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ORIGINAL PAPER

Dynamic Changes in Cytosolic ATP Levels in Cultured Glutamatergic Neurons During NMDA-Induced Synaptic Activity Supported by Glucose or Lactate

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Abstract We have previously shown that synaptic transmission fails in cultured neurons in the presence of lactate as the sole substrate. Thus, to test the hypothesis that the failure of synaptic transmission is a consequence of insufficient energy supply, ATP levels were monitored employing the ATP biosensor Ateam1.03YEMK. While inducing synaptic activity by subjecting cultured neurons to two 30 s pulses of NMDA (30 μM) with a 4 min interval, changes in relative ATP levels were measured in the presence of lactate (1 mM), glucose (2.5 mM) or the combination of the two. ATP levels reversibly declined following NMDA-induced neurotransmission activity, as indicated by a reversible 10–20 % decrease in the response of the biosensor. The responses were absent when the NMDA receptor antagonist memantine was present. In the presence of lactate alone, the ATP response dropped significantly more than in the presence of glucose following the 2nd pulse of NMDA (approx. 10 vs. 20 %). Further, cytosolic Ca^{2+} homeostasis during NMDA-induced synaptic transmission is partially inhibited by verapamil indicating that voltage-gated Ca^{2+} channels are activated. Lastly, we showed that cytosolic Ca^{2+} homeostasis is supported equally well by both glucose and lactate, and that a pulse of NMDA causes accumulation of Ca^{2+} in the mitochondrial matrix. In summary, we have shown that ATP homeostasis during neurotransmission activity in cultured neurons is supported by both glucose and lactate. However, ATP homeostasis seems to be negatively affected by the presence of lactate alone, suggesting that glucose is needed to support neuronal energy metabolism during activation.

Keywords Neuron · ATP · Glucose · Lactate

Introduction

The repetitive depolarization of neurons during neurotransmission activity is costly in terms of consumption of ATP and constitutes the primary burden on the energy-producing machinery of neurons [1]. The adult brain relies primarily on oxidation of glucose from the blood to supply the needed ATP; however, at the cellular level, other substrates such as lactate may be important [2]. The questions if, when and to what degree glutamatergic neurons employ lactate as a substrate for energy production has been addressed numerous times since the original proposal of the astrocyte-neuron lactate shuttle hypothesis in 1994 by Pellerin and Magistretti [3]. The model has sparked considerable debate, especially so the idea that lactate may be the major, or even the only fuel for neuronal energy metabolism (see [4–7] for most recent summaries). Cultured mouse cerebellar neurons (CCNs), primarily consisting of glutamatergic granule cells, have an obligatory need for glucose being present either alone or

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alongside lactate to sustain their energy metabolism and proper synaptic function in terms of re-uptake of released neurotransmitter glutamate [8]. In addition, the oxidation of glucose but not of lactate was up-regulated during neurotransmission activity [8].

Thus, we investigated the ATP homeostasis in these cultures during neurotransmission activity to test the hypothesis that glucose is needed to maintain cytosolic ATP. To this end, we expressed the FRET-based ATP biosensor Ateam1.03YEMK [9] in CCNs and induced neurotransmission activity by two 30 s pulses of NMDA (30 μM) separated by 4 min. As shown by Ca^{2+} imaging experiments, this treatment induces a depolarization-coupled elevation of Ca^{2+} in both the cytosol and the mitochondrial matrix indicating energy-demanding synaptic activity. We show that when lactate (1 mM) is present alone, the dip in cytosolic ATP following the 2nd pulse of NMDA is significantly larger than those observed when glucose (2.5 mM) is present alone or in combination with lactate. We conclude that in these cells under the present experimental conditions, glucose may be both needed and sufficient to sustain ATP homeostasis during neurotransmission activity.

Experimental Procedures

Reagents

NMDA was from Tocris (Bristol, UK), memantine from Abcam (Cambridge, UK) and verapamil from Sigma (Munich, Germany). All other chemicals used were of the proper quality and purity and provided by commercial vendors.

Cell Cultures

CCNs were prepared from P7 wild type NMRI mice as described previously [10] and used for experiments on DIV 7-9.

Plasmids, Transfection and Setup for Imaging ATP and Cytosolic Calcium Dynamics

ATeam1.03YEMK and ATeam1.03R122K/R126K-constructs [9] were obtained from Addgene (Addgene plasmids #28004 and #28005, respectively) and subcloned using Gateway cloning (Invitrogen/Thermo Fischer Scientific, Waltham, USA) into a vector containing a CMV-promoter for expression in mammalian cells. The red fluorescent calcium indicator R-GECO1 plasmid [11] was obtained from Addgene (Addgene plasmid #32444). The anti-apoptotic Bcl-xl construct was a kind gift from Dr. Judith Stegmüller. CCNs grown on 25 mm poly-D-lysine coated coverslips were transfected 3–4 days prior to imaging with the plasmids of interest using the calcium phosphate method. Briefly, culturing medium was removed and stored for later. The cells were washed twice in DMEM without glutamine and pyruvate and incubated 45 min at 37 °C. 4 μg Ateam1.03YEMK plasmid (or the mutated sensor Ateam1.03R122K/R126K) and 1.5 μg Bcl-xl plasmid dissolved in water were consecutively mixed with 2.5 M $CaCl_2$ and 2× HBSS (concentrations of the final mixture in mM: 125 $CaCl_2$, 20 HEPES, 135 NaCl, 5 KCl, 0.7 Na_2HPO_4 and 7.5 glucose adjusted to pH 7.08 at room temperature) and incubated for 6 min. For simultaneous calcium and ATP monitoring 4 μg R-GECO1 plasmid was co-transfected. Plasmid solutions were added to the cell culture medium and cells were incubated for 18 min. Thereafter cells were washed twice and cultured an additional 3–4 days in their previous culturing medium.

