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Ca^{2+} regulation of mitochondrial function in neurons ☆

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ABSTRACT

Calcium is thought to regulate respiration but it is unclear whether this is dependent on the increase in ATP demand caused by any Ca^{2+} signal or to Ca^{2+} itself. $[Na^+]_i$, $[Ca^{2+}]_i$ and $[ATP]_i$ dynamics in intact neurons exposed to different workloads in the absence and presence of Ca^{2+} clearly showed that Ca^{2+}-stimulation of coupled respiration is required to maintain $[ATP]_i$ levels. Ca^{2+} may regulate respiration by activating metabolite transport in mitochondria from outer face of the inner mitochondrial membrane, or after Ca^{2+} entry in mitochondria through the calcium uniporter (MCU). Two Ca^{2+}-regulated mitochondrial metabolite transporters are expressed in neurons, the aspartate–glutamate exchanger ARALAR/AGC1/Slc25a12, a component of the malate–aspartate shuttle, and the ATP-Mg/Pi exchanger SCaMC-3/APC2/Slc25a23, with $S_{0.5}$ for Ca^{2+} of 300 nM and 3.4 μM, respectively. The lack of SCaMC-3 results in a smaller Ca^{2+}-dependent stimulation of respiration only at high workloads, as caused by veratridine, whereas the lack of ARALAR reduced by 46% basal OCR in intact neurons using glucose as energy source and the Ca^{2+}-dependent responses to all workloads: a reduction of about 65–70% in the response to the high workload imposed by veratridine, and completely suppression of the OCR responses to moderate (K^+-depolarization) and small (carbachol) workloads, effects reverted by pyruvate supply. For K^+-depolarization, this occurs in spite of the presence of large $[Ca^{2+}]_{mit}$ signals and increased formation of mitochondrial NAD(P)H. These results show that ARALAR-MAS is a major contributor of Ca^{2+}-stimulated respiration in neurons by providing increased pyruvate supply to mitochondria. In its absence and under moderate workloads, matrix Ca^{2+} is unable to stimulate pyruvate metabolism and entry in mitochondria suggesting a limited role of MCU in these conditions. This article was invited for a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

In muscle, exercise causes fluctuations in workload involving changes in ATP consumption rates due to muscle contraction. Exercise is coupled to about 100-fold increases in oxygen consumption rates [1,2]. Neurons are also subject to changes in workload, as a result of graded changes in the ionic composition of the cytosol which follow the opening of various types of ionic channels. To restore the resting state, neurons consume vast amounts of ATP in pumping these ions out of the cell or into intracellular organelles [3]. As in muscle, this is tightly associated with ATP production by the cell.

In neurons using glucose oxidation as the main metabolic pathway, an increase in workload is necessarily associated with increased glucose oxidation and increased ATP production by mitochondria. The classical principles of chemiosmotic coupling dictate that increased ATP production by mitochondria is coupled to increased oxygen consumption in the respiratory chain and increased substrate supply to mitochondria. However, this is not the only mechanism governing changes in mitochondrial function caused by changes in the workload. It has also become apparent that Ca^{2+} regulation of mitochondrial function plays an important role in maintaining ATP homeostasis.

Abbreviations: MAS, malate/aspartate shuttle; MCU, mitochondrial calcium uniporter; OCR, oxygen consumption rate; PTP, permeability transition pore; ANT, adenine nucleotide translocase; AdN, adenine nucleotides

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In excitable cells, Ca^{2+} regulates cell function both by activation of ATP consumption (contraction, movement, ion transport, Ca^{2+} pumps) [4,5] and by activating ATP production through stimulation of OXPHOS and other means such as stimulation of glycogen breakdown (glycogen phosphorylase kinase is Ca^{2+} dependent) [6].

Ca^{2+} regulation of oxidative phosphorylation is thought to occur thanks to two different mechanisms: Ca^{2+} entry in mitochondria or Ca^{2+}-activation of metabolite transport. Calcium entry in mitochondria involves the recently identified mitochondrial calcium uniporter (MCU) complex, composed by the calcium channel proteins MCU [7,8] and MCUb, a dominant negative component of the oligomeric channel [9], together with regulatory subunits MICU1 [10], MICU2 [11] MCUR [12] and EMRE [13]. Ca^{2+} entry in mitochondria is followed by the activation of three matrix dehydrogenases and complex V, F_1F_0-ATP synthase [14–16]. In skeletal muscle mitochondria Ca^{2+} increases the activity of all complexes of the mitochondrial respiratory chain, in addition to complex V, although the mechanisms responsible for this increase are yet unknown [2]. The generation of a MCU-KO mouse has confirmed that MCU is essential in Ca^{2+}-induced increase in respiration of isolated skeletal muscle mitochondria [17].

Ca^{2+} activation of mitochondrial metabolite transporters occurs by the action of Ca^{2+} on the external side of the inner mitochondrial membrane rather than in the matrix. There are two types of such mitochondrial transporters, the aspartate–glutamate carriers (AGCs) and the ATP-Mg/Pi transporters (APCs or SCaMCs) [18–23]. Ca^{2+}-activation of these transporters does not require Ca^{2+} entry in mitochondria [19,24–28].

