) (Boyman *et al.* 2013). With the known stoichiometry for mitochondrial ATP production and exchange for ADP (3-$4H^+$/ATP) (Watt *et al.* 2010), this implies that removing 1 Ca^{2+} from mitochondria costs approximately 1ATP (i.e. the same as removing it by efflux across the plasma membrane) (Carafoli, 2012). By removing Ca^{2+} from the media, both Ca^{2+} signalling and Ca^{2+}-induced workload

in response to KCl and carbachol were abolished and so, not unexpectedly, the increase in respiration and the fall in cytosolic ATP was smaller in Ca^{2+}-free media. However, incubation with BAPTA-AM, which maintained the workload but blocked Ca^{2+} signalling, also decreased the respiratory response. This showed that Ca^{2+} regulation is required to increase respiration and maintain cytosolic ATP levels in response to any workload (Llorente-Folch *et al.* 2013).

Mechanisms involved in Ca^{2+} regulation of mitochondrial respiration upon an increase in workload

To determine the specific role of Ca^{2+}-dependent mitochondrial carriers in the regulation of OXPHOS, we studied the effects of selective removal of SCaMC-3 and Aralar/AGC1 in response to different stimuli aiming to unmask the contribution of matrix *versus* extra-mitochondrial Ca^{2+}.

Large workloads. Studies in our laboratory have revealed that SCaMC-3 and Aralar/AGC1-MAS are involved in the Ca^{2+}-dependent regulation of mitochondrial respiration at high workloads, such as those imposed by veratridine stimulation, in which the MCU complex and the mitochondrial dehydrogenases pathway also operate.

SCaMC-3/APC2. Deficiency of SCaMC-3 decreased the veratridine-induced stimulation of mitochondrial respiration in the presence of Ca^{2+} (Llorente-Folch *et al.* 2013). This confirms that SCaMC-3 is recruited at large workloads in which a high cytosolic Ca^{2+} concentration activates the carrier (Amigo *et al.* 2013) and the cytosolic ATP/ADP ratio falls. These conditions thermodynamically favour exchange of cytosolic ATP-Mg^{2-} or $HADP^{2-}$ for mitochondrial Pi (Joyal & Aprille, 1992; Aprille, 1993), increasing mitochondrial respiration.

ATP-Mg^{2-}/Pi carrier activity probably favours OXPHOS stimulation by increasing the total adenine nucleotide pool of mitochondria exerting a mass action ratio effect on complex V or the ANT, (Aprille, 1993; Satrustegui *et al.* 2007; Glancy & Balaban, 2012; Amigo *et al.* 2013). Moreover, ADP probably comprises the adenine nucleotide transported by SCaMC-3 and may allosterically activate tricarboxylic acid cycle enzymes (Gabriel *et al.* 1986; Nicholls *et al.* 1994) and inhibit pyruvate dehydrogenase kinase, preventing PDH inactivation (Hucho, 1974; Pratt & Roche, 1979). Thus, an increase in ADP would promote oxidative metabolism and increase the supply of reduced cofactors to the respiratory chain in response to cytosolic Ca^{2+} signals. It is also possible that the entry of adenine nucleotides is a protective mechanism against an early opening of the permeability transition pore (PTP) which would cause an immediate failure of OXPHOS. Adenine nucleotides inhibit PTP opening (Chinopoulos & Adam-Vizi, 2012; Traba *et al.* 2012; Giorgio *et al.* 2013) and also bind Ca^{2+} (Haumann *et al.* 2010), lowering the free matrix Ca^{2+} concentration, which would decrease the probability of PTP opening. This mechanism has been characterized in transformed cells which over-express SCaMC-1, promoting cell survival by desensitizing mitochondrial permeability transition (Traba *et al.* 2011).