All NMDA stimulations were performed with 30 μM NMDA in the presence of 10 μM L-glycine in Mg^{2+}-free imaging buffer containing (in mM) 10 HEPES, 1 $CaCl_2$, 135 NaCl, 5 KCl, buffered to pH 7.4 at room temperature. Changes in $[ATP]_c$ in CCNs upon NMDA stimulation was monitored using fluorescence microscopy (Zeiss Axiovert 200 M) using a 40× oil immersion objective (Zeiss FLUAR 40×/1.30 oil). Excitation filters used for imaging where 436/25 (CFP, FRET) and 550/25 (R-GECO1) and emission filters 480/40 (CFP), 535/30 (FRET) and 605/70 (R-GECO1). Fluorescence of the biosensors was excited with a X-cite Series120 lamp (Exfo Excelitas Technologies). Fluorescence emission was monitored using a charge-coupled device (CCD) camera (AxioCam MRm, Zeiss); the exposure times were optimized individually for each experiment but keeping the ratio of exposure times of CFP and FRET channels constant. Cells were continuously perfused at 1 ml/min at room temperature. Images were acquired every 15 s. Image analysis was performed using Fiji [12]. The background-subtracted FRET/CFP emission ratio was calculated from each ROI in Microsoft Excel.

Measurement of Cytosolic Calcium Dynamics Employing fura-2

$[Ca^{2+}]_c$ was measured in CCNs grown on 12 mm poly-D-lysine coated coverslips and loaded with 5 μM Fura-2 AM (dissolved in DMSO and 0.01 % Pluronic F-127) for 30 min at room temperature and 20 min de-esterification in imaging buffer. Imaging was performed employing a light microscope (Olympus BX51WI) with a water immersion objective (Olympus LUMPlanFl/IR 40×/0.8 W). Excitation at 340 and 380 nm was alternated using a monochromator (Polychrome V, TILL Photonics) and emission was monitored at 510 nm using a CCD camera. CCNs were superfused with Mg^{2+}-free

imaging buffer (4 ml/min) and images were acquired every 2 s with 20 ms exposure time using the TILL Vision Software. Image analysis was performed by subtracting the background and calculating the 340/380 nm ratios from each individual neuron in Microsoft Excel.

Lentiviral Calcium Biosensor Plasmids and Transduction of CCNs

For production of lentiviral mito-GEM-GECO1 calcium biosensor construct, we used PCR to subclone the entire reading frame into pENTR-D/TOPO vector as described by the manufacturer (Life Technologies). CMV-mito-GEM-GECO1 plasmid used as template was a gift from Robert Campbell (Addgene plasmid #32461; ref. [11]). The insert was then transferred to lentiviral expression plasmid pLX301 using the Gateway LR Clonase II enzyme mix (Life Technologies). pLX301 was a gift from David Root (Addgene plasmid #25895; ref. [13]). Correct insert was verified by sequencing. Viral particles were made by co-transfecting HEK293 cells with packaging plasmid psPAX2 (a gift from Didier Trono; Addgene plasmid #12260) and envelope plasmid VSV-G (a gift from Bob Weinberg; Addgene plasmid #8454; ref. [14]) by standard calcium phosphate precipitation method. Three days post transfection the culture medium was cleared for cellular debris by centrifugation at 3000×*g* for 5 min. The supernatant containing viral particles were then used directly to transduce cultured neurons. Typically, 100 µl of the supernatant containing the lentiviral particles was added to the culture medium (total volume 2 ml) on DIV 2 to transduce CCNs. The cultures were used for imaging experiments on DIV 7-8.

Measurement of Mitochondrial Calcium Dynamics

The imaging was performed using an inverted microscope DM-IRE2 with confocal laser scanning system LCS-SP2 (Leica, Heidelberg, Germany) employing a water immersion objective (HCX PL APO 63×/1.4). The microscope was equipped with a motorized xy-stage plate (Live cell Instrument, Seoul, Korea) and a LDH type pulsed diode laser (PicoQuant, Berlin, Germany). The excitation wavelength was 405 nm and the resulting emission from the ratiometric mito-GEM-GECO1 sensor was collected in the intervals 450–490 nm (Ca^{2+} bound, R) and 525–550 nm (unbound state, R_0), respectively, employing two PMT channels [11]. The imaging was performed at room temperature on CCNs grown on 25 mm poly-D-lysine coated coverslips in imaging buffer as above, and images were acquired every 5 s. The cultures were superfused at a rate of approx. 1.7 ml/min and subjected to one 30 s pulse of NMDA and glycine, as above. Image analysis was performed employing Fiji [12] and the background-subtracted emission ratio (R/R_0) was calculated from each ROI (chosen in mitochondria-rich regions) in Microsoft Excel so that an increase in R/R_0 corresponds to an increase in matrix Ca^{2+} [11]. The ratio was normalized to one at the onset of the experiment.

