The distinct roles of calcium in rapid control of neuronal glycolysis and the tricarboxylic acid cycle

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Impact Statement
When neurons are stimulated, calcium influx instructs mitochondria to increase energy metabolism, but it is increased energy demand in cytosol rather than Ca\(^{2+}\) signaling that leads to increased neuronal glycolysis.
ABSTRACT

When neurons engage in intense periods of activity, the consequent increase in energy demand can be counteracted by the coordinated activation of glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. However, the trigger for glycolytic activation is unknown and the role for Ca\(^{2+}\) in the mitochondrial responses has been debated. Using genetically-encoded fluorescent biosensors and NAD(P)H autofluorescence imaging in acute hippocampal slices, here we find that Ca\(^{2+}\) uptake into the mitochondria is responsible for the buildup of mitochondrial NADH, probably through Ca\(^{2+}\) activation of dehydrogenases in the TCA cycle. In the cytosol, we do not observe a role for the Ca\(^{2+}\)/calmodulin signaling pathway, or AMPK, in mediating the rise in glycolytic NADH in response to acute stimulation. Calcium, nevertheless, is a major contributor to glycolysis, although not strictly necessary. Aerobic glycolysis in neurons is triggered mainly by the energy demand resulting from either Na\(^{+}\) or Ca\(^{2+}\) extrusion.

INTRODUCTION

Energy demand in neurons is proportional to the frequency of action potentials, which can increase dramatically upon neuronal stimulation. During neuronal activity, Na\(^{+}\) and Ca\(^{2+}\) enter the cell via specific voltage-gated ion channels in the plasma membrane. This, in turn, accelerates Na\(^{+}\) and Ca\(^{2+}\) pumping and leads to higher ATP consumption, which is replenished by oxidative phosphorylation (OXPHOS) and glycolysis (Attwell and Laughlin, 2001; Yu et al., 2017). However, whether the upregulation of ATP synthesis is provoked simply by the degradation of ATP (and increases in ADP), or by a feedforward signal such as elevation of intracellular Ca\(^{2+}\) or AMP\(^{1}\), is still not fully understood.

Both as an energy burden and as a feedforward signal, intracellular Ca\(^{2+}\) is a strong candidate for coordinating the fast-metabolic responses to increased neuronal activity. Calcium handling is metabolically expensive and compartmentalized, involving several transporters at the plasma membrane and the endoplasmic reticulum. In addition, Ca\(^{2+}\) uptake into the mitochondria can directly dissipate the mitochondrial membrane potential (Duchen, 1992; Berndt et al., 2015) and increase

\(^{1}\) AMP could act as a feedforward signal via AMPK activation (Herzig and Shaw, 2018), but, like ADP, it could also directly activate the glycolytic enzyme phosphofructokinase (Passonneau and Lowry, 1962).
OXPHOS while activating several dehydrogenases in the tricarboxylic acid (TCA) cycle (Denton et al., 1972; Denton et al., 1978; McCormack and Denton, 1979; Denton, 2009; Wescott et al., 2019). When elevated in the cytosol, Ca^{2+} can also modulate the activity of enzymes through Ca^{2+}/calmodulin-dependent signaling (Singh et al., 2004; Marinho-Carvalho et al., 2009; Schmitz et al., 2013), or direct allosteric regulation of cytoskeletal interactions (Chen-Zion et al., 1993).

Using genetically encoded fluorescent biosensors, NAD(P)H autofluorescence imaging, and extracellular O_2 measurements, we investigated the effects of mitochondrial Ca^{2+} uptake on NADH production and consumption via the TCA cycle and the electron transport chain, respectively. Since NADH production in the cytosol is also increased upon acute neuronal stimulation, we tested if several Ca^{2+} signaling pathways, or Ca^{2+} itself, were necessary for this response. Finally, we assessed whether the energy demand resulting from Na^{+} or Ca^{2+} extrusion could trigger aerobic glycolysis in stimulated neurons.

RESULTS

Fast cytosolic NADH responses are independent of mitochondrial NADH responses

To monitor the metabolic changes in response to neuronal stimulation, we measured the NADH/NAD^{+} ratio in the cytosol using Peredox (Hung et al., 2011), which reflects the overall activity of glycolysis. We also imaged mitochondrial NADH in a population of dentate granule cells (DGCs) of the mouse hippocampus using UV-excited autofluorescence (Chance et al., 1962). This is known as the NAD(P)H signal because of a small contribution of the spectrally indistinguishable cofactor NADPH. In neurons, this signal is thought to predominantly reflect NADH changes in the mitochondria, because both the NADH concentration and the fraction of brighter protein-bound NADH, are higher in the mitochondrial matrix compared to the cytosol (reviewed by Kann and Kovács, 2007; Shuttleworth, 2010; Yellen, 2018). We also expressed the Ca^{2+} sensor RCaMP1h (Akerboom et al., 2013) in the cytoplasm of DGCs to simultaneously monitor neuronal activity.

The NADH pool in one compartment (e.g. mitochondria) can potentially influence the NADH pool in the other (e.g. cytosol) due to their connection via the malate-aspartate shuttle (MAS), whose components are highly expressed in neurons (reviewed by McKenna et al., 2006). Therefore, we first tested
whether the NADH signals in the cytosol could occur independently from the responses in the mitochondria.

The production of NADH in the mitochondria is preferentially fueled by pyruvate, which can be produced either from glycolysis or from lactate re-oxidation (via the lactate dehydrogenase reaction). Pyruvate is transported into the mitochondria via the mitochondrial pyruvate carrier (MPC) (Bricker et al., 2012; Herzig et al., 2012), where it is converted to acetyl-CoA by the enzyme pyruvate dehydrogenase and then further catabolized in the TCA cycle. We hypothesized that by inhibiting the MPC, pyruvate should fail to enter mitochondria and fuel the TCA cycle, and, as a result, the mitochondrial NAD(P)H signals (but not glycolysis) should be attenuated and pyruvate should accumulate in the cytosol.

We first confirmed that MPC inhibition leads to cytosolic pyruvate accumulation by expressing the pyruvate-sensitive FRET sensor Pyronic (San Martín et al., 2014) in the cytosol of DGCs. When slices were treated with a high affinity inhibitor of the MPC (UK5099; 2 µM), the sensor reported a steady accumulation of cytosolic pyruvate over 30 minutes, with a signal comparable to that produced by exogenous addition of 10 mM pyruvate to the bathing solution (Figure 1a, Figure 1–Supplement 1).

Starving the mitochondria of pyruvate should also hamper the activity of the TCA cycle, thus diminishing NADH production in the mitochondria. To test this hypothesis, we assessed the effect of UK5099 on the NADH dynamics in the mitochondria of stimulated neurons.

In control conditions, we observed the typical NAD(P)H signals in response to electrical stimulation of DGC axons, consisting of a prompt and brief (1-2 s) negative deflection (“dip”), followed by an “overshoot” that can last several minutes (Figure 1b Left; Shuttleworth et al., 2003; Brennan et al., 2006; Brennan et al., 2007; Ivanov et al., 2014). The dip reflects an oxidative phase, triggered by the dissipation of the electrochemical proton gradient across the inner mitochondrial membrane (ΔµH+) by Ca2+ and/or ADP uptake into the matrix (Duchen, 1992; Berndt et al., 2015; reviewed by Yellen, 2018). The subsequent overshoot (reductive phase) is the result of NADH production by several dehydrogenases in the TCA cycle, but while some studies suggest that it is activated by mitochondrial Ca2+ (Duchen, 1992; Kann et al., 2003), others dispute this for a variety of reasons, often involving
measurements in different conditions (Shuttleworth et al., 2003; Kasischke et al., 2004; Baeza-Lehnert et al., 2019).

MPC inhibition severely impaired NADH production in the mitochondria, both at baseline (Figure 1 – Supplement 2) and especially during periods of intense neuronal activity. Stimulation of the DGCs revealed a dramatic ~7-fold reduction of the overshoot in the NAD(P)H signal (Figure 1b Right; Figure 1 – Supplement 3).

Importantly, UK5099 provided a tool to abolish the mitochondrial NADH overshoot almost completely without compromising the health of the slices, as revealed by a low basal RCaMP signal and an unaltered Ca²⁺ spike in response to stimulation (Figure 1 – Supplement 2). Using this manipulation, we tested the independence of the cytosolic NADH transient. We co-expressed the genetically encoded biosensors Peredox and RCaMP1h to monitor the cytosolic NADH/NAD⁺ ratio and the Ca²⁺ level, respectively, in individual DGCs. As previously reported (Díaz-García et al., 2017), antidromically stimulating DGCs induced a fast spike in RCaMP1h fluorescence lifetime and a slower increase in Peredox lifetime, consistent with a transient buildup of the NADH/NAD⁺ ratio in the cytosol (NADH_{CYT}) (Figure 1c Left, Figure 1 – Supplement 4). This reflects an increase in glycolytic NADH production in the cytosol (Díaz-García et al., 2017). The cytosolic NADH/NAD⁺ ratio, however, was not immune to MPC inhibition.

After MPC inhibition, there was reduction but not abolition of the NADH_{CYT} transients: baseline Peredox lifetime decreased by 3.4 ± 1.6% (Figure 1 – Supplement 4) and the magnitude of the NADH_{CYT} transients (expressed as the ΔPeredox/ΔRCaMP lifetime ratio) was attenuated by almost 40% (Figure 1c Right), but far less than the ~7-fold reduction in the mitochondrial overshoot transient. Are the reduced transients evidence that cytosolic and mitochondrial NADH responses to stimulation are inextricably connected? Not necessarily: an alternative explanation is that by accumulating pyruvate in the cytosol, MPC inhibition indirectly drives increased NADH re-oxidation via the LDH reaction. Indeed, in the continuous presence of an LDH inhibitor to oppose the pyruvate-driven re-oxidation, further

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2 MPC inhibition by application of UK5099 steadily decreased the baseline NAD(P)H signal by 8.3 ± 2.9% from the initial values after ~35 min (Figure 1b Left, Figure 1 – Supplement 2b), reflecting an overall decrease in the activity of the TCA cycle. Consistent with this, baseline O₂ levels increased by 25 ± 16%, indicating that O₂ consumption at baseline was also diminished in the presence of UK5099 (Figure 1 – Supplement 2c), probably because mitochondrial NADH fuels most of the O₂ consumption by the electron transport chain.
application of UK5099 did not change the magnitude of the NADH<sub>CYT</sub> transient in response to stimulation (Figure 1d, Figure 1 – Supplement 5). This result indicates that the stimulation-induced acceleration of neuronal glycolysis, as detected by NADH production in the cytosol, is not affected by impaired NADH production in the mitochondria. MPC inhibition still abolished the mitochondrial NAD(P)H transients in the presence of the LDH inhibitor, by about 8-fold (similar to the effect without LDH inhibition; Figure 1e, Figure 1 – Supplement 6). Taken together, these results show that NADH transients in the cytosol develop independently from transients in the mitochondria.

**Calcium entry into mitochondria is required for strong activation of TCA metabolism**

The NAD(P)H autofluorescence signals and the NADH<sub>CYT</sub> transients, which report on different biochemical reactions, are both rapidly triggered by neuronal stimulation. Calcium ions, which enter the cytosol via voltage-gated Ca<sup>2+</sup> channels during action potentials, could facilitate the concerted glycolytic and mitochondrial responses. The rise in [Ca<sup>2+</sup>]<sub>CYT</sub> can propagate to the mitochondria, where Ca<sup>2+</sup> transiently dissipates the inner mitochondrial membrane (Duchen, 1992) and activates several dehydrogenases in the TCA cycle (McCormack et al., 1990).

Many studies have explored the effects of Ca<sup>2+</sup> on the different phases of the NAD(P)H response, yielding contradictory results. Experiments using Ca<sup>2+</sup>-free extracellular solutions have been particularly hard to reconcile, with reports showing either a complete elimination of both phases of the NAD(P)H transient (Duchen, 1992) or the preservation of the entire signal, depending on the stimulation paradigm (e.g. with kainate, Shuttleworth et al., 2003). We hypothesized that Ca<sup>2+</sup> can contribute to both the dip and the overshoot of the NAD(P)H signal, although not necessarily to the same extent.

To test this hypothesis, we sought to disrupt the expression of the mitochondrial Ca<sup>2+</sup> uniporter (MCU), an ion channel located at the inner mitochondrial membrane that constitutes the dominant path for mitochondrial Ca<sup>2+</sup> uptake (Kirichok et al., 2004; Baughman et al., 2011; De Stefani et al., 2011). This approach has been proven effective in diminishing Ca<sup>2+</sup> influx into the mitochondria—but not the cytosolic Ca<sup>2+</sup> spike—in axons (Ashrafi et al., 2020) and cardiomyocytes (Kwong et al., 2015).

We optimized the knockdown of MCU (MCU-KD) by expressing Cre recombinase under the Dock10 promoter (Kohara et al., 2014), in DGCs of adult hemizygous Mcu<sup>fl/fl</sup> mice (derived from Mcu<sup>fl/fl</sup> mice;
(Kwong et al., 2015)), which resulted in a strong reduction of the mitochondrial Ca$^{2+}$ transients monitored with a mitochondrially targeted sensor RCaMP1h (mitoRCaMP; Figure 2b, Figure 2 – Supplement 1 and 2).

The smaller mitochondrial Ca$^{2+}$ influx in MCU-KD neurons leads to smaller mitochondrial NADH transients. Indeed, the prominent NAD(P)H overshoots observed in DGCs from control $Mcu^{fl/Δ}$ mice (~2.1—4.8% for 25—200 pulse stimulations) were strongly attenuated in MCU-KD neurons from $Mcu^{fl/Δ}$ Dock10Cre mice (Figure 2a). The NAD(P)H overshoot was almost abolished in the 25 pulse stimulation, and strongly reduced but not quite abolished when stimulating with 200 pulses (Figure 2b), perhaps reflecting Ca$^{2+}$ entry into the mitochondria by different routes. The strong effect of Ca$^{2+}$ deprivation on the NAD(P)H overshoot together with a delayed recovery from the dip (Figure 2 – Supplement 3) are consistent with a major role for [Ca$^{2+}$]$_{MITO}$ in activating dehydrogenases in the TCA cycle to resupply mitochondrial NADH after neuronal stimulation.

