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'Mild mitochondrial uncoupling' induced protection against neuronal excitotoxicity requires AMPK activity

Petronela Weisová [a,b], Ujval Anilkumar [a], Caitriona Ryan [a], Caoimhín G. Concannon [a], Jochen H.M. Prehn [a,*], Manus W. Ward [a,1]

[a] Department of Physiology and Medical Physics, Centre for the Study of Neurological Disorders, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland
[b] Max F. Perutz Laboratories, University of Vienna, Dr. Bohr-Gasse 9, 1030 Vienna, Austria

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ABSTRACT

The preconditioning response conferred by a mild uncoupling of the mitochondrial membrane potential ($\Delta\psi_m$) has been attributed to altered reactive oxygen species (ROS) production and mitochondrial Ca^{2+} uptake within the cells. Here we have explored if altered cellular energetics in response to a mild mitochondrial uncoupling stimulus may also contribute to the protection. The addition of 100 nM FCCP for 30 min to cerebellar granule neurons (CGNs) induced a transient depolarization of the $\Delta\psi_m$, that was sufficient to significantly reduce CGN vulnerability to the excitotoxic stimulus, glutamate. On investigation, the mild mitochondrial 'uncoupling' stimulus resulted in a significant increase in the plasma membrane levels of the glucose transporter isoform 3, with a hyperpolarisation of $\Delta\psi_m$ and increased cellular ATP levels also evident following the washout of FCCP. Furthermore, the phosphorylation state of AMP-activated protein kinase (AMPK) (Thr 172) was increased within 5 min of the uncoupling stimulus and elevated up to 1 h after washout. Significantly, the physiological changes and protection evident after the mild uncoupling stimulus were lost in CGNs when AMPK activity was inhibited. This study identifies an additional mechanism through which protection is mediated upon mild mitochondrial uncoupling: it implicates increased AMPK signalling and an adaptive shift in energy metabolism as mediators of the preconditioning response associated with FCCP-induced mild mitochondrial uncoupling.

1. Introduction

Mitochondria have long been recognized as the key site for oxidative phosphorylation and ATP production within cells, however, in recent years much more research has focused on their roles in cellular life/death decisions [1]. It is generally accepted that the mitochondrial membrane potential ($\Delta\psi_m$) is an important factor in the maintenance of cellular energy and ion homeostasis, and that mitochondria 'buffer' cytosolic Ca^{2+} elevations by a $\Delta\psi_m$ dependent uptake of Ca^{2+} into mitochondria [2,3].

Neurotoxicity resulting from glutamate receptor over-activation ('excitotoxicity') has been linked to the pathophysiology of several acute and chronic neurological disorders [4]. Excitotoxic injury is predominantly mediated by the activation of Ca^{2+} permeable N-methyl-D-aspartate (NMDA) receptors. The $\Delta\psi_m$ dependent uptake of Ca^{2+} into the mitochondrial matrix has been shown to greatly reduce cytosolic Ca^{2+} elevations during excitotoxic injury [5-7]. Interestingly, it has been shown that a controlled depolarization of $\Delta\psi_m$ by administration of respiratory chain inhibitors or by 'mitochondrial uncoupling' using the protonophore carbonyl cyanide p-(trifluromethoxy) phenyl-hydrazone (FCCP), is able to delay or inhibit excitotoxic injury [5,6,8]. This protection is evident despite increased cytosolic Ca^{2+} levels in response to these treatments [5,6]. 'Mild mitochondrial uncoupling' has also been suggested to be of potential therapeutic value in disorders where energy stress is known to have a major role, including ischemic stroke [9-11]. Such interventions have been shown to protect different cell types against stimuli that would otherwise be lethal by decreasing mitochondrial Ca^{2+} uptake and by inducing a mild oxidative stress within the cell [12-14]. However, it is not known whether energy sensing pathways in addition to inhibition

Abbreviations: AICAR, 5-amino-4-imidazolecarboxamide riboside; AMP, 5′-adenosine monophosphate; AMPK, 5′-adenosine monophosphate-activated protein kinase; ANOVA, analysis of variance; ATP, adenosine 5′-(tetrahydrogen triphosphate); BSA, bovine serum albumin; Ca^{2+}, calcium; CGNs, cerebellar granule neurons; DIV, days *in vitro*; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; GLUT 1, glucose transporter 1; GLUT 3, glucose transporter 3; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]; HK-II, hexokinase II; HRP, horse radish peroxidase; kDa, kilodaltons; $\Delta\psi_m$, mitochondrial membrane potential; NAD^+/NADH, nicotinamide adenine dinucleotide; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; PFK-2, phosphofructokinase-2; PBS, phosphate buffered saline; RNA, ribonucleic acid; ROS, reactive oxygen species; SEM, standard error of the mean; TMRM, tetramethylrhodamine methylester; UCP, uncoupling protein

* Corresponding author at: Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, 123 St Stephen's Green, Dublin 2, Ireland. Tel.: +353 1 402 2255; fax: +353 1 402 2447.
E-mail address: jprehn@rcsi.ie (J.H.M. Prehn).
[1] Present address: Science Foundation Ireland, Wilton Plaza, Dublin 2, Ireland.

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of mitochondrial Ca^{2+} uptake or reactive oxygen species (ROS) production may also contribute to the protective effects of mild mitochondrial uncoupling.

A number of energy sensing pathways have been shown to be activated in response to mild mitochondrial uncoupling [13,15-17]. These pathways alter energy metabolism by stimulating glucose uptake, the oxidation of NADH, and promoting respiratory activity [15,17]. The ancient energy sensor, AMP-activated protein kinase (AMPK) has been shown to be activated in neurons in response to ischemia, glucose deprivation, and hypoxia, i.e. in models where energetic stress is known to contribute to injury [18-22]. Indeed, we have previously shown that excitotoxic injury acutely depletes cellular ATP levels, activating AMPK and resulting in an increase in glucose uptake and utilization [20]. Moreover, we observed that the duration and level of the AMPK activity within a cell following energetic stress may be pivotal in determining if a cell can tolerate a stimulus, or if the cell enters cell death when AMPK activity is over–activated [21,23].

Here we have investigated the role of AMPK signalling and altered cellular energetics in the preconditioning response associated with 'mild mitochondrial uncoupling' in response to FCCP. We have identified that primary neurons with modest changes in mitochondrial function were protected from excitotoxic injury, and that an increase in AMPK activity was required for this process.

2. Experimental procedure

2.1. Materials

Fetal calf serum and minimal essential medium were from Invitrogen (Bio Sciences, Dun Laoghaire, Ireland). Glutamate, glycine, FCCP, and rotenone were from Sigma Aldrich (Tallaght, Dublin, Ireland). Fluo-4 acetoxymethyl ester (Fluo-4 AM) and tetramethylrhodamine methyl ester (TMRM) were purchased from Invitrogen (Bio Sciences, Dun Laoghaire, Ireland). Compound C was obtained from Calbiochem (Merck Biosciences, Nottingham, UK). Aminoimidazole carboxamide ribonucleotide (AICAR) was from Cell Signaling (Isis, Wicklow, Ireland).

2.2. Preparation of primary cerebellar granule neurons

Cerebellar granule neurons (CGNs) were prepared as described previously [24]. Cells were plated on poly-L-lysine-coated glass Willco dishes (Amsterdam, The Netherlands), 6-well and 24-well plates. The following plating volumes were used for this study: 2 Millions/well in 6 well plates for Western blotting; 750,000/well in 24 well plates for ATP assays and GLUT3 translocation assays on the flow cytometer; 1 Millions/well on Willco dishes for confocal imaging studies. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Cells were pre-treated with FCCP at day 7 in culture. All animal work was carried out with ethical approval from the RCSI and under licenses by the Irish Department of Health and Children.

