

Glycerol-3-Phosphate Shuttle Is a Backup System Securing Metabolic Flexibility in Neurons

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Electrical activity in neurons is highly energy demanding and accompanied by rises in cytosolic Ca^{2+} . Cytosolic Ca^{2+} , in turn, secures energy supply by pushing mitochondrial metabolism either through augmented NADH (nicotinamide adenine dinucleotide) transfer into mitochondria via the malate–aspartate shuttle (MAS) or via direct activation of dehydrogenases of the TCA cycle after passing into the matrix through the mitochondrial Ca^{2+} uniporter (MCU). Another Ca^{2+} -sensitive booster of mitochondrial ATP synthesis is the glycerol-3-phosphate shuttle (G3PS), whose role in neuronal energy supply has remained elusive. Essential components of G3PS are expressed in hippocampal neurons. Single neuron metabolic measurements in primary hippocampal cultures derived from rat pups of either sex reveal only moderate, if any, constitutive activity of G3PS. However, during electrical activity neurons fully rely on G3PS when MAS and MCU are unavailable. Under these conditions, G3PS is required for appropriate action potential firing. Accordingly, G3PS safeguards metabolic flexibility of neurons to cope with energy demands of electrical signaling.

Key words: ATP; calcium; glycerol-3-phosphate; metabolism; mitochondria; shuttle

Significance Statement

Ca^{2+} ions are known to provide a link between the energy-demanding electrical activity and an adequate ATP supply in neurons. To do so, Ca^{2+} acts both from outside and inside of the mitochondrial inner membrane. Neuronal function critically depends on this regulation, and its defects are often found in various neurologic disorders. Although interest in neuronal metabolism has increased, many aspects thereof have remained unresolved. In particular, a Ca^{2+} -sensitive NADH (nicotinamide adenine dinucleotide) shuttling system, the glycerol-3-phosphate shuttle, has been largely ignored with respect to its function in neurons. Our results demonstrate that this shuttle is functional in hippocampal neurons and safeguards ATP supply and appropriate action potential firing when malate aspartate shuttle and mitochondrial Ca^{2+} uniporter are unavailable, thereby ensuring neuronal metabolic flexibility.

Introduction

Electrical activity because of postsynaptic potentials and action potential firing receives the highest share in energy consumption in neurons. In addition to the ATP demand itself, the major link between these electric signals and neuronal ATP supply is provided by Ca^{2+} ions (Attwell and Laughlin, 2001; Harris et al., 2012). Activity-dependent rises in cytosolic Ca^{2+} may stimulate neuronal metabolism by the following two main mechanisms:

(1) boosting activity of the glutamate/aspartate exchanger from outside of the mitochondrial inner membrane (Aralar/AGC1) which drives the malate–aspartate shuttle (MAS; Llorente-Folch et al., 2015); and (2) influx into mitochondria and ensuing activation of dehydrogenases of the TCA cycle (Denton, 2009).

The relative importance of one or the other of these Ca^{2+} -sensitive bioenergetic pathways has remained a matter of debate. While some authors favored a role of mitochondrial Ca^{2+} influx (Griffiths and Rutter, 2009; Bas-Orth et al., 2020; Díaz-García et al., 2021), others endorsed cytosolic Ca^{2+} as prime regulator (Szibor et al., 2020; Pérez-Liébana et al., 2022). The latter view is supported by the observation that mice lacking mitochondrial calcium uniporter (MCUs) developed a surprisingly mild phenotype (Pan et al., 2013). Ca^{2+} -dependent fine-tuning of mitochondrial metabolism is further enriched by the availability of a glycerol-3-phosphate shuttle (G3PS) system (Mráček et al., 2013; Liu et al., 2021). This shuttle relies on cytosolic glycerol-3-phosphate dehydrogenase (GPD1) that oxidizes nicotinamide adenine dinucleotide (NADH) by converting the glycolysis intermediate

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dihydroxyacetone phosphate (DHAP) to G3P. Subsequently, mitochondrial GPD2 reconverts G3P to DHAP and transfers electrons in a flavin adenine dinucleotide (FAD)-dependent manner to coenzyme Q to feed the electron transfer chain (ETC) via complex III (McKenna et al., 2006; Liu et al., 2021).

Expression of cytosolic GPD1 as well as mitochondrial GPD2 has been reported for the brain (Lovatt et al., 2007; Cahoy et al., 2008; Pardo and Contreras, 2012; Liu et al., 2021). In particular, neurons were reported to express these two enzymes at similar levels as astrocytes (Lovatt et al., 2007), and comparably high expression levels were detected in substantia nigra, hippocampus, and cerebellum, but less so in cortical areas (Liu et al., 2021). In any case, the relevance of G3PS has been questioned, for the brain in general (McKenna et al., 2006; Pardo and Contreras, 2012) and for cortical neurons in particular (Liu et al., 2021). As a consequence, the contribution of G3PS to the metabolic adaptation of neurons to electrical activity has remained unexplored, despite the fact that the transfer of electrons to complex III of ETC through this bioenergetic pathway is known to be regulated by Ca^{2+} (Mráček et al., 2013).

To assess whether at all and, if so, how G3PS might contribute to bioenergetic responses of neurons to electrical activity, we used hippocampal neurons in primary cell culture that were shown to have G3PS available (Martano et al., 2016). Single-cell metabolic measurements were combined with electrical field stimulation (EFS), as the latter triggers neuronal responses relying on synaptic transmission as well as action potential firing and thus represents quasisiological means of activation (Mermelstein et al., 2000; Virdee et al., 2017). The results reveal that G3PS operates in central neurons, although activity-dependent energetic needs are rather met by MAS and intramitochondrial actions of Ca^{2+} on the TCA cycle. Nevertheless, when these latter systems fail to supply ATP, G3PS can compensate for their unavailability.

Materials and Methods

Primary cell culture of hippocampal neurons. Pregnant Sprague Dawley rats were provided by the Department of Biomedical Research, Division for Laboratory Animal Science and Genetics, Medical University of Vienna, Himmerberg, Austria. Neonatal animals of either sex were killed by decapitation in full accordance with all rules of the Austrian animal protection law (see http://www.ris.bka.gv.at/Dokumente/BgblAuth/BGBLA_2012_I_114/BGBLA_2012_I_114.pdf) and the Austrian animal experiment by-laws (see http://www.ris.bka.gv.at/Dokumente/BgblAuth/BGBLA_2012_II_522/BGBLA_2012_II_522.pdf) which implement European (DIRECTIVE 2010/63/EU; see <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF>) into Austrian law (all information was accessed on November 19, 2018). The responsible animal welfare body is the Ethics Committee of the Medical University of Vienna for Research Projects Involving Animals. Brains were removed to dissect the hippocampi in ice-cold buffer. Primary cocultures of hippocampal neurons and glial cells were prepared after enzymatic digestion of the tissue with papain and mechanical dissociation with Pasteur pipettes (trituration) as described previously (Hotka et al., 2020). Neurons were cultured for at least 12 d at 37°C and 5% CO_2 in DMEM-high glucose (20 mM glucose; D5796, Sigma-Aldrich) supplemented with 10% gamma-irradiated fetal bovine serum (catalog #S 0415, Biochrom). For the subset of experiments seen in Figure 7, neurons were cultured for 14 d at 37°C in an atmosphere composed of 2% O_2 , 5% CO_2 , and 93% N_2 . Neurons were kept in 2 mM glucose.

Drugs and chemicals. Final concentrations used in experiments are indicated in parentheses preceding the compounds: (0.01%) dimethylsulfoxide (DMSO; catalog #D2650); (30 μM) glutamate (catalog #G1251); (1 μM) isradipine (catalog #I6658); (50 μM) tolbutamide

(tolbu; catalog #T0891); (300 μM) diazoxide (diaz; catalog #D9035); (1 μM) oligomycin (catalog #O4876); (10 μM) rotenone (rot; catalog #R8875); (1 μM) 3-nitropropionic acid (3NP; catalog #N5636); (1 μM) antimycin A (AA; catalog #A8674); (10 μM) Ru360 (catalog #557440); (100 μM) aminooxyacetate [O-(carboxymethyl)hydroxylamine hemihydrochloride (AOA); catalog #C13408]; (20 nM) GSK-2837808A (referred to as “GSK”; catalog #5.33660); (100 μM) N-(4-(1H-benzimidazol-2-yl)-phenyl)-succinamic acid (iGP-1), an inhibitor of mitochondrial GPDH 1 (catalog #5.30655); (100 nM and 10 μM) carbonylcyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; catalog #C2920); (2 mM) 2-deoxy-D-glucose (2DG; catalog #D8375); (10 μM) 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; catalog #C127); (2 mM) sodium pyruvate (catalog #P8574); (10 mM) sodium L-lactate (catalog #71718); (30 μM) BAPTA-AM (catalog #A1076); and bulk chemicals were purchased from Sigma-Aldrich (catalog numbers in parentheses). Isradipine was dissolved from 10 mM stock solutions in DMSO to a final concentration of 1 μM in aqueous buffer. Hence, 0.01% DMSO was also added to the respective control solutions. For some experiments, cells were pretreated in the incubator (37°C/5% CO_2) for 15 min (BAPTA-AM, AOA + pyruvate) and 10 min (Ru360), respectively. Except for BAPTA-AM, compounds were then also present at the same concentrations in the external solutions used for superfusion of the cells in imaging experiments.

Transfections. Transfections were performed using Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer instructions with some modifications. In brief, neuronal cultures >12 d *in vitro* were incubated in 500 μl of antibiotic-free medium, containing 3 μl of Lipofectamine and 1 μg of plasmid DNA, for 3 h. The transfection medium was replaced with original medium, and cultures were kept for 3 more days in the incubator. Experiments were performed on day 3 after transfection.

Electric field stimulation. Field stimulation was performed using two platinum electrodes of 1 mm diameter, placed in the culture dishes 10 mm apart. External voltage of 20 V amplitude and 1.5 ms duration (if not stated otherwise) was applied using a stimulator (model S44, Grass Medical Instruments) that allowed for the generation of single pulses and also trains of pulses at desired frequencies. Measurements were performed on neurons positioned in the center between the two electrodes.

Imaging. For imaging purposes, neurons and glial cells were cocultured in glass bottom dishes (model P35GC-1.5–14-C, MatTek). Before each experiment, the culturing medium was replaced by an external solution composed of the following (in mM): 140 NaCl, three KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES, 20 glucose, with pH adjusted to 7.4 by NaOH. Experiments were performed at room temperature, and cells were superfused continuously using an eight reservoir drug application system (Octaflo II) with a micromanifold that held eight channels converging into a 100- μm -diameter quartz outlet. Neurons were imaged using a confocal microscope (model A1R, Nikon) equipped with a focus clamp.

The ATP/ADP ratio was determined in neurons expressing the genetically encoded fluorescence sensor PercevalHR (referred to as “Perceval”), with excitation at wavelengths of 403 and 488 nm, while the emission was detected at 525 nm. The quotient F_{488}/F_{403} reflects the intraneuronal ATP/ADP ratio. GW1-PercevalHR was a gift from Gery Yellen, Department of Neurobiology, Harvard Medical School, Boston, USA (plasmid #49082, Addgene).

Cytosolic pH was monitored using the genetically encoded indicator GW1-pHRed (pHRed; plasmid #31473, Addgene), with excitation/emission wavelengths of 562/595 nm. GW1-pHRed was a gift from Gary Yellen (plasmid #31473, Addgene).

The NADH/NAD⁺ ratio was assessed in neurons expressing the genetically encoded fluorescence indicator Peredox, with excitation at wavelengths of 403 and 561 nm, while the emitted fluorescence was detected at 595 nm. The quotient F_{561}/F_{403} reflects the intraneuronal NADH/NAD⁺ ratio. pcDNA3.1-Peredox-mCherry was a gift from Gary Yellen (plasmid #32383, Addgene). Before the experiments, the performance of Peredox was evaluated under our experimental conditions. Peredox was tested by using the reaction mediated by lactate dehydrogenase (LDH) in the course of pyruvate or lactate application.

