The regulation of neuronal mitochondrial metabolism by calcium

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1. Abstract

Calcium signalling is fundamental to the function of the nervous system, in association with changes in ionic gradients across the membrane. While 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, while an increase in matrix Ca^{2+} stimulates the citric acid cycle and the 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 KO neurons reflects 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 (MCUC) in work-induced stimulation of respiration of CNS neurons is not yet clarified, abnormal mitochondrial Ca^{2+} signaling causes pathology. Indeed, loss of function mutations in MICU1, a regulator of MCUC, 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.

2. Introduction

In this review, we focus on the role played by Ca^{2+} ions in the modulation of cellular respiration and the mechanisms involved. This role for Ca^{2+} is ubiquitous, and most probably can be generalised to all cell types, as 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 about 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 adenosine 5′-triphosphate (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 (MCU), (ii) Ca^{2+}-dependent activation of mitochondrial metabolite transporters (CaMCs), 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. As 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 involve particularly those related to the mitochondrial Ca^{2+} uniporter complex (MCUC), that has only been recently characterised at the molecular level, thus only few studies directly addressed its role in the regulation of OXPHOS.

2.1 Cell metabolism and ATP homeostasis

Specialised processes in differentiated cells such as neuronal transmission, muscle contraction, cellular motility and secretion consume ATP. 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 (NCLX, the Na^+/Ca^{2+} exchanger, Na^+/K^+ ATPase pump; PMCA, the Ca^{2+}/H^+ ATPase exchanger) and within the

organelles (SERCA, 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, probably most thoroughly studied in cardiac and skeletal muscle (Balaban 2002; 2006; Glancy et al., 2013).

Neurons are also subject to changes in workload. It is well documented that most 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 like 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 sufficient supply of oxygen and nutrients and it is very sensitive to altered mitochondrial function (Whittaker et al., 2011; Kann et al., 2012). Further, mitochondrial fission and redistribution to regions of increased metabolic demand has 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 (ΔμH) and mainly used for ATP synthesis (Mitchell & Moyle, 1969).

Regulation of OXPHOS in response to work was initially thought to be carried out by a simple feedback of the ATP hydrolysis products ADP and Pi on the 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 to increased oxygen consumption by the respiratory chain and 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)

2.2 Calcium signaling 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 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 endoplasmic reticulum (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, **(see fig. 1).** This $[Ca^{2+}]$c-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 recently described by Glancy and Balaban (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, Ca^{2+}, through its association with calmodulin, activates phosphorylase kinase, which in turn activates glycogen phosphorylase, initiating glycogen breakdown and so increasing glucose supply. This is 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 (reviewed in 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 depolarisation 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). Resolution of these questions is confounded by two opposing actions of Ca^{2+}, as it 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.

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

The inner mitochondrial membrane maintains a high membrane potential ($\Delta\Psi$) (around 150-180mV 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. As 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 signaling 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; that 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., 2012b; Bai et al., 2013; Plovanich et al., 2013; Patron et al., 2014), MCUR1 (Mitochondrial Calcium Uniporter Regulator) (Mallilankaraman et al., 2012a) (but see Paupe et al., 2015) and EMRE (Essential MCU Regulator) (Sancak et al., 2013). Although the topology of MCU was initially a matter of debate (reviewed in Drago et al., 2011), it is likely that its N- and C-terminal domains span into the mitochondrial matrix and that its 9-aa linker (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 been also proposed to mediate Ca^{2+}/H^+ exchange, firstly as a Ca^{2+} efflux pathway (Jiang et al., 2009; 2013; Tsai et al., 2013), but recently also as Ca^{2+} influx pathway alternative to the MCU (Doonan et al., 2014). Besides, other routes of Ca^{2+} entry into mitochondria, such as the rapid mode (RaM) 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 need to be further investigated (Bondarenko et al., 2013; 2014).