On the other hand, the magnitude of the initial NAD(P)H dip remained unaffected in neurons lacking MCU, except for a small ~19% decrease when stimulated with 25 pulses (Figure 2 – Supplement 3). This suggests that Ca$^{2+}$ influx through MCU is only a minor contributor to the dissipation of the mitochondrial membrane potential ($ΔΨ_m$), which promotes the oxidation of NADH by Complex I (seen as the dip in autofluorescence). Consistent with the preservation of the NAD(P)H dip, the magnitude of the transient decrease in the tissue [O$_2$] was only marginally affected by MCU-KD when the slices were stimulated with 25 pulses (Figure 2 – Supplement 3).

The diminished mitochondrial Ca$^{2+}$ transient in the MCU-KD also produced some decrease in the magnitude of NADH$_{CYT}$ transient (Figure 2c, d). This decrease was ameliorated by LDH inhibition (as seen previously for the case of MPC inhibition), but the MCU-KD transient was still only ~57% of the control hemizygote (Figure 2e, Figure 2 – Supplement 5), indicating that NADH re-oxidation by LDH cannot be the sole cause for this difference. A potential explanation for the remaining effect of MCU-KD on the NADH$_{CYT}$ transient could be that reoxidation of cytosolic NADH by the MAS runs faster in MCU-KD neurons during stimulation, because it is not inhibited by the usual increases in [Ca$^{2+}$]$_{MITO}$ (Bak et al., 2012). In any case, our experiments show that despite the strong effect of knocking out $Mcu$ on the overshoot of the mitochondrial NAD(P)H autofluorescence signal, robust NADH increases were
consistently elicited in the cytosol of stimulated neurons, especially when NADH re-oxidation through LDH was prevented.

**Calcium elevation in the cytosol is a major contributor to the NADH\textsubscript{CYT} transients**

The amplitude of the NADH\textsubscript{CYT} responses, reflecting a temporary increase in aerobic glycolysis, correlates very closely with the cytosolic Ca\textsuperscript{2+} transient elicited during stimulation (Díaz-García et al., 2017). However, the mechanism linking these two events is still unknown. To test the role of Ca\textsuperscript{2+} in the cytosolic NADH\textsubscript{CYT} response, we used three orthogonal approaches to diminish the rise in [Ca\textsuperscript{2+}]\textsubscript{CYT} upon stimulation while preserving other ionic fluxes. First, we blocked L-type Ca\textsuperscript{2+} channels with 3 µM isradipine (reviewed in Catterall et al., 2005; Striessnig et al., 2015), which reduced the cytosolic Ca\textsuperscript{2+} transient in response to stimulation by 51 ± 10% (Figure 3 – Supplement 3), and this reduced the NADH\textsubscript{CYT} transient by 36 ± 13% (Figure 3a). We then added 20 µM of the non-selective Ca\textsuperscript{2+} channel inhibitor CdCl\textsubscript{2}, which further reduced the Ca\textsuperscript{2+} transient by 81 ± 7% from the original value while the magnitude of the NADH\textsubscript{CYT} transients dropped 71 ± 11% (Figure 3a).

Second, we applied 100 µM of the cell-permeable Ca\textsuperscript{2+} chelator EGTA-AM. This manipulation preserves the Ca\textsuperscript{2+} influx upon stimulation, but prevents the rise in the intracellular concentration of free Ca\textsuperscript{2+} ions. The effect on the NADH\textsubscript{CYT} transient was similar to the previous results with inhibition of Ca\textsuperscript{2+} influx: after ~1h in EGTA-AM, the stimulus-induced change in the Ca\textsuperscript{2+} signal was reduced by 87 ± 6% and the NADH\textsubscript{CYT} transient was reduced by 73 ± 11% (Figure 3b). 3

Finally, we perfused the slices with a nominally Ca\textsuperscript{2+}-free ACSF, by replacing all extracellular CaCl\textsubscript{2} with a concentration of MgCl\textsubscript{2} chosen to match the charge screening effects on the plasma membrane (Hille et al., 1975), to avoid changes in the effective voltage-dependence of ion channels. Additionally, 1 mM EGTA was included to ensure the chelation of any residual Ca\textsuperscript{2+} in the extracellular space within the slice. As expected, the transient RCaMP signal almost disappeared in the absence of extracellular Ca\textsuperscript{2+} (a 91 ± 5% reduction) and, just as with previous manipulations, the NADH\textsubscript{CYT} dropped by 73 ± 11% from

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3 The reduction in the RCaMP signal corroborated the expected decrease in free [Ca\textsuperscript{2+}], although this experiment required homogenous Ca\textsuperscript{2+} chelation throughout the soma and small processes during stimulation. As Ca\textsuperscript{2+} buffering seemed less effective in the dendrites compared to the somata, the average RCaMP responses in the slice must be kept ≤0.6 ns, otherwise the less attenuated Ca\textsuperscript{2+} spike in the dendrites would trigger some NADH\textsubscript{CYT} response that could be detected in the soma as well. A control experiment was performed since 0.1% DMSO was needed in the solution to reach the working concentration of EGTA-AM (Figure 3 – Supplement 1). This concentration of DMSO did not change the NADH\textsubscript{CYT} transients during the time that our experiments typically lasted.
the initial responses (Figure 3c). A variation of this experiment using a nominal zero Ca\(^{2+}\) solution without EGTA was similarly effective in decreasing the NADH\(_{\text{CYT}}\) transient (Figure 3 – Supplement 2).

Once again we tested the role of NADH re-oxidation via the LDH reaction, since depriving the mitochondria of a Ca\(^{2+}\) transient would diminish their pyruvate consumption. We repeated each of the Ca\(^{2+}\) manipulations in the presence of 2 µM of the LDH inhibitor GSK-2837808; they still decreased the NADH\(_{\text{CYT}}\) transients by more than 50% but not to zero (Figure 3 – Supplement 3).

Overall, these results indicate that Ca\(^{2+}\) elevation resulting from the activation of several types of Ca\(^{2+}\) channels (including the L-type), is a major contributor to the glycolytic response in stimulated neurons but it is not strictly required.

Calcium is known to modulate many signaling pathways that can act as a feedforward mechanism to promote energy production in anticipation of another episode of neuronal activity. Could neuronal stimulation trigger glycolysis via one of these pathways? One ubiquitous signaling pathway in mammalian cells is the Ca\(^{2+}\)/calmodulin axis, which has many downstream targets that promote glucose utilization (Marsin et al., 2000; Marinho-Carvalho et al., 2009; Schmitz et al., 2013; Singh et al., 2004; Xie et al., 2014; Kim et al., 2016). If this is a major signal in triggering the NADH\(_{\text{CYT}}\) transients, we would expect them to decrease by inhibiting the Ca\(^{2+}\)/calmodulin complex with E6-berbamine or calmidazolium. However, these drugs caused the opposite effect, increasing the NADH\(_{\text{CYT}}\) transients relative to the Ca\(^{2+}\) spike (Figure 4 a,b). Both drugs increased the baseline for RCaMP by ~3% but did not hamper the Ca\(^{2+}\) responses to stimulation (Figure 4 – Supplement 1a,b). These results suggest that Ca\(^{2+}\) does not activate the fast glycolytic response via Ca\(^{2+}\)/calmodulin dependent signaling.

We also tested if AMPK, a protein kinase that senses the cellular energy status through AMP levels and is also modulated by Ca\(^{2+}\) (Hawley et al., 2005; Woods et al., 2005; reviewed by Herzig and Shaw, 2018), could be responsible for the enhanced glycolysis upon stimulation. The application of the inhibitor dorsomorphin (also known as Compound C) rapidly increased both RCaMP and Peredox baselines by ~8% and 3%, respectively. Dorsomorphin decreased the Ca\(^{2+}\) transient by 18 ± 10% (Figure 4 – Supplement 1c) but a commensurate decrease in NADH\(_{\text{CYT}}\) transients was also observed, keeping the \(\Delta\text{Peredox}/\Delta\text{RCaMP}\) ratio unaffected (Figure 4c). Furthermore, the NADH\(_{\text{CYT}}\) transients became briefer in the presence of dorsomorphin due to a faster time to peak and recovery to baseline.
Taken together, these results show that AMPK is not necessary to increase neuronal glycolysis upon stimulation, although it can modulate the duration of this metabolic response.

**Energy demand from Na$^+$ or Ca$^{2+}$ pumping triggers aerobic glycolysis**

While the cytosolic NADH/NAD$^+$ ratio is sensitive to Ca$^{2+}$-dependent signaling pathways, inhibition of these pathways did not prevent the NADH$_{CYT}$ transients in response to stimulation. More importantly, Ca$^{2+}$ itself may not be necessary if the fast glycolytic response is simply reactive to energy demand resulting from Ca$^{2+}$ extrusion. Restoring [Ca$^{2+}$]$_{CYT}$ to pre-stimulation levels requires the activity of ion pumps that move Ca$^{2+}$ out of the cytosol at the expense of ATP hydrolysis. The resulting ADP, either by itself or in combination with AMP (which can be produced from ADP via the adenylate kinase reaction) could then trigger neuronal glycolysis. Other ions that enter during stimulation such as Na$^+$ should also contribute to energy demand, though pumping of Na$^+$ ions requires less ATP per ion.

If the main consequence of Ca$^{2+}$ entry during stimulation is to increase energy demand, we should be able to restore the NADH$_{CYT}$ transients by increasing energy demand independent of Ca$^{2+}$. We tested this hypothesis by boosting Na$^+$ influx, which would lead to a greater ATP hydrolysis by the Na$^+$/K$^+$ ATPases.

We started by preventing the cytosolic Ca$^{2+}$ elevation in DGCs with a nominally Ca$^{2+}$-free external solution (supplemented with EGTA), in the continuous presence of the LDH inhibitor GSK-2837808A to maximize the glycolytic NADH$_{CYT}$ transients. Then we applied α-pompidolotoxin, a toxin that slows down voltage-gated Na$^+$ channel inactivation (Konno et al., 1998; Schiavo et al., 2010) and should increase Na$^+$ influx during action potentials, increasing energy demand upon stimulation. Indeed, in the absence of a Ca$^{2+}$ spike, the application of 10 µM α-pompidolotoxin induced a large ~3.8-fold increase in the magnitude of the Peredox transient, recovering ~81% of the initial response in regular ACSF (Figure 5a), although with slightly faster kinetics (Figure 5a, Figure 5 – Supplement 1). This is consistent with the hypothesis of a metabolic response that is reactive to energy demand, and constitutes direct evidence that although Ca$^{2+}$ largely determines the magnitude of the cytosolic transients in physiological conditions, it is not strictly necessary for triggering these responses. This is fundamentally different from the overshoot of NAD(P)H transients, which is not recovered by a similar
manipulation, even if a higher concentration of α-pompilidotoxin was applied (Figure 5 – Supplement 2).

As a corollary of this experiment, the increase in glycolysis should be prevented by diminishing the ADP surge associated with ion pumping. We sought evidence for this by inhibiting the Na⁺/K⁺ pumps with strophanthidin. In virtue of its relatively high octanol/water partition coefficient (Dzimiri et al., 1987), strophanthidin provides extra convenience over other cardiac glycosides in that it blocks the activity of the Na⁺/K⁺ pumps regardless of the subcellular localization of the pumps (Galva et al., 2012). Indeed, co-application of 10 µM strophanthidin reversed the increases in NADH_CYT transients produced by α-pompilidotoxin (3 µM), and in some cases decreased the transients even below pre-α-pompilidotoxin levels (Figure 5c). These results confirmed that ATP hydrolysis by ion pumping is the main factor that triggers neuronal glycolysis in response to stimulation.

DISCUSSION

Mitochondrial Ca²⁺ uptake is required for increasing mitochondrial NADH upon neuronal stimulation

Calcium orchestrates increases in both glycolysis and the TCA cycle in response to acute neuronal stimulation. Changes in these metabolic pathways reflect the compartmentalization of Ca²⁺ dynamics: while Ca²⁺ influx through voltage-gated ion channels promotes glycolysis in the cytosol via the energy associated to the restoration of ionic gradients at the plasma membrane, further Ca²⁺ uptake into the mitochondria is required for increasing NADH production in this organelle.

We studied the role of mitochondrial Ca²⁺ elevations by knocking down the mitochondrial calcium uniporter (Mcu) gene in DGCs, which nearly abolished the Ca²⁺ entry into neuronal mitochondria, as seen also by Ashrafi and colleagues (2020). This diminished mitochondrial Ca²⁺ entry caused a strong attenuation of the reductive phase of the NAD(P)H autofluorescence signal (i.e. the overshoot after initial dip), consistent with a key role for mitochondrial Ca²⁺ in activating several dehydrogenases in the TCA cycle (reviewed by McCormack et al., 1990).

The absence of MCU, however, only marginally decreased the prompt oxidation of mitochondrial NADH upon stimulation (the rapid dip). This suggests that the direct contribution of mitochondrial Ca²⁺ to mitochondrial depolarization and subsequent NADH-fueled proton pumping is negligible compared to that of ADP, which is produced by Na⁺, K⁺ and Ca²⁺ pumping at the plasma membrane during periods
of activity. Our results, however, contrast with Duchen (1992), who completely abolished both phases of the NAD(P)H signal in isolated neurons by blocking the MCU with ruthenium red. This compound interferes with Ca²⁺ pumping and, thus, ADP production (Watson et al., 1971), so it is possible that these effects might have led to the absence of an initial dip in Duchen's study.