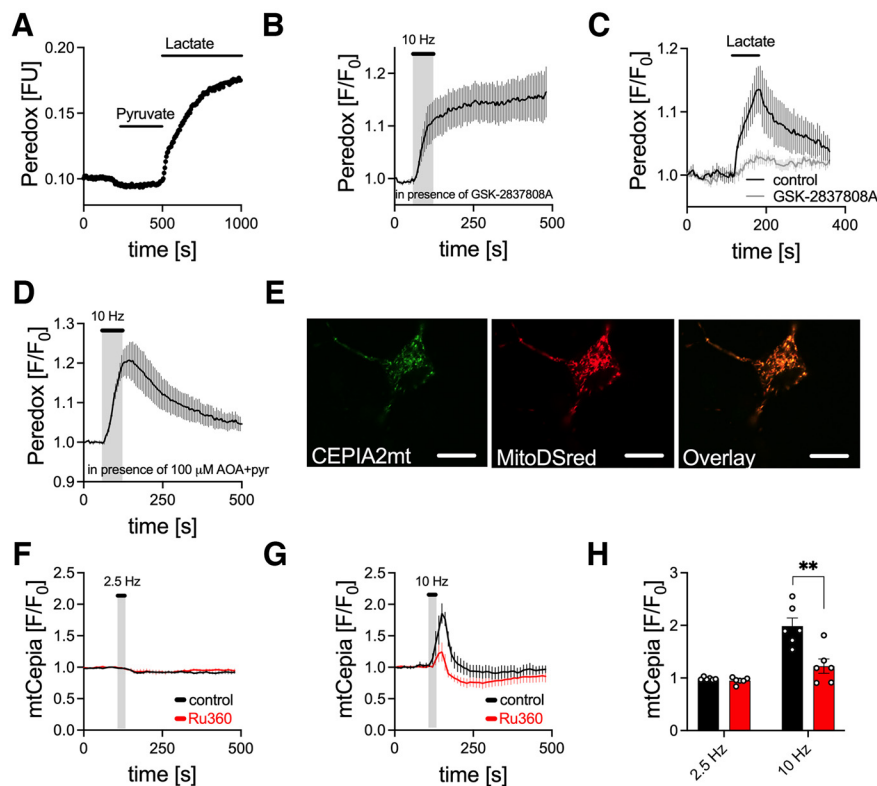


Figure 1. Characterization of Peredox and CEPIA2mt responses in hippocampal neurons. Cytosolic NADH/NAD⁺ ratio and mitochondrial Ca²⁺ were determined in somata of single hippocampal neurons by fluorescence signals of Peredox and CEPIA2mt, respectively. EFS (rectangular 1.5 ms pulses of 20 V for 40 s) was applied at 2.5 and 10 Hz, as indicated. For each neuron, all fluorescence values over time were normalized to the last value before EFS or drug application. **A**, Time course of fluorescence values with 10 mM pyruvate and 10 mM lactate being present as indicated (representative of three independent experiments). **B**, Time course of normalized fluorescence values in the presence of 20 nM GSK-2837808A ($n = 5$ neurons from two independent preparations). **C**, Time course of normalized fluorescence values under control conditions (black trace, $n = 5$ neurons) and in the presence of GSK-2837808A (gray trace, $n = 4$ neurons). Cultures were obtained from two independent preparations. **D**, Time course of normalized fluorescence values in the presence of 100 μ M AOA and 2 mM pyruvate ($n = 4$ neurons from two independent preparations). **E**, Original micrographs showing the localization of the mitochondrial Ca²⁺ indicator CEPIA2mt (left), the mitochondrially targeted red fluorescent protein mitoDSred (middle), and their overlay (right). Scale bars, 15 μ m. **F**, **G**, Time course of normalized CEPIA2mt fluorescence intensities under control conditions (black traces) and in the presence of 10 μ M Ru360 (red traces, $n = 6$ neurons from two independent preparations). **H**, Peak CEPIA2mt fluorescence intensity values during 2.5 Hz (**F**) and 10 Hz (**G**) EFS, respectively. ** $p < 0.01$ (unpaired t test).

As expected, the application of pyruvate (10 mM) decreased the NADH/NAD⁺ ratio, while the application of lactate (10 mM) led to an increase (Fig. 1A). Next, the effect of EFS on the NADH/NAD⁺ ratio was tested. Brief application of a 10 Hz pulse sharply increased the NADH/NAD⁺ ratio (see Fig. 4C). This transient increase in NADH/NAD⁺ ratio has been shown to reflect increased activity of neuronal glycolysis (Díaz-García et al., 2017). Given the controversy as to whether neurons can increase their own glycolytic activity or whether their metabolism is more dependent on lactate shuttling from astrocytes (Bak and Walls, 2018), and given that such differences may depend on the experimental model, we verified whether Peredox could be used to monitor glycolytic activity in our model system. The EFS-induced increase in Peredox fluorescence was not abolished by the LDH blocker GSK-2837808A (Fig. 1B), while GSK abolished the NADH increase seen on lactate application (Fig. 1C). Therefore, the EFS-induced increase in cytosolic NADH/NAD⁺ ratio does not require lactate import from extracellular space. Since the effect of GSK is mostly visible in the declining phase of the EFS-induced NADH/NAD⁺ transient (compare Figs. 1B, 4C), neurons appear to act as lactate exporters under our conditions. The stimulation-induced rise in NADH was not diminished by aminooxyacetic acid (AOA), an inhibitor of the MAS enzyme aspartate transaminase, which was applied along with pyruvate (AOA + pyr; Fig. 1D). Therefore, the transient

increase in Peredox fluorescence is not caused by a reverse activity of MAS. From these results, we conclude that the sensor reliably reports cytosolic NADH/NAD⁺ changes that reflect glycolytic activity in cultured neurons.

Intracellular Ca²⁺ was measured using Fluo-4 AM (catalog #F14201, Thermo Fisher Scientific). Neurons were incubated for 15 min at 37°C in 1 μ M Fluo-4 AM-containing buffer solution followed by a washout with extracellular solution. Neurons were excited by 488 nm, and emission was detected at 525 nm.

Mitochondrial Ca²⁺ was measured using the genetically encoded fluorescence sensor CEPIA2mt. The expression vector pCMV CEPIA2mt was a gift from Masamitsu Iino (plasmid #58218, Addgene). Excitation/emission wavelengths for experiments with CEPIA2mt were 488/525 nm. Localization of the mitochondrial Ca²⁺ indicator was verified using the genetically encoded fluorescent indicator “mito-DsRed” (Fig. 1E). The expression vector mitochondrial pDsRed2 (catalog #632421) was from Clontech. Excitation/emission wavelengths for experiments with mito-DsRed were 562/595 nm.

To measure mitochondrial membrane potential (Ψ_{mt}) with tetramethylrhodamine methyl ester (TMRM; catalog #T668, Thermo Fisher Scientific), cultures were equilibrated for 1 h in 1 nM TMRM at 37°C. The incubation period was not followed by a washout, and the dye was present in all perfusing solutions. Neurons were excited at 562 nm, and emission was detected at 595 nm. The terms hyperpolarization and depolarization of Ψ_{mt} were defined as any increase or decrease of the TMRM fluorescence relative to the value before the application of test solutions or compounds.

For the experiments seen in Figure 7, neurons were imaged at 37°C in a heated stage top incubator (Ibidi) filled with 2% O₂, 5% CO₂, and 93% N₂. Before each experiment, the culture medium was replaced by external solution composed of the following (in mM): 140 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, and 2 glucose, with pH adjusted to 7.4 by NaOH.

Electrophysiology experiments. Current-clamp measurements were performed using a Multiclamp 700B amplifier (Molecular Devices) and the Clampex 10.5 software, which is part of the pCLAMP 10 electrophysiology data acquisition and analysis software package (Molecular Devices). Signals were low-pass filtered at 10 kHz and were digitized with a digitizer (model Digidata 1440A, Molecular Devices) at a sampling rate of 20 kHz. Patch pipettes were made of borosilicate capillaries (GB150-8P, Science Products) with a horizontal puller (model P97, Sutter Instrument). Tip resistances were between 2 and 4 M Ω . The pipette solution was composed of the following (in mM): 120 potassium gluconate, 1.5 sodium gluconate, 3.5 NaCl, 1.5 CaCl₂, 0.25 MgCl₂, 10 HEPES, and 5 EGTA, with pH adjusted to 7.3 by KOH. All recordings were made in perforated patch mode using backfilling of the pipettes with amphotericin B, which was added to the pipette solution just before experiments. The pipette tip had been filled by capillary force with amphotericin B-free solution. Experiments were started only when the series resistance had dropped to the lowest achievable level (between 20 and 30 M Ω), which usually required ≥ 15 min. Experiments were performed at room temperature (22–24°C), and cells were superfused continuously using a drug application system (DAD-12 Drug Application System, ALA Scientific Instruments) with a micromanifold that held 12 channels converging into a 100- μ m-

diameter quartz outlet. The tip of the outlet was positioned in close proximity ($\sim 250\ \mu\text{m}$) to the patch-clamped cell. The external solution was composed of the following (in mM): 140 NaCl, three KCl, 2 CaCl_2 , 2 MgCl_2 , 10 HEPES, and 20 glucose, with pH adjusted to 7.4 by NaOH. To suppress synaptic activity during current injections, a cocktail of synaptic blockers (10 μM bicuculline, 10 μM CNQX, 50 μM AP5) was added to the external solution. Test compounds in external solution were applied from reservoirs connected to 1 of the 12 micromanifold channels.

Data analysis. In all imaging experiments, fluorescence intensity was calculated as the mean intensity from a defined region of interest positioned at neuronal somata. Neurons were taken into analysis regardless of their neurotransmitter phenotype. All fluorescence data measured in time were normalized to unity by dividing the whole trace by a representative control value obtained just before stimulation and drug application, respectively. Averaged normalized data traces are displayed in the figures together with SEMs.

In patch-clamp experiments, numbers of action potentials fired during 2 s current injections were analyzed. In addition, resting membrane potential was determined before current injection.

Statistics. GraphPad Prism version 9.1.2 was used to prepare graphs and to perform statistical analyses subsequent to testing for normal distribution of the data. *N* values represent the numbers of individual neurons in imaging as well as electrophysiological experiments. An unpaired *t* test was used for comparison of two conditions, while a one-way ANOVA with Tukey's multiple-comparisons test was used whenever three or more experimental conditions were compared. For comparison of neuronal NADH/NAD⁺ ratio signals seen in Figure 8, two-way ANOVA was used. For paired comparisons of responses before and after treatment, a repeated-measures one-way ANOVA with Geisser–Greenhouse correction followed by Tukey's multiple-comparisons test was used. One, two, and three asterisks indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Statistically nonsignificant differences (p values ≥ 0.05) are not labeled.

The data used in this study were all obtained from at least two independent cell culture preparations. Each preparation contained neurons derived from hippocampi of 2 female and 2 male rat pups. The *n* numbers in figure legends represent the numbers of individual neurons. To avoid cross-contamination from the perfusion of cells, only one experiment was performed per culture dish. In experiments in transfected neurons, the field of view contained one neuron only; therefore, *n* numbers also represent the numbers of independent experiments with respect to culture dishes. In culture dishes exposed to fluorescent dyes, multiple neurons were imaged per one field of view, and the total number of neurons was derived from at least three independent preparations.

Results

G3PS is active in unstimulated primary hippocampal neurons

To maintain adequate ATP production, neuronal mitochondria may use both cytosolic and matrix-made NADH to fuel their respiratory activity. Cytosolic NADH can enter the mitochondrial matrix via two main redox shuttling systems, MAS and G3PS. To assess the activity of these redox shuttling systems, cytosolic NADH/NAD⁺ was monitored using the genetically encoded ratiometric indicator Peredox (Hung et al., 2011).

The involvement of MAS in neuronal metabolism can be studied by either pharmacological or genetic means (Llorente-Folch et al., 2013; Díaz-García et al., 2017; Szibor et al., 2020). Advantages of pharmacological inhibition over genetic silencing include the speed of action and the possibility of pre versus post comparisons. The inhibition of MAS by 100 μM AOA can be expected to prevent NADH translocation into mitochondria, thereby leading to a rise of NADH in cytosol. Within 15 min of AOA exposure, the cytosolic NADH/NAD⁺ ratio rose by 50%

compared with baseline (Fig. 2A); this confirms high MAS activity in neurons (Díaz-García et al., 2017). To quantify the activity of G3PS, neurons were exposed to iGP-1, a specific membrane-permeant inhibitor of mitochondrial, but not cytosolic, GPD (Orr et al., 2014). This resulted in a much slower rise in cytosolic NADH/NAD⁺ ratio (Fig. 2A). The rate of increase in Peredox fluorescence in iGP-1 was not different from that under control conditions and was significantly smaller than that in AOA (Fig. 2B), while the baseline values were comparable under all conditions (Fig. 2C). Hence, if at all, G3PS displays far less constitutive activity than MAS.

High cytosolic NADH/NAD⁺ ratio may impede glycolysis, which starves mitochondria from pyruvate and interferes with the entire neuronal sugar metabolism (Gellerich et al., 2010). However, the cytosolic NADH/NAD⁺ ratio can be recovered by coapplication of pyruvate (Díaz-García et al., 2017). In the presence of 100 μM AOA plus 2 mM pyruvate (AOA + pyr), the NADH/NAD⁺ ratio was comparable to that measured before AOA application (Fig. 2D,E).

Previously, 1–5 mM AOA has been used to block MAS in cells in culture (McKenna et al., 2006). Therefore, these higher concentrations have been tested as well and rapidly led to the saturation of the NADH/NAD⁺ ratio indicator Peredox (Fig. 2D). Moreover, with these higher AOA concentrations the addition of pyruvate was not able to bring the NADH/NAD⁺ ratio back to baseline levels. Accordingly, unequivocal interpretation of results obtained in 1 or 5 mM AOA plus 2 mM pyruvate would be impossible. Therefore, in subsequent experiments neurons were always incubated in 100 μM AOA plus pyruvate to enable continuous glycolysis when assessing the role of MAS. Of course, a potentially confounding residual MAS activity must be considered when interpreting these results.

As there was negligible G3PS activity while MAS was available, we investigated potential interactions between the two shuttle systems. Nevertheless, in neurons in which MAS was inhibited continuously (AOA plus pyruvate), the NADH/NAD⁺ response to iGP remained marginal (Fig. 2F,G), suggesting that the activity of G3PS is not increased by MAS inhibition. Likewise, in neurons with MCU being blocked by the selective membrane permeant inhibitor Ru360 (10 μM ; Matlib et al., 1998), the increase in Peredox fluorescence over time in response to iGP-1 remained negligible (Fig. 2F,G). In separate experiments, it was confirmed that the incubation of neurons in 10 μM Ru360 for 10 min largely reduced the rise in mitochondrial Ca^{2+} induced by 10 Hz EFS (Fig. 1F–H). To complete these interaction experiments, MAS and MCU were blocked at the same time. Subsequent addition of iGP led to a rapid increase in cytosolic NADH/NAD⁺ ratio that reached a plateau within 3 min (Fig. 2F,G), thus indicating that G3PS took over to support mitochondrial respiration when MAS and MCU activities were suppressed. The plateau is most likely caused by two neurons in which iGP application led to Peredox saturation. For easier comparison, traces in Figure 2F show normalized values only. Nevertheless, absolute baseline values obtained before the application of iGP did not reveal significant differences between neurons with MAS, MCU, or both being blocked for 10 min (Fig. 2H).