Data and Statistical Analysis

All data are presented as mean with standard errors of the mean (SEM). Unpaired *t* test or one-way ANOVA was performed with Tukey's multiple comparison test where appropriate. Statistical significance was set to a *p* value of * <0.05 and *** <0.001.

Results

To probe the changes in cytosolic ATP levels upon neurotransmission activity, CCNs expressing the ATP biosensor Ateam1.03YEMK were superfused in a physiological medium and subjected to pulses of NMDA (30 µM) plus L-glycine (10 µM; co-agonist at NMDA receptors), a protocol that is known to induce synaptic activity including vesicular release of neurotransmitter glutamate as well as a bioenergetic response [8, 15]. A dip in the cytosolic ATP level was observed following a 30 s pulse of NMDA (Fig. 1a). At the end of the experiment, a combination of azide (10 mM; blocks mitochondrial respiration) and iodoacetate (1 mM; blocks glycolysis) was added to collapse the ATP level to a minimum; this always caused the fluorescence ratio of the biosensor to decrease to approximately 0.4. To test that the observed dip in cytosolic ATP level coincides with Ca^{2+}-linked neurotransmission activity and is indeed caused by a decrease in ATP levels, we co-expressed the Ca^{2+} biosensor R-GECO1 alongside either Ateam1.03YEMK (Fig. 1a) or a mutated form of the ATP biosensor (Fig. 1b) that is incapable of binding ATP (Ateam1.03R122K/R126K; ref. [9]) and subjected the cultures to a similar protocol as described above. The cells expressing both the functional and the mutated ATP biosensor responded to NMDA pulses with rises in cytosolic Ca^{2+} levels (Fig. 1a, b) but showed no response of the mutated ATP biosensor (Fig. 1b), indicating that the observed responses are indeed caused by NMDA-induced ATP changes and not by an artifactual response of the ATP biosensor. Finally, to ascertain that the observed changes in Ca^{2+} and ATP levels are caused by an NMDA receptor-mediated mechanism, we blocked the NMDA receptor by adding the non-competitive NMDA receptor antagonist memantine (100 µM; [16]) which completely abolished both the Ca^{2+} response as well as the decrease in ATP level upon addition of NMDA (Fig. 1a).

To probe the ability of glucose and lactate to support cytosolic ATP levels upon neurotransmission activity,

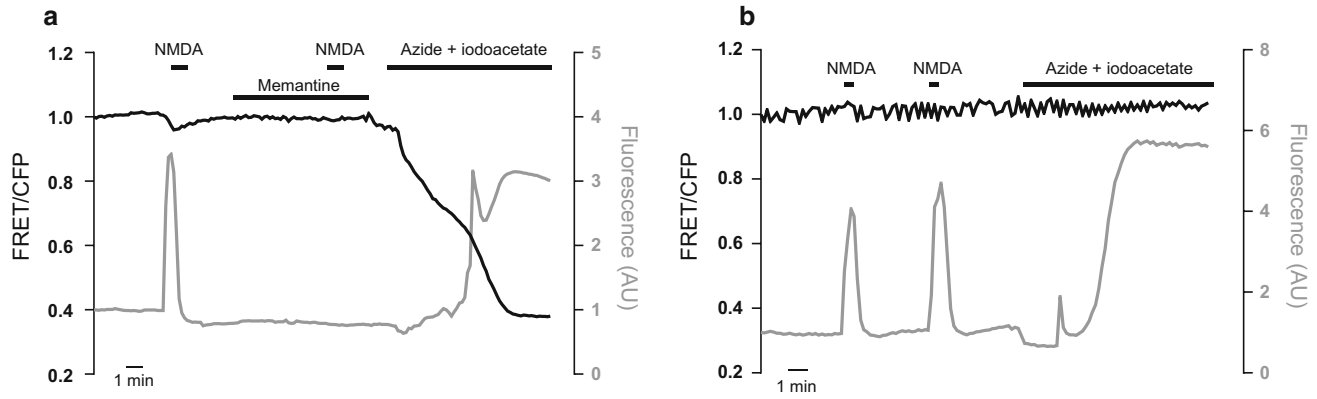

Fig. 1 Reversible decreases in cytosolic ATP levels upon neurotransmission activity. **a** Simultaneous FRET/CFP time course and Ca^{2+} monitoring collected from a single neuron expressing the biosensors Ateam1.03YEMK and R-GECO1 in the presence of 2.5 mM glucose during two 30 s NMDA pulses (30 μM NMDA, 10 μM L-glycine). The non-competitive NMDA receptor antagonist memantine (100 μM) was added to show that the response to NMDA is caused by activation of the NMDA receptor. **b** FRET/CFP time course and Ca^{2+} monitoring collected from a single neuron expressing the mutated ATP biosensor Ateam1.03R122K/R126K (ATP binding site is mutated) and the calcium biosensor R-GECO1. The absence of a change in the ATP signal despite clear Ca^{2+} signals shows that the observed response to NMDA is caused by a relative change in cytosolic ATP levels and not an intrinsic change in the fluorescent signal of the biosensor

CCNs expressing the ATP biosensor were superfused in the presence of either 2.5 mM glucose or 1 mM lactate or the combined presence of both substrates (Fig. 2a, b); a representative trace from these experiments are shown in Fig. 2a. These concentrations of substrates were chosen to replicate the conditions used in previous experiments (e.g. [8]). To mimic neurotransmission activity, the cultures were subjected to two 30 s pulses of NMDA with a 4 min interval in between. Reversible decreases in cytosolic ATP levels were observed following each pulse of NMDA; however, for all experimental conditions the ATP level did not always recover to initial baseline level.