2.3. Determination of cellular injury—Hoechst staining of nuclear chromatin

Neurons cultured on 24-well plates were stained live with Hoechst 33258 (Sigma) at a final concentration of 1 μg/ml following treatments. After incubation for 10–15 min in dark, nuclear morphology was observed using an Eclipse TE 300 inverted microscope (Nikon, Dusseldorf, Germany) and a 20× or 40× dry objective. For each time point and treatment cells were analyzed for pyknotic nuclei morphology in four subfields of each cell culture (up to 2000 cells per time point). All experiments were performed at least three times with similar results.

2.4. Characterization of changes in Ca^{2+} dynamics and $\Delta\psi_m$ by confocal microscopy

CGNs on Willco dishes were loaded with 20 nM TMRM or with 100 nM TMRM for the detection of membrane potential changes in response to FCCP (Ward et al. [24]). CGNs were exposed to different FCCP concentrations in the presence of TMRM to monitor changes in $\Delta\psi_m$. The entire volume of the medium was exchanged. For TMRM and Ca^{2+} imaging experiments, cells were co-loaded with 100 nM TMRM and Fluo-4 AM (3 μM) for 30 min at 37 °C in the dark in experimental buffer containing in mM; 120 NaCl, 3.5 KCl, 0.4 KH_2PO_4, 20 HEPES, 5 $NaHCO_3$, 1.2 Na_2SO_4, 1.2 $CaCl_2$, and 15 glucose at pH 7.4. The Willco dishes with cells were mounted in a non-perfusion (37 °C) holder and placed on the stage of an LSM 510 Meta confocal microscope (Zeiss, Oberkochen, Germany). CGNs were exposed to severe energetic stress by addition of glutamate and glycine (100 and 10 μM for 10 min) on stage of the LSM 510 microscope. Fluo-4 AM (kDa for Ca^{2+}: 345 nM) was excited at 488 nm, and the emission was collected through a 505–550 nm barrier filter; TMRM was excited at 543 nm and the emission was collected through a 560 nm long-pass filter. Images were taken in 1–2 min intervals and the resulting fluorescent images processed using MetaMorph Software version 7.1, release 3 (Molecular Devices, Berkshire, UK). For evaluation of $\Delta\psi_m$ a region of interest (ROI) was marked around the whole cell and the integrated total flourescence intensity measured and analyzed using Metamorph software. The initial values of baseline in each experiment were averaged and this value was normalized to 100% to allow for a comparison of responses between cells and experiments.

2.5. Western blotting

Preparation of cell lysates and Western blotting was carried out as described previously [20]. Blots were probed with a rabbit polyclonal antibody against phospho-Thr 172-AMPK (Cell Signaling, Isis, Ireland; 1:1,000); a rabbit polyclonal total AMPKα (Cell Signaling, 1:1000) at 4 °C overnight or a mouse, monoclonal anti-β-actin antibody (Sigma ; 1:5000) for 1 h at room temperature. Membranes were washed with Tris-buffered saline solution containing 0.1% Tween-20 three times for 10 min. Immunoreactivity was detected with anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies (1:5000, Promega, Madison, WI) for 2 h at room temperature. Blots were washed and developed using ECL chemiluminescence detection reagent (Amersham Biosciences, Buckinghamshire, UK). Images were acquired using a FujiFilm LAS-3000 imaging system (Fuji, Sheffield, UK).

2.6. Immunocytochemistry

For immunofluorescence analysis, cells were fixed on Willco dishes with formalin, washed three times with PBS, and then incubated with blocking solution (PBS with 20% fetal calf serum and 1% BSA) for 1 h at room temperature. Surface levels of GLUT 3 were detected using a rabbit polyclonal anti-GLUT 3 antibody (Millipore, Abcam). The antibody was used at a concentration of 1:250 in PBS containing 20% horse serum and 1% BSA. After incubation, cells were washed twice with PBS and incubated with Alexa Fluor 488 goat anti-rabbit IgG (H + L) antibody (Molecular Probes, Invitrogen, Oregon) diluted 1:250. The secondary antibody was detected using confocal microscopy as described above.

2.7. Flow cytometry analysis of plasma membrane GLUT 3 levels

CGNs were harvested from 24 well plates following FCCP pretreatment (100 nM for 30 min and left for recovery in media for a time indicated) and fixed in 1% formalin for 20–25 min at 4 °C. After incubation with the GLUT 3 antibody (1: 250) diluted in PBS + 0.1%

BSA for 1 h, cells were washed and incubated with an Alexa Fluor 488 goat anti-rabbit IgG (H + L) antibody (Molecular Probes, Invitrogen) diluted 1:250 for 1 h. To confirm the specificity of the staining cells were incubated with secondary antibody only. After washing cells three times with PBS + 0.1% BSA, samples were analyzed by flow cytometry on a Partec CyFlow ML (Partec, Münster, Germany) followed by analysis using FloMax software. In all cases, a minimum of 10^4 events were acquired.

2.8. Measurement of ATP levels

CGNs were maintained on poly-L-lysine (5 μg/ml)-coated 24-well plates for 7 days before use. CGNs were treated and allowed to recover as indicated in the Results section. Lysis of CGNs was performed with a hypotonic lysis buffer (Tris-acetate buffer, pH 7.75). Sample from CGNs (100 μl) and 100 μl of the luciferin-luciferase reaction kit (ENLITEN ATP Assay System Bioluminescence Detection kit; Promega, Southampton, UK) were reacted to quantify ATP levels. The content of ATP was determined by a concentration standard curve, and ATP levels were normalized according to the protein concentration for each sample (μmol ATP/mg protein).

2.9. Statistics

Data are presented as means ± SEM. For statistical comparison, *t*-test or one-way analysis of variance and post-hoc Tukey's test were employed using SPSS software (SPSS GmbH Software, Munich, Germany). p-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Alterations in mitochondrial bioenergetics in response to a mild mitochondrial uncoupling stimulus

The partial uncoupling of $\Delta\psi_m$ has been shown to be protective in models were energetic stress is believed to play a significant role [9,14,17]. We utilized the protonophore FCCP, a potent 'uncoupler' of mitochondrial oxidative phosphorylation that dissipates $\Delta\psi_m$, to establish a model of 'mild mitochondrial uncoupling'. Neurons were pre-loaded for 30 min with the potential-sensitive cationic dye TMRM to assess changes in $\Delta\psi_m$. TMRM was used either at a concentration of 100 nM (presumed 'quenched' mode) or 20 nM (presumed 'non-quenched' mode) [24,25]. Treatment of CGNs that were pre-loaded with 100 nM TMRM showed a stable baseline fluorescence, indicating full equilibration of TMRM (Fig. 1A and B, stage I). Neurons were then exposed to 100 nM FCCP for 30 min with TMRM present during the treatment. Treatment with 100 nM FCCP has resulted in a rapid, transient increase in whole cell TMRM fluorescence ('de-quenching') reflective of a depolarisation of $\Delta\psi_m$ due to net movement of quenched TMRM from the mitochondrial matrix into the cytosol (Fig. 1A and B, stage II). Interestingly this FCCP-induced depolarization of $\Delta\psi_m$ was paralleled by a rapid spike in cytosolic Ca^{2+}, determined with the Ca^{2+} indicator Fluo-4 (Fig. 1C). In contrast, the addition of 1 μM FCCP resulted in a rapid and almost complete depolarization of $\Delta\psi_m$ (Fig. 1A and B stage IV/V) that was followed closely by a loss of Ca^{2+} homeostasis within the cells (Fig. 1C). The non-quenched mode, can often be utilized to more accurately monitor both hyperpolarizations and depolarizations of $\Delta\psi_m$ therefore, neurons were also loaded with 20 nM TMRM. The addition of 100 nM FCCP induced a decrease in TMRM fluorescence (Fig. 1 D, E and F) again indicating a depolarization of $\Delta\psi_m$ which remained constant for the duration of the FCCP exposure. Significantly, upon removal of the stimulus TMRM fluorescence recovered to a level above baseline indicating that $\Delta\psi_m$ hyperpolarizes following the transient uncoupling stimulus (Fig. 1 D, E and F).