Activity-induced mitochondrial ATP production requires Ca^{2+}

All results shown above have been obtained in neurons under “resting conditions” (i.e., in the absence of any trigger of electrical activity). In this respect, one has to bear in mind that

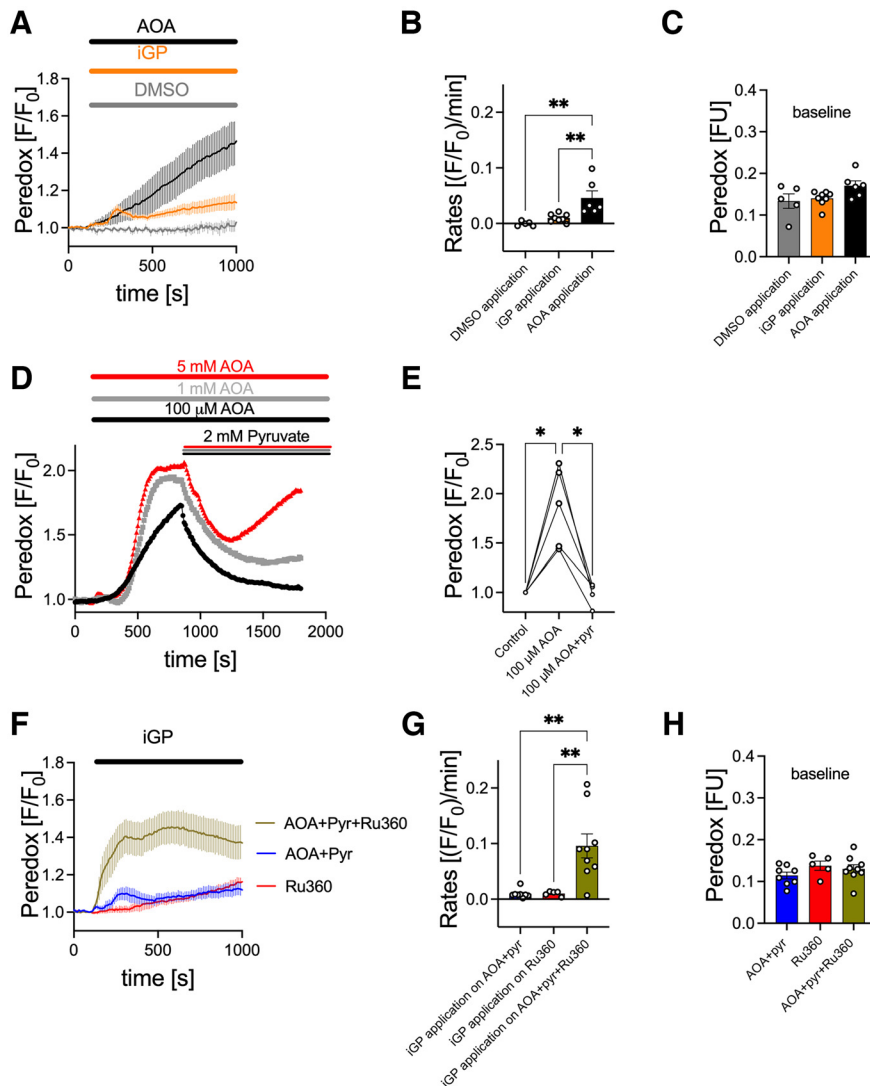


Figure 2. Activities of cytosolic NADH shuttling mechanisms in unstimulated neurons. The cytosolic NADH/NAD⁺ ratio was measured by the Peredox fluorescence intensity ratio obtained at excitation wavelengths of 403 and 561 nm in somata of single hippocampal neurons. For each neuron, all fluorescence intensity ratio values over time were normalized to the last value before drug or solvent application. **A**, One hundred micromolar AOA (black trace, *n* = 6 neurons), 100 μM iGP (orange trace, *n* = 8 neurons), or 0.01% DMSO (gray trace, *n* = 5 neurons) were present as indicated. Cultures were obtained from two to three independent preparations. **B**, Rates of changes in fluorescence intensity ratio as shown in **A** were analyzed by linear regression from the beginning of drug or solvent application to second 1000. **C**, Comparison of non-normalized baseline fluorescence intensity ratio values obtained before drug or solvent application, as shown in **A**. **D**, Time course of the Peredox fluorescence intensity ratio in single neurons in the presence of 100 μM (black trace), 1 mM (gray trace), and 5 mM (red trace) AOA, and 2 mM pyruvate, which were applied as indicated. **E**, Peredox fluorescence intensity ratio determined before and during the presence of 100 μM AOA as well as after the addition of pyruvate (*n* = 5 neurons from two independent preparations). Drugs were applied as shown in **D**. **p* < 0.05 (repeated measures one-way ANOVA with Geisser–Greenhouse correction followed by Tukey’s multiple-comparisons test). **F**, One hundred micromolar iGP was present as indicated in combination with 100 μM AOA plus 2 mM pyruvate (AOA + pyr; blue trace, *n* = 8 neurons), 10 μM Ru360 (red trace, *n* = 5 neurons), or AOA + pyruvate + Ru360 (green trace, *n* = 9 neurons); neurons had been preincubated in these latter drugs for 10 min before the application of iGP. Cultures were obtained from two to three independent preparations. **G**, Rates of changes in fluorescence intensity ratio, as shown in **F**, were analyzed by linear regression from the beginning of iGP application to the end of the apparently linear regions. **H**, Comparison of non-normalized baseline fluorescence intensity ratio values obtained before iGP application, as shown in **F**. **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 (one-way ANOVA followed by Tukey’s multiple-comparisons test).

hippocampal neurons in primary culture form dense networks with functional synaptic connections that display considerable endogenous activity (mean ± SEM firing frequency = 0.336 ± 0.082 Hz; *n* = 17). Inevitably, this introduces a substantial degree of variability. Therefore, all additional experiments were performed in neurons exposed to EFS. Electrical fields were generated via two platinum electrodes, 10 mm apart and 1 mm

above the neuron/glia monolayers. To monitor Ca²⁺ responses, cells were loaded with the cytosolic Ca²⁺ indicator Fluo-4 AM. A single rectangular 1.5 ms pulse of 20 V amplitude led to a sharp increase in cytosolic Ca²⁺. Such evoked cytosolic Ca²⁺ transients were similar to those observed in spontaneously firing neurons (Fig. 3A). The underlying electrical activity involved excitatory postsynaptic potentials and action potential propagation as evidenced by the inhibitory effects of the AMPA receptor blocker CNQX (10 μM) and the sodium channel blocker tetrodotoxin (TTX; 500 nM) on Ca²⁺ elevation triggered by 1 Hz EFS (Fig. 3B). With EFS delivered at 10 Hz, evoked Ca²⁺ responses were larger, but were still attenuated by CNQX and TTX (Fig. 3C). Such rises in cytosolic Ca²⁺ displayed proportional increases with incremental EFS frequencies ranging from 0.5 to 15 Hz (Fig. 3D,E). Thus, EFS is well suited to provide quasiphenological stimuli at graded intensities and thereby permits precise control over the resulting cytosolic Ca²⁺ responses.

To analyze metabolic responses of neurons to EFS, cytosolic NADH/NAD⁺, cytosolic ATP/ADP, and mitochondrial Ca²⁺ were monitored by use of the NADH/NAD⁺ ratiometric indicator Peredox (Díaz-García et al., 2017), the ATP/ADP ratiometric indicator PercevalHR (Tantama et al., 2013), and the high-affinity mitochondrial Ca²⁺ sensor CEPIA2mt (Suzuki et al., 2014). Delivery of EFS (1.5 ms/20 V) for periods of 40 s at either 2.5 Hz (100 pulses) or 10 Hz (400 pulses) resulted in transient increases in cytosolic Ca²⁺; amplitudes of these Ca²⁺ transients rose with increasing stimulation intensities (Fig. 4A,B). In parallel, the cytosolic NADH/NAD⁺ ratio displayed transient increases, the sizes of which correlated with incremental stimulation intensities (Fig. 4C,D) and thus with the size of Ca²⁺ transients. The monophasic increase of the Peredox signal differs from the widely used NADPH autofluorescence, which changes in a biphasic manner (Duchen et al., 2003). This difference is because of the fact that Peredox measures the cytosolic NADH/NAD⁺ ratio only, whereas UV light-induced NADPH signals originate primarily from mitochondria. An increase in cytosolic NADH/NAD⁺ ratio has been shown to reflect transient activation of neuronal glycolysis with subsequent uptake of NADH into mitochondria and its accompanying conversion to NAD⁺ via lactate dehydrogenase (for more details, see Díaz-García et al., 2017).

Mitochondrial Ca^{2+} rose when neurons were stimulated by 10 Hz EFS, but not by 2.5 Hz EFS (Fig. 4E,F). It cannot be ruled out that rises in mitochondrial Ca^{2+} during 2.5 Hz EFS might have escaped detection. However, CEPIA2mt is a mitochondrial Ca^{2+} sensor operating in the nanomolar range ($K_d = 160$ nM; Suzuki et al., 2014). Thus, mitochondrial Ca^{2+} levels lower than that would be unable to stimulate Ca^{2+} -sensitive dehydrogenases of the TCA cycle, which have K_d values in the low micromolar range (Denton, 2009). This suggests that even if there were undetected Ca^{2+} rises, they were insufficient to modulate these enzymes.

The ATP/ADP ratio exhibited transient stimulation-dependent decreases, and the extent of this effect depended on stimulation intensities. Recovery of the ATP/ADP ratio was seen after both types of stimulation (Fig. 4G,H). Thus, replenishment of the cytosolic ATP pool occurred in parallel with a declining NADH/NAD $^{+}$ ratio. In contrast, ATP/ADP recovery did not appear to be strictly correlated with increases in mitochondrial Ca^{2+} , as the latter parameter was not affected by 2.5 Hz EFS. Thus, the two stimulation intensities elicited qualitatively different metabolic responses. During larger workloads, mitochondrial ATP production may be induced by a combined action of cytosolic and matrix Ca^{2+} , while smaller workloads can be handled by an increased activity of NADH shuttles stimulated by cytosolic Ca^{2+} only.

The fluorescence intensity of Perceval may change in a pH-sensitive manner, thus creating ATP/ADP-independent changes (Tantama et al., 2013). Therefore, we monitored cytosolic pH changes induced by 10 Hz EFS using the genetically encoded pH reporter pHRed (Tantama et al., 2011). This stimulation led to a brief acidification (Fig. 5A), but the time course of evoked pH changes was shorter (return to baseline within 4 min after stimulation) than that of changes in the ATP/ADP ratio (return to baseline within 12 min after stimulation; Fig. 4G). Furthermore, the time course of the observed pH change was not altered by oligomycin (Fig. 5A). When a pH change of similar amplitude was caused by the application of 1 mM NH_4Cl (Fig. 5B), Perceval fluorescence intensity changed by 10% (Fig. 5C), which is approximately one-fourth of the change caused by 10 Hz EFS (Fig. 4G). Accordingly, changes in Perceval fluorescence because of EFS cannot be explained by drifts in cytosolic pH.

To provide direct evidence for the replenishment of cytosolic ATP being a Ca^{2+} -dependent mitochondrial process, subsequent experiments tested for effects of ATP synthase inhibition and prevention of rises in cytosolic Ca^{2+} . To this end, the recovery of the ATP/ADP ratio subsequent to 10 Hz EFS was quantified by the rate of Perceval fluorescence intensity ratio increase (Fig. 6A, B). This recovery was complete within 10–12 min under control conditions, but abolished by the ATP synthase inhibitor oligomycin (1 μM) and the blocker of L-type Ca^{2+} channels isradipine (1 μM). Likewise, there was no recovery when neurons had been incubated in the membrane-permeant Ca^{2+} chelator BAPTA-

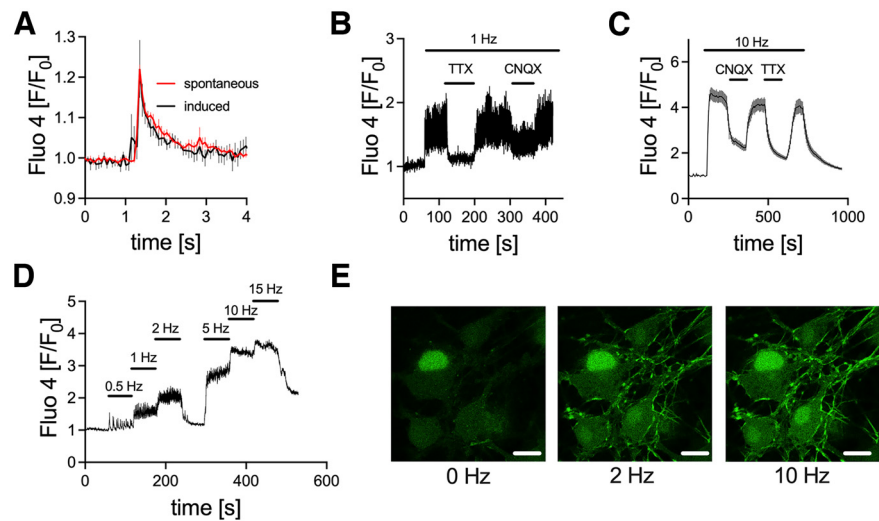


Figure 3. Changes in cytosolic Ca^{2+} in response to electrical field stimulation. Cytosolic calcium was determined in somata of single hippocampal neurons by fluorescence signals of Fluo-4 AM. EFS (rectangular 1.5 ms pulses of 20 V for 40 s) was applied at various frequencies, as indicated. For each neuron, all fluorescence values over time were normalized to the last value before EFS. **A**, Time course of normalized Fluo-4 AM fluorescence intensities that either occurred spontaneously or were evoked by single-pulse EFS (20 V, 1.5 ms; black trace; $n = 4$ neurons). **B**, Original trace of normalized Fluo-4 AM fluorescence intensity; CNQX (10 μM) and TTX (0.5 μM) were present, and 1 Hz EFS was applied as indicated. **C**, Time course of normalized Fluo-4 AM fluorescence intensities; CNQX (10 μM) and TTX (0.5 μM) were present, and 10 Hz EFS was applied as indicated ($n = 12$ neurons from two independent preparations). **D**, Original trace of normalized Fluo-4 AM fluorescence intensity; EFS was applied at increasing frequencies (0.1–15 Hz) as indicated. **E**, Original micrographs from experiment shown in **D**. Scale bars, 15 μm .