In order to perform a semi-quantitative comparison of the fluctuations in cytosolic ATP levels following NMDA stimulation, we calculated the percent decrease in the response of the ATP biosensor relative the response in the presence of azide and iodoacetate (illustrated in Fig. 2a; see figure legend for a more detailed explanation). Following the first pulse of NMDA, the decrease in cytosolic ATP levels was of a similar magnitude for all three combinations of substrates; however, following the 2nd pulse of NMDA, the reduction in the presence of lactate alone was significantly more pronounced when compared to the decrease in the presence of glucose (Fig. 2b). Thus, lactate as the only available substrate for cultured neurons seems not to support ATP homeostasis to the same extent as glucose under these experimental conditions.

To test if the differences observed in ATP homeostasis during neurotransmission activity between the glucose and lactate-fueled conditions were caused by changes in the induced cytosolic Ca^{2+} signals, we loaded the cultures with the Ca^{2+}-sensitive dye fura-2 and subjected them to a stimulation protocol similar to the one employed above. Pulses of NMDA induced a robust response in cytosolic Ca^{2+} levels (Fig. 3a) and when quantified as either peak height or area under the curve, no differences were observed in the Ca^{2+} responses to pulses of NMDA for either of the combinations of substrates (Fig. 3b). Since the depolarization induced by NMDA receptor stimulation among others causes opening of voltage-gated Ca^{2+} channels (VGCCs), the observed spikes in cytosolic Ca^{2+} following NMDA stimulation is likely to consist of Ca^{2+} originating from multiple sources, including Ca^{2+} moving through the NMDA receptor channel and a component related to VGCCs as well as Ca^{2+}-induced Ca^{2+} signaling. Thus, to test if any changes in Ca^{2+} signaling would be visible in the absence of the presumably large VGCC-related contribution, we performed similar experiments in the presence of verapamil (30 μM), a blocker of VGCCs [17]. Verapamil significantly reduced the NMDA-induced Ca^{2+} signal (Fig. 4a, b) but no differences between the three combinations of substrates were observed in the presence of verapamil (Fig. 4b). This indicates that the NMDA-induced spikes in cytosolic Ca^{2+} levels are not affected by the presence of the different substrate combinations, i.e. glucose and lactate are equally good at maintaining cytosolic Ca^{2+} homeostasis under the present experimental conditions.

It has been suggested previously that the malate-aspartate shuttle (MAS) is curbed during peak neurotransmission activity when Ca^{2+} is accumulated into the mitochondrial matrix, thus making lactate a poorer substrate (discussed in more detail below; references [18, 19]). To ascertain if a pulse of NMDA causes accumulation of Ca^{2+} into the mitochondrial matrix, we expressed the matrix-targeted, ratiometric Ca^{2+} biosensor mito-GEM-GECO1 in CCNs