By acting on both of these targets, through matrix Ca^{2+} and through stimulation of metabolite transport, Ca^{2+} may activate simultaneously both NADH supply to the respiratory chain and ATP synthesis resulting in metabolic homeostasis, i.e., increase in workload under conditions where the ATP/ADP and NADH/NAD levels remain constant [16], or only change in microdomains sensed by the appropriate targets.

ARALAR/AGC1 is the mitochondrial transporter of aspartate–glutamate present in brain and it is a component of the malate–aspartate NADH shuttle (MAS). Activation by extramitochondrial Ca^{2+} of ARALAR-MAS results in an increase in NADH production in neuronal mitochondria [25] with a requirement of about 300 nM Ca^{2+} to obtain half maximal activation of MAS. Ca^{2+}-activation of respiration of brain mitochondria on glutamate + malate had a similar requirement [29,30]. Gellerich et al. [31] have proposed that Ca^{2+}-activation of ARALAR functions as a "gas pedal" to increase pyruvate formation, thus amplifying the effects of ARALAR in terms of mitochondrial matrix NADH production and respiration. Under conditions of increased workload, such amplifying effect of Ca^{2+} on ARALAR may be required to fully activate substrate supply to mitochondria.

SCaMC-3 is the main mitochondrial ATP-Mg/Pi carrier present in brain and liver [20,21,32]. Activation by Ca^{2+} of the tumor cell SCaMC-1 or the liver cell ATP-Mg/Pi carrier SCaMC-3 results in the electroneutral uptake of either $[ATP\text{-}Mg]^{2-}$ or $HADP^{2-}$ against Pi^{2-} [28,33]. Work from Aprille's group [34,35, reviewed in 36] and Amigo et al. [32] have shown that adenine nucleotide accumulation in rat liver mitochondria through the ATP-Mg/Pi carrier SCaMC-3 results in a progressive increase in state 3 respiration. Compared to ARALAR/AGC1, SCaMC-3 requires much higher Ca^{2+} concentrations to obtain half maximal activation of ATP-Mg/Pi transport, about 3.4 μM [32], in the range of that of the mitochondrial calcium uniporter.

2. Calcium regulation of mitochondrial function is required to maintain cytosolic ATP levels upon changes in workload

Although changes in neuronal mitochondrial function in response to an increase in workload have been described before [37,38], the control of respiration by Ca^{2+} in intact neurons is still largely unknown. Hayakawa et al. [39] have described rapid Ca^{2+} dependent changes in oxygen consumption in response to high KCl in cultured Purkinje neurons, but Mathiesen et al. [40] have found no evidence for a role of cytosolic Ca^{2+} in activity-dependent rises in cerebral metabolic rate of oxygen in cerebellar Purkinje neurons in the intact brain. A confounding variable in these and other studies relates to the coincidence of the Ca^{2+}-mechanism with the classical mechanism activating mitochondrial respiration, i. e., ATP demand. Indeed, any Ca^{2+} signal involves ATP consumption in order to reestablish pre-stimulation values, and the role of Ca^{2+} versus ADP-stimulation of respiration needs to be established.

This problem has been addressed by analyzing the role of Ca^{2+} as a signaling molecule versus an inducer of ATP demand in neurons subject to different workloads: veratridine, high K^+ depolarization, and carbachol.