Aralar/AGC1. Veratridine-induced workload promoted a strong increase in mitochondrial respiration that was severely attenuated, although not completely abolished, in Aralar/AGC1 KO neurons (Llorente-Folch *et al.* 2013). This showed a major role for the Aralar/AGC1-MAS pathway in response to a high workload-induced stimulation. However, Aralar/AGC1 KO neurons still presented a Ca^{2+}-dependent response, which indicated that the Ca^{2+}-dependent dehydrogenases and SCaMC-3-dependent Ca^{2+} regulation are also signalling mechanisms engaged under these high workloads. Exogenous pyruvate, which bypasses Aralar/AGC1-MAS activity, rescued the effects of the lack of Aralar/AGC1 on respiration, clearly showing that the major role of Aralar/AGC1-MAS is to provide pyruvate to mitochondria (Llorente-Folch *et al.* 2013). These results also reveal that the accumulation of Ca^{2+} into mitochondria through the MCUC is not sufficient to fully activate mitochondrial respiration and that Aralar/AGC1-MAS activity, by providing pyruvate into mitochondria, is unambiguously required.

Small workloads. Aralar/AGC1-MAS pathway is the only Ca^{2+}-regulated mechanism responsible for upregulation of respiration in response to small Ca^{2+} signals produced by carbachol (Llorente-Folch *et al.* 2013). Small Ca^{2+} signals generated by activation of G-protein-coupled receptors and Ca^{2+} release from intracellular stores did not reach mitochondria in neurons (Pardo *et al.* 2006; Llorente-Folch *et al.* 2013) but increased neuronal mitochondrial NAD(P)H mediated by activation of Aralar/AGC1-MAS (Pardo *et al.* 2006). Carbachol stimulation resulted in a small increase in ATP demand and coupled respiration in intact neurons which, not unexpectedly, was absolutely dependent on Aralar/AGC1-MAS (Llorente-Folch *et al.* 2013).

Moderate workloads. Depolarization in response to iso-smotic high K^+ promoted an increase in cytosolic Ca^{2+} that reached the mitochondrial matrix and produced a moderate fall in cytosolic ATP levels (Llorente-Folch *et al.* 2013) (Fig. 2). This in turn stimulated mitochondrial respiration (Llorente-Folch *et al.* 2013) (Fig. 3*A* and *E*).

Interestingly, Ca^{2+}-dependent regulation of mitochondrial respiration by high K^+-induced depolarization was completely abolished in the absence of Aralar/AGC1 and the addition of external pyruvate rescued the lack of Aralar/AGC1 (Fig. 3*B* and *D*). Paradoxically, the lack of response of Aralar/AGC1 KO neurons to K^+-depolarization occurred despite increased cytosolic and mitochondrial Ca^{2+} concentrations and increased mitochondrial NAD(P)H levels (Pardo *et al.* 2006), which would be expected to increase