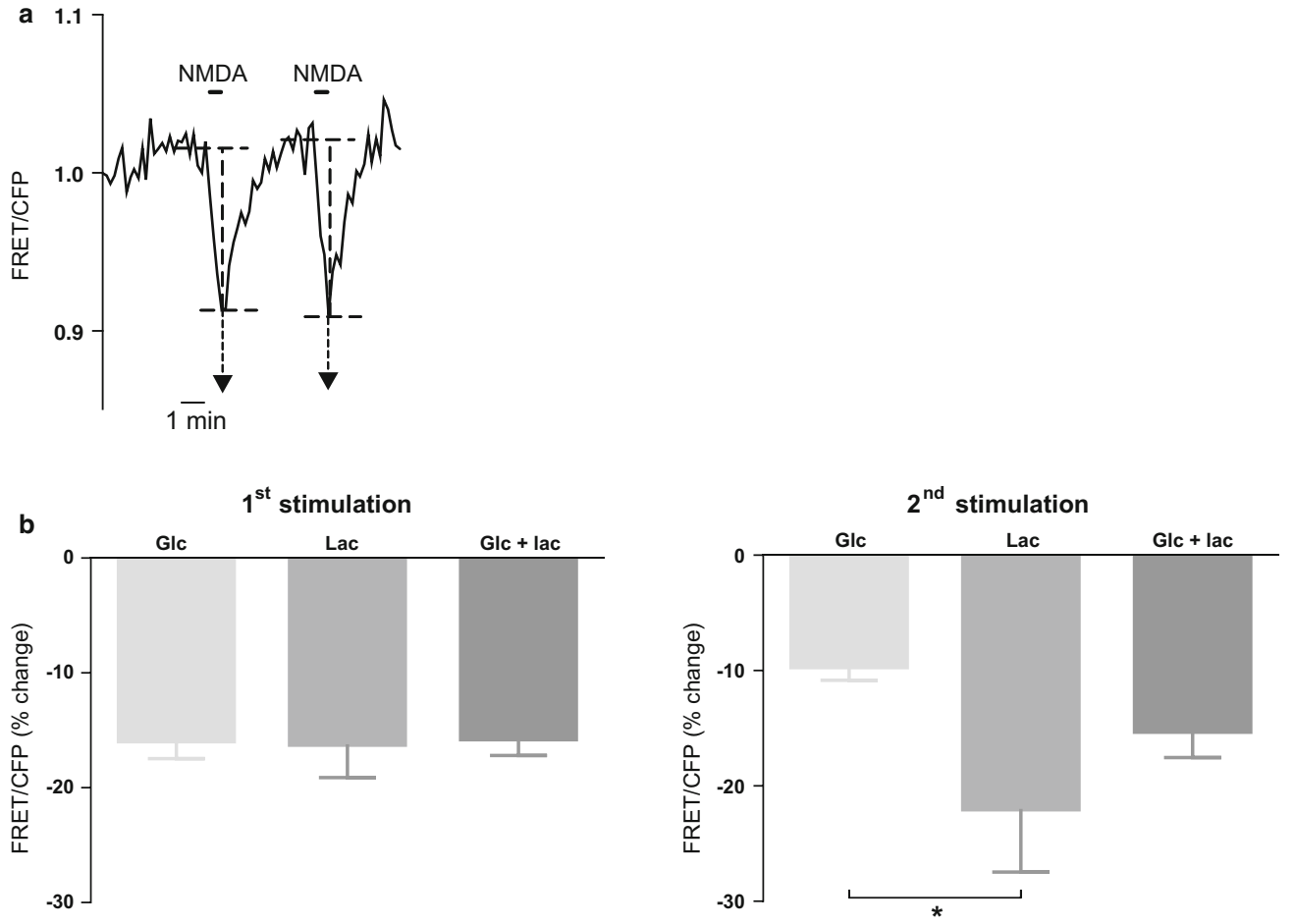

Fig. 2 Glucose sustains cytosolic ATP levels following NMDA-stimulated neurotransmission activity better than lactate. **a** Representative FRET/CFP time course collected from a single neuron expressing Ateam1.03YEMK during continuous superfusion (1 ml/min). The cultures were subjected to two 30 s pulses of NMDA (30 μM; 10 μM L-glycine) with a 4 min interval followed by 10 min in the combined presence of 10 mM azide and 1 mM iodoacetate to inhibit mitochondrial respiration and glycolysis, respectively (not shown here, see Fig. 1). Decreases in cytosolic ATP levels are evident upon addition of NMDA. The *vertical and horizontal dashed lines* indicate the maximal drop in the signal from the biosensor used to calculate the percent decrease in ATP level relative to the signal from the biosensor in the combined presence of azide and iodoacetate (not shown but indicated by the *arrowheads*). **b** Relative quantification of the decreases in cytosolic ATP levels after 30 μM NMDA-induced signaling events show no differences between glucose (2.5 mM; $n = 22$), lactate (1 mM; $n = 10$) and glucose plus lactate ($n = 13$) during the first signaling event. Following the second signaling event glucose ($n = 7$) sustains ATP levels significantly better than lactate alone ($n = 4$; p value 0.0295) and to the same extent as the combination of substrates ($n = 8$); error bars represent SEM and the performed statistics is 1-way ANOVA with Tukey's multiple comparison test

[11]. A 30 s pulse of NMDA similar to the one employed above caused a prolonged increase in the matrix Ca^{2+} level that lasted for several minutes (Fig. 5) suggesting that MAS may indeed be curbed under these experimental conditions.

Discussion

In summary, we have shown that ATP homeostasis during neurotransmission activity in cultured neurons is supported by both glucose and lactate. However, ATP homeostasis seems to be negatively affected by the presence of lactate alone, suggesting that glucose is needed to support neuronal energy metabolism during activation. Further, we have shown that Ca^{2+} homeostasis is supported equally well by both glucose and lactate.

The bulk of neuronal ATP is produced via mitochondrial oxidative phosphorylation fuelled by glucose-derived pyruvate; to what extent this pyruvate is derived from glucose or lactate in the intact brain is still an open question [2, 4]. It should be mentioned in this context, that part of the basis for the alleged superior ability of neurons over astrocytes to oxidize lactate is dissimilar expression of lactate dehydrogenase isoforms with distinct kinetic