Removing Ca²⁺ from the extracellular solution recapitulated the effects seen with Mcu knockdown: the dip of the NAD(P)H signal was only decreased by ~21% while the overshoot was almost abolished, indicating again that Ca²⁺ influx is essential for the excess in NADH production in the mitochondria. Using a similar manipulation, Duchen (1992) abrogated the full signal, while Kann et al. (2003) reported only a partial decrease (~59%) in the overshoot, and Shuttleworth et al. (2003) observed the preservation of the full NAD(P)H transient. It is possible that differences between cultured cells, acute or organotypic brain slices, cell types, or even the stimulation paradigm, may contribute to differences among studies. Apparently, the NAD(P)H overshoot can be Ca²⁺-independent in certain experimental circumstances, possibly produced by rapid pyruvate uptake into the mitochondria upon stimulation, rather than TCA stimulation by Ca²⁺ (Baeza-Lehnert et al., 2019).

**OXPHOS is largely preserved in neurons despite the loss of the NAD(P)H overshoot**

Although the NAD(P)H overshoot has been universally observed in metabolic studies with neuronal stimulation, it is apparently not essential for maintenance of oxidative phosphorylation. We found that oxygen utilization, a sensitive indicator of flux through OXPHOS (Hall et al., 2012; Ivanov et al., 2014), was not altered by MCU knockdown, even though the NAD(P)H overshoot is practically eliminated. This agrees with results on isolated brain and heart mitochondria from MCU-KD animals (Szibor et al., 2020). What accounts for the continued ability of mitochondria to engage in OXPHOS? Of course, an overshoot of NADH is not required to prevent mitochondrial NADH levels from becoming limiting for OXPHOS; all that is required is maintenance of NADH levels. Even without augmented TCA cycle production of NADH, the increased supply of reducing equivalents produced in glycolysis and transferred to mitochondria via the malate-aspartate shuttle (MAS) may be substantial. Both the diminished mitochondrial Ca²⁺ due to knockdown of MCU and the still-increased cytosolic Ca²⁺ will contribute to increased MAS function (Pardo et al., 2006; Bak et al., 2012; Llorente-Folch et al., 2013).

Mitochondrial fuels whose metabolism is less dependent on Ca²⁺ stimulation may also contribute to production of mitochondrial NADH. For instance, we find that in contrast to the NAD(P)H overshoot,
the late reductive phase of increased FADH$_2$ (seen as a prolonged decrease in FAD$^+$ autofluorescence) is preserved after MCU knockdown or when the MPC is blocked (Figure 2 – Supplement 3, and Figure 1 – Supplement 2 and 3, respectively). Pyruvate unavailability may be partially compensated by the use of alternative mitochondrial fuels, such as glutamate and glutamine (Tildon et al., 1985; McKenna et al., 1993; Westergaard et al., 1995; Olstad et al., 2007; Divakaruni et al., 2017). Indeed, these amino acids have been shown to sustain flux through the TCA cycle from $\alpha$-ketoglutarate to oxaloacetate, ultimately resulting in aspartate accumulation as a result of oxaloacetate transamination (Erecińska et al., 1988; Erecińska et al., 1990; Sonnewald and McKenna, 2002; reviewed by McKenna, 2007).

The preservation of the O$_2$ transients in the DGC layer contrasts with the previously reported impairment of ATP production in MCU-deficient axon terminals during sustained activity (Ashrafi et al., 2020). The contributions of MCU and the NAD(P)H overshoot to OXPHOS, as well as to glutathione regeneration, may differ among cellular compartments (i.e. somata, dendrites/spines and axons/synaptic terminals), especially when facing high energy demands.

**Neuronal glycolysis is triggered not by Ca$^{2+}$ signaling but rather by ATP hydrolysis**

In addition to OXPHOS, neurons also respond to stimulation by increasing the rate of glycolysis, which we found to be strongly associated with the rise in [Ca$^{2+}$]$_{CYT}$. We considered a potential role for Ca$^{2+}$ acting as a feedforward signal through protein kinases, which may trigger ATP production in anticipation for future episodes of activity and energy demand, but we tested multiple known Ca$^{2+}$ signaling pathways and were unable to find one that was important for the glycolytic response.

We pharmacologically inhibited the Ca$^{2+}$/CaM signaling pathway, which led to increased [Ca$^{2+}$]$_{CYT}$ at baseline, probably reflecting some inhibition of the “housekeeping” Ca$^{2+}$-ATPase isoform PMCA1 (Brini et al., 2013). However, the NADH/NAD$^+$ ratio remained responsive to rises in the [Ca$^{2+}$]$_{CYT}$ in the presence of either of the two inhibitors tested (E6-berbamine and calmidazolium). In fact, the amplitude of the NADH$_{CYT}$ transient relative to the Ca$^{2+}$ spike was even higher when the Ca$^{2+}$/CaM complex was inhibited, indicating that this signaling pathway is not responsible for the glycolytic response to acute stimulation.

We also explored a connection between Ca$^{2+}$ and AMPK, a protein kinase that promotes glucose utilization (Marsin et al., 2000; Wu et al., 2013) and can be activated by CaMKK (Hawley et al., 2005;
Woods et al., 2005). More importantly, its main activator, AMP, reflects the depletion of the ATP pool (reviewed by Herzig and Shaw, 2018), which can be a consequence of neuronal activity (Gerka et al., 2019). However, even though AMPK seems poised for the integration of two signals derived from acute neuronal stimulation, its inhibition did not prevent the glycolytic NADH<sub>CYT</sub> transients in the soma of DGCs. Overall, AMPK seems irrelevant for neuronal lactate production, as observed in cultured cortical neurons (Muraleedharan et al., 2020), however, it promotes glucose uptake and glycolysis during prolonged periods of activity in synaptic terminals (Ashrafi et al., 2017), suggesting that AMPK signaling may be tailored to cope with local energy demands.

A more universal and ancient mechanism for adjusting energy supply in the face of demand could be the direct activation of glycolysis by consumption of ATP. The buildup of ADP and/or AMP can directly activate phosphofructokinase (PFK) (Passonneau and Lowry, 1962; Erecińska and Silver, 1989) and drive glycolysis. These mechanisms could act in neurons despite their low levels of Pfkb3, the enzyme that produces the potent PFK activator fructose-2,6-bisphosphate (F2,6BP) (Herrero-Mendez et al., 2009). This is not unprecedented: in skeletal muscle fibers, failure to increase F2,6BP after repetitive stimulation can be compensated by AMP, ADP and other allosteric modulators of PFK (Wegener and Krause, 2002).

Our experiments provide evidence that neuronal glycolysis can indeed be driven by ATP hydrolysis rather than by Ca<sup>2+</sup> signaling: we were able to elicit NADH<sub>CYT</sub> transients by increasing Na<sup>+</sup> influx, in the absence of extracellular Ca<sup>2+</sup>. Calcium is not strictly necessary for producing these glycolytic transients; the activity of the ATP-consuming ion pumps is sufficient, specifically the Na<sup>+</sup>,K<sup>+</sup>-ATPase (NKA; sodium pump) in the case of the Na<sup>+</sup>-only transients. Under these conditions the Na<sup>+</sup> pump activity is also necessary, as the Na<sup>+</sup>-driven glycolytic responses were reversed by the sodium pump inhibitor, strophanthidin.

The effectiveness of the low concentration of strophanthidin used here (10 µM) to block the NADH<sub>CYT</sub> transients suggests that the NKA isoform α3 could mediate the coupling between neuronal activity and glycolysis. This isoform is more expressed in neurons than in any other cell type in the brain (Zhang et al., 2014; Zeisel et al., 2015; Hrvatin et al., 2018), and confers the high sensitivity of axonal preparations to cardiotonic steroids (Marks and Seeds, 1978; Sweekner, 1979; Urayama and Sweekner, 1988; Sweekner, 1989). However, given the apparent low affinity of this NKA isoform for Na<sup>+</sup>
(K<sub>0.5</sub>=30—70 mM; Munzer et al., 1994; Crambert et al., 2000; Hamada et al., 2003), it only seems poised to counteract Na<sup>+</sup> accumulation after strong neuronal activity (Munzer et al., 1994; Azarias et al., 2013), or with the α-pompidolotoxin-induced augmentation of Na<sup>+</sup> influx used here.

Calcium is surprisingly important for promoting glycolysis in control ACSF, even though it seems unlikely that Ca<sup>2+</sup> accounts for most of the total ion pumping after neuronal excitation. It is possible that Ca<sup>2+</sup> extrusion is better coupled to glycolysis than Na<sup>+</sup> extrusion (Gover et al., 2007; Ivannikov et al., 2010; Fernández-Moncada and Barros, 2014), perhaps due to interactions between Ca<sup>2+</sup> ATPases (PMCs) and glycolytic enzymes at the plasma membrane (reviewed by Dhar-Chowdhury et al., 2007; Bruce, 2018; James et al., 2020). In addition, this coupling might require a relatively calmodulin-insensitive isoform like PMCA2 (Elwess et al., 1997; reviewed by Brini et al., 2013), which would be consistent with the preservation of the NADH<sub>CYT</sub> transient after the application of calmidazolium, a drug that delays Ca<sup>2+</sup> clearance through PMCs as a result of inhibiting calmodulin (Markram et al., 1995; Scheuss et al., 2006). However, although Ca<sup>2+</sup> ATPases typically present a higher affinity for Ca<sup>2+</sup> when compared to the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, they also exhibit a lower turnover rate, so their contribution to Ca<sup>2+</sup> extrusion would depend on the density and localization of transporters in the membrane (Blaustein and Lederer, 1999; Brini and Carafoli, 2011), and likely on the strength of stimulation as well.

Future work is required to identify the precise routes of Ca<sup>2+</sup> extrusion coupled to neuronal glycolysis and to fully understand the biological significance of this process. Glycolysis may provide a fast and localized ATP supply via the phosphoglycerate kinase and pyruvate kinase reactions near the site of high energy demand, as well as additional ATP production from the oxidation of reducing equivalents shuttled into the mitochondria. In addition, the GAPDH-derived NADH<sub>CYT</sub> transients may also reflect some contribution from the pentose phosphate pathway since some intermediates can be re-introduced into the glycolytic pathway, thus providing the much needed antioxidant capacity for neurons (Herrero-Mendez et al., 2009).

In summary, our work provides novel evidence on how neurons cope with moment to moment energy demands during bouts of action potentials, highlighting the different roles of Ca<sup>2+</sup> in coordinating increases in the TCA cycle and glycolysis. Considering the differences in morphology, Ca<sup>2+</sup> buffering capacity, ion channels, and other cellular components, we anticipate variations in the metabolic
responses—and their regulation—in small compartments (e.g. dendrites/spines and axons/synaptic terminals), as well as in different cell-types in the brain (e.g. interneurons and astrocytes).
# MATERIALS AND METHODS

## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and Virus Strains** | | |
| AAV9.Syn.RCaMP1h.WPRE.SV40 | Penn Vector Core | Discontinued |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| NBQX (6-Nitro-7-sulfamoylbenzo[f]quinoxaline-2,3-dione, Disodium Salt) | Toronto Research Chemicals | Cat#N550005; CAS:479347-86-9 |
| D-AP5 (D-(-)-2-Amino-5-phosphonopentanoic acid) | Abcam | Cat#ab120003; CAS:79055-68-8 |
| Isradipine | Abcam | Cat#ab120142; CAS:75695-93-1 |
| α-pompidilidotoxin | Alomone Labs | Cat#P-170 |
| EGTA-AM | Anaspec Inc | Cat#AS-84100; CAS:99590-86-0 |
| Calmidazolium | Cayman Chemical | Cat#14442; CAS:57265-65-3 |
| Dorosomorphin dihydrochloride | Tocris Bioscience | Cat#3093; CAS:1219168-18-9 |
| E6-berbamine | Santa Cruz | Cat#sc-221573; CAS:73885-53-7 |
| UK5099 | Santa Cruz | Cat#sc-361394; CAS:56396-35-1 |
| | Tocris Bioscience | Cat#5185 |
| GSK-2837808A | Tocris Bioscience | Cat#5189; CAS:1445879-21-9 |
| MgCl₂ (1M solution) | Teknova | Cat#M0304 |
| Picrotoxin | Sigma-Aldrich | Cat#P1675; CAS:124-87-8 |
| Sodium pyruvate | Sigma-Aldrich | Cat#P8574; CAS:113-24-6 |
| CdCl₂ | Sigma-Aldrich | Cat#202908; CAS:10108-64-2 |
| Strophanthidin | Sigma-Aldrich | Cat#56626; CAS:66-28-4 |
| **Experimental Models: Organisms/Strains** | | |
| C57BL/6NCrl mice | Charles River | RRID:IMSR_CRL:27 |
| Edil3³(fSox2-cre)¹Amc/J mice (Sox2-Cre) | The Jackson Laboratory | Stock No: 004783 |
| Mcu⁻/⁻ mice | Kwong et al. (2015) | |
| Dock10Cre mice | Kohara et al. (2014) | |
| Mcu⁻/⁺ mice | This paper | |
| Mcu⁻/⁺ Dock10Cre mice | This paper | |
| **Recombinant DNA** | | |
| AAV.CAG.Peredox.WPRE.SV40 | Mongeon et al. (2016) | Addgene #73807 |
| AAV.Syn.RCaMP1h.WPRE.SV40 | Akerboom et al. (2013) | |
| pZac2.1-CaMKII-mito-GCaMP6s | Li et al. (2014) | |
Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. The synaptic blocker NBQX was obtained from Toronto Research Chemicals (Toronto, ON). The mitochondrial pyruvate carrier blocker UK5099 was purchased either from Tocris Bioscience (Bristol, UK) or Santa Cruz (Dallas TX). Another drug from Santa Cruz was E6-berbamine, an inhibitor of the Ca\(^{2+}\)/calmodulin signaling pathway. Calmidazolium, a drug with similar effects on the Ca\(^{2+}\)/calmodulin complex, was obtained from Cayman Chemical (Ann Arbor, MI). The L-type calcium channel blocker isradipine and the NMDA-glutamate receptor inhibitor D-AP5 were obtained from Abcam (Cambridge, MA). Stock solutions of MgCl\(_2\) (1M) were purchased from Teknova (Hollister, CA). dorsomorphin dihydrochloride (Compound C) and GSK-2837808A were obtained from Tocris (Bristol, UK), EGTA-AM from Anaspec Inc (Fremont, CA) and α-pompidolotoxin from Alomone Labs (Jerusalem, Israel).