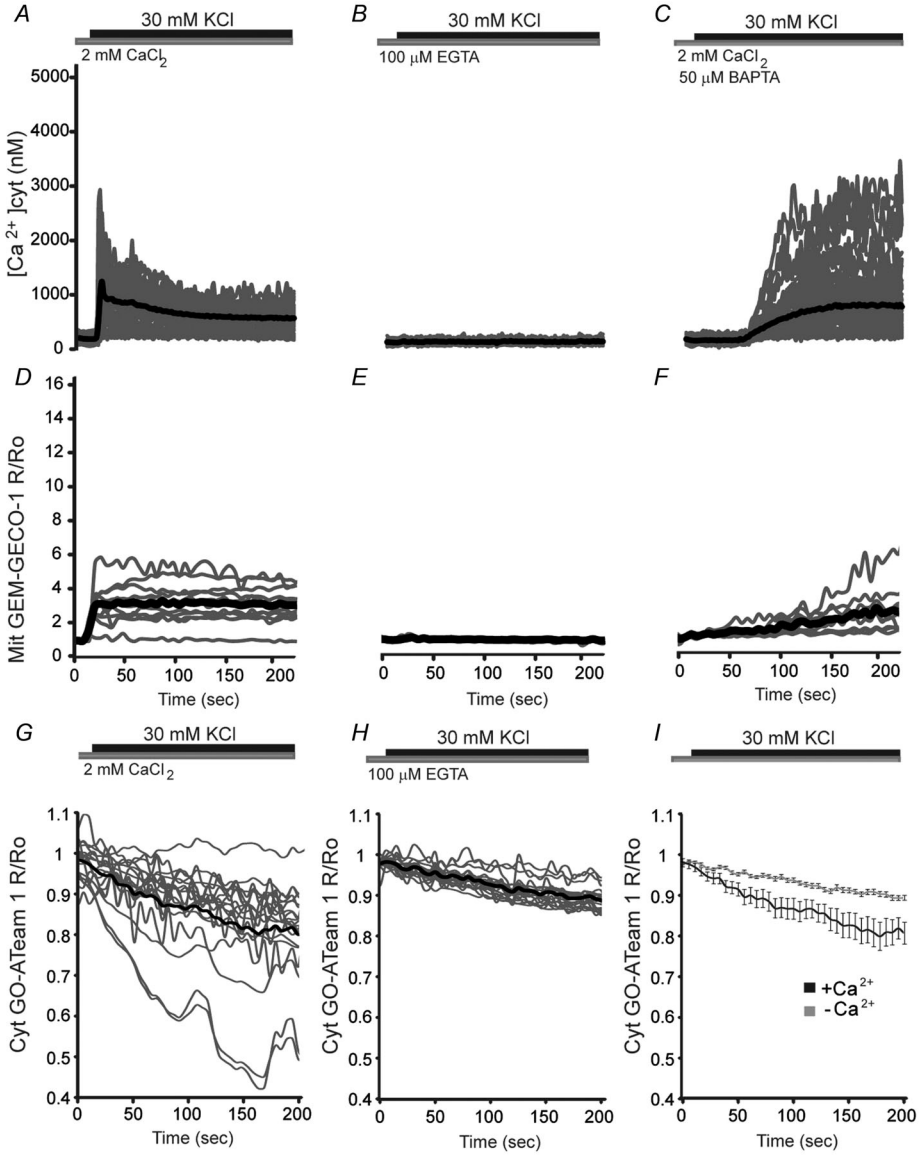

Figure 2. Changes of cytosolic and mitochondrial Ca^{2+} and cytosolic ATP in cortical neurons in response to potassium
Changes in $[Ca^{2+}]_{cyt}$, in Fura-2 loaded neurons (***A***–***C***) or $[Ca^{2+}]_{mit}$, in neurons transfected with Mit GEM-GECO1 probe (***D***–***F***) obtained by stimulation with 30 mM KCl in 2 mM Ca^{2+} medium (*A* and *D*), Ca^{2+} free medium in the presence of the Ca^{2+} chelator EGTA (***B*** and ***E***), or in 50 μM BAPTA-AM preloaded neurons, an intracellular Ca^{2+} chelator that preserves the workload preventing Ca^{2+} signalling, in 2 mM Ca^{2+} medium (***C*** and ***F***). Recordings from at least 60 cells per condition and two independent experiments were used for cytosolic Ca^{2+} imaging and a minimum of 15 cells and eight independent experiments were used for mitochondrial Ca^{2+} imaging. Individual cell recordings (in grey) and average (thick black trace) were shown. ***G***–***I***, cytosolic ATP levels after a switch from HCSS medium to isosmotic high K^+ medium in which 30 mM NaCl was replaced by 30 mM KCl either in 2 mM Ca^{2+} medium (***G***) or 100 μM EGTA medium (***H***). Comparison between the two previously mentioned conditions is shown in (***I***). The drop in ATP values with respect to basal levels 200 s after high K^+ stimulation was 18.6 ± 0.3% in the presence and 9 ± 0.1% in the absence of Ca^{2+} (**$P = 0.009$, two-tailed unpaired Student's *t* test). Data are expressed as the mean ± SEM (modified from Llorente-Folch *et al*. 2013).

respiration. Moreover, wild-type and Aralar/AGC1 KO neurons showed a similar activation of PDH after 5 min in isosmotic 30 mM KCl (1.20 ± 0.09-fold *versus* 1.21 ± 0.06-fold increase in the PDH/P-PDH ratio in wild-type and Aralar/AGC1 KO neuronal cultures, respectively) (Fig. 4*A*–*C*). Thus, the absence of stimulation of mitochondrial respiration in Aralar/AGC1 KO neurons is not a result of differences in PDH dephosphorylation compared to wild-type. Treatment with the PDH kinase inhibitor dichloroacetic acid (DCA), 1 h before K^+ stimulation, also causes a similar maximal PDH dephosphorylation in wild-type and Aralar/AGC1 KO neurons (1.35 ± 0.01-fold *versus* 1.39 ± 0.05-fold increase in PDH/P-PDH ratio in wild-type and Aralar/AGC1