3.2. A mild mitochondrial uncoupling provides protection to neurons against glutamate induced energetic stress (excitotoxicity)

In order to identify if 'mild mitochondrial uncoupling' preconditioned (protected) cells against a subsequent 'toxic' insult we utilized a model of glutamate induced injury that has been extensively characterized by us previously [7,20,21,24,26]. We first established that a stimulation of CGNs with 100 nM FCCP for 30 min did not induce a significant level of injury (Fig. 2A). Of note, an incubation of CGNs with 1 μM FCCP (a concentration that induced an almost complete depolarization of $\Delta\psi_m$) for 30 min resulted in 68.6 ± 1.5% cell death within a 24 h period.

Next, CGNs were pre-treated with FCCP (100 nM for 30 min) and allowed to recover for a period of 1 h. Cells were then stimulated with glutamate and glycine (100 μM/10 μM) for 10 min and cell death was assayed after 24 h (Fig. 2B). The pre-treatment of the CGNs cultures with 100 nM FCCP induced on average 31.5 ± 8.0% less cell death than that identified in cells pre-treated with vehicle (Fig. 2C, D). Interestingly, protection was still evident 72 h after the glutamate stimulus indicating that the preconditioning response provided long-lasting protection rather than delaying the onset of injury to a later time point (Suppl Fig. 1). In order to understand the preconditioning response further, CGNs were exposed to glutamate at 1, 4 and 24 h after the FCCP-preconditioning stimulus, and the cell death assayed for each stimulus after an additional 24 h (Fig. 2E). We found that the protective response induced by the mild uncoupling stimulus was transient and no longer evident after 24 h of FCCP washout (Fig. 2E).

We next investigated alterations in cytosolic Ca^{2+} levels in neurons exposed to glutamate following the mild uncoupling stimulus. In agreement with a previous report [6], we detected that FCCP-preconditioned neurons displayed significantly elevated cytosolic Ca^{2+} levels at baseline as well as during and 20 min following glutamate exposure when compared to vehicle-pre-treated controls (Fig. 3A–C). Interestingly, on examination of mitochondrial bioenergetics using 20 nM TMRM at a single cell level in neurons pre-treated with FCCP, a significant increase in the baseline TMRM fluorescence was identified in cells pre-treated with FCCP compared to non-treated (Fig. 3D–F). In contrast to the experiments using FCCP alone shown in Fig. 1D, we observed a transient de-quenching of TMRM fluorescence in response to glutamate. TMRM fluorescence changes induced by glutamate may also be sensitive to changes in plasma membrane potential or mitochondrial volume [25,26].

3.3. Mild mitochondrial uncoupling induces activation of AMPK, increased expression of GLUT3 at the plasma membrane and increased intracellular ATP levels

In a previous investigation we found that AMPK became activated following an excitotoxic stimulus and that this elevation in AMPK activity was closely linked to increased glucose transporter 3 (GLUT 3) cell surface expression, a hyperpolarization of $\Delta\psi_m$, and increased ATP availability in neurons tolerant to the glutamate toxicity [20]. Therefore, we wished to establish if similar signalling pathways were activated in the model of mild mitochondrial uncoupling described above.

On examination, the phosphorylation status of AMPK (Thr172) was found to be increased significantly 5 min after a preconditioning exposure to FCCP (100 nM), with the phosphorylation state continuing to increase up to 30 min after the addition of FCCP (Fig. 4A). As positive controls, neurons were also exposed to the pharmacological AMPK activator, AICAR (2.5 mM, 60 min), and to the complex I inhibitor, rotenone (5 nM, 30 min) [27,28]. The increase in AMPK signalling following the addition of FCCP was associated with an increase in GLUT 3 expression at the plasma membrane (Fig. 4B and C), a response that could be mimicked by the stimulation of AMPK activity with AICAR (Fig. 4B and C).