AM (30 μM for 15 min; Fig. 6A,B). This confirms that L-type Ca^{2+} channels and rises in cytosolic Ca^{2+} adjust mitochondrial ATP production to energetic needs caused by neuronal electrical activity (Hotka et al., 2020).

For the sake of clarity, Figure 6 presents the time course of normalized traces of Perceval fluorescence intensity ratio only. However, baseline values might differ between single cells and chosen experimental conditions. Therefore, throughout this work, normalized Perceval traces will be accompanied by corresponding baseline values obtained from original, non-normalized signals. Compared with control neurons, oligomycin-treated and BAPTA-AM-treated neurons display lower ATP/ADP baseline values (Fig. 6C).

G3PS drives activity-induced mitochondrial ATP production during combined inhibition of MCU and MAS

Mitochondria are equipped with three Ca^{2+} -sensitive mechanisms by which they adapt their respiratory activity to meet the energetic needs of a neuron. While inhibition of MAS and G3PS is expected to prevent cytosolic Ca^{2+} from stimulating mitochondrial respiration, blockage of the mitochondrial Ca^{2+} uniporter can impede activation of Ca^{2+} -sensitive enzymes of the TCA cycle by matrix Ca^{2+} . The involvement of each Ca^{2+} -sensitive pathway in ATP regeneration following EFS may be studied using selective inhibitors. Interference with any one of these pathways might result in lower respiration and consequently in slower ATP production compared with control conditions. However, none of the compounds used (AOA + pyr, Ru360, or iGP) affected ATP recovery after neuronal stimulation at 2.5 Hz (Fig. 6D): rates of ATP resynthesis were unchanged (Fig. 6E) and the ATP/ADP ratio reached baseline levels by the end of each experiment (Fig. 6D). ATP recovery remained unaffected even when two of the three Ca^{2+} -sensitive

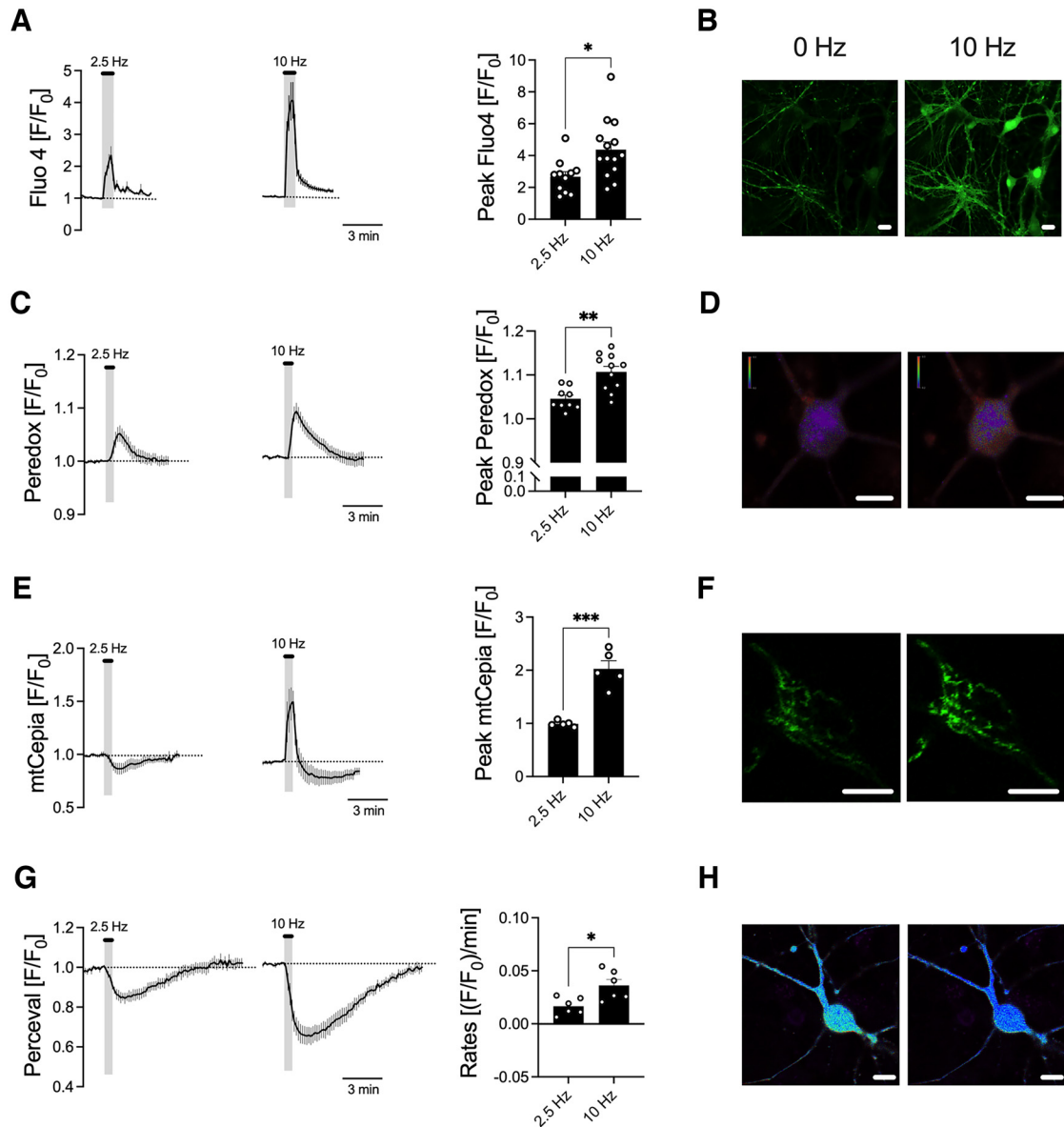


Figure 4. Metabolic responses of neurons to electrical field stimulation. Cytosolic Ca^{2+} , cytosolic NADH/NAD^+ ratio, mitochondrial Ca^{2+} , and cytosolic ATP/ADP were determined in somata of single hippocampal neurons by fluorescence signals of Fluo-4 AM, Peredox, CEPIA2mt, and PercevalHR, respectively. To trigger neuronal firing with defined intensity, the neurons were stimulated by EFS. EFS (rectangular 1.5 ms pulses of 20 V for 40 s) was applied at 2.5 and 10 Hz, respectively, as indicated. For each neuron, all fluorescence values over time were normalized to the last value before EFS. **A**, Time course and peak values of Fluo-4 AM fluorescence intensities ($n = 11$ –14 neurons from two independent preparations). **C**, Time course and peak values of Peredox fluorescence intensity ratio obtained at excitation wavelengths of 403 and 561 nm ($n = 9$ –11 neurons from four independent preparations). **E**, Time course and peak values of CEPIA2mt fluorescence intensities ($n = 5$ neurons from three independent preparations). **G**, Time course and rate of increase of PercevalHR fluorescence intensity ratio obtained at excitation wavelengths of 403 and 488 nm ($n = 6$ neurons from three independent preparations). The increase in fluorescence intensity ratio subsequent to EFS was analyzed by linear regression from the minimum value to the last point. **B**, **D**, **F**, **H**, Original micrographs illustrate the fluorescence change of the respective indicators Fluo-4 AM (**B**), Peredox (**D**), CEPIA2mt (**F**), and PercevalHR (**H**) in response to 10 Hz EFS. Scale bars, 15 μm . * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (unpaired t test).

mechanisms were blocked simultaneously (Fig. 6D,E). However, when MCU, MAS, and G3PS were inhibited at the same time, the rate of ATP synthesis was significantly reduced and close to zero (Fig. 6D,E). When MCU, MAS, and L-type Ca^{2+} channels were blocked concomitantly, the ATP/ADP ratio also failed to recover (Fig. 6D,E). This suggests that G3PS activity is driven by Ca^{2+} influx via channels of this type. Baseline values were comparable across all experimental conditions tested (Fig. 6F). Similar results were obtained in neurons stimulated with EFS at 10 Hz (Fig. 6G–I) and when MAS was completely blocked by 1 mM (instead of 0.1 mM) AOA (Fig. 5D). These results highlight the activity of

substituting mechanisms that ensure the robustness of mitochondrial bioenergetics.

G3PS drives activity-induced mitochondrial ATP production through complex III

All the previous experiments were relying on partial inhibition of MAS only. To overcome this limitation and to further distinguish between MAS and G3PS activities, inhibitors of mitochondrial ETC complexes were used. ETC can be separated into two main functional parts. The first of these two comprises complexes I, III, and IV; in this pathway, NADH donates electrons at the level of complex I, which are then transferred to complexes

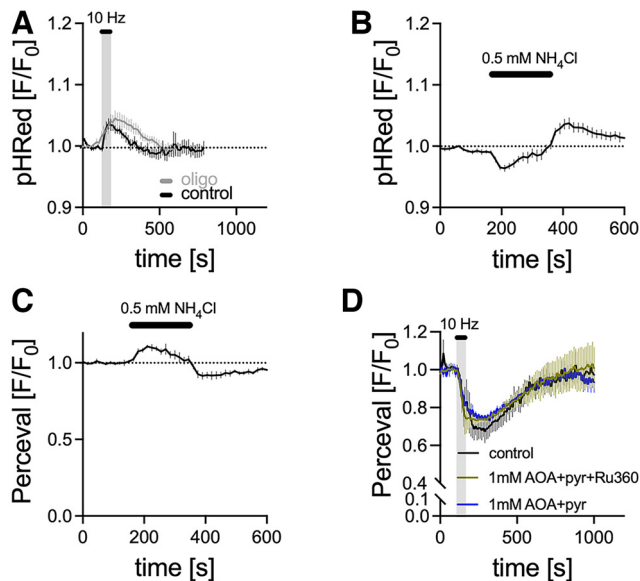


Figure 5. Comparison of EFS-induced changes in Perceval and pHRed. Cytosolic pH and cytosolic ATP/ADP were determined in single hippocampal neurons by fluorescence signals of pHRed and PercevalHR, respectively. EFS (rectangular 1.5 ms pulses of 20 V for 40 s) was applied at 10 Hz, as indicated. For each neuron, all fluorescence values over time were normalized to the last value before EFS or NH_4Cl application. **A**, Time course of normalized pHRed fluorescence intensities under control conditions (black trace, $n=6$ neurons from three independent preparations) and in the presence of $1 \mu\text{M}$ oligomycin (gray trace, $n=5$ neurons from two independent preparations), respectively. **B**, Time course of normalized pHRed fluorescence intensities; 0.5 mM NH_4Cl was present as indicated ($n=8$ from three independent preparations). **C**, Time course of normalized PercevalHR fluorescence intensity ratio obtained at excitation wavelengths of 403 and 488 nm; 0.5 mM NH_4Cl was present as indicated ($n=8$ neurons from three independent preparations). **D**, Time course of normalized PercevalHR fluorescence intensity ratio under control conditions (black trace, $n=5$ neurons), in the presence of 1 mM AOA + pyr (blue trace, $n=4$ neurons) and 1 mM AOA + pyr + Ru360 (green trace, $n=4$ neurons). Cultures were obtained from two independent preparations.

III and IV. The other part of ETC relies on complex II, which is passing electrons to complex III and IV and uses FADH_2 instead of NADH as the electron donor (Mráček et al., 2013). While NADH generated by MAS and/or the TCA cycle delivers electrons to complex I, G3PS bypasses this complex and directly feeds proton transport via complex III. Therefore, differences in sensitivities toward the inhibition of complex I and III, respectively, can be used to distinguish between these two shuttle systems without touching the enzymes being involved.

In neurons with MAS and MCU being blocked by AOA + pyr plus Ru360, ATP/ADP recovery subsequent to 10 Hz EFS was seen even when complex I was inhibited by $10 \mu\text{M}$ rotenone and also when complex II was repressed by 1 mM 3NP (Fig. 6J, K). This shows that the activities of complexes I and II are not required for ATP synthesis when MAS and MCU are inhibited. However, the addition of the complex III inhibitor Antimycin A (AA) ($1 \mu\text{M}$) entirely prevented recovery of the ATP/ADP ratio (Fig. 6J, K). An equivalent effect was obtained when AA was replaced by $100 \mu\text{M}$ iGP (Fig. 6J, K). This confirms that G3PS fueled mitochondrial respiration through complex III when MAS and MCU were blocked. Under all these conditions, baseline values of ATP/ADP ratio were the same (Fig. 6L).