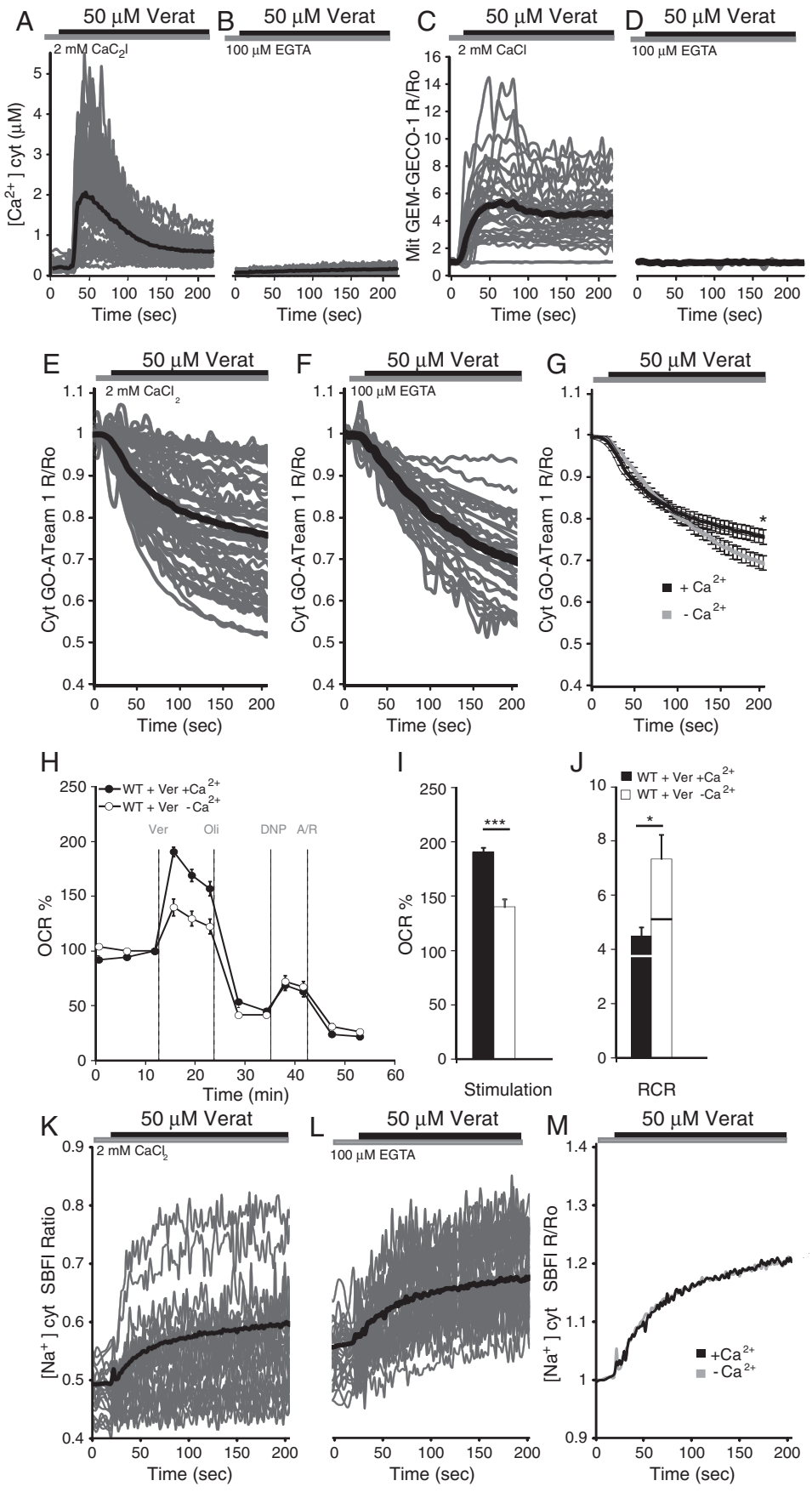
Veratridine induces a robust increase in $[Na^+]_i$ and $[Ca^{2+}]_i$, a large increase in $[Ca^{2+}]_{mit}$ in intact cortical neurons, and a large increase in respiration (Fig. 1). In a Ca^{2+}-free medium the increase in $[Na^+]_i$ was the same, but that of $[Ca^{2+}]_i$ and $[Ca^{2+}]_{mit}$ was abolished. The increase in respiration was also much lower. Cytosolic ATP dropped after veratridine addition in these neurons, but the drop was larger in a Ca^{2+}-free than in a Ca^{2+}-containing medium (Fig. 1E–G). These results showed that the smaller respiratory response to veratridine in Ca^{2+}-free medium is not due to a smaller ATP demand, but to the absence of a Ca^{2+}-regulatory mechanism.

A similar strategy was employed to establish the role of Ca^{2+} in the response to KCl and carbachol. As for veratridine, the increase in respiration caused by KCl or carbachol was strongly reduced in Ca^{2+}-free medium, which also abolished K^+-induced $[Ca^{2+}]_i$ and $[Ca^{2+}]_{mit}$ signals, and carbachol-induced $[Ca^{2+}]_i$ signals. Not surprisingly, K^+ or carbachol-induced drop in cytosolic ATP was smaller or unchanged in Ca^{2+}-free conditions, and this could explain by itself the smaller increase in respiration in a Ca^{2+}-free medium. However, incubation with BAPTA-AM, which maintained the workload but blocked Ca^{2+} signaling also resulted in a lower respiratory response to these agents indicating that Ca^{2+}-regulation is required to increase respiration and maintain cytosolic ATP levels in response to any workload [41].

3. Role of the mitochondrial calcium uniporter (MCU) and the Ca^{2+}-regulated mitochondrial carriers in OCR stimulation in response to different workloads

Having found that Ca^{2+} regulation of respiration is required to maintain ATP levels in response to workload in neurons using glucose as energy source, the role of matrix versus extramitochondrial Ca^{2+} in stimulating respiration was analyzed next.

Fig. 1. Changes in cytosolic and mitochondrial Ca^{2+}, cytosolic ATP, oxygen consumption and cytosolic Na^+ in primary neuronal cultures in response to veratridine. A, B, Changes in $[Ca^{2+}]_{cyt}$, in Fura-2 loaded neurons and C, D changes in $[Ca^{2+}]_{mit}$ in neurons transfected with Mit-GEM-GECO1 probe, obtained by stimulation with 50 μM veratridine (Ver or Verat) in 2 mM Ca^{2+} (A, C) or Ca^{2+}-free medium (B, D). Recordings from at least 60 cells per condition and two independent experiments were used for $[Ca^{+2}]_i$ (A, B) and a minimum of 15 cells and 8 independent experiments for $[Ca^{2+}]_{mit}$ imaging (C, D). Individual cell recordings (gray) and average (thick black trace) were shown. E–G, Cytosolic ATP in neurons transfected with cyt-GO-ATeam1 probe stimulated with veratridine in 2 mM Ca^{2+} medium (E), Ca^{2+}-free medium plus 100 μM EGTA (F) and comparison of the two conditions (G). H, Veratridine-induced stimulation of oxygen consumption rate (OCR) in *aralar* WT neurons under the mentioned Ca^{2+} conditions: 2 mM Ca^{2+} medium (filled circles or black bars), Ca^{2+}-free medium plus 100 μM EGTA (empty circles or white bars). OCR was measured using a Seahorse XF24 Extracellular Flux Analyzer. The sequential injections at different time points of veratridine (Ver, 50 μM) and the metabolic inhibitors oligomycin (Oli, 6 μM), 2,4-dinitrophenol (DNP, 0.5 mM) and antimycin A/rotenone (A/R, 1.0 μM both) are indicated by dashed lines. I, J, Stimulation of respiration (indicated as percentage of basal values) and respiratory control ratio (RCR) at the assayed conditions (n = 9–11 experiments, $^*p < 0.05$, $^{***}p < 0.001$, Student's t-test). RCR in non-stimulated state is represented with horizontal lines for each experimental condition. K–M, Changes in $[Na^+]i$, in individual SBFI-loaded neurons by stimulation with 50 μM veratridine in 2 mM Ca^{2+} medium (K) or Ca^{2+}-free medium (L), and comparison of mean values (M).
Taken from Llorente-Folch et al. [41].