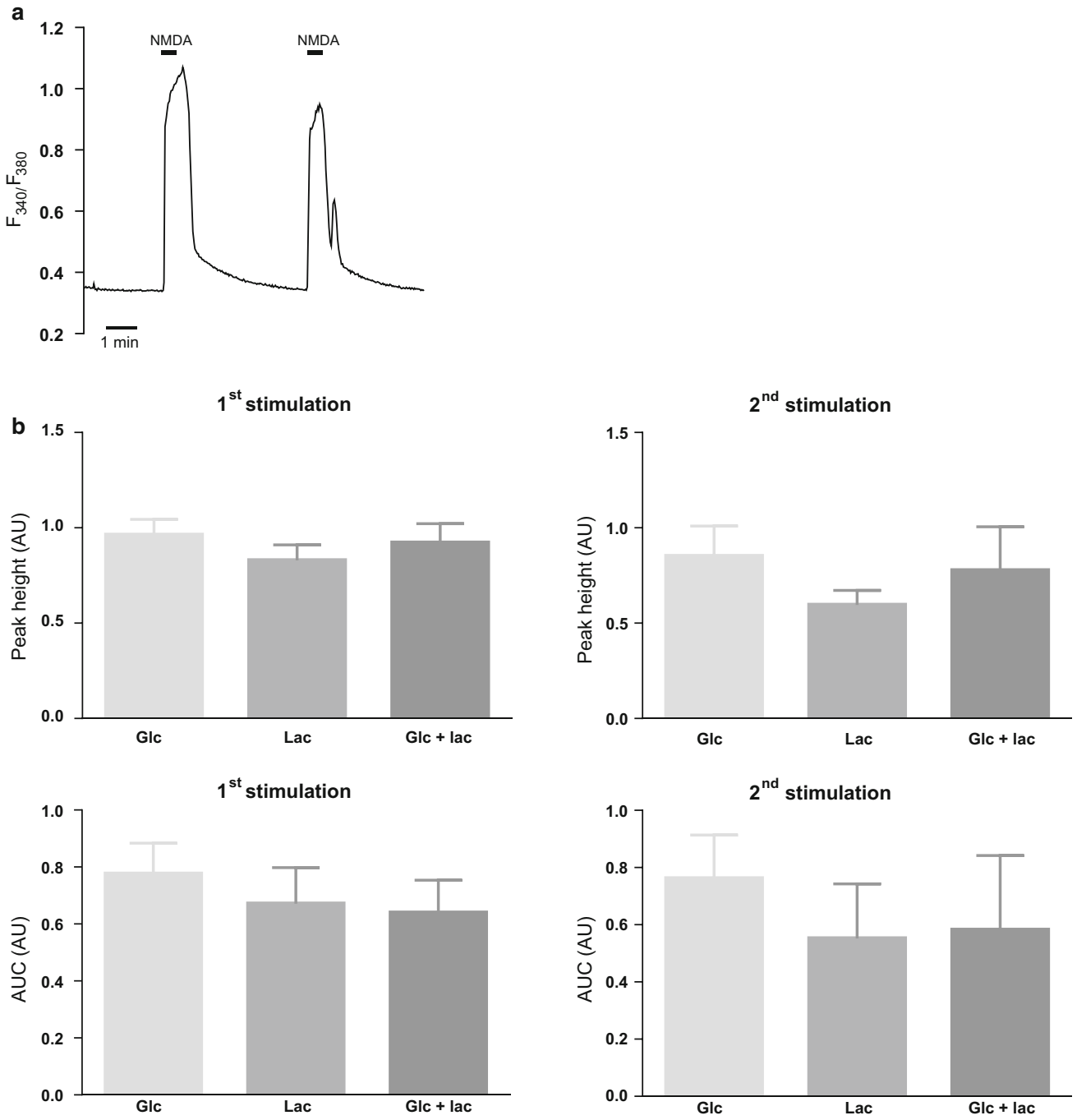

Fig. 3 Calcium increases to an equal extent during NMDA-stimulated neurotransmission activity in the presence of glucose, lactate or a combination of glucose and lactate. **a** Representative calcium trace (Fura-2 ratio 340/380 nm) time course of two 30 s 30 μM NMDA stimulations during continuous superperfusion of CCNs loaded with Fura-2 in the presence of 2.5 mM glucose. **b** Quantification of the peak calcium signals and area under the curve for 2.5 mM glucose, 1 mM lactate and 2.5 mM glucose + 1 mM lactate after first ($n = 12$; 6; 8, respectively, each averaged from 15 to 50 cells) and second ($n = 3$ for all groups, each averaged from 15 to 50 cells) NMDA signaling events. There were no significant differences with ANOVA and Tukey's multiple comparisons test

parameters in astrocytes and neurons [20]; yet, in situ at 37 °C these differences in kinetics, that have been mostly determined in vitro at room temperature, may not be relevant [21]. However, the expression pattern of different MCT isoforms with distinct kinetic properties seem compatible with lactate transfer from astrocytes to neurons [2].

Cultured neurons vividly metabolize both glucose and lactate [8, 18, 22–25]; however, we have found that