We prepared stock solutions of calmidazolium (100 mM), E6-berbamine (33 mM), EGTA-AM (100 mM), GSK-2837808A (10 mM), isradipine (50 mM), strophanthidin (500 mM) and UK5099 (20 mM) in DMSO. The final concentration of DMSO in the experiments was kept ≤0.04%, except for EGTA-AM (0.1%), for which control experiments with 0.1% DMSO-only solution were performed to rule out interferences from the organic solvent in the recordings.

We used Cd\(^{2+}\) as a non-selective blocker of high voltage activated Ca\(^{2+}\) channels (Swandulla and Armstrong, 1989; Catterall et al., 2005), at a concentration that avoid significant off-target effects on Na\(^+\) channels (DiFrancesco et al., 1985) or the Na\(^+\)/Ca\(^{2+}\) exchanger (Hobai et al., 1997).

Experiments with sequential application of α-pompidolotoxin and strophanthidin were challenging because the prolonged exposure to a nominal Ca\(^{2+}\)-free solution, plus LDH inhibition, caused
spontaneous elevations of the Peredox lifetime in some cells (Figure 5 – Supplement 3). Furthermore, in slices treated with α-pompidolidotoxin, extensive inhibition of the Na⁺ pumps caused cell swelling and death upon stimulation. It was necessary to lower the concentrations of both α-pompidolidotoxin and strophanthidin to ensure the preservation of neuronal viability.

**Animals**

Brain slice experiments were performed using brains of male and female wild-type mice (C57BL/6NCrl; Charles River Laboratories), *Mcu<sup>fl/fl</sup>*, *Mcu<sup>fl/Δ</sup>* and *Mcu<sup>fl/Δ</sup>* Dock10Cre (see below). Animals were housed in a barrier facility in individually ventilated cages with *ad libitum* access to standard chow diet (PicoLab 5053). All experiments were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. The Harvard Medical Area Standing Committee on Animals approved all procedures involving animals.

**Generation of *Mcu<sup>fl/Δ</sup>* and *Mcu<sup>fl/Δ</sup>* Dock10Cre mice**

We selectively inactivated the *Mcu* gene in DGCs by crossing conditional knockout *Mcu<sup>fl/fl</sup>* mice (Kwong et al., 2015) with a Cre-driver line specific to DGCs (Kohara et al., 2014). Male *Mcu<sup>fl/fl</sup>* mice (Kwong et al., 2015) were crossed with female Dock10Cre mice (Kohara et al., 2014) to generate *Mcu<sup>fl/+</sup>* Dock10Cre mice, which were backcrossed with *Mcu<sup>fl/fl</sup>* mice to obtain *Mcu<sup>fl/fl</sup>* Dock10Cre mice. Additional adjustments were necessary to maximize the consistency of the MCU-KD phenotype. First, we produced hemizygous *MCU<sup>fl/Δ</sup>* mice so that strong knockdown of *Mcu* would require deletion of only a single copy of the gene to compensate for the limited efficiency of Cre-dependent recombination (Bao et al., 2013). Male *Mcu<sup>fl/fl</sup>* mice were mated with female Sox2-Cre<sup>+/−</sup> mice (Hayashi et al., 2002; obtained from The Jackson Laboratory) to generate an offspring with 50% of *Mcu<sup>wt/Δ</sup>* mice (without the Sox2-Cre transgene). Finally, the crossing of male *Mcu<sup>wt/Δ</sup>* mice with female *Mcu<sup>fl/fl</sup>* Dock10Cre mice produced a 25% offspring of each experimental genotype: *Mcu<sup>fl/Δ</sup>* and *Mcu<sup>fl/Δ</sup>* Dock10Cre (with *Mcu* selectively deleted in DGCs). Other combinations from the above-mentioned genotypes were able to produce experimental mice (although with different proportions). In all crossings, the Dock10Cre transgene was present in the female parent.

We also worked with adult mice (82 ± 12 days-old, N= 69) instead of juveniles to permit more complete knockdown in the face of the late, postnatal expression of the Dock-10 promoter (Jaudon et al., 2015)
and the slow turnover of mitochondrial proteins (with a half-time of ~25 days; Beattie et al., 1967; Menzies and Gold, 1971).

**DNA Plasmids and Viral vectors**

*Construction of AAV.Syn.mito-RCaMP1h.WPRE.SV40*

We created an AAV plasmid that targets the sensor RCaMP1h into the mitochondrial matrix by inserting a portion of the precursor of the mitochondrial protein COX8 in the N-terminus of the fluorescent protein, as previously reported for GFP (Rizzuto et al., 1995) and GCaMP6s (Li et al., 2014).

A 101 bp EcoRI-BamHI DNA fragment from pZac2.1-CaMKII-mito-GCaMP6s (obtained from Dr. Shinghua Ding), containing the mitochondrial leader sequence, was subcloned into the backbone of EcoRI-BamHI DNA fragment (5569 bp) of AAV.Syn.RCaMP1h.WPRE.SV40 (obtained from Dr. Loren Looger), using T4 DNA ligase.

*Construction of AAV.CAG.Pyronic.WPRE.SV40*

We cloned the pyruvate sensor Pyronic into an AAV plasmid with the CAG promoter. The Pyronic insert, 2272 bp BamHI-HindIII DNA fragment, from Pyronic/pC3DNA3.1(-) (Addgene# 51308, deposited by San Martín et al., 2014) was subcloned into the backbone of BamHI-HindIII DNA fragment (4696 bp) of AAV.CAG.Laconic.WPRE.SV40 (AAV construct from Díaz-García et al. (2017), derived from San Martín et al. (2013) - Addgene# 44238), using T4 DNA ligase. The resultant plasmid, AAV.CAG.Pyronic.WPRE.SV40, was used to clone in an optimal Kozak sequence (CCACC). A 771 bp amplicon containing the optimized Kozak sequence was generated by using PCR and was amplified from pC3DNA3.1-Pyronic (Forward Primer: 5’ GAATTGGATCCATCATGGTGAGCAAGGGCGAGGAGAC 3’ and Reverse Primer: 5’ GGGCGAATTCCAGGAGCGGAGGAGAC 3’). The amplicon was double digested with BamHI-EcoRI and was subcloned, using T4 DNA ligase, into the backbone of BamHI-EcoRI DNA fragment (6211 bp) of AAV.CAG.Pyronic.WPRE.SV40.

*Production of AAV particles*

Custom-made adeno-associated vectors (AAV) were used for biosensor expression in brain tissue. For the expression of Peroxid in the hippocampus, we used the AAV8 serotype (obtained from the Penn Vector Core, University of Pennsylvania, PA) and the universal promoter CAG (Mongeon et al., 2016).
For expression of the pyruvate sensor Pyronic, we used the AAV9 serotype (obtained from the Viral Core Facility from Children Hospital in Boston, MA) and the universal promoter CAG.

For expression of the Ca\(^{2+}\) sensor RCaMP1h, we used the AAV9 serotype and the neuron-specific promoter synapsin. For 2p-FLIM experiments, we used viral batches from three different suppliers: the Penn Vector Core, University of Pennsylvania, PA, the Viral Core Facility from Children Hospital in Boston, MA, and the Center for Genomics and System Biology, New York University, Abu Dhabi, UAE (kindly provided by Dr. G. Fishell and Dr. J Dimidschstein). For Ca\(^{2+}\) imaging in autofluorescence experiments, AAV9.Syn.RCaMP1h.WPRE.SV40 was produced in our laboratory using a protocol reported elsewhere (Kimura et al., 2019).

**Biosensor expression in hippocampus**

For sensor expression in the hippocampus, mice at postnatal day 1 or 2 were anesthetized using cryoanesthesia. Following confirmation of anesthesia, the viral mix was loaded onto a pulled glass capillary pipette (Wiretrol II, Drummond Scientific Company, Broomall, PA) and the pups were intracranially injected with 150 nl of the AAV mix, twice per hemisphere, at the following coordinates with respect to lambda: (i) 0 mm in the anterior-posterior direction, ±1.9 mm in the medial-lateral axis, and ±2.0 mm in the dorsal-ventral direction (ii) 0 mm in the anterior-posterior direction, ±2.0 mm in the medial-lateral axis and ±2.3 mm in the dorsal-ventral direction. Viral injections were delivered at a rate of 50 nl/min using an UltraMicroPump III (WPI, Sarasota, FL) microinjector. After injections, we waited ~2 min before gently pulling out the pipette, as a precaution to avoid spilling virus outside the target area. The pups recovered on a heat pad (covered by a paper towel) and/or under a heat lamp, before returning them to their cages. As a post-surgery care, one subcutaneous injection of ketoprofen (10mg/kg) was delivered for up to 3 days. Acute brain slices were suitable for imaging from 2 weeks to 4 months post-injection.

Alternatively, some intracranial injections were performed in adult mice (after postnatal day 45). Mice were administered dexamethasone sodium phosphate (8 mg/kg) by an intramuscular injection to the hind leg, 1-2 hours before the surgery. Animals were anesthetized with isoflurane (induction: 4-5%, maintenance: 1-3%). Following confirmation of anesthesia, the mice were placed on a heated pad to maintain the body temperature at 37\(^{\circ}\)C. Local anesthetics (10 mg/kg lidocaine and 2.5 mg/kg bupivacaine) were injected subcutaneously at the incision site, and the analgesic ketoprofen (10
mg/kg) and 0.5 ml of sterile 0.9% NaCl solution were also injected subcutaneously prior to the surgery. A small incision was performed in the skin to expose the skull, and a small hole was drilled over the desired area on the right hemisphere. The stereotactic coordinates with respect to lambda, for a single 2 µl viral injection, were the following: 3.39 mm in the anterior-posterior direction, -2.2 mm in the medial-lateral axis, and -2.4 mm in the dorsal-ventral direction. At the end of the surgery, a subcutaneous injection of buprenorphine SR (0.75 mg/kg) was delivered. We waited at least 2 weeks post-injection for experiments in acute hippocampal slices.

For 2p-FLIM experiments, the viral titer (genome copies/ml) used for injecting AAV8.CAG.Pyronic.WPRE.SV40 was $1.64 \times 10^{14}$ gc/ml. The viral titers in the Peredox:RCaMP1h mix varied depending on the AAV providers (in parenthesis): $2.05 \times 10^{12}$ gc/ml of AAV8.CAG.Peredox.WPRE.SV40 (UPenn) and $1.87 \times 10^{10}$ gc/ml of AAV9.Syn.RCaMP1h.WPRE.SV40 (Abu Dhabi), $4.1 \times 10^{12}$ gc/ml of AAV8.CAG.Peredox.WPRE.SV40 (UPenn) and $2.55 \times 10^{14}$ gc/ml of AAV9.Syn.RCaMP1h.WPRE.SV40 (BCH), or $3.08 \times 10^{12}$ gc/ml of AAV8.CAG.Peredox.WPRE.SV40 (UPenn) and $2.01 \times 10^{13}$ gc/ml of AAV9.Syn.RCaMP1h.WPRE.SV40 (UPenn).

For autofluorescence experiments, the viral stocks were diluted in sterile 0.9% NaCl solution prior to the injections, to achieve the following titers: $1.45 \times 10^{13}$ gc/ml for AAV9.Syn.RCaMP1h.WPRE.SV40 (laboratory-made), and $4.83 \times 10^{12}$ gc/ml for AAV9.Syn.mito-RCaMP1h.WPRE.SV40 (BCH). Several dilutions were tested until the bleedthrough of RCaMP fluorescence into the green channel was negligible.

**Mouse hippocampal slice preparation**

Mice between 14 and 24 days-old were anesthetized with isoflurane, decapitated, and the brain was placed in ice-cold slicing solution containing (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH2PO4, 25 NaHCO3, 0.5 CaCl2, 7 MgCl2, 75 sucrose and 25 D-glucose (335-340 mOsm/kg). A different slicing solution was used for 2—4 months-old adult mice (Ting et al., 2014), consisting of (mM): 93 N-Methyl-D-Glucamine, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 10 MgSO4, 0.5 CaCl2, 25 D-glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate (~310 mOsm/kg; pH 7.4 adjusted with HCl).

Brains were glued by the dorsal side on a specimen tube holder and embedded in warm PBS with 2% low-melting agarose. The agarose was quickly congealed using a chilling block. The specimen tube was
inserted in a chamber containing the same slicing solution (previously oxygenated) and horizontal slices were cut at a thickness of 275 µm using a Compressome slicer (VF-300-Z, Precisionary, Natick, MA). Alternatively, brains were glued by the dorsal side in a chamber containing the same slicing solution and horizontal slices were cut at a thickness of 275 µm using a vibrating slicer (7000smz-2, Campden Instruments, Loughborough, England).

Slices were immediately transferred to a chamber filled with artificial cerebrospinal fluid (ACSF) at 37ºC, containing (in mM): 120 NaCl, 2.5 KCl, 1 NaH₂PO₄, 26 NaHCO₃, 2 CaCl₂, 1 MgCl₂ and 10 D-glucose (~290 mOsm/kg). All solutions were continuously bubbled with a mix of 95% O₂ and 5% CO₂, for adequate oxygenation and pH buffering around 7.4. Slices were incubated at 37ºC for 35 min and then at room temperature for at least 30 min before the experiments, which were executed in the next 5 hours after slicing.