A
50 μM Verat
2 mM CaC2l
[Ca2+] cyt (μM)
Time (sec)
B
50 μM Verat
100 μM EGTA
Time (sec)
C
50 μM Verat
2 mM CaCl
Mit GEM-GECO-1 R/Ro
Time (sec)
D
50 μM Verat
100 μM EGTA
Time (sec)
E
50 μM Verat
2 mM CaCl2
Cyt GO-ATeam 1 R/Ro
Time (sec)
F
50 μM Verat
100 μM EGTA
Cyt GO-ATeam 1 R/Ro
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100 μM EGTA
Time (sec)
M
50 μM Verat
[Na+] cyt SBFI R/Ro
+Ca2+
- Ca2+
Time (sec)

Table 1.
Glucose utilization in WT and ARALAR-KO cultured neurons. Cortical neurons were cultured for 9–10 DIV (days in vitro) and subsequently incubated for 24 h in free-serum B27-supplemented Neurobasal-A medium with 10 mM glucose. Experiments were performed at a final glucose concentration of 5 mM, 240 μM lactate and 367 μM pyruvate. Lactate and glucose contents were determined enzymatically [51–54]. Pyruvate concentration in the media was determined using a kit from Instruchemie BV following the manufacturer's instructions. Taken from Llorente-Folch et al. [41].

Genotype	Glucose consumed (μmol/mg/h)	Lactate formed (μmol/mg/h)	% Lac/Gluc	Pyr consumed (μmol/mg/h)
WT	0.87 ± 0.211	1.09 ± 0.22	1.25	1.26 ± 0.07
Aralar KO	0.67 ± 0.17	1.03 ± 0.31	1.54	1.74 ± 0.08***

*** $p < 0.001$.

One way to address this point was by studying the effects of selective removal of the two main Ca^{2+} regulated mitochondrial carriers expressed in neurons, ARALAR/AGC1/Slc25a12, the aspartate–glutamate exchanger and component of the malate–aspartate shuttle (MAS), and SCaMC-3/Slc25a23, the ATP-Mg/Pi exchanger.

The first of these carriers is clearly operating under resting conditions, as there is a drop in basal respiration of about 46% in ARALAR-KO versus control neurons [41]. In addition, glucose consumption tended to decrease and pyruvate utilization increased (Table 1). This is consistent with the major role of MAS in the oxidation of glucose in neurons. In contrast, SCaMC-3 does not play a role in respiration under basal conditions.

3.1. SCaMC-3 regulates Ca^{2+}-dependent respiration only at high workloads

The strong Ca^{2+}-dependent increase in neuronal respiration in response to veratridine is dependent on the presence of SCaMC-3, with a 10–15% reduction in the response in SCaMC-3 KO neurons (Fig. 2A–D). However, SCaMC-3 deficiency does not affect the responses to smaller workloads (high K^+, carbachol) [41], or to veratridine in a Ca^{2+}-free medium (Fig. 2A–D). This confirms that SCaMC-3 is only recruited at high cytosolic Ca^{2+} concentrations, as those that activate the carrier [32] and large workloads in which mitochondrial ATP may fall and/or cytosolic ADP levels increase. In these conditions, $[ATP\text{-}Mg]^{2-}$ or $HADP^{2-}$ uptake in mitochondria in exchange of Pi will be thermodynamically favored [42,43], resulting in an increase in the mitochondrial pool of adenine nucleotides and respiration.

The mechanism whereby respiration increases is, however, unclear. It is known that the uptake of either ATP or ADP changes the total content but not the distribution of adenylates in ATP, ADP and AMP which is under the control of the ANT (adenine nucleotide translocase) plus complex V [36]. An increase in the adenine nucleotide pool size may affect ATP synthase activity through mass action ratio, and this by itself could explain the increase in neuronal coupled respiration. However, as this only occurs at high workloads which involve a robust uptake of Ca^{2+} in mitochondria, it is also possible that the entry of adenine nucleotides is required as a protective mechanism against an early opening of the PTP (permeability transition pore) which would cause an immediate inhibition of respiration. Adenine nucleotides are major PTP blockers [44,45,28] and also bind calcium [46]. Liver mitochondria incubated with millimolar ADP or ATP concentrations have a substantial increase in calcium retention capacity which depends upon the presence of SCaMC-3 [32]. Adenine nucleotides in the matrix of neuronal mitochondria may lower the free matrix Ca^{2+} concentration which would also result in a reduced PTP opening and in an increased respiration.