Neuronal glucose metabolism in primary cell culture critically depends on glucose and oxygen concentrations in culture media (Kleman et al., 2008; Zhu et al., 2012). To be able to exclude that the role of G3PS activity described above is only because of the

chosen culture conditions, ATP/ADP measurements were repeated in neurons kept in 2 mM glucose and 2% O_2 for 14 d. Neurons were then imaged at 37°C in a stage-top incubator filled with a mixture of 2% O_2 , 5% CO_2 , and 93% N_2 . In agreement with the previous results, neuronal ATP/ADP recovery following 10 Hz EFS was sensitive only to a combined inhibition of MAS, MCU, and G3PS. ATP/ADP ratio recovery in neurons with functional G3PS was lost when L-type Ca^{2+} channels were blocked by isradipine and thus relied on transmembrane calcium influx (Fig. 7A, B). Under all these conditions, the baseline ATP/ADP ratio was the same (Fig. 7C).

As above, ATP/ADP recovery following 10 Hz EFS with MAS and MCU being blocked was insensitive toward complex I and II inhibition by rotenone and nitropropionic acid, respectively, but was prevented by either iGP or antimycin A (Fig. 7D, E). These inhibitors also affected basal respiration as evidenced by low baseline ATP/ADP levels (Fig. 7F).

G3PS contributes to cytosolic redox balance

Activity in redox shuttling systems is exemplified best by the responses of cytosolic NADH/NAD^+ to neuronal stimulation. EFS raises glycolytic activity (Díaz-García et al., 2017), which is reflected by a transient increase in the cytosolic NADH/NAD^+ ratio (Fig. 8A). This ratio is determined by an equilibrium between cytosolic NADH production and loss. Accordingly, inhibition of any of the NADH shuttling systems can be expected to result in exaggerated NADH/NAD^+ responses. In line with this assumption, partial suppression of MAS by $100 \mu\text{M}$ AOA + pyr enhanced the EFS-induced surge in NADH/NAD^+ (Fig. 8A). However, the rise in NADH/NAD^+ ratio caused by 2.5 Hz EFS was not substantially affected when G3PS was repressed by iGP, or when the mitochondrial Ca^{2+} uniporter was blocked by Ru360 (Fig. 8A). When either MCU and G3PS or MCU and MAS were halted at the same time, responses of the cytosolic NADH/NAD^+ ratio were the same as under control conditions (Fig. 8A, B). Unexpectedly, the NADH/NAD^+ rise caused by 2.5 Hz EFS in the presence of AOA + pyr + Ru360 was significantly smaller than that in AOA + pyr only. A similar response pattern was observed with 10 Hz EFS, and more details are provided below. Combined inhibition of MCU, MAS, and G3PS led to a significant enhancement of the NADH/NAD^+ ratio rise triggered by 2.5 Hz EFS (Fig. 8B). Thus, G3PS becomes a relevant regulator of the cytosolic NADH/NAD^+ ratio as soon as the other two Ca^{2+} -dependent metabolic mechanisms fail.

A fourfold increase in EFS intensity changed the pattern of NADH/NAD^+ ratio responses. As above, the suppression of G3PS by iGP failed to affect the time course of the NADH/NAD^+ ratio surge, and the inhibition of MAS (AOA + pyr) led to a marked augmentation (Fig. 8C). In contrast to the 2.5 Hz stimulation, at 10 Hz EFS Ru360 blunted the NADH/NAD^+ surge, which led to a decrease in the cytosolic NADH/NAD^+ ratio below baseline (Fig. 8C). This is most likely related to the finding that 10 Hz, but not 2.5 Hz, EFS elicited an increase in mitochondrial Ca^{2+} (Fig. 4E), which stimulates NADH production within the mitochondrial matrix. Accordingly, blockage of mitochondrial Ca^{2+} uptake with Ru360 prevents this mechanism from occurring, while ATP recovery remains unaltered (Fig. 6G). As the cytosolic NADH/NAD^+ ratio reflects a balance between production and mitochondrial uptake, the time course of NADH/NAD^+ ratio in Ru360-treated neurons can be assumed to reflect increased NADH shuttling from cytosol into mitochondria to compensate for the lack of Ca^{2+} -

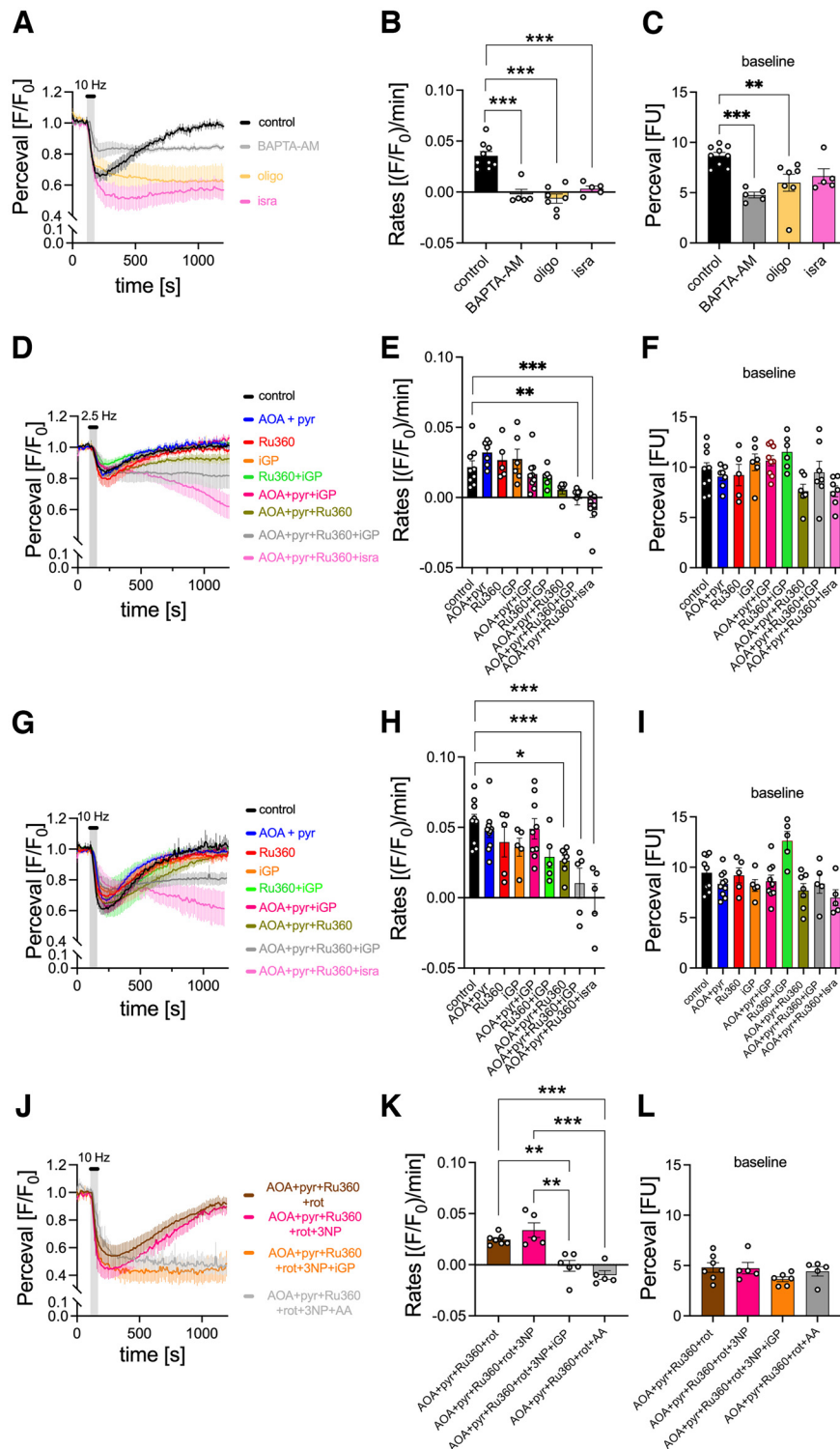


Figure 6. Mitochondrial ATP production in responses to electrical field stimulation. Cytosolic ATP/ADP was determined in single neurons by the PercevalHR fluorescence intensity ratio obtained at excitation wavelengths of 403 and 488 nm. EFS (rectangular 1.5 ms pulses of 20 V for 40 s) was applied at 2.5 and 10 Hz, respectively, as indicated. For each neuron, all fluorescence values over time were normalized to the last value before EFS. **A**, Time course of normalized fluorescence values obtained under control conditions (black trace; $n = 9$ neurons), in the presence of either 1 μ M oligomycin (orange trace; $n = 7$ neurons) or 1 μ M isradipine (isra; pink trace; $n = 5$ neurons), and in neurons loaded with 30 μ M BAPTA-AM (gray trace; $n = 5$ neurons), respectively. Cultures were obtained from three independent preparations. **B**, Rates of changes in fluorescence intensity ratio were analyzed by linear regression from 100 s after the start of 10 Hz EFS to the end of the traces shown in **A**. **C**, Comparison of non-normalized baseline fluorescence intensity ratio values obtained before EFS as shown in **A**. **D**, Time course of normalized fluorescence values obtained under control conditions (black trace; $n = 9$ neurons) or in the presence of 100 μ M AOA plus 2 mM pyruvate (AOA + pyr; blue trace; $n = 7$ neurons), 10 μ M Ru360 (red trace; $n = 5$ neurons), 100 μ M iGP (orange trace; $n = 6$ neurons), (AOA + pyr + plus iGP; purple trace; $n = 9$ neurons), Ru360 plus iGP

dependent NADH synthesis by the mitochondrial TCA cycle.

Coapplication of the G3PS inhibitor iGP with Ru360 let the 10 Hz-induced NADH/NAD⁺ ratio rise reappear, whereas simultaneous inhibition of MCU and MAS depressed the cytosolic NADH/NAD⁺ ratio even further (Fig. 8D). In both of these conditions, ATP resynthesis after EFS was going on at similar rates (Fig. 6G, H). However, when ATP production relies on MAS, less NADH is consumed than under involvement of G3PS instead (Mráček et al., 2013). Therefore, removal of MAS from the ATP-producing machinery with blocked MCU results in a further decline of cytosolic NADH/NAD⁺ ratio as more NADH is shuttled by G3PS. In contrast, removal of G3PS brings the cytosolic NADH/NAD⁺ ratio up as NADH translocation into mitochondria becomes more efficient in relation to ATP synthesis. When MCU,

(Ru360 + iGP; light green trace; $n = 6$ neurons), AOA + pyr + Ru360 (dark green trace; $n = 6$ neurons), AOA + pyr + Ru360 + iGP (gray trace; $n = 7$ neurons), and AOA + pyr + Ru360 + isra (pink trace; $n = 7$ neurons), respectively. Cultures were obtained from three independent preparations. **E**, Rates of changes in fluorescence intensity ratio were analyzed by linear regression from 100 s after the start of 2.5 Hz EFS to the end of the traces shown in **D**. **F**, Comparison of non-normalized baseline fluorescence intensity ratio values obtained before EFS, as shown in **D**. **G**, Time course of normalized fluorescence values obtained under control conditions (black trace; $n = 9$ neurons) or in the presence of 100 μ M AOA + 2 mM pyr (blue trace; $n = 10$ neurons), 10 μ M Ru360 (red trace; $n = 5$ neurons), 100 μ M iGP (orange trace; $n = 5$ neurons), AOA + pyr + iGP (purple trace; $n = 9$ neurons), Ru360 + iGP (light green trace; $n = 5$ neurons), AOA + pyr + Ru360 (dark green trace; $n = 7$ neurons), AOA + pyr + Ru360 + iGP (gray trace; $n = 5$ neurons), and AOA + pyr + Ru360 + 1 μ M isra (pink trace; $n = 5$ neurons), respectively. Cultures were obtained from three independent preparations. **H**, Rates of changes in fluorescence intensity ratio were analyzed by linear regression from 100 s after the start of 10 Hz EFS to the end of the traces shown in **G**. **I**, Comparison of non-normalized baseline fluorescence intensity ratio values obtained before EFS, as shown in **G**. **J**, Time course of normalized fluorescence values obtained in 100 μ M AOA + 2 mM pyr + 10 μ M Ru360 + 10 μ M rotenone (brown trace; $n = 7$ neurons), AOA + pyr + Ru360 + rot + 1 mM 3NP (purple trace; $n = 5$ neurons), AOA + pyr + Ru360 + rot + 3NP + 100 μ M iGP (orange trace; $n = 6$ neurons), AOA + pyr + Ru360 + rot + 3NP + 1 μ M AA (gray trace; $n = 5$ neurons) respectively. Cultures were obtained from two to three independent preparations. **K**, Rates of changes in fluorescence intensity ratio were analyzed by linear regression from 100 s after the start of 10 Hz EFS to the end of the traces shown in **J**. **L**, Comparison of non-normalized baseline fluorescence intensity ratio values obtained before EFS, as shown in **J**. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, respectively (one-way ANOVA followed by Tukey's multiple-comparisons test).

MAS, and G3PS were blocked at the same time, the NADH/NAD⁺ ratio rise triggered by 10 Hz EFS was enhanced (Fig. 8D) as described for 2.5 Hz EFS above. This confirms that G3PS decisively controls the cytosolic NADH/NAD⁺ ratio once the other Ca²⁺-dependent regulatory pathways are out of order.