Efflux pathways are also essential to equilibrate mitochondrial and cytosolic Ca^{2+} (see review Takeuchi et al., 2014). The major pathway is a Na^+/Ca^{2+} exchanger which is distinct from the exchanger at the plasma membrane and was recently characterized as NCLX (Palty et al., 2010) while 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 (ICDH) and α-ketoglutarate dehydrogenase (α-KGDH), whilst pyruvate dehydroganase (PDH) is activated by the Ca^{2+}-sensitive phosphatase activity (PDP) (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 OX¬PHOS through effects on the 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 matrix ADP content, which decreases F1-FoATPase activity.

2.4 Signalling by extramitochondrial calcium

AGCs AND SCaMCs

Aspartate-glutamate carriers (AGCs) 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, are 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 oxidises 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 state 3 respiration rate when using glutamate plus malate as substrates in the presence of physiological cytosolic free-Ca^{2+} concentration (Gellerich et al., 2012, 2013), and promoting Ca^{2+} dependent pyruvate oxidation in the mitochondria (Gellerich et al., 2012; 2013) **(fig. 1).** Interestingly, the mitochondrial pyruvate carrier, recently characterised at the molecular level (Bricker et al., 2012; Herzig et al., 2013)**,** has a low affinity for pyruvate (Km 0.6 mM in rat liver, Paradies et al., 1983), and so may limit pyruvate oxidation (Schell & Rutter, 2013). This may be important regarding the role of Aralar/AGC1-MAS activity in increasing pyruvate concentration, favoring 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 about 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 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 due to 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. In fact, 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).

3. Calcium regulation of mitochondrial respiration in intact neurons. Basal state

The contribution of Ca^{2+} signaling to respiration in intact neurons may be inferred from the effects of the disruption of the 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 about 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 seems unlikely as 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 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, as 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), but this may be a consequence of the lack of MAS itself, which decreases pyruvate supply to mitochondria, rather than to a lack of Ca^{2+} signaling through Aralar/AGC1. Indeed, Aralar/AGC1 is not absolutely dependent on Ca^{2+}; in Ca^{2+}-free media, MAS activity is attenuated by about 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 kept in mind that these considerations apply to the basal state in cultured neurons, 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).

4. Calcium regulation of mitochondrial respiration in intact neurons. Response to workloads

In order to characterise the role of Ca^{2+} in the regulation of energy metabolism, one has to consider the double role of Ca^{2+} in regulating OXPHOS, as an inducer of workload (i.e, as an inducer of ATP utilization to restore Ca^{2+} levels) and as a signal molecule, as a regulator of mitochondrial transporters or dehydrogenases. To dissect these aspects, we applied different stimuli in order to produce different workloads due to 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 mobilises 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+}, while that induced by carbachol primarily exerts their 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 while preventing Ca^{2+} signaling (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 be also regulated by Ca^{2+} itself. In all cases analyzed, 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 since 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+}, 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 ($3\text{-}4H^+$/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+} signaling and Ca^{2+}-induced workload in response to KCl and carbachol were abolished, so not surprisingly, 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+} signaling, 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).

5. Mechanisms involved in Ca^{2+} regulation of mitochondrial respiration upon 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 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 confirmed that SCaMC-3 is recruited at large workloads in which 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.

It is likely that ATP-Mg^{2-}/Pi carrier activity favors 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 is likely to be the adenine nucleotide transported by SCaMC-3 and it may allosterically activate TCA cycle enzymes (Gabriel et al., 1986; Nicholls et al., 1994) and inhibit pyruvate dehydrogenase kinase (PDK), 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 characterised 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 which was severely attenuated, though 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 signaling 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 revealed 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.

<u>**Small workloads**</u>

Aralar/AGC1-MAS pathway is the only Ca^{2+}-regulated mechanism responsible for up-regulation 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 (GPCR) 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 small increase in ATP demand and coupled respiration in intact neurons which, not surprisingly, was absolutely dependent on Aralar/AGC1-MAS (Llorente-Folch et al., 2013).