3.2. Ca^{2+}-stimulation of neuronal respiration caused by high workloads is highly dependent on ARALAR-MAS

In contrast to SCaMC-3 deficiency, which causes a mild reduction in the response to veratridine, the lack of ARALAR-MAS causes a very drastic reduction of Ca^{2+}-dependent veratridine-induced stimulation of neuronal respiration (about 65–70% reduction) (Fig. 3A–C). The lack of ARALAR-MAS also reduced veratridine-stimulated respiration in a Ca^{2+}-free medium (about 40% reduction, Fig. 3B, C) indicating that ARALAR-MAS is recruited upon an increase in workload also in the absence of calcium signaling. Indeed, aspartate/glutamate exchange through ARALAR, and ARALAR-MAS activity do not have an absolute requirement for Ca^{2+}, and MAS is also active in a Ca^{2+}-free medium [20,25,26]. The requirement for ARALAR-MAS in workload-induced stimulation of respiration is consistent with the role of MAS in aerobic glucose utilization. In fact, activation of ARALAR-MAS activity results in an increased supply of pyruvate to mitochondria, as external pyruvate reverts the effects of ARALAR deficiency (Fig. 3E–H).

Although in a weaker way, ARALAR-KO neurons still respond to veratridine with an increase in OCR which is also Ca^{2+}-dependent, i.e., larger in the presence than in the absence of Ca^{2+} (Fig. 3C). This indicates that MCU-dependent and possibly SCaMC-3-dependent Ca^{2+}-regulations are the Ca^{2+} signaling mechanisms involved. The relative contribution of each one has still to be defined.

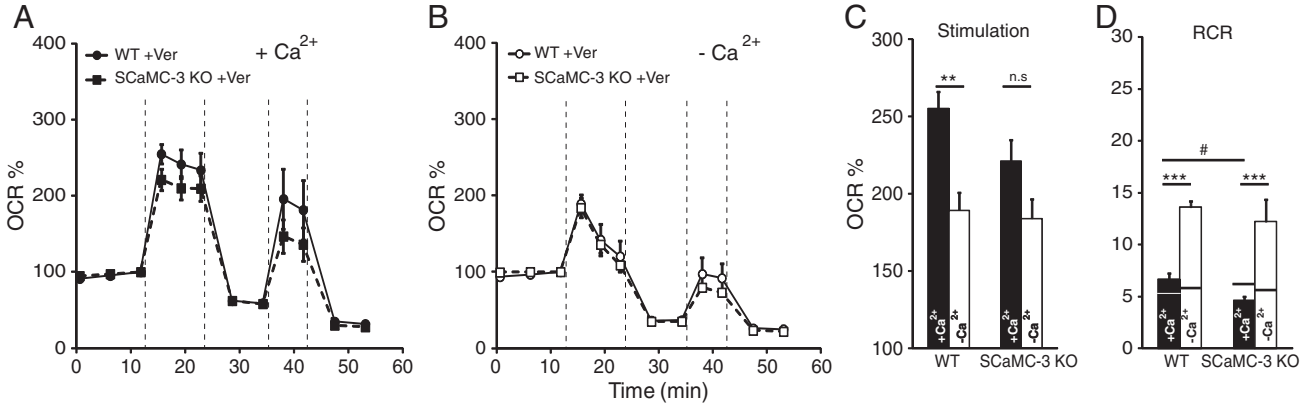

Fig. 2. OCR responses to veratridine in SCaMC-3-deficient neurons. Respiratory profiles in the presence (A) or absence of 2 mM Ca^{2+} (B), stimulation of mitochondrial respiration as OCR % (C) and RCRs (D) of *SCaMC-3*-WT and *SCaMC-3*-KO neurons upon 50 μM veratridine (Ver) stimulation in 2.5 mM glucose are shown. OCR was measured as described in Fig. 1. In D, RCR in non-stimulated state is represented with horizontal lines for each experimental condition. Data are expressed as mean ± S.E.M. from n = 5–6 experiments in WT and SCaMC-3 KO cultures, Student's t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
Taken from Llorente-Folch et al. [41].