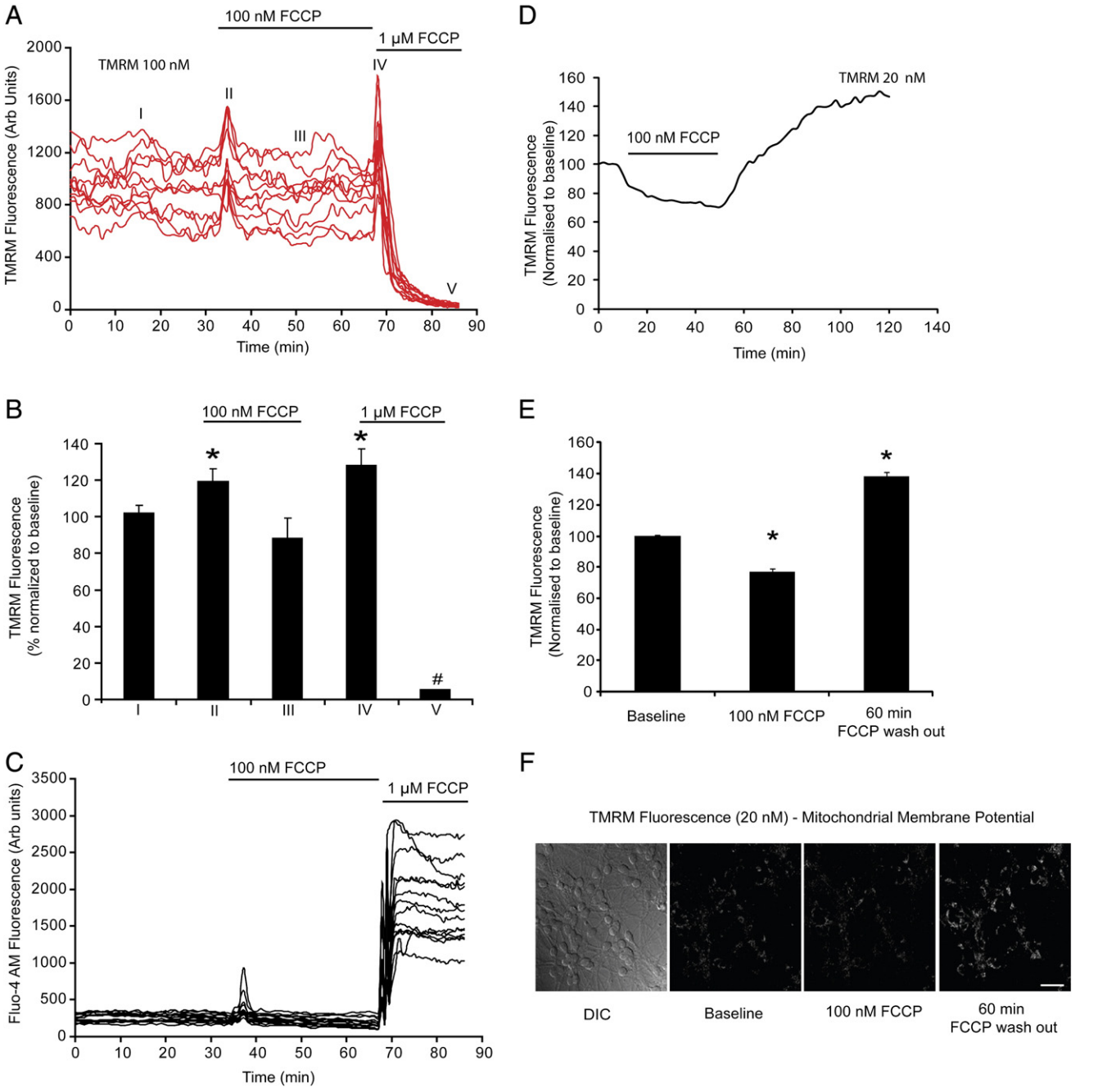

Fig. 1. Model of FCCP-induced 'mild mitochondrial uncoupling'. A–C) CGNs were loaded with TMRM (100 nM) and Fluo-4 (3 μM for 30 min at 37 °C) prior to stimulation with 100 nM FCCP for 30 min followed by a sequential addition of a fully depolarizing concentration of FCCP (1 μM) and monitored over time on a Zeiss LSM 510 confocal microscope. A) Representative traces for TMRM fluorescence in quenched mode (100 nM) in neurons prior to and during the 2 additions of FCCP as described above. B) Quantification of the TMRM at different stages of FCCP treatment as indicated in A (I, II, III, IV and V). Data are presented as mean ± SEM. *$p<0.01$ represents difference between I and II, III, IV. #$p<0.001$ is difference between stages I and V. C) Representative traces for Fluo-4 AM fluorescence in neurons prior to and during stimulation with varying FCCP concentrations (100 nM for 30 min and 1 μM FCCP). D, E) CGNs were loaded with a non-quenching concentration of TMRM (20 nM) prior to the addition of vehicle of 100 nM FCCP and $\Delta\psi_m$ was monitored over time on a Zeiss LSM 510 confocal microscope. *$p<0.001$ difference between vehicle pre-treated ($n=21$) and FCCP pre-treated ($n=26$) neurons. F) Representative images of TMRM fluorescence in FCCP pre-treated neurons. Bar, 30 μm.

Furthermore cellular ATP levels were found to have a marked increase 1 h following FCCP (100 nM) washout (Fig. 4D). Conversely, the exposure of neurons to a toxic FCCP concentration (1 μM) induced a prolonged ATP depletion (Fig. 4D). This data indicated that the preconditioning of neurons through mild mitochondrial uncoupling (FCCP) results in an activation of signalling pathways that increase cellular energetics within the cell. Interestingly acute treatment with AICAR led to only a modest increase in cellular ATP levels after 30 min of treatment (Fig. 4D), which quickly returned to baseline levels after 60 min (Fig. 4D) of AICAR treatment.

Previously, a recovery in ATP levels has been reported following a mild mitochondrial uncoupling stimulus in fibroblasts which was accompanied by an increase in mitochondrial mass and an induction of genes related to mitochondrial energy metabolism [29]. To determine whether increased ATP availability observed in our model of mitochondrial uncoupling was related to changes in selected genes believed to play a role in cellular metabolism (glycolytic flux and mitochondrial metabolism), we examined mRNA levels of several key enzymes and transporters (Suppl Fig. 2). No major increase in expression levels for a range of genes involved