Moderate workloads

Depolarisation in response to isosmotic high K^+ promoted an increase in cytosolic Ca^{2+} which reached the mitochondrial matrix and produced a moderate fall in cytosolic ATP levels **(fig. 2** from Llorente-Folch et al., 2013). This in turn stimulated mitochondrial respiration (Llorente-Folch et al., 2013 **and fig. 3 A, 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, D).** Paradoxically, the lack of response of Aralar/AGC1 KO neurons to K^+-depolarization occurred in spite of the fact that cytosolic and mitochondrial Ca^{2+} concentrations and mitochondrial NAD(P)H levels increased (Pardo et al., 2006), which would be expected to increase respiration. Moreover, wild-type and Aralar/AGC1 KO neurons showed a similar activation of PDH after five minutes 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 due to differences in PDH dephosphorylation compared to wild-type. Treatment with the PDH kinase inhibitor DCA (dichloroacetic acid), an hour 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 KO neurons respectively, **fig. 4 A-C).** However, DCA treatment increases basal respiration only in wild-type, but not in Aralar/AGC1 KO neurons, and this increase in respiration caused by DCA was not increased any further by 30 mM KCl **(fig. 3A).** However, DCA treatment did not change basal or K^+-stimulated respiration in Aralar/AGC1 KO neurons, **(fig. 3 B, E).**

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 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.

6. 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 due to 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 a surprisingly mild phenotype (Pan et al., 2013) and of human diseases due to mutations in *MICU1*, the Ca^{2+} sensitive regulatory subunits of MCUC, may shed light of the role of MCUC in 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, since MCU KO mitochondria do not take up any measurable Ca^{2+} over a 10 to 20 minute 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 (about 15%) in MCU KO mice. Whether compensatory mechanisms explain the mild effect of MCU deficiency on mitochondrial metabolism remains to be established. In fact, MCU knockout generated a viable phenotype only in a mixed background as a hypomorph, but has proven to be embryonic lethal in pure inbred strains, including the C57BL/6 background (Murphy et al., 2014).

In contrast to the mild phenotype of the MCU knockout 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 7 families resulting in nonsense-mediated decay and loss of protein. Cellular and mitochondrial Ca^{2+} homeostasis were analyzed in primary 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 (> μM) extramitochondrial Ca^{2+} levels. Such a dual role of MICU1 has been previously shown in cellular models (Mallilankaraman et al., 2012a; Csordas et al., 2013), and the molecular details of the regulatory mechanism have been recently further refined by the description of MICU1/MICU2 heterodimers (Patron et al., 2014). Importantly, while all studies agree that MICU1 (and consequently MICU2) loss leads to a significant increase in basal resting mitochondrial $[Ca^{2+}]$, no clear metabolic consequences have yet been demonstrated. This might due to the highly glycolytic phenotype of human fibroblasts (Logan et al., 2014) and cancer cell lines (Mallilankaraman et al., 2012a; Csordas et al., 2013), but similarly, primary hepatocytes, where cellular energy metabolism relies more on OXPHOS, have also shown unaltered basal respiration following knock-down of MICU1 (Csordas et al., 2013). On the other hand, 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, e.g. see 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 resting membrane potential. Thus, although results from the MCU KO mice and MICU1 deficient models suggest that intramitochondrial Ca^{2+} signaling 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 now available models.

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 small, moderate and strong signals we have studied (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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Figure legends.

Figure 1. Schematic representation of Ca^{2+}regulation of mitochondrial respiration

TCA enzymes are highly sensitive to changes in [Ca^{2+}] which presumably binds directly to ICDH, α-KGDH, whilst PDH is activated by the Ca^{2+}-sensitive PDP. Complex IV and complex III may also be regulated by intramitochondrial 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.

PDH: pyruvate dehydrogenase; P-PDH: phosphorilated pyruvate dehydrogenase; PDK: pyruvate dehydrogenase kinase; PDP: pyruvate dehydrogenase phosphatase; Pyr: pyruvate; AcCoA: acetyl coenzyme A; ANT: adenine nucleotide translocase; MCU: mitochondrial calcium uniporter; PTP:permeability transition pore; TCA: tricarboxylic acid cycle; IDH: isocitrate dehydrogenase; α -KGDH: α-ketoglutarate dehydrogenase; NCLX: Na$^+$/Ca^{2+} exchanger; NHX:Na$^+$/H$^+$ exchanger;