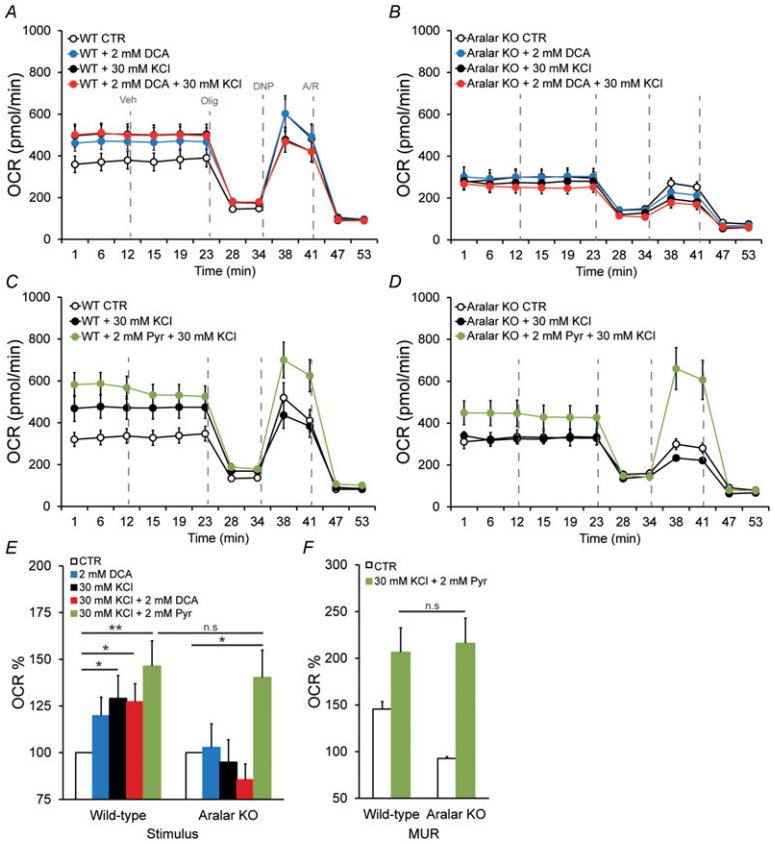

Figure 3. OCR responses to potassium in Aralar/AGC1-deficient neurons
Cellular OCR was measured using a Seahorse XF24 Extracellular Flux Analyser (Seahorse Bioscience, North Billerica, MA, USA) (Qian and Van Houten, 2010). Cortical neurons were plated in XF24 V7 cell culture at 1.0×10^5 cells/well and incubated for 9–10 days in a 37°C, 5% CO_2 incubator in serum-free B27-supplemented neurobasal medium with high levels of glucose. To study OCR, cells were equilibrated for 1 h in 2.5 mM glucose HCSS in the presence of 2 mM $CaCl_2$. Next, neurons were either maintained in the same medium or stimulated with 30 mM KCl in 2.5 mM glucose in Ca^{2+}-containing isosmotic HCSS medium in which 30 mM NaCl was replaced by 30 mM KCl at the start of the respirometry experiments. Calibration of respiration took place after the vehicle (veh) injection in port A. Substrates were prepared in the same medium in which the experiment was conducted and were injected from the reagent ports automatically to the wells at the times indicated. Mitochondrial function in neurons was determined through sequential addition of 6 μM oligomycin (Olig), 0.5 mM 2,4-dinitrophenol (DNP) and 1 μM antimycin/1 μM rotenone (A/R). This allowed the determination of basal oxygen consumption (BS), oxygen consumption linked to ATP synthesis (ATP), non-ATP linked oxygen consumption (leak), mitochondrial uncoupled respiration (MUR) and non-mitochondrial oxygen consumption (NM) (Qian and Van Houten, 2010; Brand and Nicholls, 2011). Respiratory profiles are shown for control (***A***) and Aralar/AGC1-deficient neurons (***B***) upon K^+ stimulation, in neurons pre-treated or not with 2 mM DCA for 1 h, in the presence of 2 mM Ca^{2+}. Respiratory profiles are shown for control (***C***) and Aralar/AGC1-deficient neurons (***D***) upon K^+ stimulation, with or without the addition of 2 mM pyruvate just before starting the experiment, in the presence of 2 mM Ca^{2+}. Stimulation of mitochondrial respiration (***E***) and maximal uncoupled respiration (MUR) (***F***) is shown upon K^+ stimulation after 2 mM DCA pre-teatment or 2 mM pyruvate addition. Data correspond to four or five and two to four independent experiments in wild-type and Aralar/AGC1 KO cultured neurons, respectively (one-way ANOVA, $^*P \leq 0.05$; $^{**}P \leq 0.01$). KCl, isosmotic high K^+, 30 mM; Pyr, pyruvate, 2 mM.