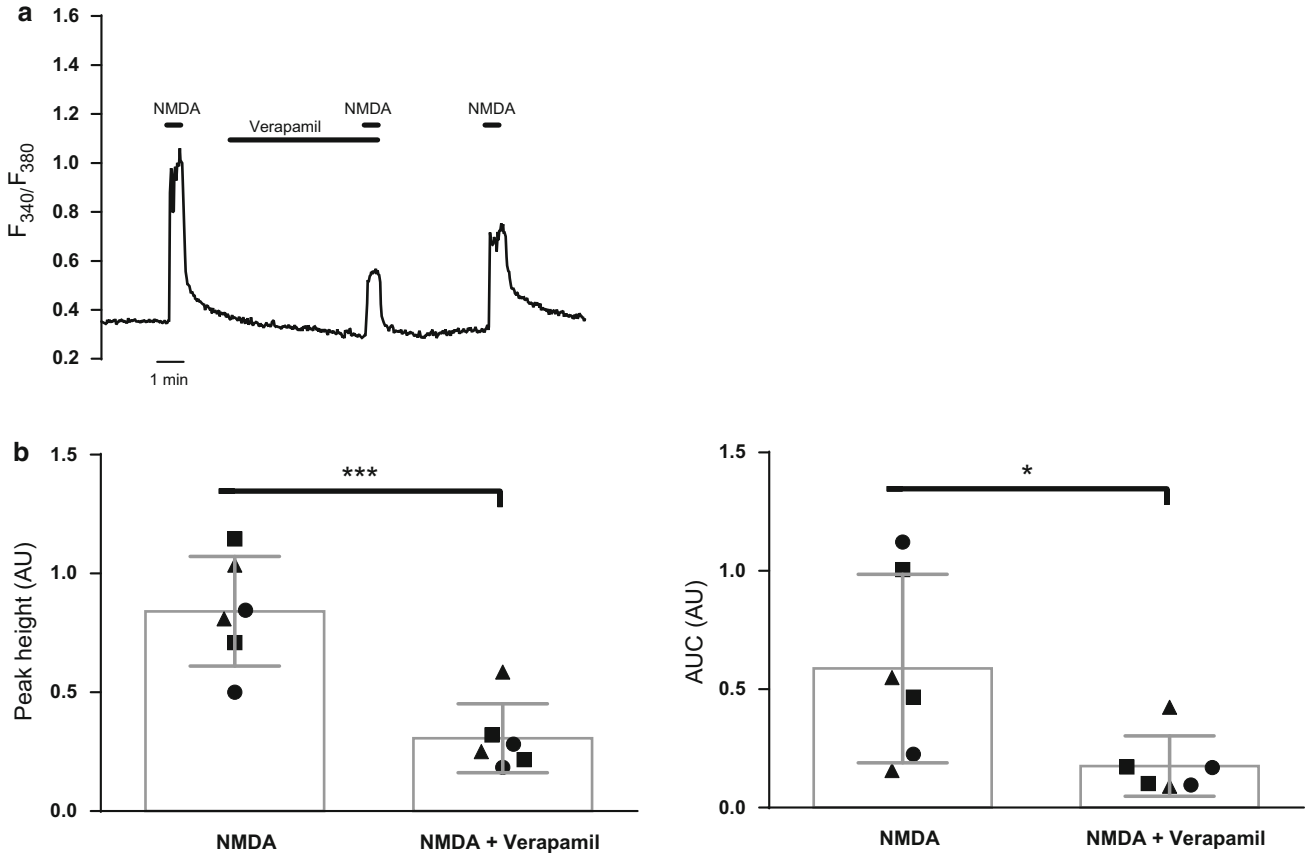

Fig. 4 NMDA-stimulated neurotransmission activity involves calcium ion flux through voltage-gated calcium channels. **a** Raw trace of calcium in Fura-2 loaded CGNs during three 30 µM NMDA stimulations under continuous superperfusion in the presence or absence of 30 µM verapamil. **b** Quantification of initial NMDA stimulation and following verapamil + NMDA stimulation of peak calcium signals ($p = 0.0007$) and area under the curve ($p = 0.0364$). Data are pooled with 2.5 mM glucose (*circle*), 1 mM lactate (*opened square symbol*) or 2.5 mM glucose + 1 mM lactate (*triangle symbol*) as substrates, *error bars* represent SEM and statistics are unpaired *t* test

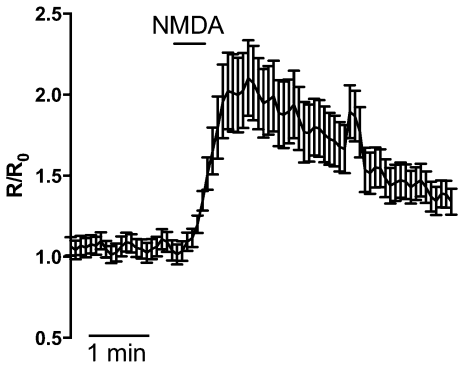

Fig. 5 A 30 s pulse of NMDA induces a prolonged increase in mitochondrial matrix Ca^{2+}. CCNs were transduced employing a lentiviral expression system to express the mitochondrially targeted Ca^{2+} biosensor mito-GEM-GECO1 and stimulated by a 30 s pulse of 30 µM NMDA during continuous superfusion (see "Experimental Procedures"). The figure shows a representative experiment ($n = 3$). The trace shown represents the average response of 21 individual ROIs in a single coverslip ± SEM

glucose, but not lactate, is metabolized to a greater extent when cultured neurons are repetitively depolarized [8, 18]. Further, if exogenous lactate is not supplied in such experiments, cultured neurons release lactate to the extracellular medium following depolarization events [22]. This may be consistent with the opinion presented in a recent review by Hertz et al. [26] in which both neurons and astrocytes release and accumulate lactate during brain activation until a new intra-extracellular equilibrium is reached; lactate may then be dispersed within the astrocytic syncytium and eventually to some extent be discharged from the brain accounting for the observed uncoupling of glucose and oxygen consumption during brain activation [27]. Further, we have previously found that lactate alone is a poor substrate in terms of supporting synaptic transmission, since re-uptake of released neurotransmitter glutamate in cultured glutamatergic neurons fails [8]. In addition, it was recently found that glucose is most