Individual Ca²⁺-sensitive metabolic components can substitute each other

Partial inhibition of MAS alone (by 100 μ M AOA) did not result in increased activity of G3PS, nor did the lack of MAS in the presence of added pyruvate compromise oxidative phosphorylation (Fig. 6G, H). Similar results were obtained when MAS was completely inhibited by 1 mM AOA + pyr (Fig. 5D). This points toward a substituting pathway other than G3PS being involved. Therefore, we monitored mitochondrial Ca²⁺.

Under control conditions, mitochondria were taking up Ca²⁺ only when stimulated at higher (10 Hz), but not lower (2.5 Hz) intensities (Fig. 4E). However, when MAS activity was inhibited, mitochondria accumulated Ca²⁺ with both 2.5 and 10 Hz EFS (Fig. 9A–C). Thus, at moderate stimulation intensities mitochondrial Ca²⁺ uptake appeared to compensate for a lack of MAS. This led to the question as to how this increased mitochondrial Ca²⁺ accumulation during MAS inhibition arose. As treatment of neurons with AOA + pyr did not alter amplitudes of cytosolic Ca²⁺ transients triggered by 2.5 Hz EFS (Fig. 9D), this was not because of an increase in transmembrane Ca²⁺ influx.

Depolarization of the mitochondrial membrane potential by 20 mV was shown to decrease the activity of the mitochondrial Na⁺/Ca²⁺/Li⁺ exchanger (NCLX) and to enhance mitochondrial Ca²⁺ uptake (Kostic et al., 2018). Accordingly, mitochondrial membrane potential was measured using the lipophilic cationic dye TMRM. The application of AOA + pyr (but not of 2 mM pyruvate alone) depolarized neuronal mitochondria partially, as evidenced by a decrease in TMRM fluorescence intensity. A similar change in TMRM fluorescence was observed when mitochondria were exposed to 100 nM FCCP (Fig. 9E,F). Subsequently, complete mitochondrial depolarization was induced by 10 μ M FCCP to provide a positive control for comparison (Fig. 9E). Thus, the inhibition of MAS is accompanied by mitochondrial depolarization.

To corroborate that depolarization of mitochondria might suffice to promote mitochondrial Ca²⁺ uptake, experiments were conducted in the absence and presence of 100 nM FCCP. Indeed, such a moderate mitochondrial depolarization enabled Ca²⁺ influx into mitochondria with 2.5 Hz EFS, an effect that was not observed in the absence of FCCP (Fig. 9G,I). With 10 Hz EFS, 100 nM FCCP slightly prolonged mitochondrial

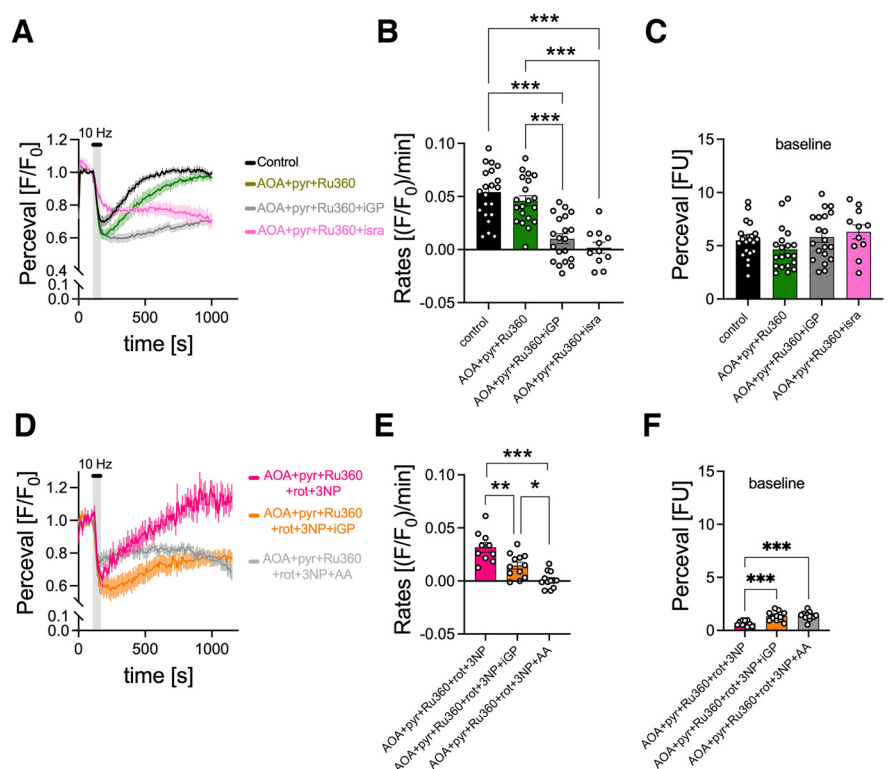


Figure 7. Mitochondrial ATP production in responses to electrical field stimulation in neurons cultured in quasiphsiological conditions. Cytosolic ATP/ADP was determined at 37°C in single neurons by the PercevalHR fluorescence intensity ratio obtained at excitation wavelengths of 403 and 488 nm. EFS (rectangular 1.5 ms pulses of 20 V for 40 s) was applied at 2.5 and 10 Hz, respectively, as indicated. For each neuron, all fluorescence values over time were normalized to the last value before EFS. **A**, Time course of normalized fluorescence values obtained under control conditions (black trace; $n = 22$ neurons) or in the presence of 100 μ M AOA + pyr + Ru360 (dark green trace; $n = 20$ neurons), AOA + pyr + Ru360 + iGP (gray trace; $n = 20$ neurons), and AOA + pyr + Ru360 + 1 μ M isradipine (AOA+pyr+Ru360+isra; pink trace; $n = 11$ neurons), respectively. Cultures were obtained from three independent preparations and kept in 2 mM glucose and 2% O₂. **B**, Rates of changes in fluorescence intensity ratio were analyzed by linear regression from 100 s after the start of 10 Hz EFS to the end of the traces shown in **A**. **C**, Comparison of non-normalized baseline fluorescence intensity ratio values obtained before EFS as shown in **A**. **D**, Time course of normalized fluorescence values obtained in 100 μ M AOA + 2 mM pyr + 10 μ M Ru360 + 10 μ M rot + 1 mM 3NP (purple trace; $n = 10$ neurons), AOA + pyr + Ru360 + rot + 3NP + 100 μ M iGP (orange trace; $n = 12$ neurons), and AOA + pyr + Ru360 + rot + 3NP + 1 μ M AA (gray trace; $n = 12$ neurons), respectively. Cultures were obtained from two independent preparations and were kept in 2 mM glucose and 2% O₂. **E**, Rates of changes in fluorescence intensity ratio were analyzed by linear regression from 100 s after the start of 10 Hz EFS to the end of the traces shown in **D**. **F**, Comparison of non-normalized baseline fluorescence intensity ratio values obtained before EFS as shown in **D**. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (one-way ANOVA followed by Tukey's multiple-comparisons test).

Ca²⁺ transients, but left their peaks unaltered (Fig. 9H,I). Thus, during neuronal activity of lower intensity (2.5 Hz EFS), partial mitochondrial membrane depolarization is sufficient to allow for mitochondrial Ca²⁺ accumulation.

To provide additional evidence for mitochondrial Ca²⁺ uptake to enable ATP synthesis when NADH shuttling is compromised, neurons were incubated in the nonmetabolizable glucose analog 2DG (2 mM). This leaves MAS unaltered, but prevents glycolysis and thus the generation of NADH (Fig. 10A) and pyruvate in response to EFS. As a consequence, the respiratory chain of mitochondria is deprived of pyruvate as a fuel, and ATP resynthesis after 2.5 and 10 Hz EFS is blocked (Fig. 10B–E). Reintroduction of pyruvate, however, is sufficient for ATP synthesis to reoccur (Fig. 10B–E). Under these conditions, both MAS and G3PS can operate, but no cytosolic NADH is available. Therefore, mitochondria have to rely on NADH generated by the TCA cycle within the matrix.

In neurons exposed to 2DG plus pyruvate, ATP recovery after 2.5 and 10 Hz EFS was prevented by the blockage of MCU by

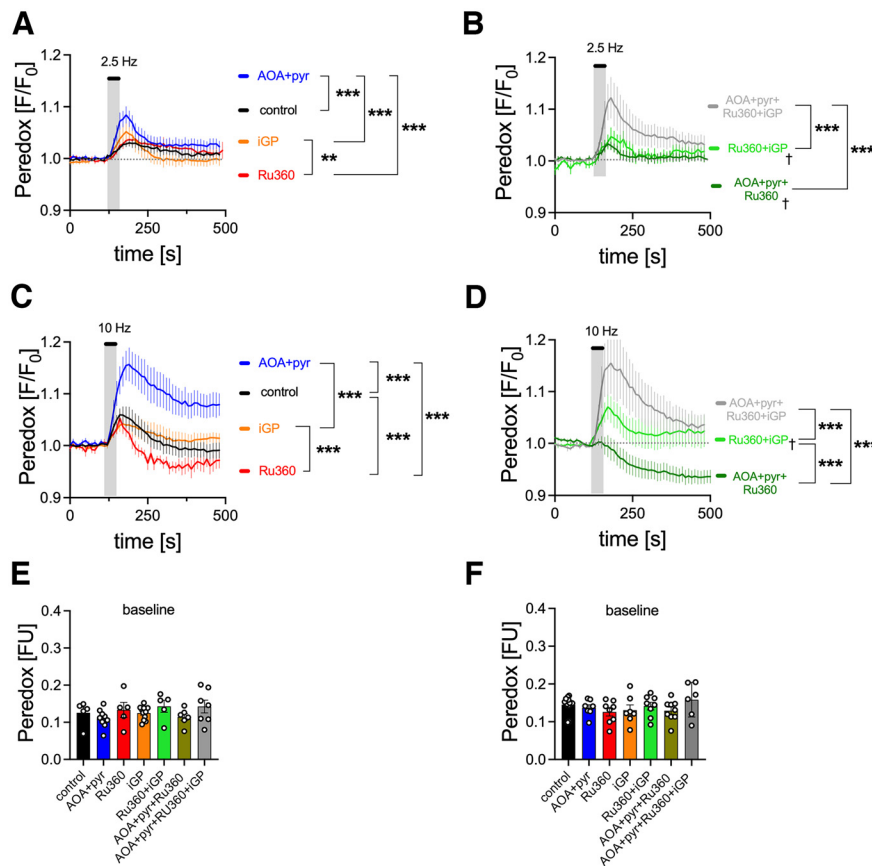


Figure 8. Changes in cytosolic NADH/NAD⁺ in responses to electrical field stimulation. The cytosolic NADH/NAD⁺ ratio was measured by the Perodox fluorescence intensity ratio obtained at excitation wavelengths of 403 and 561 nm. EFS (rectangular 1.5 ms pulses of 20 V for 40 s) was applied at 2.5 and 10 Hz, respectively, as indicated. For each neuron, all fluorescence values over time were normalized to the last value before EFS. **A**, Time course of normalized fluorescence values obtained under control conditions (black trace; $n = 5$) or in the presence of 100 μ M AOA + 2 mM pyr (blue trace; $n = 9$ neurons), 10 μ M Ru360 (red trace; $n = 5$ neurons), and 100 μ M iGP (orange trace; $n = 9$ neurons), respectively. **B**, Time course of normalized fluorescence values in the presence of Ru360 + iGP (light green trace; $n = 5$ neurons), AOA + pyr + Ru360 (dark green trace; $n = 6$ neurons), and AOA + pyr + Ru360 + iGP (gray trace; $n = 7$ neurons), respectively. Cultures were obtained from three or four independent preparations. **C**, Time course of normalized fluorescence values obtained under control conditions (black trace; $n = 11$ neurons) or in the presence of AOA + pyr (blue trace; $n = 11$ neurons), Ru360 (red trace; $n = 8$ neurons), and iGP (orange trace; $n = 7$ neurons), respectively. Cultures were obtained from three independent preparations. **D**, Time course of normalized fluorescence values in the presence of Ru360 + iGP (light green trace; $n = 8$ neurons), AOA + pyr + Ru360 (dark green trace; $n = 9$ neurons), and AOA + pyr + Ru360 + iGP (gray trace; $n = 6$ neurons), respectively. Cultures were obtained from three independent preparations. **E**, Comparison of non-normalized baseline fluorescence intensity ratio values obtained before 2.5 Hz EFS as shown in **A** and **B**. **F**, Comparison of non-normalized baseline fluorescence intensity ratio values obtained before 10 Hz EFS as shown in **C** and **D**. $^{**}p < 0.01$ and $^{***}p < 0.001$, respectively (two-way ANOVA followed by Tukey's multiple-comparison with time and solvent/drug as variables for all points from the beginning of EFS to the end of traces). $tp > 0.05$ versus control (two-way ANOVA followed by Tukey's multiple-comparisons test).

Ru360 and of L-type Ca²⁺ channels by isradipine, respectively (Fig. 10B–E). Baseline values of the ATP/ADP ratio were not affected under these conditions (Fig. 10F). Hence, when NADH supply by the cytosol is disabled, transmembrane Ca²⁺ influx into the cytosol via L-type channels and ensuing uptake into mitochondria via MCU is required to enable mitochondrial NADH synthesis and ATP production.

In summary, during MAS inhibition mitochondria experience partial depolarization and concomitant Ca²⁺ uptake in response to neuronal activity. Mitochondrial Ca²⁺ stimulates the TCA cycle to produce NADH inside of mitochondria, thereby feeding mitochondrial respiration most efficiently via complex I. Alternatively, G3PS may step in when MAS activity is blocked, but this pathway is less effective in fueling ETC

than the direct stimulation of the TCA cycle by calcium. Therefore, G3PS can be viewed as a metabolic backup system to ensure neuronal energy supply when alternative mechanisms such as MAS and MCU are defective.