The Ca²⁺-free ACSF contained (in mM): 120 NaCl, 2.5 KCl, 1 NaH₂PO₄, 26 NaHCO₃, 1 EGTA, 4.38 MgCl₂ (~4.1 free) and 10 D-glucose. In these experiments, the control solution was modified to include EGTA, as follows (in mM): 120 NaCl, 2.5 KCl, 1 NaH₂PO₄, 26 NaHCO₃, 1 EGTA, 3 CaCl₂ (~2 free as in regular ACSF), 1 MgCl₂ and 10 D-glucose. The concentrations for divalent ions in the presence of EGTA were estimated using Chelator (Schoenmakers et al., 1992), through the Maxchelator website (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/CaMgATPEGTA-TS.htm). An alternative, simpler, nominal zero Ca²⁺ ACSF was also tested (in mM): 120 NaCl, 2.5 KCl, 1 NaH₂PO₄, 26 NaHCO₃, 4.1 MgCl₂ and 10 D-glucose. The regular 2 mM CaCl₂ was substituted by 3.1 free MgCl₂ to preserve the charge screening effect of divalent ions on the membrane surface (Hille et al., 1975).

For experiments, a brain slice was attached to a poly-lysine coated coverslip and the recordings were performed in a chamber with a continuous supply of oxygenated ACSF at a flow rate of 5 ml/min. The solution was maintained at 33—34ºC using inline heaters (Warner Instruments, Hamden, CT) or custom-made heaters. To prevent degassing in perfusion line, solutions were preheated at 38ºC in a waterless bead bath (Cole-Parmer, Vernon Hills, IL) for 2p-FLIM experiments, or in water bath (VWR) for autofluorescence experiments.

Dentate granule cells were stimulated with a concentric bipolar electrode CBBEC75 (FHC, Bowdoin, ME) placed in the hilus. During the experiments, the ACSF contained NBQX (5 µM), D-AP5 (25 µM) and
picrotoxin (100 µM) to block synaptic activity. Stimulation was delivered in trains of 25 to 200 brief (0.1 ms) pulses at a frequency of 50 Hz, using an A360 stimulus isolation unit (WPI, Sarasota, FL). The stimulation intensity was adjusted to reliably evoke spike activity, which typically ranged from 750—1500 µA for antidromic stimulation (in autofluorescence experiments, the stimulation intensity was always set at 1000 µA).

**Two-photon fluorescence lifetime imaging microscopy**

Lifetime imaging data were acquired with a modified Thorlabs Bergamo II microscope (Thorlabs Imaging Systems, Sterling, VA), with hybrid photodetectors R11322U-40 (Hamamatsu Photonics, Shizuoka, Japan); the light source was a Chameleon Vision-S tunable Ti-Sapphire mode-locked laser (80 MHz, ~75 fs; Coherent, Santa Clara, CA). The objective lens used for brain slice imaging was an Olympus LUMPLFLN 60x/W (NA 1.0). An excitation wavelength of 790 nm was used for the Peredox and RCaMP sensors. Fluorescence emission light was split with an FF562-Di03 dichroic mirror and bandpass filtered for green (FF01-525/50) and red (FF01-641/75) channels (all filter optics from Semrock, Rochester, NY). For the Pyronic sensor, excitation was at 850 nm, and emission light was split with an FF506-Di03 dichroic mirror and bandpass filtered for CFP (FF01-475/35) and YFP (FF01-542/27) channels. The photodetector signals and laser sync signals were preamplified and then digitized at 1.25 gigasamples per second using a field programmable gate array board (PC720 with FMC125 and FMC122 modules, 4DSP, Austin, TX).

Laboratory-built firmware and software performed time-correlated single photon counting to determine the arrival time of each photon relative to the laser pulse; the distribution of these arrival times indicates the fluorescence lifetime (Yellen and Mongeon, 2015; Mongeon et al., 2016). Lifetime histograms were fitted using nonlinear least-squares fitting in MATLAB (Mathworks, Natick, MA), with a two-exponential decay convolved with a Gaussian for the impulse response function (Yasuda et al., 2006). Microscope control and image acquisition were performed by a modified version of the ScanImage software written in MATLAB (Pologruto et al., 2003) (provided by B. Sabatini and modified by G.Y.).
**Lifetime imaging quantification**

Image analysis was performed using MATLAB software developed in our laboratory. Regions of interest (ROIs) were defined around individual cells, and photon statistics were calculated for all pixels within the ROI. Typical ROIs encompassed 100-900 pixels, in images of 128x128 pixels acquired at a scanning rate of 2 ms per line. Lifetime values were calculated as a standardized “tau8” value, which minimizes the variability of the fits by restricting the averaging to the approximate time window of the actual data (Díaz-García et al., 2019). Most data points in the time series plots of lifetimes are for the mean value of 20 sequentially acquired frames, except that for RCaMP1h (Calcium) data in the 10 seconds after stimulation, the data points represent individual frames.

Bleedthrough of green Peredox fluorescence into the red RCaMP optical channel was corrected using the ratio of measured red to green fluorescence observed when only Peredox was expressed, approximately 6.0% (Díaz-García et al., 2017). Similarly, RCaMP1h expression alone led to some signal in the green optical channel, probably due to an immature fluorophore, in direct experiments approximately 4.4% of the red fluorescence intensity, and having a tau8 of ~0.675 ns; these values were used to correct the baseline in dual expression experiments.

Only neurons with a stable baseline for RCaMP and Peredox lifetimes were considered for analysis.

**Wide-field fluorescence microscopy and electrical stimulation**

Autofluorescence signals in brain slices (also expressing a red Ca^{2+} biosensor) were visualized with an Olympus BX51WI upright microscope using an LUMPPlanFl/IR 60x/0.90W objective. The excitation light was delivered by an AURA light engine (Lumencor, Beaverton, OR) at 365, 480 and 575nm to excite NAD(P)H, FAD^+ and RCaMP1h (or mito-RCaMP1h), respectively. The times of exposure were 100 ms (excitation at 365 nm), 50—85 ms (excitation at 480 nm), and 5—85 ms (excitation at 575 ms). The fluorescence emission light was split with an FF395/495/610-Di01 dichroic mirror and bandpass filtered with an FF01-425/527/685 filter (all filter optics from Semrock, Rochester, NY). Images were collected with a CCD camera (IMAGO-QE, Thermo Fisher Scientific), at a rate of 1 frame every two seconds in non-stimulated conditions, alternating the excitation wavelengths in each frame. During the fast response to stimulation, the acquisition rate was temporarily increased to 6 frames per second, for a total duration of 10 s (starting 2 s prior to each stimulation), and then returned to the initial lower-
frequency acquisition rate. Image acquisition and analysis was performed using laboratory-built software written in MATLAB (Mathworks, Natick, MA). This software communicated with an electrophysiology setup by sending an external trigger to a protocol written in pClamp 10 (Molecular Devices, San Jose, CA), which in turn controlled a A360 stimulus isolation unit (WPI, Sarasota, FL) via a Digidata 1321A digitizer (Molecular Devices, San Jose, CA). In pilot experiments, some recordings of local field potentials in the DGC layer were collected and amplified via a Multiclamp 700B (Molecular Devices, San Jose, CA) to confirm the stimulation, but they were not performed routinely in subsequent experiments.

For analysis, the DGC layer was identified in an image obtained with transmitted light, and an ROI was drawn around the somata. Typical ROIs encompassed 4000—4500 pixels, in images of 172×130 pixels acquired at 16 bits per pixel and a binning of 8. The average fluorescence (as Arbitrary Units; A.U.) per pixel was calculated in each ROI (A.U./px), as well as the relative fluorescence intensity changes (ΔF/F).

Unlike many stimulation paradigms used for recording autofluorescence signals in brain slices (Shuttleworth et al., 2003; Brennan et al., 2006; Brennan et al., 2007; Ivanov et al., 2014), ours prevented the signaling through ionotrophic receptors by direct electrical stimulation of the axons and the inclusion of synaptic blockers in the ACSF. Therefore, we measured signals in response to backpropagating action potentials in the somata. By limiting our analysis to the DGC layer, we minimized the contribution of astrocytes to these signals: this region in the dentate gyrus is densely packed with neurons, and its neuron-to-astrocytes ratio is higher than the average value for the whole hippocampus (Lana et al., 2017; Keller et al., 2018).

**Extracellular O₂ recordings**

These measurements were executed simultaneously with the imaging of the autofluorescence signals. The Clark type oxygen glass microsensors, with tip diameters of 10 µm or 25 µm (Unisense, Aarhus, Denmark), were pre-polarized overnight for the initial calibration, which was performed in ACSF saturated with 20% or 95% O₂, and an anoxic solution of 0.1 M sodium ascorbate and 0.1M NaOH (following the manufacturer instructions). Subsequent calibrations were performed at least once a week using two points: zero (anoxic solution) and 20% O₂ in ACSF, both at 33—34⁰C. For routine calibrations the pre-polarization times ranged between 1—2 h, or until the signal was stable for 10 min. The microelectrode was connected to an OXY-Meter amplifier (Unisense, Aarhus, Denmark), and
the signal acquired with the Sensor Trace software (Unisense, Aarhus, Denmark) at a rate of 1Hz. The microsensor tip was inserted ~140 µm into the slice, in the DGC layer, and the signal was calibrated and expressed as [O$_2$] in µM. The data was exported to an Excel file, and then analyzed offline using laboratory-built software written in MATLAB (Mathworks, Natick, MA). Interpretation of the O$_2$ changes in terms of ATP synthesis are made with the assumption that the proton leak across the mitochondrial membrane (independent of ATP synthesis and Ca$^{2+}$ pumping) is unchanged.

**STATISTICAL ANALYSIS**

Statistical analyses were performed using GraphPad InStat v3.06 (GraphPad Software, San Diego, CA). Data were tested for normality with a Kolmogorov-Smirnov test. If the data did not fulfill all the assumptions for parametric tests (paired or unpaired Student’s t-test, one-way or repeated measures ANOVA with a Student-Newman-Keuls post-test), an equivalent non-parametric test was used. For comparisons of two populations with normal distributions but different SDs, a Welch’s t-test was used. The alternatives used in this study were: the Mann-Whitney test (for unpaired comparisons between two groups), the Wilcoxon matched pairs test (similar but for paired comparisons), the Kruskal-Wallis test (for multiple comparisons) and the Friedman test for repeated measures, with a Dunn’s post test. The selected tests and post-tests, as well as the descriptive statistics, are indicated in the figure legends and throughout the manuscript. Values are expressed as mean ± SD for individual neurons, except for autofluorescence experiments and O$_2$ recordings, where the standard deviation is calculated using the number of slices.

Graphics were constructed using Origin 9.1 (OriginLab, Northampton, MA) and Microsoft Excel (Microsoft, Redmond, WA). Datasets were represented as box plots comprising the 25—75% (Q2—Q3) interquartile range, with whiskers expanding to the lower (Q1) and upper (Q4) quartiles of the distribution (5—95%). The median of the distribution is represented by a horizontal line inside the box, and the mean is represented by a cross symbol (×).

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Figure 1. The inhibition of the mitochondrial pyruvate carrier reveals the independence of NADH transients in the cytosol and mitochondria.
a. Left: Representative traces of two different readouts of the Pyronic sensor, expressed in the cytosol of a dentate granule cell (DGC). The lifetime of the donor species mTFP (black line, left Y-axis) is overlapped to the change in the ratio of intensities between the donor and the acceptor species (ΔmTFP/Venus), expressed as the percent change over the initial mTFP/Venus value (gray line, right Y-axis). Both readouts transiently increase in response to a brief exposure to 10 mM pyruvate for 2 min in the bath solution. The application of 2 µM UK5099, an inhibitor of the mitochondrial pyruvate carrier, steadily increased the Pyronic signal to a plateau after 30—35 min. The bars indicate the times of application of both pyruvate and UK5099. Right: Quantification of Pyronic mTFP lifetimes before and after the treatment with UK5099. Data points obtained before and after the application of UK5099 are connected by lines. Box plots represent the 25—75% (Q2—Q3) interquartile range, and the whiskers expand to the lower (Q1) and upper (Q4) quartiles of the distribution (5—95%). The median of the distribution is represented by a horizontal line inside the box, and the mean is represented by a cross symbol (×). The data were compared using a paired Student’s t’s test (Nneurons= 82, Nslices= 7 and Nmice= 4).

b. Left: Representative trace of the NAD(P)H autofluorescence signal and RCaMP1h fluorescence, simultaneously recorded from a population of DGCs in an acute hippocampal slice. A stimulating electrode was placed in the hilus of the dentate gyrus of the hippocampus, and a train of depolarizing pulses was delivered to the DGC axons (antidromic stimulation) before and after the treatment. Treating with UK5099 reduces the baseline and the responses induced by stimulation. Right: Quantification of the normalized amplitudes of the NAD(P)H signal overshoot, with or without UK5099. The baseline before each stimulation (Fbaseline-) was subtracted from the putative trace, and the difference between the baseline and the peak (ΔF=Fpeak-Fbaseline-) is presented as a percentage change over the baseline (ΔF/Fbaseline-). The data were compared using a paired Student’s t’s test (Nslices= 10 and Nmice= 6).

c. Left: Representative trace of Peredox and RCaMP1h lifetimes simultaneously recorded in a DGC. The Peredox lifetime at baseline, and the metabolic transients in response to neuronal stimulation, were recorded before and after the application of UK5099. Right: The NADH_CYT transient was decreased in the presence of UK5099. The Peredox lifetime change from the baseline to the peak of the transient was divided by the magnitude of the RCaMP1h transient (ΔPeredox/ΔRCaMP), as the metabolic responses are correlated with the Ca²⁺ spikes (Díaz-García et al., 2017). The data were compared using a Wilcoxon matched pairs test (Nneurons= 48, Nslices= 7 and Nmice= 6).

d. Representative trace of Peredox and RCaMP1h lifetimes simultaneously recorded in a DGC, with sequential application of the LDH inhibitor GSK-2837808A (LDHi) and UK5099. The data were compared using a Wilcoxon matched pairs test (Nneurons= 52, Nslices= 6 and Nmice= 5).