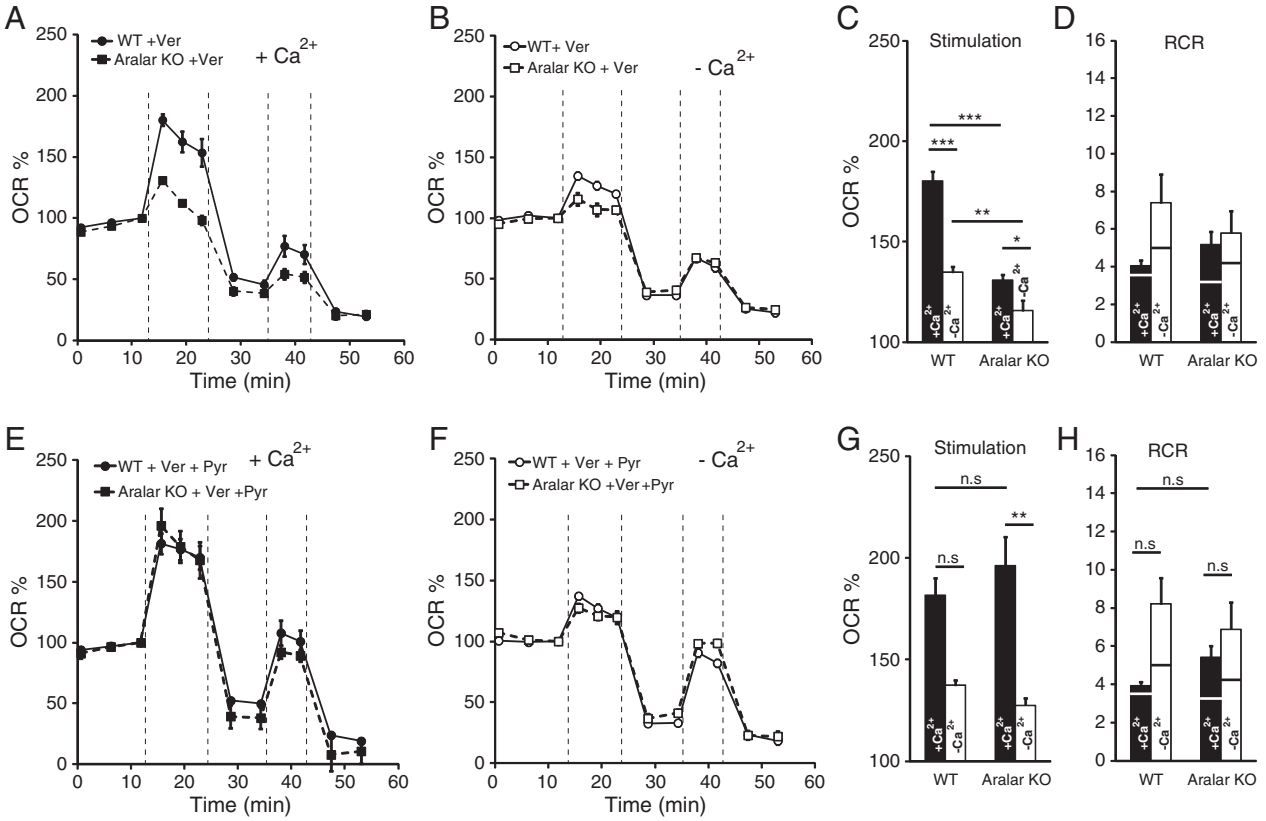

Fig. 3. OCR responses to veratridine in ARALAR-deficient neurons. A–D, Respiratory profiles in the presence or absence of 2 mM Ca^{2+}, stimulation of mitochondrial respiration and RCRs of *aralar* WT and *aralar* KO neurons. E–H, Corresponding data of veratridine-induced stimulation of mitochondrial respiration in the presence of 2 mM pyruvate (Pyr) in *aralar* WT and *aralar* KO neurons. OCR determinations were performed as described in Fig. 1. RCR in non-stimulated state is represented with horizontal lines for each experimental condition. Data are expressed as mean ± S.E.M. from n = 4–12 experiments in WT and ARALAR KO cultures. Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001.
Taken from Llorente-Folch et al. [41].

Taken together, Ca^{2+}-dependent regulation of respiration in response to high workloads recruits all three mechanisms, ARALAR-MAS, SCaMC-3 and MCU-dependent effects of Ca^{2+} in the mitochondrial matrix. However, ARALAR-MAS exerts the largest influence on the final response.

3.3. *ARALAR-MAS is the only mechanism of Ca^{2+}-regulation of neuronal respiration operating at low and moderate workloads*

Pardo et al. [25] showed that small Ca^{2+} signals, as generated by activation of G-protein-coupled receptors (GPCR) and Ca^{2+} release from intracellular stores, caused an increase in neuronal mitochondrial NAD(P)H mediated by activation of ARALAR-MAS. These small Ca^{2+} signals do not reach mitochondria [25,41], and result in a small increase in ATP demand and coupled respiration in intact neurons which, not surprisingly, is absolutely dependent on ARALAR-MAS [41].

In the case of a moderate workload, as that produced by high K^+ depolarization, the scenario is quite different. ATP demand is larger and the cytosolic Ca^{2+} signal is also larger and reaches mitochondria [25,41]. In these conditions, mitochondrial NAD(P)H increases regardless of the presence of ARALAR [25]. Therefore, current concepts in the field would predict that both the ARALAR-MAS and MCU pathways would be recruited in response to high K^+-induced workload. However, high K^+-stimulated respiration is absolutely dependent on ARALAR-MAS, with no stimulation of respiration in ARALAR-KO neurons (Fig. 4), an effect reverted by the supply of exogenous pyruvate [41]. This very surprising result suggests that the full respiratory response to a moderate workload requires ARALAR-MAS to supply pyruvate to mitochondria, and does not take place in its absence even though