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,D), Ca^{2+} free medium in the presence of the Ca^{2+} chelator EGTA (B,E), or in 50 µM BAPTA-AM preloaded neurons, an intracellular Ca^{2+} chelator which preserves the workload preventing Ca^{2+} signaling, in 2 mM Ca^{2+} medium (C,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 8 independent experiments 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 sec. after high K^+ stimulation were 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 mean ± S.E.M. (modified from Llorente-Folch et al., 2013)

Figure 3. OCR responses to potassium in Aralar/AGC1-deficient neurons

Cellular Oxygen Consumption Rate (OCR) was measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience) (Qian and Van Houten, 2010). Cortical neurons were plated in XF24 V7 cell culture at 1.0 x 10^5 cells/well and incubated for 9-10 days in a 37ºC, 5 % CO_2 incubator in serum-free B27-supplemented Neurobasal medium with high levels of glucose. To study OCR a cells were equilibrated for an hour in 2.5 mM glucose HCSS in the presence of 2 mM $CaCl_2$. Then, 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; for review see Brand and Nicholls, 2011).

A,B Respiratory profiles in control (A) and Aralar/AGC1-deficient neurons (B) upon K^+ stimulation, in neurons pretreated or not with 2 mM DCA for an hour, in the presence of 2 mM Ca^{2+}. C, D Respiratory profiles in 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+}. E, F Stimulation of mitochondrial respiration (E) and maximal uncoupled respiration (MUR) (F) upon K^+ stimulation after 2 mM DCA preteatment or

2 mM pyruvate addition. <u>Data correspond to 4-5 to 2-4 independent experiments in wild-type and Aralar/AGC1 KO cultured neurons respectively (one-way ANOVA, *$p<0.05$; **$p< 0.01$).</u>

DCA: dichloroacetic acid, 2 mM; KCl: isosmotic high K^+, 30 mM; Pyr: pyruvate, 2mM

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. PDK, whose activity is negatively controlled by NAD/NADH, ADP/ATP and Pyr/AcCoA ratios, phosphorylates the enzyme in Ser (293) residue inactivating the complex. On the other hand, PDP, which is positively regulated by intramitochondrial Ca^{2+}, dephosphorylates PDH-E1 recovering the active form. DCA inhibits PDK favoring the active form of PDH. B. Representative western blot against PDH (anti-PDH subunit E1, mouse monoclonal antibody, 1:5000,Invitrogen) and p-Ser(293) PDH (anti-PDH, rabbit polyclonal antibody, 1:2000, Novus Biological) and IRDye secondary antibodies optimized for use with Oddysey (800 CW goat anti-rabbit IgG and 680 RD goat anti-mouse IgG, 1:50000, LI-COR). Neurons were obtained in control conditions, or after 5 min exposure to isosmotic 30 mM KCl, or in these same conditions prior pre-treatment for an hour with 2 mM DCA. Merge combines both green and red fluorescences to denote the PDH/P-PDH ratio. C. Western-blotting quantification expressed like fold increase in anti-PDH/anti-p-Ser(293) PDH ratio compared to control situation. Data correspond to n= 3-5 independent experiments in wild-type and Aralar/AGC1 KO cultured neurons, respectively (Student´s t-test, *$p<0.05$).

PDH: pyruvate dehydrogenase; P-PDH: phosphorylated pyruvate dehydrogenase; PDK: pyruvate dehydrogenase kinase; PDP: pyruvate dehydrogenase phosphatase; Pyr: pyruvate; AcCoA: acetyl coenzyme A; DCA: dichloroacetic acid, 2 mM; KCl: isosmotic high K^+, 30 mM.

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

A. The relationship between cytosolic and mitochondrial [Ca^{2+}] during evoked Ca^{2+} signals. MICU1/2 defective cells have 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 resulting in