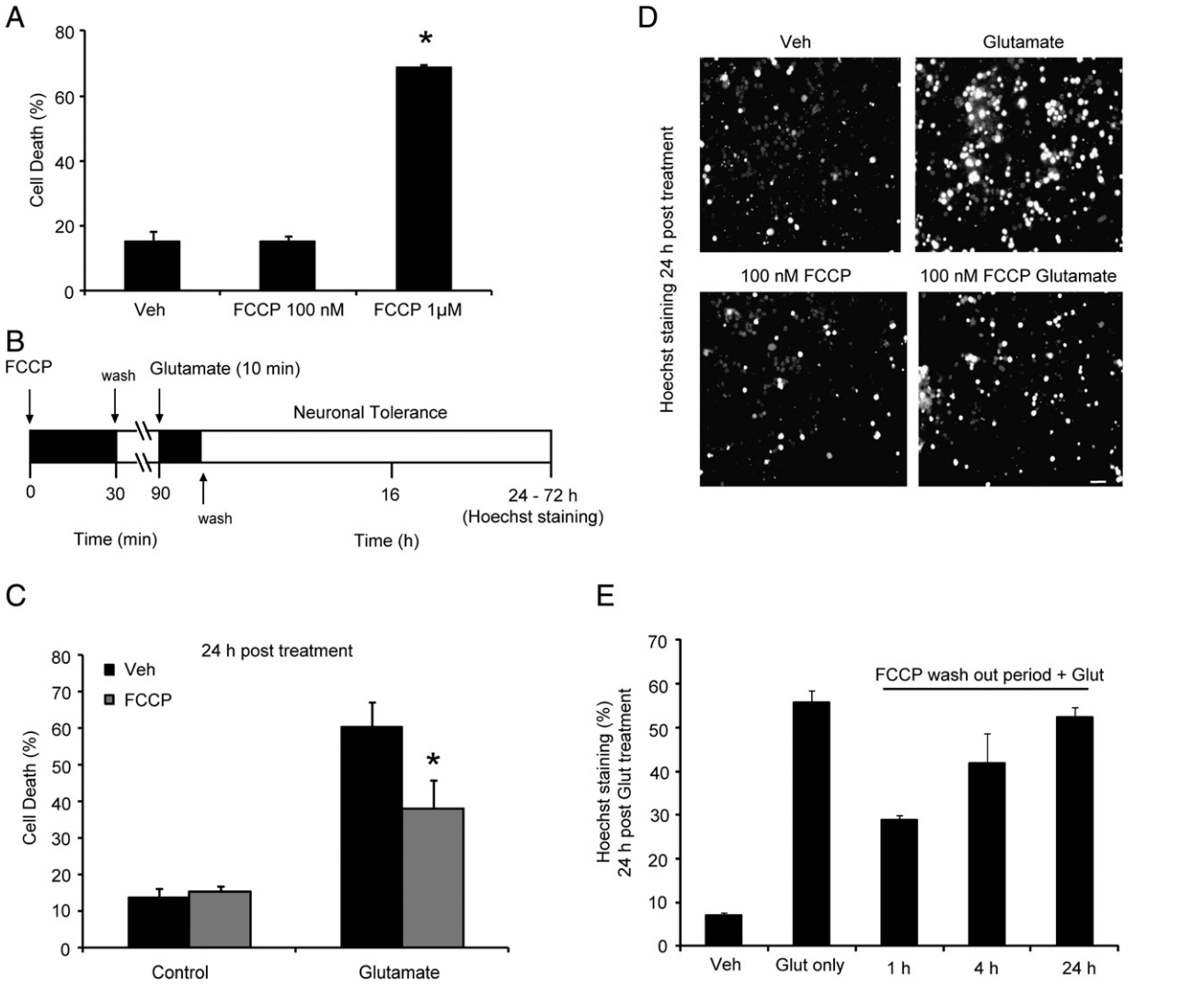

Fig. 2. 'Mild mitochondrial uncoupling' of CGN cultures with FCCP provides acute protection against glutamate overexcitation. A) CGNs were treated with FCCP (100 nM or 1 μM) for 30 min. The compound was washed off and viability examined after 24 h. Nuclei were stained with Hoechst 33358 (1 μg/ml) with uniformly stained nuclei counted as healthy (viable neurons) and condensed nuclei counted as apoptotic (n = 3 experiments in triplicate) *p < 0.001 difference between 100 nM and 1 μM FCCP. B) Flowchart depicting the experimental conditions used; CGNs were pre-stimulated for 30 min with FCCP (100 nM) in media, the cells were washed and 60 min later stimulated with glutamate (glutamate/glycine 100 μM/10 μM). Injury in the cultures was assessed after 24 and 72 h. C) The viability of CGNs was measured using Hoechst staining in cultures pre-treated with vehicle or FCCP and stimulated with glutamate. (n = 5) *p < 0.05 difference between CGN cultures pre-treated with FCCP compared to vehicle-treated cultures. D) Hoechst staining in CGN cultures 24 h after cells were exposed to vehicle or 100 nM FCCP (left panel) or pre-treated with vehicle or 100 nM FCCP and exposed to glutamate for 10 min (right panel). Bar, 40 μm. E) CGNs were preconditioned with FCCP (100 nM for 30 min) and exposed to glutamate/glycine (10 min) at 1, 4 and 24 h after the stimulus. Twenty four hours after each of the excitation timepoint cellular viability was determined by Hoechst staining of condensed pyknotic nuclei and imaged using an Eclipse TE 300 inverted microscope (Nikon, Germany). Data are presented as mean ± SEM with experiments repeated twice in quadruplicate (n = 8).

in cellular metabolism was observed, particularly 1 h following the FCCP treatment where many of the major physiological changes were evident (Suppl Fig. 2), however a moderate increase in hexokinase II (*hkII*) expression could be detected 1 h following FCCP treatment (Suppl Fig. 2).

3.4. Mild mitochondrial uncoupling stimulus accelerates the recovery of cellular ATP in response to glutamate excitation

We next investigated whether preconditioning with FCCP led to improved cellular bioenergetics during and following glutamate excitation in CGNs. Interestingly, we observed that preconditioning with FCCP failed to alter the acute decrease in ATP levels in response to glutamate, but accelerated the recovery of cellular ATP levels 90 min after termination of glutamate excitation (Fig. 5). These data suggested that the preconditioning effect of FCCP increased the ability of CGN to cope with increased energy demands.

3.5. Inhibition of AMPK during a mild mitochondrial uncoupling stimulus re-sensitizes CGNs to glutamate excitotoxicity

In order to determine if AMPK activity did indeed regulate the preconditioning response identified following a 'mild mitochondrial uncoupling' stimulus, CGN cultures were also incubated with compound C, a known inhibitor of AMPK signalling [20,30,31] during the preconditioning stimulus. FCCP-induced mitochondrial uncoupling induced a robust increase in the phosphorylation state of AMPKα (Thr172) (Fig. 6A). Significantly, on examination of AMPK phosphorylation by Western blot analysis in cultures co-incubated with compound C and FCCP, we found that AMPKα (Thr172) phosphorylation levels were greatly reduced and similar to that of vehicle-treated cultures (Fig. 6A). A densitometric analysis of the phospho-(Thr172) AMPK levels further highlighted this inhibitory effect (Fig. 6B). Furthermore in neuronal cultures co-incubated with compound C and FCCP the increase in GLUT 3 surface expression was lost (Fig. 6C), as was the increase in baseline TMRM fluorescence