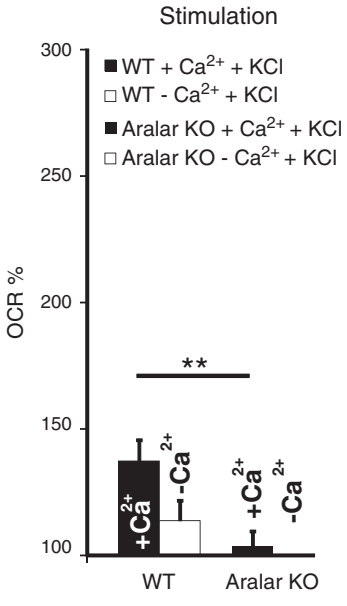

Fig. 4. OCR-responses to 30 mM KCl in ARALAR-deficient cortical neurons. Stimulation of mitochondrial respiration in response to 30 mM KCl obtained in *aralar* WT and *aralar* KO cultures in the presence or absence of 2 mM Ca^{2+}. OCR was measured at the conditions indicated as described in Fig. 1. Stimulation of respiration by KCl in the presence or absence of 2 mM Ca^{2+} is indicated as percentage of basal values. Data are expressed as mean ± S.E.M. from n = 4–24 experiments in *aralar* WT and ARALAR KO respectively. Student's t-test, *p < 0.05, **p < 0.01, ***p < 0.001.
Taken from Llorente-Folch et al. [41].

Ca^{2+} entry in mitochondria is maintained [24]. Intriguingly, the production of NAD(P)H in neuronal mitochondria is the same in control and ARALAR-KO neurons under high K-depolarization [24], but in spite of it, respiration is only stimulated in control, but not ARALAR-KO, neurons.

A possible explanation of this finding is that the MCU-driven metabolic changes in mitochondria are not sufficient by themselves to trigger the increase in respiration. NAD(P)H production in mitochondria may be activated by way of Ca^{2+}-stimulation of matrix dehydrogenases, but this by itself will not increase respiration if it is not coupled to the entry of pyruvate in mitochondria, which is the main role of ARALAR-MAS.

4. Regulation of pyruvate supply to mitochondria: Ca^{2+}-pull versus Ca^{2+}-push mechanisms

Theoretically, Ca^{2+} in the matrix could "pull" pyruvate into mitochondria independently of ARALAR-MAS (Fig. 5). These are the conditions met by mitochondria containing a pyruvate dehydrogenase (PDH) complex undergoing a Ca^{2+}-dependent activation by dephosphorylation. The increased oxidation of pyruvate will "pull" pyruvate into mitochondria simply by mass action ratio on the mitochondrial pyruvate carrier which is driven by the ΔpH [47–49]. However this does not appear to be the case in neurons, at least under moderate workloads. There are two PDH phosphatases which activate PDH, PDP-1, which is Ca^{2+}-dependent, and PDP-2, which is Ca^{2+}-independent [50]. Unlike astrocytes which have a highly phosphorylated PDH under basal conditions, and express Ca^{2+}-dependent PDP-1, neurons have a relatively unphosphorylated PDH under basal conditions, and their main PDH phosphatase is PDP-2, suggesting that a full activation of PDH does not depend on matrix Ca^{2+} in neurons [51]. Thus the matrix Ca^{2+} "pull" mechanism of pyruvate entry in mitochondria will not occur under moderate workloads. The entry of pyruvate in mitochondria in these conditions is provided by other means, particularly by ARALAR-MAS. Ca^{2+}-activation of ARALAR-MAS will function to "push" pyruvate into mitochondria by changing the cytosolic redox state of NAD/NADH, and possibly, by conversion of lactate to pyruvate. It is likely that pyruvate entry in mitochondria is required for full activation of neuronal PDH, by its inhibitory action on PDH kinases (PDK1 and PDK3 in neurons, [51]) and this may be necessary for the full activation of PDH and respiration in intact neurons.

It could be argued that the lack of the matrix Ca^{2+} "pull" mechanism of pyruvate entry in ARALAR-KO neurons undergoing a moderate workload is artifactual and due to limited pyruvate levels in these cells. While this possibility cannot be fully ruled out, it is also obvious that at higher workloads as those produced by veratridine, ARALAR-KO neurons do show an increase in coupled respiration, indicating that pyruvate limitation is overcome in these conditions. In this setting the activation of respiration must be taking place exclusively through Ca^{2+} entry in mitochondria and activation of mitochondrial dehydrogenases.

Our results do not imply that the pull and push mechanisms are mutually exclusive. On the contrary, the activation of ARALAR-MAS probably synergizes with that of MCU-mitochondrial dehydrogenases to obtain a full respiratory response. Further work will be required to address this issue.

5. Conclusions

Together, these results underscore the roles of MCU-$[Ca^{2+}]_{mit}$, ARALAR-MAS, and SCaMC-3 in upregulating oligomycin-sensitive respiration in cerebral cortex neurons in response to workloads produced by increases in Na^+ and/or Ca^{2+} and robust-to-small Ca^{2+} signals. These roles may vary in neurons from the adult brain due to changes in enzyme and transporter composition. In this regard, Mathiesen et al., [40] found no evidence for a role of cytosolic Ca^{2+} in activity dependent rises in cerebral metabolic rate of oxygen ($CMRO_2$) in cerebellar Purkinje neurons in the intact brain.