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 endoplasmic reticulum mitochondria tethering was 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*)
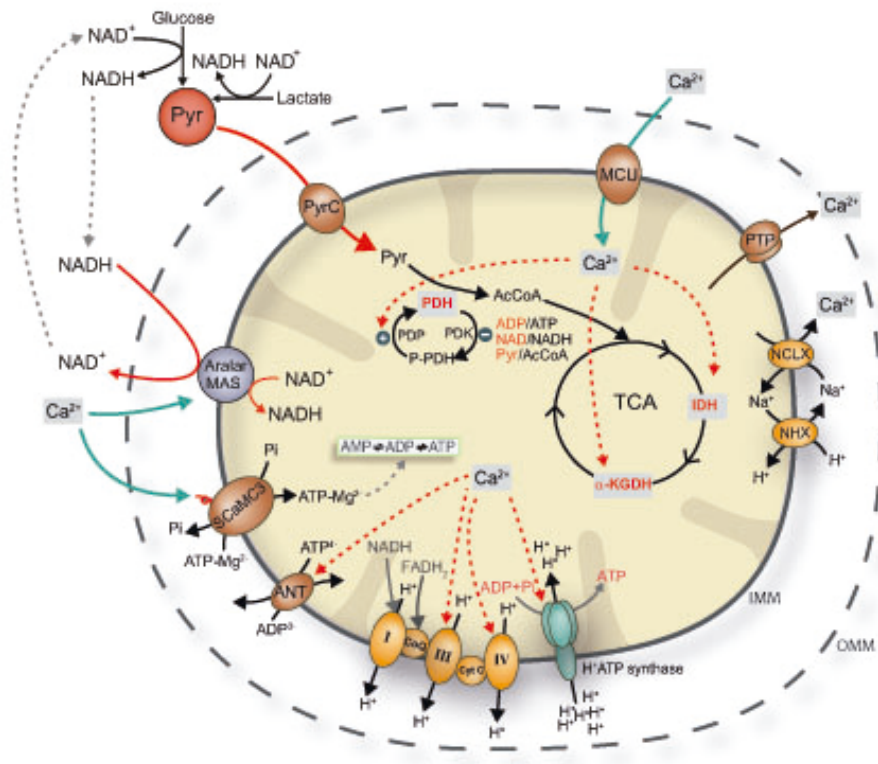
Figure 1. Llorente-Folch I