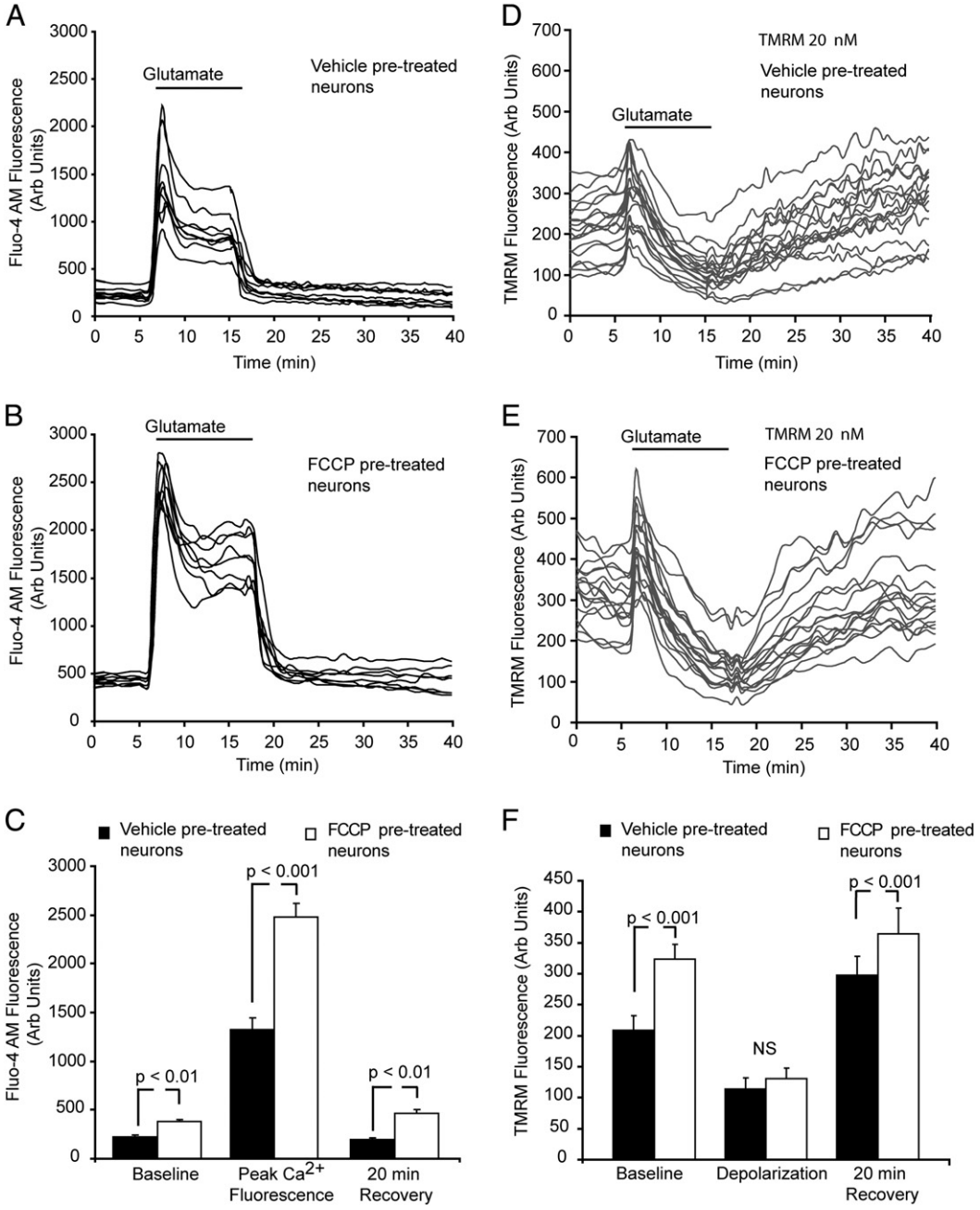

Fig. 3. Increase in $[Ca^{2+}]_c$ and mitochondrial membrane potential in FCCP-pre-treated CGNs during glutamate excitation. A) CGNs pre-treated with vehicle or B) FCCP (100 nM 30 min) were loaded with Fluo-4 AM (3 μM) for 30 min at 37 °C in dark prior to stimulation with glutamate/glycine (100 μM/10 μM for 10 min) and cytosolic Ca^{2+} responses were monitored over time on a Zeiss LSM 510 confocal microscope. Traces are representative of responses from 3 separate experiments. C) Average Fluo-4 AM peak area in vehicle pre-treated and FCCP pre-treated neurons following glutamate excitation represented as mean ± SEM. *$p<0.001$ difference between vehicle pre-treated ($n=19$) and FCCP pre-treated ($n=25$) neurons. Data are from $n=3$ separate experiments with 6–12 neurons in a single field. D) CGNs pre-treated with vehicle or E) FCCP (100 nM for 30 min) pre-treated neurons were loaded with TMRM (20 nM) prior to exposure to excitotoxicity (glutamate/glycine 100 μM/10 μM for 10 min) and $\Delta\psi_m$ was monitored over time on a Zeiss LSM 510 confocal microscope. F) Average TMRM fluorescence in vehicle pre-treated and FCCP pre-treated neurons following glutamate excitation represented as mean ± SEM. *$p<0.001$ difference between vehicle pre-treated ($n=23$) and FCCP pre-treated ($n=31$) neurons. Data are from $n=3$ separate experiments each with 7–12 neurons in a single field.

(Fig. 6D). Treatment with compound C also reduced the FCCP-induced increase in ATP availability (Fig. 6E) after 1 h of preconditioning. Most significantly, the uncoupling induced neuroprotection response was also lost in those CGN cultures co-incubated with FCCP and compound C prior to glutamate excitation (Fig. 6F).

4. Discussion

In this study a 'mild mitochondrial uncoupling' stimulus induced by low concentrations of FCCP has been shown to be sufficient to induce a rapid activation of AMPK in primary neurons. Furthermore, our data has provided evidence that the neuroprotection against glutamate excitotoxicity achieved through FCCP preconditioning required AMPK signalling.

The idea that mild mitochondrial uncoupling could be important in neuronal survival has been previously supported by several other observations [11-13,15,17,32,33]. Some pioneering work of Nicholls and co-workers [5,34,35] as well as Reynolds and co-workers [6] suggested that a controlled decrease in $\Delta\psi_m$ is able to diminish a detrimental Ca^{2+} influx during an excitotoxic insult. Further studies

went on to show that partial uncoupling of mitochondria decreased loading of Ca^{2+} to the mitochondria and inhibited cell death induced by excitotoxicity [6,36]. Much research has focused on the association of such preconditioning responses with oxidative stress signalling within the cell [13,32,37]. While the role of oxidative stress in preconditioning responses is well established in the field, other work by Nicholls and co-workers has suggested that oxidative stress may not be essential to the development of injury during periods of energetic stress [38,39].

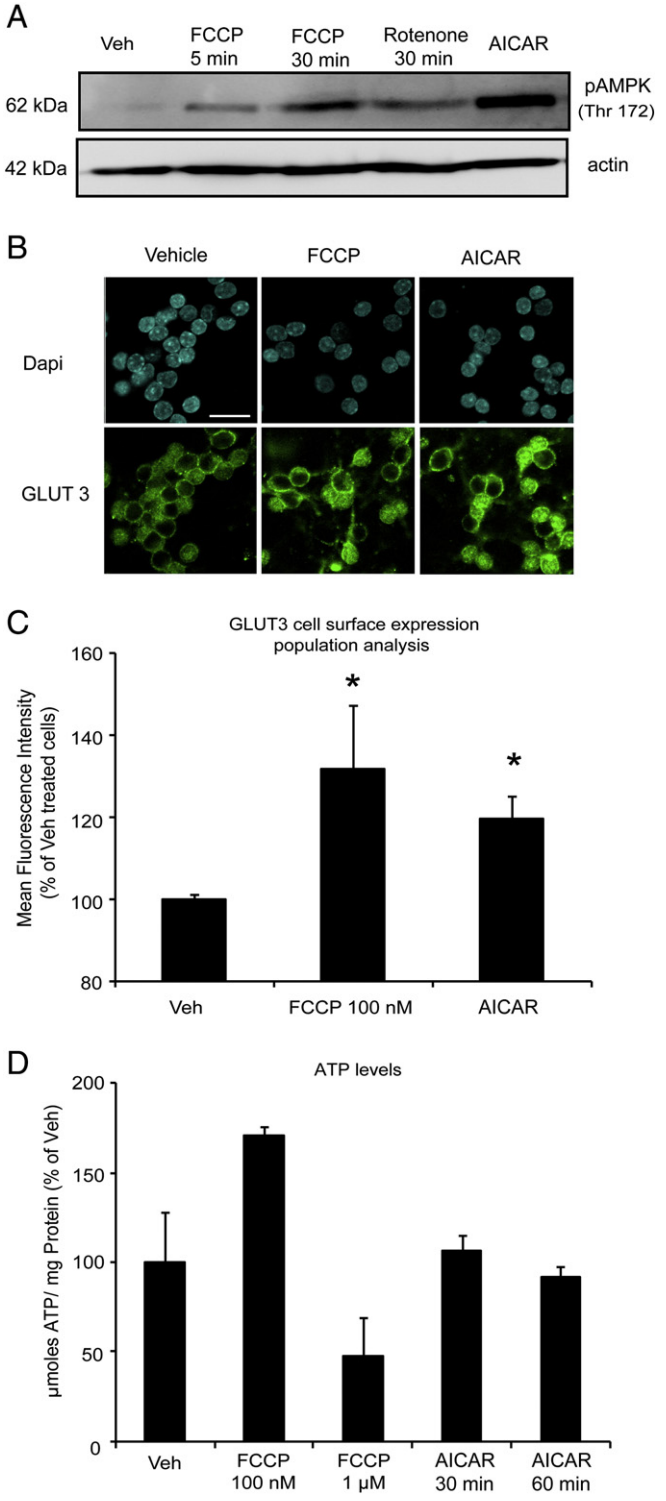

Fig. 4. 'Mild mitochondrial uncoupling' increases AMPK activation, cell surface glucose transporter isoform 3 expression and cellular ATP levels. A) CGN cultures were treated with FCCP (100 nM) for 5 or 30 min, lysed, and the phosphorylation status of AMPK (Thr 172) examined by Western blotting. Treatment with the complex I inhibitor rotenone (5 nM, 30 min) and the AMPK activator AICAR (2.5 mM, 60 min) served as additional positive controls. The observed responses are similar to that obtained in 2 separate experiments. Actin was used as a loading control. B) Immunofluorescence of the cell surface expression of GLUT 3 in vehicle- and 100 nM FCCP-treated neurons (30 min treatment). Nuclei were stained with Dapi (blue) and an Alexa Fluor 488-labeled secondary antibody (green) was used to visualize GLUT 3 expression. AICAR (2.5 mM for 60 min) treated neurons served as an additional positive control. Bar, 20 µm. C) Population analysis (flow cytometry analysis) of GLUT 3 surface expression for vehicle and neurons treated with FCCP after 30 min of treatment. Data presented as mean ± SEM. $p<0.001$ difference between vehicle-treated neurons and neurons 30 min post-FCCP treatment. Experiments were carried out in triplicate from 3 separate cultures and data presented as mean ± SEM. For flow cytometry experiments a minimum of 10^4 events were collected per sample. Scale is shown from 80 to 160% to highlight the differences between groups. D) Neurons were lysed and their ATP content measured (µmol ATP/mg protein) 60 min after a 30 min pre-treatment with 100 nM FCCP, or 30 and 60 min after treatment with AICAR (2.5 mM). Treatment with FCCP at a concentration of 1 µM for 90 min served as a positive control for decline in ATP levels. Data are presented as mean ± SEM with experiments repeated twice in triplicate (n = 6).