G3PS secures electrical activity of neurons

Action potential firing is a major energy-consuming function in neurons (Attwell and Laughlin, 2001; Harris et al., 2012). To reveal whether the availability of G3PS might be essential for electrical signaling in neurons, action potential firing in response to depolarizing currents was investigated. Numbers of action potentials fired during 2 s current injections were proportional to current amplitudes (Fig. 11A,B). To verify that a shortage in ATP supply may affect action potential firing, neurons were exposed to 1 μ M oligomycin; this lowered the number of action potentials evoked by current injection significantly (Fig. 11A–C). In addition, oligomycin led to a hyperpolarization (Fig. 11A,D).

Combined inhibition of MAS and MCU had no major impact on cytosolic ATP/ADP because of compensatory activation of G3PS (Fig. 6). Accordingly, exposure of neurons to AOA + pyr + Ru360 failed to alter the number of action potentials fired in response to current injection with increasing amplitudes, but this number was significantly reduced when iGP was coapplied (Fig. 11E–G). This decrease in membrane excitability was accompanied by a hyperpolarization combined inhibition of MCU, MAS, and G3PS (Fig. 11H). These results prove that G3PS secures neuronal electrical activity by providing an energy supply backup mechanism.

In highly active neurons of the substantia nigra, the failure of mitochondrial ATP synthesis is transduced into a decrease in spontaneous firing rates through an activation of K_{ATP} channels (Lutas et al., 2014).

To test whether an analogous mechanism might be involved in the effects shown

above, current injection was performed in the presence of diazoxide (300 μ M) and/or tolbutamide (50 μ M), an activator and inhibitor of K_{ATP} channels (Foster and Coetzee, 2016), respectively. While tolbutamide left action potential firing in response to current injection unaltered (Fig. 11M–P), diazoxide caused a reduction similar to that seen in the presence of either oligomycin or AOA + pyr + Ru360 + iGP. This restriction in excitability induced by diazoxide was fully reverted by the addition of tolbutamide (Fig. 11I–K). The resting membrane potential was not affected by diazoxide and/or tolbutamide (Fig. 11L). Finally, application of oligomycin in the continuous presence of tolbutamide did not alter action potential firing triggered by current injections (Fig. 11M–O); nor

did it affect the membrane potential (Fig. 11P). This confirms that K_{ATP} channels are the link between compromised mitochondrial energy supply and loss of membrane excitability in hippocampal neurons.

Discussion

Metabolic flexibility of primary hippocampal neurons

Electrical signaling of neurons is highly energy demanding, and a link between neuronal activity and sufficient ATP supply is provided by Ca^{2+} ions (Harris et al., 2012). Two Ca^{2+} -sensitive metabolic mechanisms have been described as major determinants of activity-dependent mitochondrial ATP production in neurons: transfer of NADH from the cytosol to mitochondria via MAS and mitochondrial Ca^{2+} uptake through MCU (Denton, 2009; Griffiths and Rutter, 2009; Llorente-Folch et al., 2015; Szibor et al., 2020; Díaz-García et al., 2021; Pérez-Liébana et al., 2022). The present results add a new player to the system of Ca^{2+} -regulated mitochondrial ATP supply in neurons: G3PS.

MAS and MCU are differentially engaged in activity-dependent neuronal ATP production in mitochondria; MAS operates at all intensities of electrical activity (2.5 and 10 Hz), but MCU contributes at higher intensity (10 Hz) only. Nevertheless, each of these two mechanisms is sufficient to maintain activity-dependent energy supply: when one of these two mechanisms fails, the other one can fully compensate for the failure. Even when both systems were compromised at the same time, neurons continued to produce ATP in response to electrical stimulation. In this latter situation, Ca^{2+} -dependent G3PS was sufficient to substitute the other two mechanisms.

Mitochondrial ATP production is required for neuronal signaling, as evidenced by the decline in stimulation-evoked action potential firing because of ATP synthase inhibition. The link between impaired mitochondrial energy supply and reduced excitability is provided by K_{ATP} channels as the effects of oligomycin on action potentials and membrane potential were abolished by a respective channel blocker. Activation of K_{ATP} channels has been found to interfere with spontaneous activity in neurons of the substantia nigra when mitochondria failed to synthesize ATP (Lutas et al., 2014). As expected from the results above, simultaneous inhibition of MAS and MCU did not interfere with action potential firing, but an additional block of G3PS diminished neuronal excitability. Thus, G3PS secures electrical signaling in hippocampal neurons when alternative Ca^{2+} -dependent support mechanisms of mitochondrial function cease.

Obviously, the availability of three Ca^{2+} -dependent pathways that fuel mitochondrial ATP production during electrical activity

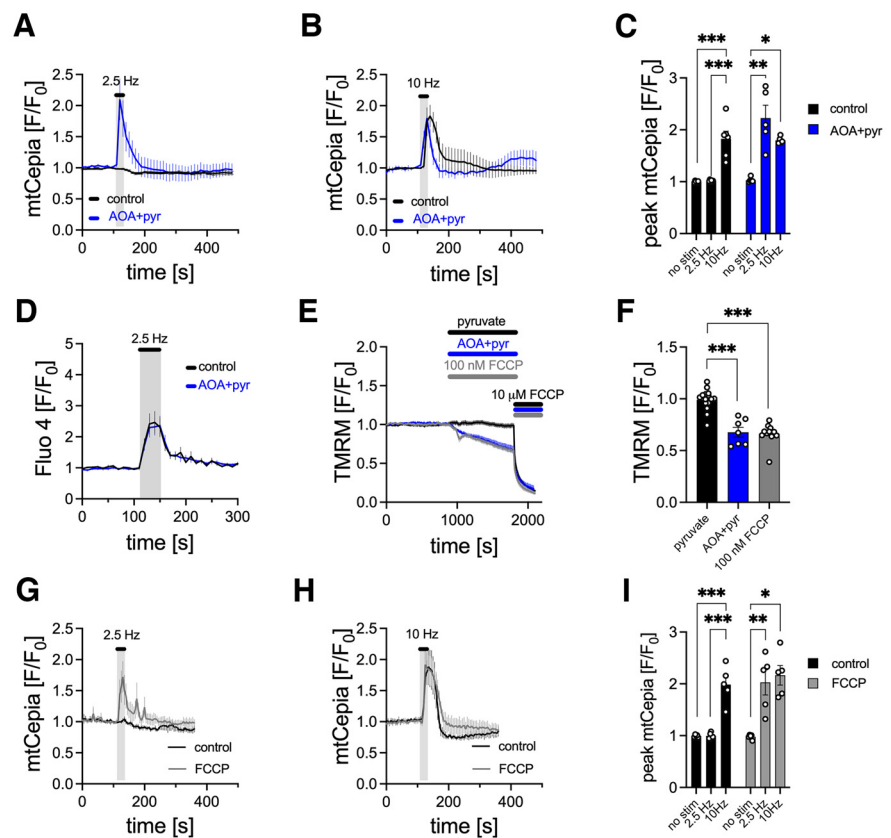


Figure 9. Interrelation between mitochondrial membrane potential and mitochondrial Ca^{2+} handling. Mitochondrial Ca^{2+} and mitochondrial membrane potential were determined by fluorescence signals of CEPIA2mt and TMRM. EFS (rectangular 1.5 ms pulses of 20 V for 40 s) was applied at 2.5 and 10 Hz, respectively, as indicated. For each neuron, all fluorescence values over time were normalized to the last value before EFS. **A, B**, Time course of normalized CEPIA2mt fluorescence intensity values obtained under control conditions (black trace; $n = 6$ neurons) and in the presence of 100 μ M AOA + 2 mM pyr (blue trace; $n = 5$ neurons), respectively (Fig. 1E, mitochondrial localization of CEPIA2mt). Cultures were obtained from three independent preparations. **C**, Peak CEPIA2mt fluorescence intensity values before (no stim) or during 2.5 and 10 Hz EFS, respectively, as shown in **A** and **B**. **D**, Time course of normalized Fluo-4 AM fluorescence intensities under control conditions (black trace) and in the presence of 100 μ M AOA + 2 mM pyr (blue trace; $n = 8$ from two independent preparations). **E**, Time course of normalized TMRM fluorescence intensity values obtained in presence of pyruvate (black trace; $n = 15$ neurons), AOA + pyr (blue trace; $n = 7$ neurons), and 100 nM FCCP (gray trace; $n = 11$ neurons), respectively. At the end of each trace, 10 μ M FCCP was applied to fully depolarize mitochondria. Cultures were obtained from two independent preparations. **F**, Changes in normalized TMRM fluorescence intensity values determined at second 1800 within the traces shown in **E**. **G, H**, Time course of normalized CEPIA2mt fluorescence intensity values obtained under control conditions (black trace; $n = 5$ neurons) and in the presence of 100 nM FCCP (gray trace; $n = 5$ neurons), respectively. Cultures were obtained from two independent preparations. **I**, Peak CEPIA2mt fluorescence intensity values before (no stim) or during 2.5 and 10 Hz EFS, respectively, under control conditions and in presence of 100 nM FCCP as shown in **G** and **H**. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, respectively (one-way ANOVA followed by Tukey's multiple-comparisons test).

provides considerable metabolic flexibility to neurons. Nevertheless, this side-by-side existence of three systems subserving one cellular function, mitochondrial ATP synthesis, appears to be redundant. Such a potential redundancy triggers the question of whether the three pathways operate identically with respect to ATP production. While MAS and matrix Ca^{2+} feed ETC with NADH, G3PS generates $FADH_2$ instead. Moreover, the mitochondrial GPD2 delivers $FADH_2$ to the intermembrane space, whereas NADH arises from the MAS and TCA cycle within the matrix. In consequence, matrix NADH drives ETC via electron delivery to complex I, whereas intermembrane $FADH_2$ passes electrons onto complex III (McKenna et al., 2006; Liu et al., 2021). This is also evident from the present results: ATP synthesis with MAS and MCU being blocked was insensitive to the complex I and II blockers rotenone and 3NP, respectively, but was abrogated by the complex III inhibitor AA (Fig. 6J,K). As complex I provides a major proportion

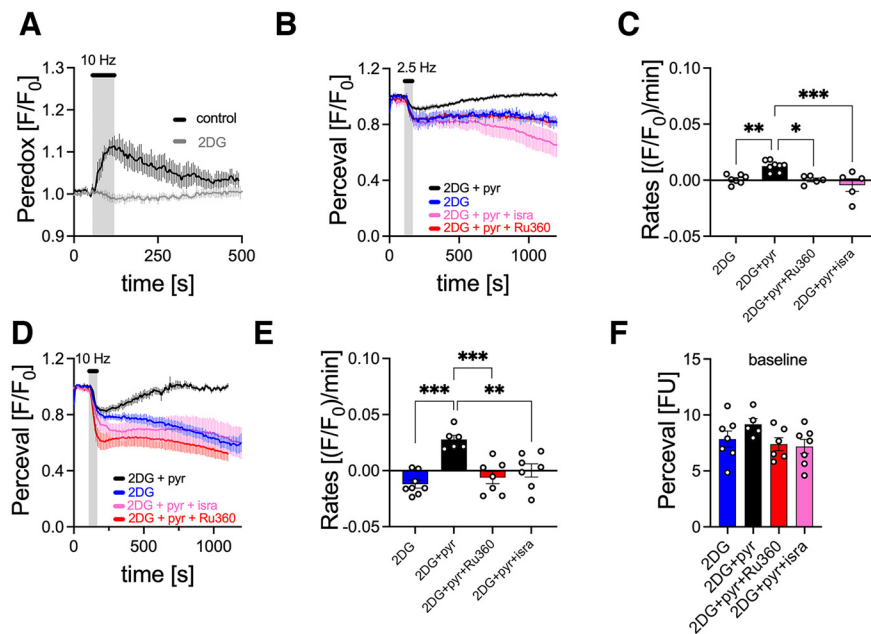


Figure 10. Interrelation between cytosolic NADH, mitochondrial Ca^{2+} uptake, and mitochondrial ATP synthesis. **A**, Time course and peak values of Peredox fluorescence intensity ratio obtained at excitation wavelengths of 403 and 561 nm under control conditions (black trace; $n = 4$ neurons) and in the presence of 2DG (gray trace; $n = 5$ neurons). Cultures were obtained from two independent preparations. **B**, Time course of normalized PercevalHR fluorescence intensity ratio obtained at excitation wavelengths of 403 and 488 nm in the presence of 2 mM 2DG (blue trace; $n = 7$ neurons), 2DG + pyr (black trace; $n = 8$ neurons), 2DG + pyr + isra (pink trace; $n = 5$ neurons), and 2DG + pyr + 10 μM Ru360 (red trace; $n = 5$ neurons), respectively. Cultures were obtained from two independent preparations. **C**, Rates of changes in fluorescence intensity ratio were analyzed by linear regression from 100 s after the start of 2.5 Hz EFS to the end of the traces shown in **B**. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, respectively (one-way ANOVA followed by Tukey's multiple-comparisons test). **D**, Time course of normalized PercevalHR fluorescence intensity ratio in 2DG (blue trace; $n = 8$ neurons), 2DG + pyr (black trace; $n = 6$ neurons), 2DG + pyr + isra (pink trace; $n = 7$ neurons), and 2DG + pyr + Ru360 (red trace; $n = 7$ neurons), respectively. Cultures were obtained from three independent preparations. **E**, Rates of changes in fluorescence intensity ratio were analyzed by linear regression from 100 s after the start of 10 Hz EFS to the end of the traces shown in **D**. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, respectively (one-way ANOVA followed by Tukey's multiple-comparisons test). **F**, Comparison of non-normalized baseline Perceval fluorescence intensity ratio values obtained in 2 mM 2DG (blue bar; $n = 7$ neurons), 2DG + pyr (black bar; $n = 5$ neurons), 2DG + pyr + 10 μM Ru360 (red bar; $n = 6$ neurons), and 2DG + pyr + 1 μM isradipine (isra; pink bar; $n = 7$ neurons) before EFS, as shown in **D**. Cultures were obtained from two independent preparations.

of the proton gradient that drives ATP synthase (Zickermann et al., 2009; Ripple et al., 2013), its exclusion from ETC operation reduces the efficiency of proton pumping decisively. Accordingly, the fueling of ATP synthesis through G3PS is less efficient than that via either MAS or MCU. Presumably, this is the reason why G3PS can serve as ultimate backup mechanism for activity-dependent energy supply only.