effective (compared to lactate or pyruvate) in supporting fast neuronal network oscillations in organotypic hippocampal slice cultures [28]. To add to this, a recent paper employed a near-infrared 2-deoxyglucose analogue to study glucose metabolism in neurons and astrocytes in vivo [29]; these authors suggest that neurons take up about twice the amount of glucose as astrocytes in behaving mice. However, taking into account that neurons spend some 70–80 % of the ATP produced in the brain [1], that still leaves room for shuttling of energy substrates from astrocytes to neurons such as lactate. Interestingly, these authors link their findings to the preferential expression of hexokinase in neurons [29], a notion compatible with the suggested importance of mitochondrially bound hexokinase for ATP production in neurons, but not astrocytes (e.g. as discussed in ref. [30]). Further, it has been shown that uptake of 2-deoxyglucose in synaptic terminals isolated from rats infused with 2-deoxyglucose is increased following bicuculline-induced seizures [31] suggesting that neurons take up glucose in an activity-dependent manner in vivo. In contrast, using fluorescent glucose analogues it has been suggested that glucose is at least equally well taken up by astrocytes as by neurons and neuronal activity results in increased astroglial uptake [32–34]. Indeed, the study of glucose and lactate metabolism and transport in the brain is difficult, and ridded with technical challenges such as complex interpretation of cellular accumulation of fluorescent glucose analogues, as discussed by DiNuzzo et al. [35]. Finally, it should be noted that lactate might act to modulate neuronal function via the recently characterized G protein-coupled lactate receptor, GPR81 [36]. On this backdrop, it seemed pertinent to study the ability of glucose and lactate as substrates to support neuronal ATP homeostasis specifically during neurotransmission activity.

The data presented here show that both glucose and lactate supports cytosolic ATP homeostasis in cultured glutamatergic neurons, i.e. pyruvate derived from both sources must be oxidized in neuronal mitochondria to produce the ATP needed for neurotransmission activity. On a technical note, it is important to realize that ATP dynamics measured with a reporter of ATP only, do not reveal the dynamics of buffering via phosphocreatine; however, no biosensors are currently available that fulfills this requirement. In addition, the concentrations of substrates were chosen to replicate previous experiments rather than to reflect equicaloric amounts of the two substrates (e.g. ref. [8]). In reality, equicaloric amounts of substrates would only result in similar energy production within the cell if the kinetics of transport and metabolism of glucose and lactate would be identical, which is not the case; glucose and lactate do not share the same transporters nor do they share the same metabolic pathways in terms of producing pyruvate as a substrate for mitochondrial oxidative metabolism [2]. Finally, one has to bear in mind that the cells are continuously superfused with fresh medium, thus the availability of substrates remains the same throughout the experiment and the vast majority of the glucose and lactate will be flowing past the cells.

The finding that lactate when present alone does not support ATP homeostasis following the 2nd pulse of NMDA to the same degree as glucose, suggests that differences in metabolism of these substrates are present. This may be consistent with the model presented by Bak et al. [18], and independently supported by Conteras et al. [19] that the MAS is curbed during peak neurotransmission activity. Briefly, when cytosolic Ca^{2+} levels increase sufficiently to surpass the threshold of the inwardly directed flow of Ca^{2+} into the mitochondrial matrix, MAS activity is curbed since the outward transport of α-ketoglutarate is limited due to substrate competition between the transport system and the now activated α-ketoglutarate dehydrogenase complex [18]. We show here that a pulse of NMDA causes a prolonged increase in mitochondrial matrix Ca^{2+} (Fig. 5), thus it is likely that MAS activity is curbed during these experimental conditions. If this is the case, why may lactate be a poorer substrate than glucose or glucose plus lactate? Most likely, during depolarization when the NADH/NAD^+ ratio is high (i.e. MAS is curbed), lactate metabolism via lactate dehydrogenase yielding pyruvate is limited whereas anaerobic glycolysis (if glucose is present) is prevailing, as the high NADH/NAD^+-redox ratio favors the conversion of pyruvate to lactate [18]. Following the depolarization event, full MAS activity returns, the cytosolic NADH/NAD^+-redox ratio drops again and both glucose and lactate may be metabolized [18]. This of course requires a very fast switching between the two redox states, and whether this is possible remains to be established. Alternatively, the mitochondrial matrix Ca^{2+} level stays high during high frequency neuronal firing thus curbing MAS activity under these conditions. The latter possibility also remains to be explored, and is not reflected in the experimental protocol employed here subjecting the cultures to 30 s pulses of NMDA. As an additional aspect, compartmentalized signaling-metabolism coupling may play a role as well. Thus, we have suggested that two distinct pools of mitochondria exist in (post-synaptic) neurons; one that is metabolizing pyruvate derived from lactate at a steady pace, and one that is metabolizing glucose-derived pyruvate, with only the latter pool of mitochondria being sensitive to activation by cytosolic-mitochondrial Ca^{2+} signaling [22]. The pool of mitochondria metabolizing pyruvate derived from lactate may at least to some extent be fuelled by glucose-derived pyruvate [22] supporting the present finding that glucose alone is sufficient to support neuronal ATP homeostasis.

Conclusion

In conclusion we suggest that glucose is both needed and sufficient to sustain neuronal ATP homeostasis, and that lactate when present as the only substrate may not be able to fully support ATP production. The latter situation is unlikely to occur in vivo under normal physiological function of the brain and could explain why the brain may be dispersing the lactate produced during brain activation [27].

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