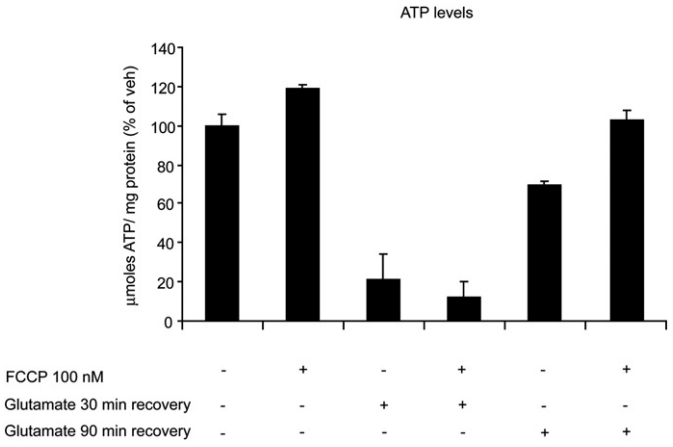

Fig. 5. 'Mild mitochondrial uncoupling' with FCCP accelerates the recovery of cellular ATP in response to glutamate excitation. CGNs were treated with FCCP (100 nM for 30 min followed by 1 h recovery), and then exposed to glutamate. Controls were sham-exposed. ATP levels (µmol ATP/ mg protein) were determined 30 min and 90 min after termination of the glutamate. Data are presented as mean ± SEM with experiments repeated twice in triplicate (n = 6).

Here we hypothesised that the induction of a mild energetic stress could be an adaptive response, activating compensatory energetic pathways that prevent against ATP loss during a subsequent severe energetic stress such as that evident during glutamate toxicity. On examination of previously published data by others using similar cellular systems [27,39] we successfully established a model where $\Delta\psi_m$ and cell survival were not significantly inhibited by mild mitochondrial 'uncoupling' with FCCP. Addition of 250 nM FCCP has been shown to be sufficient to induce uncontrolled respiration in cerebellar granule neurons [39], and FCCP concentrations between 10 and 100 nM increased respiration and produced changes in $\Delta\psi_m$ almost below detection level (2–5 mV) [39]. Guided by these previous observations, we found that a transient uncoupling of respiration with 100 nM FCCP was sufficient to induce significant neuroprotection against glutamate toxicity (Fig. 2). However, when considering the use of FCCP as a preconditioning agent, it is important to note that excessive uncoupling may prove to be detrimental to cellular viability [26]. Furthermore, it should be noted that the timing of the preconditioning stimulus is of critical importance. For example it has been shown that even a 'mild respiratory inhibition' using rotenone potentiates and accelerates cell death when performed during an acute exposure to glutamate [27].