e. Representative trace of the NAD(P)H autofluorescence signal and RCaMP1h fluorescence, simultaneously recorded from a population of DGCs in an acute hippocampal slice. Treating with 2 µM GSK-2837808A (LDH inhibitor) increases the baseline while preserving the waveform of the NAD(P)H responses to stimulation. The subsequent application of 2 µM UK5099, as in panel (d), diminished both phases of the NAD(P)H responses, especially the overshoot. The data were compared using a paired t-test (Nslices= 4 and Nmice= 3).
Figure 1 – Supplement 1. The rise in [pyruvate]_{CYT} due to MPC inhibition almost saturates the Pyronic sensor.

a. Representative images of DGCs expressing the Pyronic sensor, pseudocolored according to the lifetime of the donor species (mTFP). Cells perfused in ACSF only (Control), or in the presence of the MPC inhibitor UK5099, are shown before and after the application of 10 mM pyruvate as in Figure 1a. Images in the bottom were taken at the peak value of the response to pyruvate. The color scale bar to the right shows the range of lifetime for Pyronic mTFP.

b. The change in the Pyronic mTFP lifetime (LT) in response to external pyruvate application was diminished in DGCs after incubating with UK5099. Data points obtained before and after the application of UK5099 are connected by lines. The data were compared using a paired Student’s t’s test ($N_{neurons}$ = 82, $N_{slices}$ = 7 and $N_{mice}$ = 4).
Figure 1 – Supplement 2. The inhibition of the mitochondrial pyruvate carrier slows down the TCA cycle and decreases O₂ consumption at baseline.

a. **Top:** Low magnification image of an acute brain slice of the hippocampus, showing the placement of the stimulating electrode in the hilus (H) and the O₂ electrode in the layer of dentate granule cells (DGCs). The red square represents the area used for imaging the NAD(P)H autofluorescence signals. **Bottom:** The identification of the area of interest (dashed-line polygon drawn in the DGC layer), was performed in infrared-illuminated/differential interference contrast images, obtained at high magnification (60X objective) and a pixel binning of 8.

b. **Left:** Representative traces of NAD(P)H and FAD⁺ autofluorescence signals, extracellular oxygen concentration and RCaMP fluorescence, simultaneously recorded from a population of DGCs in a hippocampal slice. The shaded area corresponds to the application of 2 µM of the MPC inhibitor UK5099. **Right:** Effect of UK5099 treatment on the baseline level of each signal. Data points obtained before and after the application of UK5099 are connected by lines (N_{slices}=10 and N_{mice}=6 for NAD(P)H and FAD⁺ autofluorescence signals, along with RCaMP fluorescence). As shown in the representative trace, there were some experiments with oxygen and autofluorescence signals recorded simultaneously, but the full dataset for O₂ measurements contained stand-alone O₂ recordings as well (N_{slices}=10 and N_{mice}=5).
Figure 1 – Supplement 3. After MPC inhibition, O₂ consumption and FADH₂ production are less affected than mitochondrial NADH production in acutely stimulated DGCs

a. The traces represent the average normalized NAD(P)H signals (mean ± SD) before and after 2 μM UK5099 (as in Figure 1b), elicited by antidromic stimulation. The baseline before each stimulation (F_{baseline}) was subtracted from the putative trace.

The difference between the baseline and the dip (ΔF=F_{dip}-F_{baseline}), presented as percent change over the baseline (ΔF/F_{baseline}), was compared between the two conditions. Other panels show the comparisons for the time from the beginning of the stimulation to the minimum value of the initial dip and the recovery time from that minimum value to 50% of the amplitude of the dip. Additionally, we compared the overshoot/dip ratio, which reflects the relative contributions of the reductive and the oxidative phases to the NAD(P)H signal (re/ox), independently of changes in the baseline autofluorescence (Ivanov et al., 2014). This comparison also shows a decreased mitochondrial NADH production in stimulated neurons, following MPC inhibition.
b. Average normalized FAD\(^+\) traces, expressed as percent change from the baseline before each stimulation (F\(_{\text{baseline}}\)-i). The difference between the baseline and the initial peak, or the undershoot (\(\Delta F=F_{\text{peak or undershoot}}-F_{\text{baseline}}\)-i), were calculated as a percent change over the baseline (\(\Delta F/F_{\text{baseline}}\)-i). The time from the beginning of the stimulation to the peak, and the recovery time from the peak to 50\% of its amplitude, were also compared.

c. Average normalized O\(_2\) recordings, expressed as percent change from the baseline before each stimulation. The graphs show the difference between the baseline and the dip in the O\(_2\) signal elicited by stimulation (\(\Delta [O_2]= [O_2]_{\text{dip}}-[O_2]_{\text{baseline}}\)-i), either as the absolute change (expressed in \(\mu M\)) or as percent change over the baseline (\(\Delta [O_2]/[O_2]_{\text{baseline}}\)-i). The time from the beginning of the stimulation to the minimum value of the \([O_2]\) upon stimulation, and the recovery time from this value to 50\% of its amplitude, were also compared.

d. Average normalized RCaMP traces, expressed as percent change from the baseline before each stimulation (F\(_{\text{baseline}}\)-i). The difference between the intensities at baseline and at the peak (\(\Delta F=F_{\text{peak}}-F_{\text{baseline}}\)-i), expressed as A.U./px or as the percent change over the baseline (\(\Delta F/F_{\text{baseline}}\)-i), were compared between the recordings before and after MPC inhibition. The time from the beginning of the stimulation to the peak, and the recovery time from the peak to 50\% of its amplitude, were also compared.

Surprisingly, neither the dip of the NAD(P)H signal nor the transient increase in O\(_2\) consumption in the tissue were as attenuated as the NAD(P)H overshoot, although both were mildly decreased. A likely explanation for these changes is that MPC inhibition partially depolarizes mitochondria, slowing down events that require an energized mitochondria. These events include Ca\(^{2+}\) influx, the adenine nucleotide exchanger and Complex V (Metelkin et al., 2006; reviewed by Kann and Kovács, 2007; Zorova et al., 2018), all of which further dissipate the mitochondrial membrane potential and/or the proton gradient, contributing to NADH oxidation.

Data points obtained before and after the application of UK5099 are connected by lines (N\(_{\text{slices}}\)=10 and N\(_{\text{mice}}\)=6 for NAD(P)H and FAD\(^+\) autofluorescence signals, along with RCaMP fluorescence). Oxygen recordings were performed simultaneously to the autofluorescence signals in most of the experiments, except one (N\(_{\text{slices}}\)=10 and N\(_{\text{mice}}\)=5). A paired Student’s t test was used for comparisons between normally distributed data, or a non-parametric paired Wilcoxon test was used otherwise.
Figure 1 – Supplement 4. MPC inhibition shortens the NADH_{CYT} transient.

a. Filmstrip of the metabolic response in the cytosol of stimulated DGCs, before and after the application of 2 μM UK5099 (as in Figure 1c). The images were pseudocolored according to the lifetime of Peroxidox and RCaMP. The time stamp at -10 s corresponds to the baselines, ten seconds prior to the stimulation. A train of depolarizing pulses was delivered at time zero, which is revealed by an increase in the RCaMP lifetime because of the intracellular Ca^{2+} spike. The bottom row of images corresponds to the peak NADH response in the cytosol (+40s), in control conditions. The color scale bar to the right shows the range of lifetimes for both sensors. The UK5099-treated DGCs exhibit a lower Peroxidox lifetime at baseline and an attenuated NADH_{CYT} response to stimulation.

b—c. Effects of UK5099 on the parameters derived from the Peroxidox and RCaMP transients, respectively.

The full dataset of Figure 1c was used (N_{neurons} = 48, N_{slices} = 7 and N_{mice} = 6), except for comparisons of the time to peak and decay of the NADH_{CYT} response, when only neurons that presented a ΔPeroxidox Lifetime...
≥0.05ns after UK5099 were analyzed (N\textsubscript{neurons} = 13, N\textsubscript{slices} = 3 and N\textsubscript{mice} = 3). A paired Student’s t test was used for comparisons between normally distributed data, or a non-parametric paired Wilcoxon test was used otherwise.
Figure 1 – Supplement 5. LDH inhibition prevents the effect of UK5099 on the amplitude of the NADH<sub>CYT</sub> transient, but not on its time course.

a—b. Effects of the sequential application of 2 µM GSK-2837808A and 2 µM UK5099 (as in Figure 1d) on the parameters derived from the Peroxidox and RCaMP transients, respectively. The full dataset of Figure 1d was used (N<sub>neurons</sub> = 52, N<sub>slices</sub> = 6 and N<sub>mice</sub> = 5), except for comparisons of the time to peak and decay of the NADH<sub>CYT</sub> response, when only neurons that presented a ΔPeroxidox Lifetime ≥0.05ns during all the experiment were included (N<sub>neurons</sub> = 49, N<sub>slices</sub> = 6 and N<sub>mice</sub> = 5). For multiple comparisons of normally distributed data, a repeated measures ANOVA was used, with a Student-Newman-Keuls post-test. The data were compared using a non-parametric repeated measures ANOVA (Friedman test) with a Dunn post-test otherwise.

The ratio (after UK5099)/(before UK5099) was included for each parameter, to compare the conditions UK5099/Control and (UK5099+LDHi)/LDHi alone (as in Figure 1e). For UK5099/Control, the full dataset of Figure 1c was used (N<sub>neurons</sub> = 48, N<sub>slices</sub> = 7 and N<sub>mice</sub> = 6), except for comparisons of the time to peak and decay of the NADH<sub>CYT</sub> response, when only neurons that presented a ΔPeroxidox Lifetime ≥0.05ns after UK5099 were analyzed (N<sub>neurons</sub> = 13, N<sub>slices</sub> = 3 and N<sub>mice</sub> = 3). A Student’s t test or a Welch’s t-test...
were used for comparisons between normally distributed data, or a non-parametric Mann-Whitney test was used otherwise.

c. Effects of the sequential application of GSK-2837808A and UK5099 (as in Figure 1d) on the ratio of lifetimes $\Delta$Peredox/$\Delta$RCaMP.

d. Comparison of the size effect of LDH inhibition on the NADH$_{CYT}$ transients elicited with antidromic stimulation (data from experiments in Figure 1d, $N_{neurons}$= 52, $N_{slices}$= 6 and $N_{mice}$= 5) or synaptic stimulation (data from Figure 3F in Díaz-García et al. (2017), $N_{neurons}$= 54, $N_{slices}$= 5 and $N_{mice}$= 5), using a Mann-Whitney test.
Figure 1 – Supplement 6. LDH inhibition elevates the NAD(P)H autofluorescence at baseline but does not restore the NAD(P)H overshoot after the treatment with UK5099.

a. Representative traces of simultaneously recorded NAD(P)H and FAD+ autofluorescence signals, and RCaMP fluorescence, in a population of DGCs in an acute brain slice.

b. Effects of the sequential application of 2 µM GSK-2837808A and 2 µM UK5099 on the parameters derived from the NAD(P)H and RCaMP transients. The treatment with GSK-2837808A alone significantly elevated the baseline of the NAD(P)H autofluorescence by 31 ± 12%, likely because of the combination of two factors: an increased pyruvate availability and a higher NADH transfer from the cytosol to the mitochondria via the MAS. Since the responses to stimulation seems to occur independently from the changes in the NAD(P)H baseline. The FAD+ signal at baseline was unaltered by LDH inhibition and showed the expected trends after MPC inhibition with UK5099. Baselines were compared using a repeated measures ANOVA, with a Student-Newman-Keuls post-test (N_{slices}= 4 and N_{mice}= 3). Comparisons for other parameters were restricted to before and after the application of UK5099, in the continuous presence of an LDH inhibitor. These comparisons were performed using a paired t-test.
Figure 2. Calcium entrance via MCU is essential for the mitochondrial NAD(P)H overshoot.

a. Representative traces of the NAD(P)H autofluorescence signals and the mitoRCaMP1h transients in response to different trains of depolarizing pulses. The data correspond to simultaneously recorded signals from a population of DGCs in acute hippocampal slices.

b. Top: The decay time of the mitoRCaMP signal from the maximum to 50% of each peak was compared between from $Mcu^{fl/Δ}$ (25 pulses: $N_{slices}=17$, $N_{mice}=10$; 100 pulses: $N_{slices}=27$, $N_{mice}=12$; 200 pulses: $N_{slices}=26$, $N_{mice}=12$) and $Mcu^{fl/Δ}$Dock10Cre mice (abbreviated $Mcu^{fl/Δ}$Cre in the figure, 25 pulses: $N_{slices}=21$, $N_{mice}=9$; 100 pulses: $N_{slices}=31$, $N_{mice}=13$; 200 pulses: $N_{slices}=30$, $N_{mice}=12$). Data points represent individual slices. Only recordings with a peak amplitude ≥1% over the baseline were included for analysis. All comparisons were performed using a Mann-Whitney test.

Bottom: The NAD(P)H overshoot was diminished in Cre-expressing DGCs of $Mcu^{fl/Δ}$ mice. The datasets used for comparisons included slices that did not expressed mitoRCaMP in $Mcu^{fl/Δ}$ (25 pulses: $N_{slices}=31$, $N_{mice}=17$; 100 pulses: $N_{slices}=41$, $N_{mice}=19$; and 200 pulses: $N_{slices}=40$, $N_{mice}=19$) and $Mcu^{fl/Δ}$Dock10Cre.
(25 pulses: $N_{\text{slices}}=39$, $N_{\text{mice}}=19$; 100 pulses: $N_{\text{slices}}=49$, $N_{\text{mice}}=23$; and 200 pulses: $N_{\text{slices}}=48$, $N_{\text{mice}}=22$). All comparisons were performed using an unpaired t-test with a Welch’s correction.

c. Representative traces of Peredox and RCaMP1h lifetimes simultaneously recorded in DGCs of both genotypes. The typical stimulation protocol of 100 pulses elicited a smaller response (both in RCaMP and Peredox) in adult animals compared to juvenile mice. Increasing the number of pulses to 200 improved the detection of the NADH$_{\text{CYT}}$ transients.

d. **Left**: Comparison of the $\Delta$RCaMP lifetime transient between $Mcu^{fl/\Delta}$ mice ($N_{\text{neurons}}=197$, $N_{\text{slices}}=21$ and $N_{\text{mice}}=9$) and $Mcu^{fl/\Delta} Dock10Cre$ mice ($N_{\text{neurons}}=275$, $N_{\text{slices}}=27$ and $N_{\text{mice}}=11$). The range of the data is similar between genotypes, although the mean Ca$^{2+}$ transient is higher in $Mcu^{fl/\Delta} Dock10Cre$ mice. It is unlikely that this reflects an impaired clearance of cytosolic Ca$^{2+}$ by mitochondria, since the transients in MCU-lacking DGCs were similar to neurons from $Mcu^{fl/fl}$ mice (Figure 2 – Supplement 5).