Mutual influence of MAS and MCU

Although a key role of Ca^{2+} in the regulation of neuronal metabolism has been emphasized (Kann and Kovács, 2007; Pivovarov and Andrews, 2010), several aspects thereof have remained unresolved. For instance, the finding that MCU-KO mice have functional oxidative phosphorylation (Harrington and Murphy, 2015; Holmström et al., 2015) raised doubts on a role of matrix Ca^{2+} as regulator of mitochondrial metabolism. It has been proposed that the lack of MCU may be compensated for by mechanisms relying on extramitochondrial (i.e., cytosolic) Ca^{2+} ; Wang et al., 2020). The present results confirm that mitochondria may use both, cytosolic as well as matrix Ca^{2+} to sustain ATP production: the rate of ATP/ADP ratio recovery after EFS was the same whether the shuttling systems that rely on cytosolic Ca^{2+} , MAS, and G3PS, or on MCU, which provides matrix Ca^{2+} , were blocked (Fig. 6E,H). When all three bioenergetic pathways were

interrupted simultaneously, ATP resynthesis was no longer possible, thus indicating that cytosolic and mitochondrial Ca^{2+} can perfectly substitute each other with respect to ATP supply.

Nevertheless, stimulation of MAS by cytosolic Ca^{2+} and of TCA by mitochondrial Ca^{2+} does not occur simultaneously, but is rather mutually exclusive as both systems are linked to α -ketoglutarate. Increased TCA cycle activity raises α -ketoglutarate availability, which inhibits mitochondrial aspartate transaminase, a central component of MAS. Vice versa, blockage of MCU and the resulting lack of TCA cycle stimulation by Ca^{2+} lead to levels of α -ketoglutarate that favor the aspartate transaminase reaction (Contreras and Satrustegui, 2009). This interplay between MAS and TCA cycle was proposed to be unidirectional with TCA cycle activity controlling MAS. However, the present results revealed increased and/or prolonged stimulation evoked mitochondrial Ca^{2+} transients on MAS inhibition (Fig. 9A,B), thereby indicating that MAS TCA cycle interactions may occur in a bidirectional manner.

The shift of mitochondria from dependence on cytosolic Ca^{2+} acting on MAS toward dependence on matrix Ca^{2+} to increase ATP production was accompanied by mitochondrial depolarization. Moreover, mild mitochondrial depolarization by low concentrations of FCCP was sufficient to let EFS-induced mitochondrial Ca^{2+} transients grow. This raises the question as to how the mitochondrial membrane potential and matrix Ca^{2+} are linked to each other.

The respective coupling is provided by NCLX, which is regulated by the mitochondrial membrane potential in an allosteric manner: depolarizations limit NCLX activity and thereby reduce Ca^{2+} extrusion from the matrix (Kostic et al., 2018). Such a mechanism arms neurons with a quick way of shifting from reliance on cytosolic to reliance on matrix Ca^{2+} . When mitochondrial Ca^{2+} is raised, NADH is generated directly from the TCA cycle, and this fuels mitochondrial respiration more effectively than NADH transport from cytosol. This may explain why G3PS activity does not increase on MAS inhibition alone.

Pathologic consequences of reliance on G3PS

Having established that G3PS steps into an activity-dependent mitochondrial ATP supply of neurons as terminal backup mechanism only, one wonders whether there might be disadvantageous consequences for neurons to rely on this system. In fact, G3PS has been associated with increased mitochondrial ROS production (Mráček et al., 2013), which may, in the long run, lead to the emergence of neurodegenerative diseases (Angelova and Abramov, 2018). Moreover, neuronal glycerol formation occurs during seizures or ischemia (Nguyen et al., 2007), and patients with defective MAS

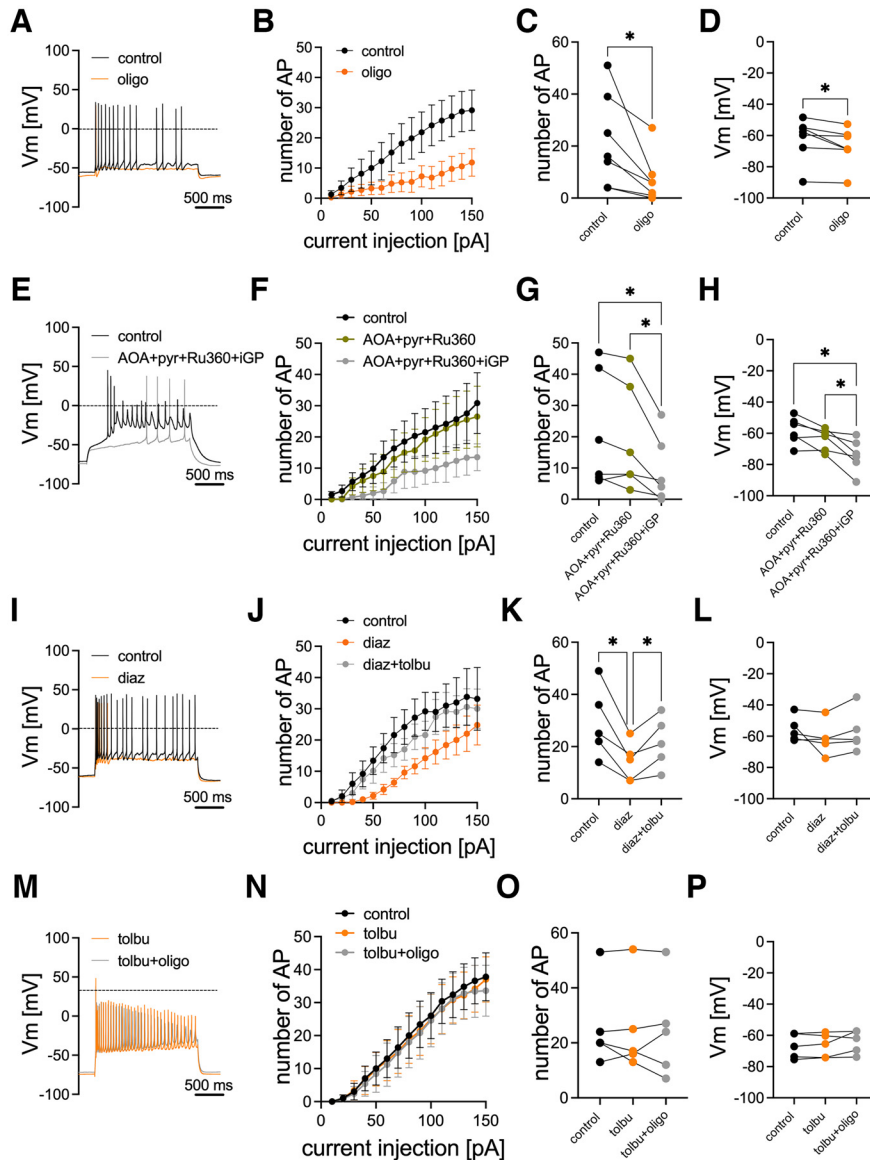


Figure 11. Metabolic regulation of action potential firing. Action potentials were triggered by 2 s current injections with increasing amplitudes in single neurons using the perforated patch configuration. **A**, Original traces with 100 pA current injection before (black trace) and during the presence of 1 μ M oligomycin (orange trace). **B**, Number of action potentials evoked by 2 s currents with increasing amplitudes ($n = 7$ neurons from two independent preparations) before (black symbols) and during the presence of oligomycin (orange symbols). **C**, Number of action potentials evoked by 100 pA currents before (black symbols) and during the presence of oligomycin (orange symbols; same neurons as in **B**). **D**, Resting membrane potential before (black symbols) and during the presence of oligomycin (orange symbols; same neurons as in **B** and **C**). **E**, Original traces with 100 pA current injection before (black trace) and during the presence of 100 μ M AOA + 2 mM pyr + 10 μ M Ru360 + 100 μ M iGP (gray trace). **F**, Number of action potentials evoked by 2 s currents with increasing amplitudes ($n = 6$ neurons from two independent preparations) before (black symbols) and during (green symbols) the presence of AOA + pyr + Ru360 + iGP (gray symbols). **G**, Number of action potentials evoked by 100 pA currents before (black symbols) and during (green symbols) the presence of AOA + pyr + Ru360 and after the addition of iGP (AOA + pyr + Ru360 + iGP; gray symbols; same neurons as in **F**). **H**, Resting membrane potential before (black symbols) and during (green symbols) the presence of AOA + pyr + Ru360 and after addition of iGP (AOA + pyr + Ru360 + iGP; gray symbols; same neurons as in **F** and **G**). **I**, Original traces with 100 pA current injection before (black trace) and during (orange trace) the presence of 300 μ M diaz. **J**, Number of action potentials evoked by 2 s currents with increasing amplitudes ($n = 5$ neurons from two independent preparations) before (black symbols) and during (orange symbols) the presence of diaz and after subsequent addition 50 μ M tolbutamide (diaz + tolbu; gray symbols). **K**, Number of action potentials evoked by 100 pA currents before (black symbols) and during (orange symbols) the presence of diaz and after addition of tolbutamide (diaz + tolbu; gray symbols; same neurons as in **J**). **L**, Resting membrane potential before (black symbols) and during (orange symbols) the presence of diaz and after addition of tolbutamide (diaz + tolbu; gray symbols; same neurons as in **J** and **K**). **M**, Original traces with 100 pA current injection during the presence of 50 μ M tolbutamide (tolbu; orange trace) and after subsequent addition of 1 μ M oligomycin (oligo; gray trace). **N**, Number of action potentials evoked by 2 s currents with increasing amplitudes ($n = 5$ neurons from two independent preparations) before (black symbols) and during (orange symbols) the presence of tolbu and after subsequent addition of 1 μ M oligomycin (tolbu +

exhibit infantile epileptic encephalopathy (Broeks et al., 2021). One important functional aspect of MAS is its role in glutamate synthesis from glutamine (Palaiologos et al., 1988, 1989), and this cannot be substituted for by G3PS. Thus, G3PS may prevent neurons from energetic crises caused by exaggerated electrical activity, on the one hand, but potentially may lead to the endangering of neuronal function and integrity when operating for extended periods of time.

Limitations of the study

Selective interference with calcium-dependent mechanisms involved in neuronal metabolism is experimentally challenging. For instance, the inhibition of MAS inevitably leads to inhibition of glycolysis, which in turn blocks the entire metabolic machinery. To overcome this problem, pyruvate was added exogenously, and this restored cytosolic NADH/NAD⁺ ratio to baseline levels as prerequisite for additional manipulations. However, such extra pyruvate supply is artificial and will not occur *in vivo*.

Another limitation is related to the focus on neuronal somata. While these are more easily accessible for fluorescence measurements and patch-clamp recordings, their metabolic requirements may be different from those in axons and dendrites. However, subcellular differences in the Ca²⁺-dependent regulation of neuronal metabolism currently remain open for future investigations. Likewise, most of the present results were obtained in 20 mM glucose at room temperature and ambient oxygen. Atmospheric oxygen and high glucose represent nonphysiological conditions used routinely for practical reasons (Dienel, 2019), whereas low oxygen and low glucose can lead to hypoxia-inducible factor activation (Liu et al., 2011; López-Hernández et al., 2012), resulting in LDH accumulation, altered expression of glycolytic enzymes, and modulation of the neuronal redox state. Therefore, assessing G3PS activity

←
oligo; gray symbols). **O**, Number of action potentials evoked by 100 pA currents before (black symbols) and during (orange symbols) the presence of tolbu and after addition of oligomycin (tolbu + oligo; gray symbols; same neurons as in **N**). **P**, Resting membrane potential before (black symbols) and during (orange symbols) the presence of tolbu and after addition of oligomycin (tolbu + oligo; gray symbols; same neurons as in **N** and **O**). * $p < 0.05$ (repeated-measures one-way ANOVA with Geisser–Greenhouse correction followed by Tukey's multiple-comparisons test).

in both high and low oxygen/glucose may help to account for problems associated with each of these conditions. Accordingly, key findings were verified in neurons kept in 2 mM glucose at 37°C and 2% oxygen, and these conditions were maintained during experimentation. In these settings, the neuronal ATP/ADP ratio was more sensitive toward inhibition of respiration, but responses to neuronal stimulation were comparable to those at high oxygen/glucose.

Conclusion

This study demonstrates exceptional metabolic flexibility of neurons that renders them able to cope with the high energetic demands of electrical signaling. This flexibility is provided by the parallel availability of three Ca^{2+} -dependent bioenergetic systems that fuel mitochondrial ATP production: MAS, MCU, and G3PS. Among these, G3PS operates least efficiently, but secures neuronal function as the ultimate backup mechanism.

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