Surprisingly, MCU-$[Ca^{2+}]_{mit}$ appears to play a role in upregulation of respiration only in the response to the large workload produced by veratridine. Moreover, although its role is evident, it is not the main contributor to veratridine stimulated respiration. The ARALAR-MAS pathway by regulation of pyruvate supply to mitochondria in this condition vastly increases the OCR response.

The ARALAR-MAS pathway plays an outstanding role in the response to smaller workloads, being the only Ca^{2+}-regulation mechanism responsible for upregulation of respiration in response to the small Ca^{2+} signals produced by carbachol and in response to KCl-depolarization, which induces large Ca^{2+} signals in mitochondria. In all cases, ARALAR-MAS through the Ca^{2+}-activation of ARALAR in the intermembrane space provides pyruvate to mitochondria. The mitochondrial ATP-Mg/Pi carrier SCaMC-3 limits respiration only in response to high workloads and robust Ca^{2+} signals which are able to

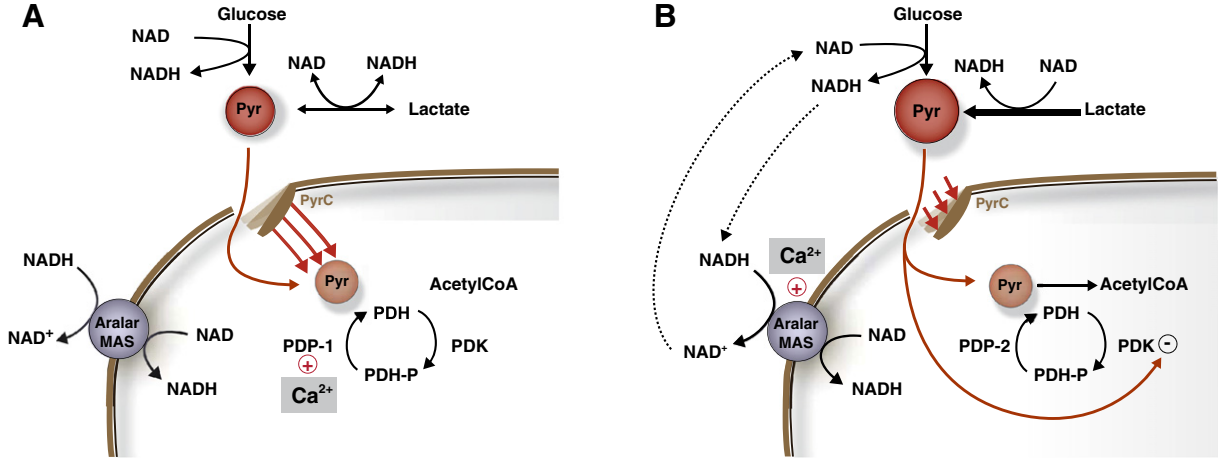

Fig. 5. Schematic representation of the Ca^{2+}-dependent "pull" and "push" mechanisms to regulate pyruvate supply to mitochondria. A, Pull mechanism: Ca^{2+} in the matrix could "pull" pyruvate into mitochondria independently of ARALAR-MAS. Ca^{2+} in the matrix could promote pyruvate dehydrogenase (PDH) complex activation by dephosphorylation. The increased oxidation of pyruvate will "pull" pyruvate into mitochondria by mass action ratio on the mitochondrial pyruvate carrier. B, Push mechanism: extramitochondrial Ca^{2+}-activation of ARALAR-MAS changes the cytosolic redox state of NAD/NADH and may promote the conversion of lactate to pyruvate. The increase in cytosolic pyruvate will function to "push" pyruvate into mitochondria by mass action ratio on the mitochondrial pyruvate carrier. Pyruvate entry would induce inhibition of PDH kinases which might be necessary for the full activation of PDH and respiration in intact neurons. PDH, pyruvate dehydrogenase (active); PDK, pyruvate dehydrogenase kinase; PDH-P, phosphorylated pyruvate dehydrogenase (inactive); PDP-1 and PDP-2, calcium dependent and Ca^{2+}-independent pyruvate dehydrogenase phosphatase, respectively; Pyr, pyruvate; PyrC, pyruvate carrier; MAS, malate–aspartate shuttle.

activate carrier activity, as produced by veratridine, suggesting a requirement of adenine nucleotide uptake in mitochondria to generate a full respiratory response under these conditions.

Although surprising, the limited role of MCU-$[Ca^{2+}]_{mit}$ in Ca^{2+}-regulated respiration in intact neurons is consistent with recent findings from skeletal muscle of MCU-KO mice [17]. Ca^{2+} uptake and Ca^{2+}-stimulated respiration in skeletal muscle mitochondria were severely blocked in MCU-KO mice, and matrix Ca^{2+} levels and basal PDH dehydrogenase activity, which is under the control of PDP-1 in muscle, were also lower, consistent with the known role of matrix Ca^{2+} activation of dehydrogenases. However, the effects of MCU deficiency were much smaller in the intact muscle, with no effects on basal respiration and small effects on muscle work, restricted to a limitation in top performance. This suggests that the role of MCU-$[Ca^{2+}]_{mit}$ in regulating mitochondrial metabolism in vivo should be re-evaluated in different tissues and cells with changing energy demands.

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