KO neurons, respectively) (Fig. 4*A*–*C*). However, DCA treatment increases basal respiration only in wild-type and not in Aralar/AGC1 KO neurons, and this increase in respiration caused by DCA was not increased any further by 30 mM KCl (Fig. 3*A*). However, DCA treatment did not change basal or K^+-stimulated respiration in Aralar/AGC1 KO neurons (Fig. 3*B* and *E*).

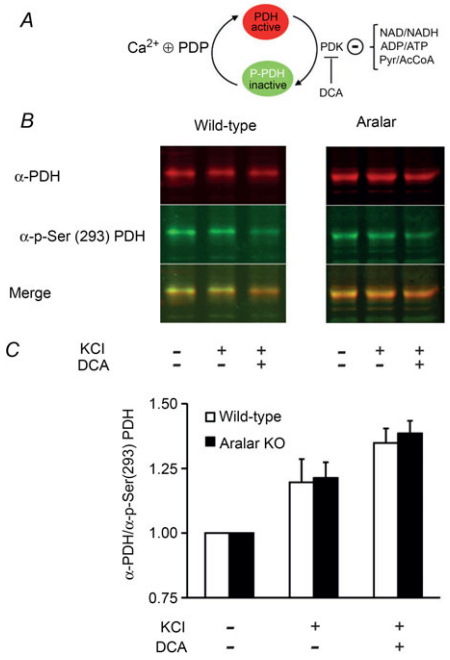

Figure 4. Pyruvate dehydrogenase complex dynamics after K^+- depolarization in wild-type and Aralar/AGC1 KO primary cortical neurons
A, scheme depicting the complex dynamics of pyruvate dehydrogenase. PDH-E1 subunit is active in its dephosphorylated state. Pyruvate dehydrogenase kinase (PDK), whose activity is negatively controlled by NAD/NADH, ADP/ATP and Pyr/AcCoA ratios, phosphorylates the enzyme in the Ser(293) residue, inactivating the complex. On the other hand, pyruvate dehydrogenase phosphatase (PDP), which is positively regulated by intramitochondrial Ca^{2+}, dephosphorylates PDH-E1, recovering the active form. DCA inhibits PDK, favouring the active form of PDH. ***B***, representative western blot against PDH (anti-PDH subunit E1, mouse monoclonal antibody, dilution 1:5000; Invitrogen, Carlsbad, CA, USA) and p-Ser(293) PDH (anti-PDH, rabbit polyclonal antibody, dilution 1:2000; Novus Biologicals, Littleton, CO, USA) and IRDye secondary antibodies optimized for use with Oddysey (800 CW goat anti-rabbit IgG and 680 RD goat anti-mouse IgG, dilution 1:50000; Li-Cor, Lincoln, NE, USA). Neurons were obtained under control conditions, or after 5 min of exposure to isosmotic 30 mM KCl, or under these same conditions prior to pre-treatment for 1 h with 2 mM DCA. Merged image combines both green and red fluorescence to denote the PDH/phosphorylated pyruvate dehydrogenase (P-PDH) ratio. ***C***, western-blotting quantification expressed as the fold increase in anti-PDH/anti-p-Ser(293) PDH ratio compared to control. Data correspond to three to five independent experiments in wild-type and Aralar/AGC1 KO cultured neurons, respectively (Student's *t* test, $^*P < 0.05$). Pyr, pyruvate; AcCoA, acetyl coenzyme A; KCl: isosmotic high K^+, 30 mM.