**Right**: The NADH$_{\text{CYT}}$ transient was decreased in DGCs lacking MCU, as revealed by the comparison of the ratio of the Peredox lifetime change, divided by the magnitude of the RCaMP1h transient (to normalize the metabolic responses to the elevation in [Ca$^{2+}]_{\text{CYT}}$).

e. **Left**: Pyruvate accumulation is partially responsible for the diminished NADH$_{\text{CYT}}$ transients in $Mcu^{fl/\Delta} Dock10Cre$ mice, but there might be other mechanisms contributing to this difference. In a subset of slices that were treated with the LDH inhibitor, the $\Delta$Peredox/$\Delta$RCaMP ratio increased in both groups but it did not equalize the transients in the two genotypes; DGCs from $Mcu^{fl/\Delta} Dock10Cre$ mice ($N_{\text{neurons}}=113$, $N_{\text{slices}}=12$ and $N_{\text{mice}}=6$) remained about ~57% of hemizygote $Mcu^{fl/\Delta}$ mice ($N_{\text{neurons}}=103$, $N_{\text{slices}}=12$ and $N_{\text{mice}}=6$). These comparisons were performed using a paired Wilcoxon test.

**Right**: Consistent with cytosolic pyruvate accumulation in the MCU-KD, LDH inhibition produced a larger increase of the $\Delta$Peredox/$\Delta$RCaMP ratio in the MCU-KD.

Mann-Whitney test was used for all comparisons except where stated otherwise.
**Figure 2 – Supplement 1. Design of the mitochondrially targeted RCaMP1h.**

**a. Top:** Schematic representation of mitoRCaMP1h. Abbreviations indicate the mitochondrial leader sequence from the Cox8 subunit (as used in Li et al., 2014), added to the RCaMP1h sensor that includes the calmodulin (CaM) and M13 peptide, and the circularly permuted mRuby (Akerboom et al., 2013). The abbreviations Nt and Ct correspond to the amino and carboxy-termini of mRuby, respectively. The size of the boxes does not reflect the length of the sequences.

**Bottom:** Amino acid sequence of the mitoRCaMP1h sensor, with colors matching the schematic representation in the top panel.

**b. Filmstrip of DGCs in an acute hippocampal slice from a juvenile mouse, expressing the RCaMP1h sensor in the mitochondria (mitoRCaMP) and imaged using 2p-FLIM. Upon stimulation at time zero, the mitoRCaMP fluorescence intensity (top) and lifetime (bottom) promptly increased and then decayed over the span of several minutes.
Figure 2 – Supplement 2. The apparent relaxation time of the normalized mitoRCaMP transients is faster in DGCs from \textit{Mcu}^{fl/Δ} Dock10Cre mice.

\textbf{a.} Fluorescence signals from a matrix-targeted Ca\textsuperscript{2+} sensor (mitoRCaMP) in hemizygous control DGCs (\textit{Mcu}^{fl/Δ}) and MCU-KD DGCs (\textit{Mcu}^{fl/Δ} Dock10-Cre). The baseline before each stimulation (\(F_{\text{baseline-i}}\)) was subtracted from the putative trace and average traces of the mitoRCaMP signal were expressed as the \(\Delta F/F_{\text{baseline-i}}\) ratio (mean \(\pm\) SD, sample sizes as in Figure 2b, Left panel). In the control DGCs, the Ca\textsuperscript{2+} signal is mainly slowly declining (\(t_{1/2}\) of about 0.5-1 min), with a smaller fast peak that is most likely from a different compartment (most likely from mistargeted sensor in the cytosol); this fast peak is more prominent in the MCU-KD DGCs because of the reduced entry into matrix. Because the fast peak appeared in all DGCs, in the graphs of Fig. 2, we used the time to decay to 50\% of the peak to summarize the effectiveness of the knockdown in MCU-KD.

\textbf{b.} Data from a, replotted with normalization from baseline to peak on a scale of 0—1. The mitoRCaMP signal of the DGC layer show an apparent faster decay in \textit{Mcu}^{fl/Δ} Dock10Cre mice when compared to \textit{Mcu}^{fl/Δ} mice.
Figure 2 – Supplement 3. The initial dip of the NAD(P)H signal, the overall FAD$^+$ signal, and the O$_2$ dip in response to stimulation, are less affected than the slow NAD(P)H overshoot in DGCs from $Mcu^{fl/\Delta}$ Dock10Cre mice.

a. Average traces of the NAD(P)H signal expressed as the $\Delta F/F_{\text{baseline}}$ ratio (mean ± SD of number of slices, sample sizes as in Figure 2b, Right panel). The initial dip of the NAD(P)H signal was marginally affected in $Mcu^{fl/\Delta}$ Dock10Cre mice compared to $Mcu^{fl/\Delta}$ mice, despite a marked attenuation of the NAD(P)H overshoot. The lack of MCU also prolonged the time to the initial dip and delayed the recovery of the fluorescence after reaching a minimum upon stimulation.

b. The dip in tissue [O$_2$] following the stimulation of the layer of DGCs was marginally decreased in $Mcu^{fl/\Delta}$ Dock10Cre mice (25 pulses: $N_{\text{slices}}=26$, $N_{\text{mice}}=13$; 100 pulses: $N_{\text{slices}}=27$, $N_{\text{mice}}=14$; 200 pulses: $N_{\text{slices}}=29$, $N_{\text{mice}}=14$), when compared to $Mcu^{fl/\Delta}$ mice (25 pulses: $N_{\text{slices}}=27$, $N_{\text{mice}}=15$; 100 pulses: $N_{\text{slices}}=28$, $N_{\text{mice}}=15$; and 200 pulses: $N_{\text{slices}}=27$, $N_{\text{mice}}=15$).

c. The late decrease in the FAD$^+$ signal (the “Undershoot”) was less attenuated than the NAD(P)H overshoot in DGCs from $Mcu^{fl/\Delta}$ Dock10Cre mice ($N_{\text{slices}}=13$, $N_{\text{mice}}=8$ for all stimulations), when compared to $Mcu^{fl/\Delta}$ mice ($N_{\text{slices}}=13$, $N_{\text{mice}}=6$ for all stimulations).

d. The time course of the cytosolic RCaMP1h signal in response to stimulation was very similar between both groups, except for a ~1.65-fold increase in the decay time to 50% of the peak of $Mcu^{fl/\Delta}$ Dock10Cre mouse ($N_{\text{slices}}=13$, $N_{\text{mice}}=8$) versus $Mcu^{fl/\Delta}$ mouse ($N_{\text{slices}}=13$, $N_{\text{mice}}=6$), when stimulating with 200 pulses. For the reasons described in the legend of Figure 2 – Supplement 2 regarding the
variability in sensor expression levels, here we focused on the kinetics of the signal and later assessed the magnitude of the $[\text{Ca}^{2+}]_{\text{CYT}}$ spike in 2p-FLIM experiments.

A Student’s t test or a Welch’s t-test were used for comparisons between normally distributed data, or a non-parametric Mann-Whitney test was used otherwise.
Figure 2 – Supplement 4. Hemizygous mice for MCU show mild impairments in the TCA cycle with respect to \( \text{Mcu}^{fl/fl} \) controls.
a. Average traces of the NAD(P)H signal expressed as the $\Delta F/F_{\text{baseline-i}}$ ratio (mean ± SD of number of slices, sample sizes for $Mcu^{0/\Delta}$ mice as in Figure 2b, Right panel; sample size for $Mcu^{0/\Delta}$: $N_{\text{slices}} = 5$, $N_{\text{mice}} = 3$). Depending on the stimulation paradigm, the magnitude and/or duration of the late overshoot were diminished in $Mcu^{0/\Delta}$ mice when compared to $Mcu^{0/\Delta}$ controls.

b. Average traces of the FAD$^{+}$ signal expressed as the $\Delta F/F_{\text{baseline-i}}$ ratio (mean ± SD of number of slices, sample sizes for $Mcu^{0/\Delta}$ mice as in Figure 2 – Supplement 3). The late FAD$^{+}$ undershoot was less affected by the deletion of one $Mcu$ allele, although its duration was still shortened in $Mcu^{0/\Delta}$ mice when compared to $Mcu^{0/\Delta}$ controls.

A Student’s t test or a Welch’s t-test were used for comparisons between normally distributed data, or a non-parametric Mann-Whitney test was used otherwise.
Figure 2 – Supplement 5. The lack of one Mcu allele slightly reduces the NADH_{CYT} transient.

a. Comparison of the ΔRcAMP lifetime transient among genotypes (sample size for Mcu^{fl/fl} mice: N_{neurons}=86, N_{slices}=9 and N_{mice}=4; sample sizes for Mcu^{fl/Δ} and Mcu^{fl/Δ}Dock10Cre mice as in Figure 2d). Only the transients in response to a 200-pulses stimulation were analyzed.

b. Comparison among neurons of different genotypes for parameters derived from the Peredox signal. For the lifetime at baseline, the sample sizes are the same as in the previous section. For the time to peak and the decay to 50% of the peak, only neurons with a ΔPeredox lifetime in response to stimulation of ≥0.05 ns were considered (sample sizes for Mcu^{fl/fl}: N_{neurons}=79; Mcu^{fl/Δ}: N_{neurons}=151; Mcu^{fl/Δ}Dock10Cre: N_{neurons}=125).

c. Representative traces of Peredox and RCaMP1h lifetimes simultaneously recorded in a DGC from an adult mouse of Mcu^{fl/fl} genotype. In this recording, the LDH inhibitor GSK-2837808A (LDHi) was applied for ~30 min, and the NADH_{CYT} transients were elicited using antidromic stimulation.

d. The lack of MCU causes a gradual decrease in the change of Peredox lifetime relative to the RCaMP spike, indicating smaller NADH_{CYT} transients in response to stimulation (sample size for Mcu^{fl/fl} mice: N_{neurons}=86, N_{slices}=9 and N_{mice}=4; sample sizes for Mcu^{fl/Δ} and Mcu^{fl/Δ}Dock10Cre mice as in Figure 2d). Differences between groups could not be rescued by LDH inhibition. The datasets for LDH inhibition corresponds to a subset of slices (sample size for Mcu^{fl/fl} mice+LDHi: N_{neurons}=30, N_{slices}=4 and N_{mice}=2; sample sizes for Mcu^{fl/Δ} and Mcu^{fl/Δ}Dock10Cre mice as in Figure 2e).

The data were compared using a Kruskal-Wallis test with a Dunn post-test.
Figure 3. The rise in $[\text{Ca}^{2+}]_{\text{CYT}}$, mainly caused by the activity of high voltage-activated Ca$^{2+}$ channels, makes a major contribution to the NADH$_{\text{CYT}}$ transients in response to stimulation.

a. Left: Representative trace from a DGC expressing Peroxid and RCaMP1h. The slice was superfused for $\sim$20 min with the L-type Ca$^{2+}$ channel inhibitor isradipine (Isra, 3 µM), and then stimulated. In the continuous presence of isradipine, 20 µM of CdCl$_2$ (Cd$^{2+}$, a non-selective blocker of voltage-activated Ca$^{2+}$ channels) was added to the ACSF. Inhibition of Ca$^{2+}$ influx was evident from the progressive reduction of the stimulus associated RCaMP1h spike. Right: The amplitude of the metabolic responses to stimulation (Peroxid lifetime change) mirrored the decrease in the RCaMP spikes (Figure 3 – Supplement 3a). The data were compared using a non-parametric repeated measures ANOVA (Friedman test) with a Dunn post-test ($N_{\text{neurons}} = 86, N_{\text{slices}} = 11$ and $N_{\text{mice}} = 6$). For all panels, only neurons showing an initial $\Delta$Peroxid lifetime response $\geq$0.05 ns were included for analysis.

b. Left: Representative trace of a DGC superfused with EGTA-AM (100 µM), a cell-permeable Ca$^{2+}$ chelator. As expected, the stimulus-induced RCaMP transients gradually diminished over time, typically stabilizing after $\sim$1h of treatment. Right: NADH$_{\text{CYT}}$ transients are strongly attenuated after effective Ca$^{2+}$
buffering by EGTA-AM (Figure 3 – Supplement 3b). The data were compared using a Wilcoxon matched pairs test ($N_{\text{neurons}} = 45$, $N_{\text{slices}} = 5$ and $N_{\text{mice}} = 5$).

c. Left: Representative trace for the effect of Ca$^{2+}$ removal from the bath solution on the metabolic transients in the cytosol. The cell-impermeant Ca$^{2+}$ chelator EGTA (1 mM) was added to the ACSF to reinforce Ca$^{2+}$ removal after switching to a nominal 0Ca$^{2+}$ solution. A modified control ACSF also contained 1 mM EGTA and an adjusted total [Ca$^{2+}$] resulting in a free concentration of 2 mM, as in any other control experiment. Effective Ca$^{2+}$ removal was confirmed by the absence of a RCaMP1h spike upon stimulation. Right: The NADH$_{\text{CYT}}$ transients were diminished in a Ca$^{2+}$-deprived ACSF. The data were compared using a Wilcoxon matched pairs test ($N_{\text{neurons}} = 31$, $N_{\text{slices}} = 7$ and $N_{\text{mice}} = 6$).
Figure 3 – Supplement 1. Control experiment for 0.1% DMSO in the ACSF, as in the EGTA-AM experiments.