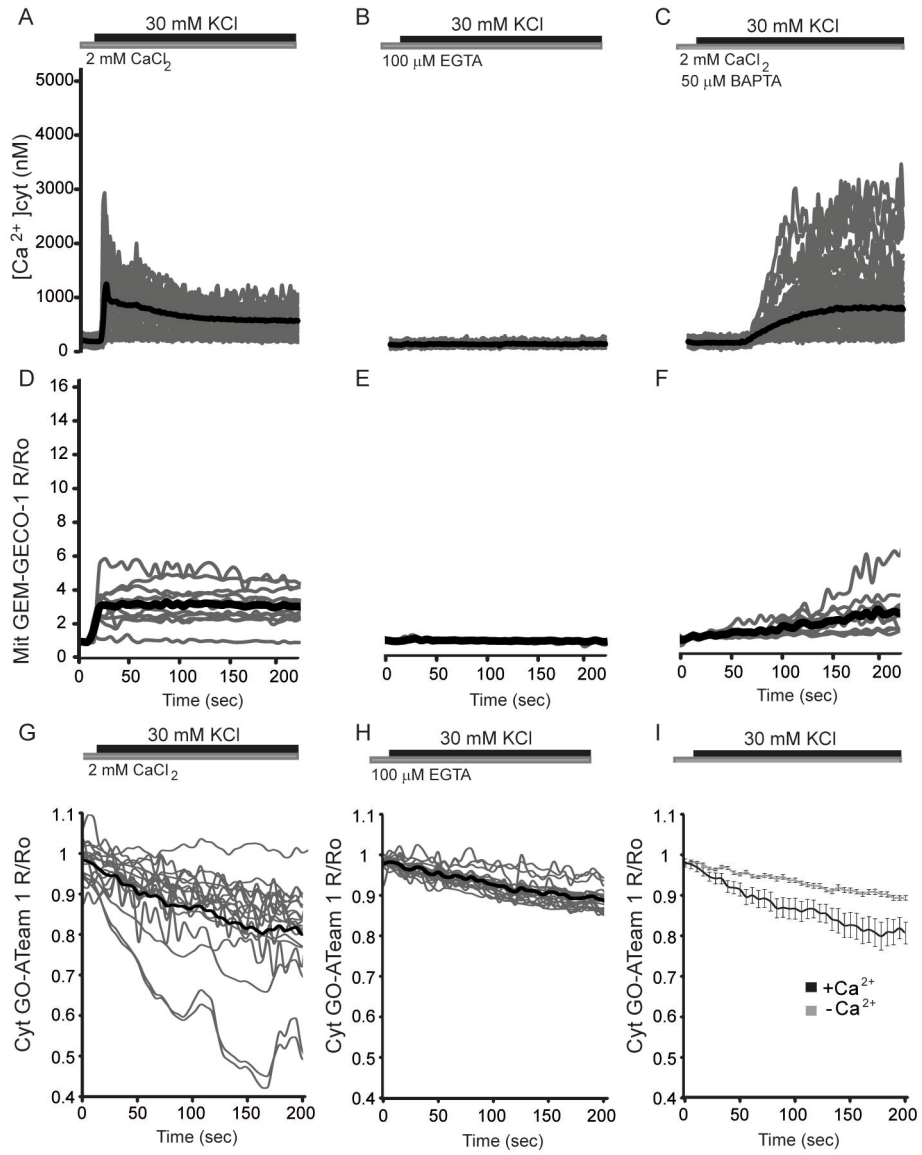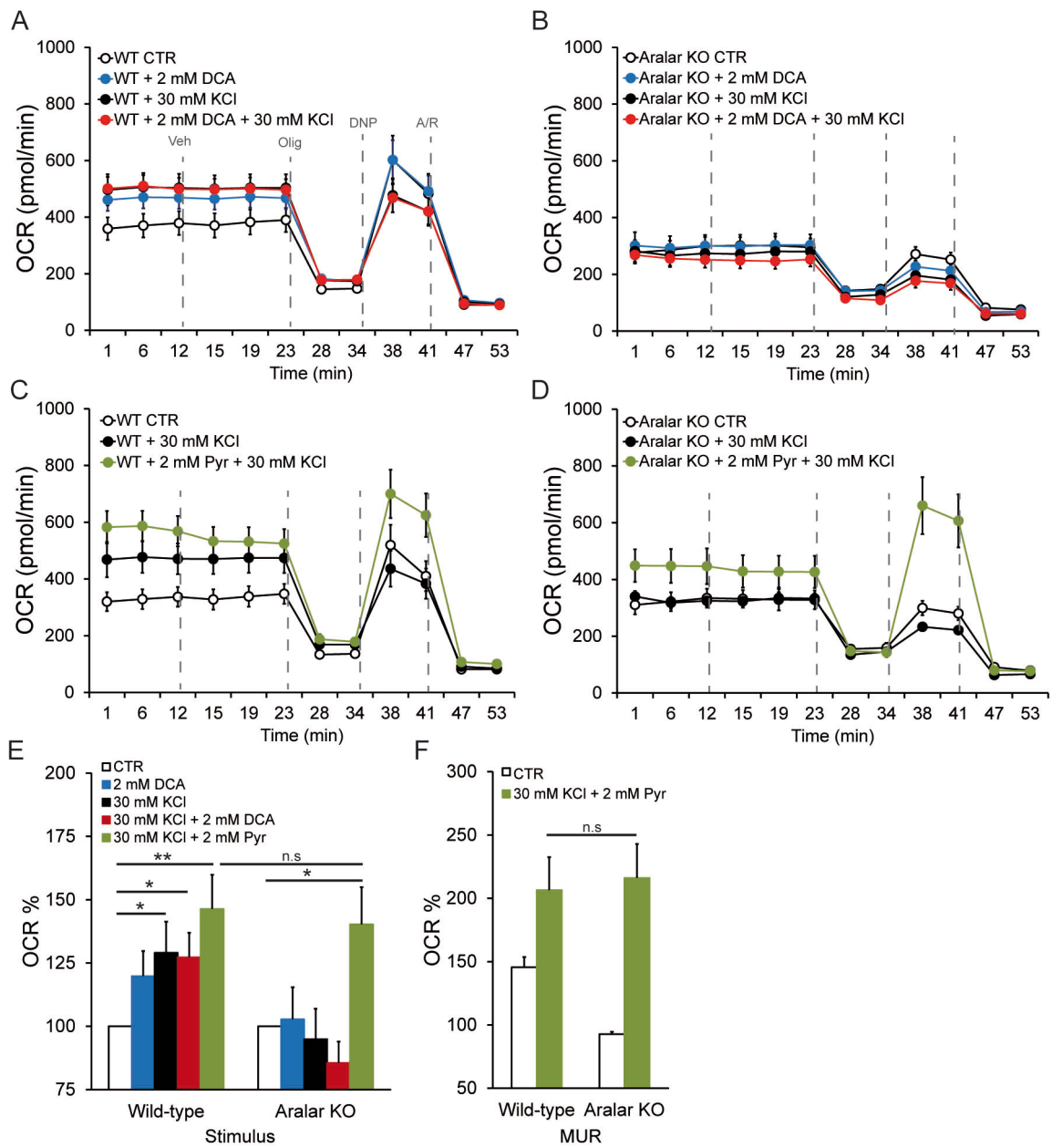Figure 2. Llorente-Folch I