The findings in wild-type neurons are consistent with a role of matrix Ca^{2+} in 'pulling' pyruvate into mitochondria during K^+-induced stimulation of respiration (Rueda *et al.* 2014). By activating PDH phosphatase and enzyme dephosphorylation, matrix Ca^{2+} will activate PDH and the decarboxylation of pyruvate, which will pull pyruvate from the cytosol to mitochondria across the pyruvate carrier by mass action ratio effects. The finding that activation of PDH by DCA results in an increase in OCR similar to that obtained by K^+-depolarization is consistent with a role of PDH activity in driving pyruvate into mitochondria.

However, K^+-induced or DCA-induced activation of respiration does not take place in Aralar/AGC1 KO neurons even though PDH is activated under both conditions (Fig. 4*A*–*C*). The lack of Aralar/AGC1-MAS activity and the diminished pyruvate levels in Aralar/AGC1 KO neurons (Pardo *et al.* 2011) reveal that activation of PDH is insufficient to increase pyruvate entry in mitochondria under this workload.

MCUC in the regulation of mitochondrial respiration

The role of the MCUC in basal respiration or workload-induced respiration in neurons is still unknown. MCU is clearly responsible for glutamate/NMDA-induced Ca^{2+} entry in neuronal mitochondria, and knockdown of endogenous MCU decreased NMDA-induced increases in mitochondrial Ca^{2+} in neurons and slightly attenuated the sensitivity to excitotoxicity (Qiu *et al.* 2013). However, this approach did not completely eliminate NMDA-induced increases in mitochondrial Ca^{2+}, either as a result of the insufficient silencing efficiency or the presence of other pathways for Ca^{2+} uptake (Qiu *et al.* 2013). Thus, the consequences of MCU silencing on neuronal respiration remain to be determined.

On the other hand, the description of an MCU KO mouse with an unexpectedly mild phenotype (Pan *et al.* 2013) and of human diseases as a result of mutations in *MICU1* (the Ca^{2+} sensitive regulatory subunits of MCUC) may shed light of the role of MCUC in the regulation of mitochondrial respiration. Mitochondria from MCU KO mice had no Ru360-inhibitable Ca^{2+} uptake in mitochondria, with no functional compensation for the rapid entry of Ca^{2+} into the matrix because MCU KO mitochondria do not take up any measurable Ca^{2+} over a 10–20 min period (Pan *et al.* 2013; Murphy *et al.* 2014). However mitochondrial Ca^{2+} levels were depleted but not completely absent, suggesting a possible slow mechanism for Ca^{2+} uptake independent of MCU (Murphy *et al.* 2014).

As discussed earlier, MCU KO mice did not present alterations in basal metabolism at the whole animal level, or any defect in respiration in mouse embryonic

fibroblast cultures, even though the Ca^{2+} content in skeletal muscle mitochondria was decreased and the PDH phosphorylation state was increased (Pan *et al.* 2013). Consistent with a role of MCU at high workloads, skeletal muscle peak performance was slightly decreased (~15%) in MCU KO mice. Whether compensatory mechanisms explain the mild effect of MCU deficiency on mitochondrial metabolism remains to be established. Indeed, MCU KO generated a viable phenotype only in a mixed background as a hypomorph, although this has proven to be embryonic lethal in pure inbred strains, including the C57BL/6 background (Murphy *et al.* 2014).

By contrast to the mild phenotype of the MCU KO mouse, loss of function mutations of *MICU1*, the first genetic human disease to be identified involving mutations of the MCUC, were associated with proximal myopathy, learning difficulties and a progressive extrapyramidal movement disorder in children (Logan *et al.* 2014). Genetic analysis identified two mutations, a splice acceptor site mutation, c.1078-1G>C, and a splice donor site mutation, c.741 + 1G>A, in *MICU1* in a total of 15 affected individuals from seven families, resulting in nonsense-mediated decay and loss of protein. Cellular and mitochondrial Ca^{2+} homeostasis were analysed in primary