Left: Representative trace of the effect of DMSO (0.1%) on the Peredox and RCaMP signals. Right: The change in Peredox lifetime elicited by stimulation was unaffected by the application of 0.1% DMSO for 30—60 min, contrary to the gradual decrease observed with EGTA-AM. The RCaMP spike decreased over time but it was not as attenuated as in EGTA-AM. The Peredox and RCaMP transients were compared using a Wilcoxon matched pairs test and a paired t-test, respectively (N_{neurons} = 17, N_{slices} = 4 and N_{mice} = 3). Only neurons showing an initial ΔPeredox lifetime response ~0.05 ns or higher were included for analysis.
Figure 3 – Supplement 2. Calcium removal from the ACSF, without addition of EGTA, is also effective in decreasing the NADH<sub>CYT</sub> transients.

*Left:* Representative trace for the effect of Ca<sup>2+</sup> removal from the ACSF on the NADH<sub>CYT</sub> transients. As in Figure 2 – Supplement 4, no EGTA was added to the solution. *Right:* Simple substitution of Ca<sup>2+</sup> by Mg<sup>2+</sup> in the ACSF was effective in decreasing the NADH<sub>CYT</sub> transients. The data were compared using a Wilcoxon matched pairs test (N<sub>neurons</sub> = 27, N<sub>slices</sub> = 5 and N<sub> mice</sub> = 3).
Figure 3 – Supplement 3. A major Ca^{2+}-dependent component of the NADH_{CYT} also occurs under LDH inhibition.

a. Comparison of the ΔPeredox/ΔRCaMP values obtained in the continuous presence of the LDH inhibitor GSK-2837808A, before and after the blockade of voltage gated Ca^{2+} channels with a combination of isradipine and cadmium (Isra+Cd^{2+}; N_{neurons}= 13, N_{slices}= 3 and N_{mice}= 3). The slices were exposed to GSK-2837808A for at least 30 min prior to the experiment. LDH inhibition should improve the ability to detect the cytosol-only component of Ca^{2+} actions on the NADH_{CYT} transients by increasing the control responses to stimulation, as well as by preventing the potential impact of pyruvate accumulation on these transients due to lower Ca^{2+} influx into the mitochondria. The effects of the manipulation on the Peredox baseline and the RCaMP spike are also included for the experiments with or without the LDH inhibitor (the sample size is reported in Figure 3 for the latter).

b—c. Comparisons for the application of the cell-permeable Ca^{2+} chelator EGTA-AM (N_{neurons}= 15, N_{slices}= 3 and N_{mice}= 3 for experiments with LDHi, sample sizes for the other group as in Figure 3), or the removal...
of Ca$^{2+}$ from the ACSF ($N_{\text{neurons}} = 136$, $N_{\text{slices}} = 22$ and $N_{\text{mice}} = 13$ for experiments with LDHi, sample sizes for the other group as in Figure 3).

For all panels, only neurons showing an initial $\Delta$Peredox lifetime response $\geq 0.05$ ns were included for analysis. A paired Student’s t test was used for comparisons between normally distributed data, or a non-parametric paired Wilcoxon test was used otherwise.

LDH inhibition partially rescued the Peredox responses in Isra+Cd$^{2+}$ and EGTA-AM, but not in 0Ca$^{2+}$+EGTA, even though some pyruvate accumulation is also expected in the last condition due to less Ca$^{2+}$-dependent pyruvate utilization in the mitochondria during stimulation. We do not have a definitive answer for this difference. It is possible that the Ca$^{2+}$ channel blockade or Ca$^{2+}$ chelation may not be complete, especially in dendrites, triggering a component of the metabolic responses that propagates to the soma, and is better revealed during LDH inhibition.
Figure 4. The inhibition of the Ca\textsuperscript{2+}-calmodulin complex, or AMPK, does not abolish the NADH\textsubscript{CYT} transients in response to stimulation.

a. Left: Representative trace of a DGC treated with E6-berbamine (10 µM), an inhibitor of the Ca\textsuperscript{2+}-calmodulin signaling pathway. Right: The magnitude of the metabolic response was expressed as the change in Peredox lifetime change divided by the change in RCaMP1h lifetime in response to stimulation (ΔPeredox/ΔRCaMP). E6-berbamine marginally elevated the NADH\textsubscript{CYT} transients. The data were compared using a paired t-test (N\textsubscript{neurons}= 37, N\textsubscript{slices}= 4 and N\textsubscript{mice}= 4). For all panels, the effect of the drugs on the metabolic transients were monitored for at least 30 min.

b. Left: Representative trace of a DGC treated with calmidazolium (20 µM), another inhibitor of the Ca\textsuperscript{2+}-calmodulin signaling pathway. Right: The effect of calmidazolium on the ΔPeredox/ΔRCaMP ratio was similar to the previous Ca\textsuperscript{2+}-calmodulin complex inhibitor. The data were compared using a Wilcoxon matched pairs test (N\textsubscript{neurons}= 24, N\textsubscript{slices}= 3 and N\textsubscript{mice}= 3).

c. Left: Representative trace of a DGC before and after the inhibition of the AMPK pathway using dorsomorphin (10 µM). Since the drug decreases the Ca\textsuperscript{2+} transients (Figure 4 – Supplement 1c), the intensity of the stimulus was adjusted to elicit strong responses, and the number of depolarizing pulses was sometimes increased to 150 (before and after treatment) to ensure effective stimulation of the soma throughout the experiment. Right: In the presence of dorsomorphin, the ΔPeredox/ΔRCaMP ratio remained unaltered. The data were compared using a paired t-test (N\textsubscript{neurons}= 17, N\textsubscript{slices}= 4 and N\textsubscript{mice}= 3).
Figure 4 – Supplement 1. Ca²⁺/CaM and AMPK signaling modulates the cytosolic [Ca²⁺] and NADH/NAD⁺ ratio at baseline, and the time course of the NADH<sub>CYT</sub> transient in response to stimulation.

a. Inhibition of the Ca²⁺/CaM axis with 10 µM E6-berbamine (E6-Berb) increases the RCaMP lifetime at baseline while keeping the stimulus-associated RCaMP spike intact. The Peredox lifetime at baseline, the amplitude, and the time to peak of the NADH<sub>CYT</sub> transient are also increased with this treatment. The sample size corresponds to Figure 4a, except for the time course parameters (time to peak and decay), where only DGCS with ΔPeredox≥0.05ns were analyzed (N<sub>neurons</sub> = 27).

b. Inhibition of the Ca²⁺/CaM axis with 20 µM calmidazolium (Calmdz) caused similar effects to E6-berbamine. Sample size as in Figure 4b; with N<sub>neurons</sub> = 20 for the time course analysis, corresponding to neurons with ΔPeredox≥0.05ns.

c. Inhibition of AMPK with 10 µM dorsomorphin (Dorsm) increased the RCaMP and Peredox baselines but decreased the RCaMP transients in response to stimulation. Sometimes the slices were stimulated with 150 pulses instead of 100 (before and after treatment) to ensure effective stimulation of the somata throughout the experiment. Sample size as in Figure 4c; with N<sub>neurons</sub> = 16 for the time course analysis, corresponding to neurons with ΔPeredox≥0.05ns.

A paired Student’s t test was used for comparisons between normally distributed data, or a non-parametric paired Wilcoxon test was used otherwise.
Figure 5. Neuronal stimulation triggers glycolysis in response to energy demand from ion pumping.

a. Left: Representative trace of Peredox and RCaMP1h lifetimes simultaneously recorded in a DGC from an acute hippocampal slice. The slice was superfused with 2 µM GSK-2837808A for at least 30 min before the experiment, and the LDH inhibitor was kept in the ACSF during the experiment. The ACSF also contained 1 mM EGTA to reinforce Ca²⁺ removal in the nominal 0Ca²⁺ condition (but [Ca²⁺] in the control ACSF was accordingly adjusted to a free concentration of 2 mM, as in any other experiment). Effective Ca²⁺ removal was confirmed by the absence of a RCaMP1h spike upon stimulation. The Peredox lifetime at baseline, and the metabolic transients in response to neuronal stimulation, were recorded after substituting the bath solution with a 0Ca²⁺ ACSF (to obtain Na⁺-only NADHCYT responses), and the further application of 10 µM α-pompidilotoxin (α-Pmtx, a toxin that prevents voltage-gated Na⁺ channel inactivation). Right: The Na⁺-only NADHCYT transient was increased in the presence of α-pompidilotoxin. The Peredox lifetime change in response to stimulation was diminished in the absence of Ca²⁺ but bounced back to higher amplitudes by increasing Na⁺ influx. The data were compared using a repeated measures ANOVA with a Student-Newman-Keuls post-test (Nneurons= 35, Nslices= 5 and Nmice= 3). For all panels, only neurons showing an initial ΔPeredox lifetime response ≥0.05 ns were included for analysis.

b. Representative trace of Peredox and RCaMP1h lifetimes in a DGC stimulated with trains of 100 and 200 electrical pulses. The two-stimulation protocol was also performed in 0Ca²⁺ ACSF before and after the application of 3 µM α-pompidilotoxin. The latter condition was followed by the application of the
Na⁺/K⁺ ATPase inhibitor strophanthidin. As in (a), the slices were exposed to the LDH inhibitor GSK2837808A from 30 min prior, until the end of the experiment. Likewise, all the solutions contained 1 mM EGTA.

c. Comparison of the Peredox lifetime changes in response to both stimulation paradigms (100 or 200 pulses) among the conditions in (b). The NADHₙₑₓₜ transients in control condition was different from the other conditions (the discontinuous line for the associated p-value applies to all comparisons). The Na⁺-only NADHₙₑₓₜ responses recorded in 0Ca²⁺ ACSF were increased slightly but significantly increased by the application of 3 µM α-pompidolotoxin, an effect that was reversed by strophanthidin. The data were compared using a non-parametric repeated measures ANOVA (Friedman test) with a Dunn post-test (Nₙₑᵤᵣₜₙₜₜₜ= 66, Nₙₛᵢₗᵋₑₜₜₜ= 10 and Nₘᵋᵋₑᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋᵋôte
Figure 5 – Supplement 1. NADH_Cyt transients elicited in 0Ca^{2+}-ACSF by boosting Na^+ influx, are briefer than those elicited in control ACSF.

a. The Peredox baseline remained unaltered during the recordings from Figure 5a, where stimulations were delivered ~10 min after changing solutions. The data were compared using a repeated measures ANOVA (sample sizes as in Figure 5a). The peak Peredox lifetime in 0Ca^{2+}-ACSF, boosted with 10 µM pompidolotoxin, occurred earlier than the peak in the control ACSF. On the other hand, the decay to 50% of the peak was similar between both groups. Comparisons were performed using a Wilcoxon matched pairs test.

b. The prolonged exposure to each condition in Figure 5c (same sample sizes) caused marginal but statistically significant effects on the Peredox baseline. Values were taken before the stimulation with 200 pulses.
Figure 5 – Supplement 2. The late overshoot in the NAD(P)H signal disappears in a Ca\(^{2+}\)-free solution, and it is not recovered by α-pompilidotoxin application.

a. Left: Representative trace of the autofluorescence and RCaMP signals in the DGC layer of an acute hippocampal slice. These experiments were performed with samples from juvenile wild-type mice. When Ca\(^{2+}\) was removed from the ACSF (as in Figure 3 – Supplement 2), the late increase ("overshoot") in the NAD(P)H autofluorescence and the late decrease ("undershoot") in the FAD\(^{+}\) signal were almost abolished. Reducing voltage-gate Na\(^{+}\) channel inactivation with 25 µM α-pompilidotoxin (α-Pmtx), which increases Na\(^{+}\) influx during action potentials and results in higher energy demand due to ion pumping, did not rescue any of these signals. As expected, the application of 1 µM Tetrodotoxin (TTX), a blocker of voltage-gate Na\(^{+}\) channels that prevents the firing of action potentials, inhibited all the
signals elicited upon stimulation. **Right**: Average traces of the NAD(P)H and FAD* signals, expressed as the \( \Delta F/F_{\text{baseline}} \) ratio (mean ± SD of number of slices, \( N_{\text{slices}} = 5 \), \( N_{\text{mice}} = 3 \)). The initial segment of the signals was zoomed in to better visualize the effects of the manipulations on the initial transient phases of both the NAD(P)H and the FAD* signals.

**b—c.** Comparisons of the parameters derived from the NAD(P)H and the FAD* signals, respectively. While \( \text{Ca}^{2+} \) removal recapitulated the effects on the NAD(P)H signal, the decrease in the FAD* undershoot was more prominent than the observed in DGCs lacking MCU. The data were compared using a repeated measures ANOVA with a Student-Newman-Keuls post-test when all the groups presented Gaussian distributions, otherwise, a non-parametric Friedman test with a Dunn post-test was performed.
Figure 5 – Supplement 3. Spontaneous oscillations in the Peredox signal may occur during the prolonged application of zero Ca\(^{2+}\)-ACSF, in the presence of EGTA and LDH inhibition.

Prolonged exposure to a nominal Ca\(^{2+}\)-free solution, plus LDH inhibition, caused spontaneous elevations of the Peredox lifetime in 25 \(\pm\) 13% of the cells in each slice (slices=10, mice=5), contrasting with only \(~\)3% of cells exposed to a brief exposure as in Figure 3c. The spontaneous elevations in Peredox lifetime occurred at any time after removing Ca\(^{2+}\) from the ACSF. Although these neurons were not included in the analysis, once the spontaneous transients cleared, the experiment resumed as usual. The continuous presence of 1 mM EGTA and 2 \(\mu\)M GSK-2837808 (LDH inhibitor) is not indicated in the figure for simplicity. In the control ACSF, the [Ca\(^{2+}\)] was adjusted accordingly to yield a free concentration of 2 mM.
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