Figure 3. Llorente-Folch I

A

Ca²⁺ ⊕ PDP

PDH active

P-PDH inactive

PDK ⊖ — NAD/NADH, ADP/ATP, Pyr/AcCoA

DCA

B

Wild-type | Aralar KO

α-PDH

α-p-Ser(293) PDH

Merge

C

KCl	–	+	+	–	+	+
DCA	–	–	+	–	–	+

α-PDH/α-p-Ser(293) PDH

1.50
1.25
1.00
0.75

☐ Wild-type
■ Aralar KO

KCl –   +   +
DCA –   –   +
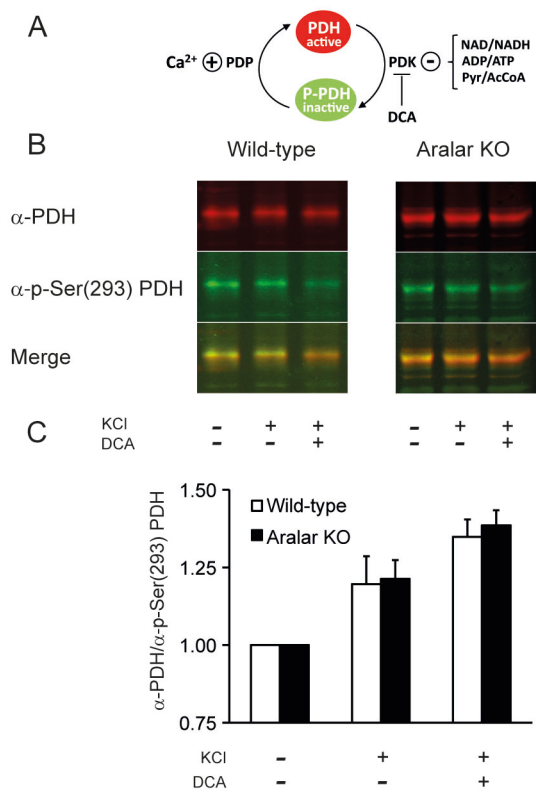
Figure 4 Llorente-Folch I

A

control | histamine (10 μM)

mitochondrial signal ◄
nuclear (cytoplasmic) signal

histamine (10 μM)

control
ΔMICU1 (8a, 6)

mitochondrial rhod-2 intensity (F/F$_0$)
cytoplasmic rhod-2 intensity (F/F$_0$)

B

Ca^{2+}

mfn2
ERMES
Miro
tethering
fission
Drp1
Fis1
MiD49
VDAC
MICU2 MICU1*
MCU
MINOS
Opa1
RC
RC
F$_O$
F$_1$
Ca^{2+} sensitive dehydrogenases
NADH+H$^+$
Ca^{2+} overload
mPTP
RC
F$_O$
F$_1$

Ca^{2+} Na$^+$ H$^+$
Na$^+$/Ca^{2+} antiport
Na$^+$/H$^+$ antiport
Ca^{2+} Na$^+$ H$^+$
Ca^{2+} extrusion, futile Ca^{2+} cycling

Figure 5. Llorente-Folch I