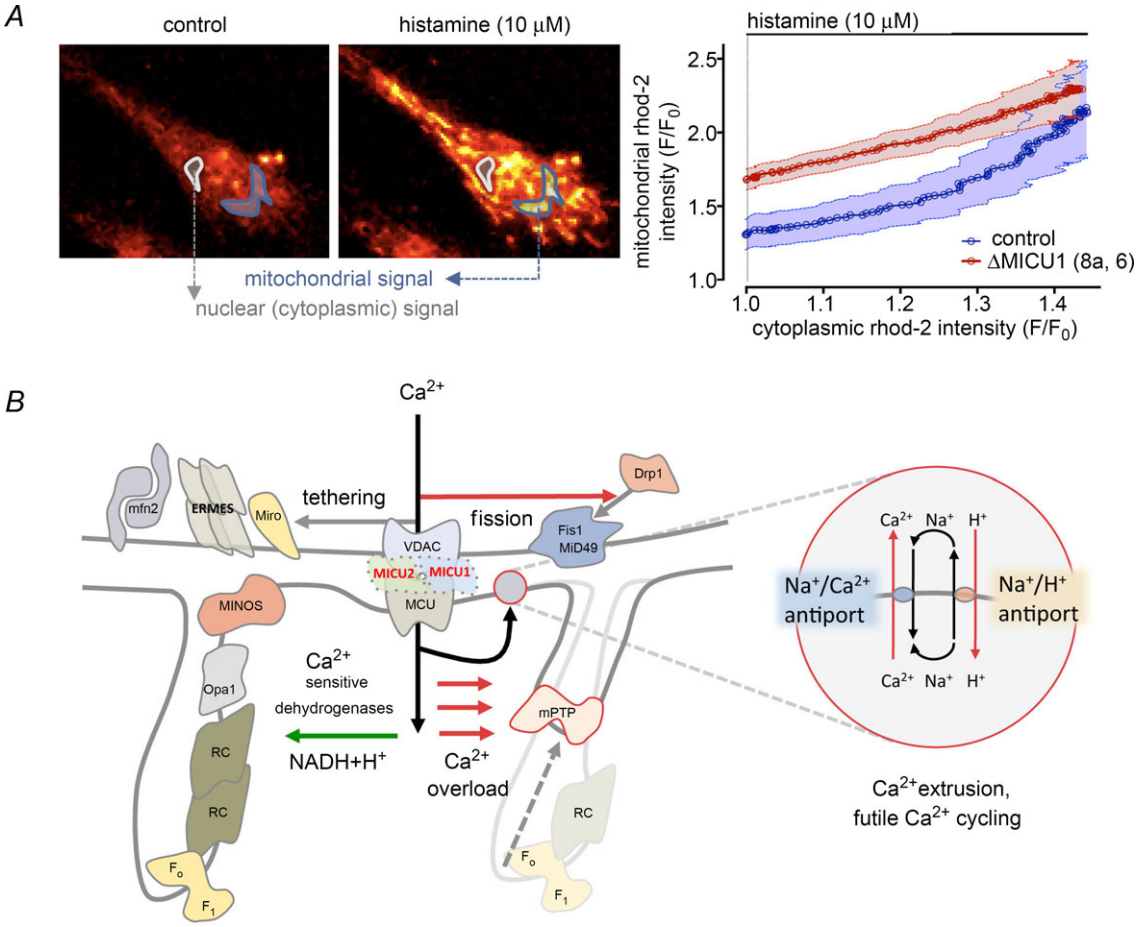

Figure 5. Consequences of MICU1/2 loss on mitochondrial Ca^{2+} homeostasis, shape and metabolic function

A, relationship between cytosolic and mitochondrial [Ca^{2+}] during evoked Ca^{2+} signals. MICU1/2 defective cells have an increased basal mitochondrial Ca^{2+} load, which follows linearly the increase in cytoplasmic [Ca^{2+}] (reproduced with permission from Logan *et al.* 2014). ***B***, increased mitochondrial Ca^{2+} load can activate mitochondrial metabolism by stimulating Ca^{2+}-dependent dehydrogenases of the mitochondrial matrix. On the other hand, chronic Ca^{2+} load in the mitochondrion might also have also a metabolic cost by resulting in futile Ca^{2+} cycling (inset) and opening of the mitochondrial permeability transition pore (mPTP), both leading to depolarization. In addition, we observed increased mitochondrial fission, which might impact on the metabolic capacity of the organelle. In the human fibroblast model of the disease, no changes in ER mitochondria tethering were observed. mfn2, mitofusin 2; ERMES, ER-mitochondria encounter structures; Miro, mitochondrial Rho GTPase; MINOS: MINOS/MitOS/MICOS complexes; RC, respiratory supercomplexes; F1/FO, F1FO ATPase.

skin fibroblasts from patients showing that *MICU1* deficiency caused loss of the physiological cooperative sigmoid regulation of mitochondrial Ca^{2+} concentration (Szabadkai & Duchen, 2008), increasing the basal Ca^{2+} load in the organelle with a significant increase in the velocity of mitochondrial Ca^{2+} uptake in response to a rise in cytosolic Ca^{2+} concentration. The increased resting mitochondrial Ca^{2+} concentration was associated with a highly fragmented mitochondrial network. The data suggested that loss of *MICU1* leads to chronic activation of the MCU channel even at resting cytosolic Ca^{2+} concentrations, highlighting the functional importance of the sigmoid dependence of mitochondrial Ca^{2+} uptake on extramitochondrial $[Ca^{2+}]$. The expression of MICU1 effectively results in a threshold effect at low, submicromolar extramitochondrial $[Ca^{2+}]$ and cooperative Ca^{2+}-mediated activation of Ca^{2+} uptake kinetics at higher ($> \mu$M) extramitochondrial Ca^{2+} levels. Such a dual role of MICU1 has been previously shown in cellular models (Mallilankaraman *et al.* 2012*a*; Csordas *et al.* 2013) and the molecular details of the regulatory mechanism were recently refined further by the description of MICU1/MICU2 heterodimers (Patron *et al.* 2014). Importantly, although all of the studies agree that MICU1 (and consequently MICU2) loss leads to a significant increase in basal resting mitochondrial $[Ca^{2+}]$, no clear metabolic consequences have been demonstrated. This might be a result of the highly glycolytic phenotype of human fibroblasts (Logan *et al.* 2014) and cancer cell lines (Mallilankaraman *et al.* 2012*a*; Csordas *et al.* 2013), although, similarly, primary hepatocytes, where cellular energy metabolism relies more on OXPHOS, have also shown unaltered basal respiration after knockdown of MICU1 (Csordas *et al.* 2013). By contrast, in the hepatocyte model, Ca^{2+}-dependent activation of oxygen consumption was blunted in the absence of MICU1. These findings might indicate the relatively minor importance of basal Ca^{2+} levels on mitochondrial metabolism (in contrast to previous findings; Cardenas *et al.* 2010) or can be attributed to a more complex role of Ca^{2+} in mitochondrial metabolism.

Ultimately, these data show that the MICU1-mediated sigmoid Ca^{2+} activation of mitochondrial Ca^{2+} uptake serves as a signal-to-noise discriminator, protecting cells from chronic futile Ca^{2+} cycling, which represents an important energy drain. In the absence of MICU1/MICU2, increased uptake of Ca^{2+} even at rest would represent chronic activation of a futile Ca^{2+} cycle, dissipating the proton motive force and preventing OXPHOS (Fig. 5). The net result on cellular metabolism in this case might not be evident from measurements of membrane potential and O_2 fluxes, particularly in cells where the reverse mode of the ATP synthase (Campanella *et al.* 2009) is also involved in maintaining the resting membrane potential. Thus, although the results from MCU KO mice and MICU1 deficient models suggest that intramitochondrial Ca^{2+} signalling makes a relatively small contribution in metabolism, its tight regulation and its conservation under evolutionary pressure are evident from the pathology of the human *MICU1* deficiency, affecting primarily muscle and neurons. The precise influence of the MCU-mediated pathway on respiration of brain neurons awaits clarification using the models now available.

On the other hand, Aralar/AGC1-MAS activity by itself or through stimulation by extramitochondrial Ca^{2+} has a strong relevance both under basal conditions and upon stimulation. Aralar/AGC1-MAS deficiency significantly compromises glucose oxidation, basal respiration and maximal uncoupled respiration (Llorente-Folch *et al.* 2013). Moreover, the Aralar/AGC1-MAS pathway, by regulation of pyruvate supply to mitochondria, plays a paramount role in the response to the small, moderate and strong signals that we have investigated (Llorente-Folch *et al.* 2013, Rueda *et al.* 2014). The roles played by MAS activity itself with respect to Ca^{2+} regulation through Ca^{2+} binding to Aralar/AGC1 remain to be established.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

All authors approved the final version of the manuscript submitted for publication.