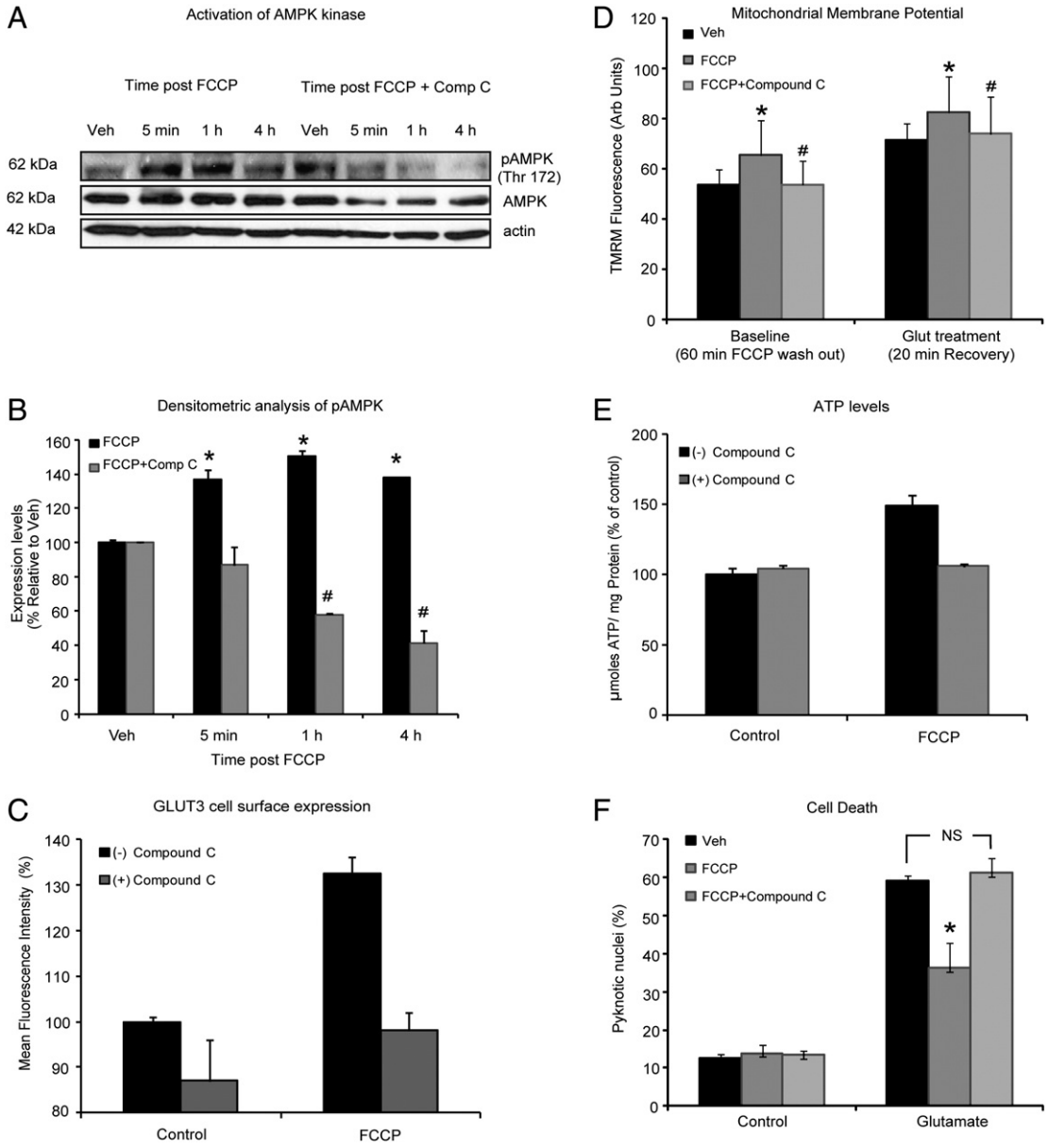

Fig. 6. 'Mild mitochondrial uncoupling' induced activation of AMPK, cellular preconditioning and its adaptative molecular events are suppressed by the AMPK inhibitor Compound C. A) Western blot analysis of AMPK activity (Thr 172) in vehicle-treated and FCCP-treated (100 nM for 30 min) neurons in the presence or absence of the AMPK inhibitor Compound C (10 μM) at indicated time points (5 min, 1 h and 4 h). Actin was used as loading control. B) Densitometric analysis of pAMPK (Thr 172) normalized to loading control and represented as % of vehicle-treated CGNs. Data are represented as mean ± SEM. *$p<0.001$ difference between vehicle-treated and FCCP pre-treated neurons at time points indicated. #$p<0.05$ difference between vehicle-treated neurons vs FCCP-treated neurons; both in the presence of Compound C. C) Immunofluorescence analysis of GLUT 3 surface expression in Control and FCCP (100 nM for 30 min followed by 1 h recovery) treated CGNs +/− Compound C (10 μM). Data presented as mean ± SEM. Experiments were carried out in triplicates. Data are presented as mean ± SEM with experiments repeated twice in triplicate ($n=6$). Scale is shown from 80 to 140% to highlight the differences between groups. D) Average TMRM fluorescence in vehicle, FCCP or Comp C plus FCCP pre-treated neurons stimulated with glutamate (100 μM/10 μM glutamate/glycine for 10 min) and monitored over time on a Zeiss LSM 510 confocal microscope. Data are represented as mean ± SEM. *$p<0.001$ difference between vehicle-treated and FCCP pre-treated neurons in baseline and recovery period indicating hyperpolarization of $\Delta\psi_{m}$. #$p<0.05$ difference between FCCP pre-treated vs Comp C plus FCCP pre-treated cultures in baseline and recovery period indicating suppression of FCCP mediated hyperpolarization of $\Delta\psi_{m}$. E) CGNs were treated with FCCP (100 nM for 30 min followed by 1 h recovery) +/− Compound C (10 μM) and ATP levels were determined (μmol ATP/mg protein). Data are presented as mean ± SEM with experiments repeated twice in triplicate ($n=6$). F) Cellular viability at 24 h for vehicle or FCCP-treated CGN cultures stimulated with glutamate in the presence and absence of compound C (10 μM). Viability was assayed with Hoechst 33358 (1 μg/ml). Data are presented as mean ± SEM *$p<0.01$, difference between vehicle and FCCP/glutamate treated neurons. N.S. No significant difference was found between vehicle-pre-treated CGNs stimulated with glutamate and CGN cultures co-treated with FCCP vs Compound C and excited with glutamate.

The present study also investigated the possible effects of mild mitochondrial uncoupling on downstream molecular signalling events. An association of AMPK with cellular protection has already been suggested in previous studies [19,40-42] with AMPK believed to be responsible for enhanced glucose uptake [20,43-45], and the stimulation of glucose utilization and glycolysis through the activation of 6-phosphofructo-2-kinase [16]. Glucose tranport is facilitated through AMPK by the activation of different glucose tranporters with GLUT 3 referred to as the neuron specific glucose transporter [46]. Previous work in this laboratory has shown a significant increase in GLUT 3 surface expression in cerebellar granule neurons following a 30-minute excitation with glutamate, and identified the importance of AMPK activation of GLUT 3 in mediating a tolerance to excitotoxicity [20]. It was also suggested that mild metabolic stress stimulated GLUT 1 expression, glucose utilization and prevented NAD^+ depletion in neurons, and this in turn protected neurons against energetic stress induced by

excitotoxicity [17]. Interestingly, in non-neuronal cells inhibition of mitochondrial respiration by nitric oxide (NO) stimulates GLUT 3 mediated glucose uptake through AMPK [45,47], thus affording cytoprotection against energy failure. Moreover, in perfused rat hearts anaerobic conditions or inhibitors of oxidative phosphorylation induced AMPK activation, and this was shown to phosphorylate and activate PFK-2, a potent stimulator of glycolysis [16]. In view of the similarities between models of mild uncoupling and other models of mitochondrial inhibition/anaerobic conditions [16,47], it is tempting to suggest that various types of mild mitochondrial stress can stimulate similar molecular pathways converging on the activation of AMPK.

Indeed, inhibition of AMPK signalling with compound C during the mild mitochondrial uncoupling stimulus abolished the neuroprotection afforded by FCCP preconditioning in our study. Unfortunately due to the transient nature (30 min exposures) of many of the experiments carried out in this study and the fact that prolonged AMPK activation also contributes to excitotoxic cell death [21], experiments utilizing genetic approaches to silence AMPK gene expression during and after the preconditioning stimulus were not feasible. It is also important to note that additional signalling pathways induced by FCCP preconditioning may have played important additional roles in stimulating ATP production, as treatment with AICAR *per se* increased AMPK activation and GLUT3 translocation, but only had a very mild effect on cellular ATP levels. These experiments suggested that AMPK is necessary, but not sufficient to stimulate ATP production in response to FCCP preconditioning. In other studies the role of endogenous uncoupling proteins (UCPs), in particular of UCP2, were reported to be neuroprotective in ischemia [13,33]. A role for UCPs in other neurological disorders and during ageing has also been reported [12,13]. The UCP isoform 4 has likewise been shown to modulate neuronal energy metabolism by increasing glucose uptake, thereby maintaining neuronal ATP levels [15]. Mitochondrial uncoupling is also linked to caloric restriction and the increased longevity of organisms [48-50]. Indeed it is now apparent that further studies are required to explore the role of AMPK in these processes.

4.1. Conclusion

Taken together our study indicates that preconditioning through 'mild mitochondrial uncoupling' with FCCP can serve as an inducer of protective compensatory mechanisms in neurons by preventing energy loss and upregulating cellular energetic flux. This preconditioning response may work in concert with other mechanisms such as increased ROS formation or decreased mitochondrial Ca^{2+} uptake, and requires the activation of AMPK, enabling an adaptive shift in energy metabolism of cells that protects against a subsequent toxic stimulus.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbabio.2012.01.016.

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References

[1] L. Galluzzi, K. Blomgren, G. Kroemer, Mitochondrial membrane permeabilization in neuronal injury, Nat. Rev. Neurosci. 10 (2009) 481–494.
[2] T.E. Gunter, K.K. Gunter, S.S. Sheu, C.E. Gavin, Mitochondrial calcium transport: physiological and pathological relevance, Am. J. Physiol. 267 (1994) C313–C339.
[3] R.J. White, I.J. Reynolds, Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure, J. Neurosci. 16 (1996) 5688–5697.
[4] D.W. Choi, Glutamate neurotoxicity and diseases of the nervous system, Neuron 1 (1988) 623–634.
[5] R.F. Castilho, O. Hansson, M.W. Ward, S.L. Budd, D.G. Nicholls, Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells, J. Neurosci. 18 (1998) 10277–10286.
[6] A.K. Stout, H.M. Raphael, B.I. Kanterewicz, E. Klann, I.J. Reynolds, Glutamate-induced neuron death requires mitochondrial calcium uptake, Nat. Neurosci. 1 (1998) 366–373.
[7] M.W. Ward, Y. Kushnareva, S. Greenwood, C.N. Connolly, Cellular and subcellular calcium accumulation during glutamate-induced injury in cerebellar granule neurons, J. Neurochem. 92 (2005) 1081–1090.
[8] B. Sengpiel, E. Preis, J. Krieglstein, J.H. Prehn, NMDA-induced superoxide production and neurotoxicity in cultured rat hippocampal neurons: role of mitochondria, Eur. J. Neurosci. 10 (1998) 1903–1910.
[9] J.D. Pandya, J.R. Pauly, V.N. Nukala, A.H. Sebastian, K.M. Day, A.S. Korde, W.F. Maragos, E.D. Hall, P.G. Sullivan, Post-injury administration of mitochondrial uncouplers increases tissue sparing and improves behavioral outcome following traumatic brain injury in rodents, J. Neurotrauma 24 (2007) 798–811.
[10] A.S. Korde, L.C. Pettigrew, S.D. Craddock, W.F. Maragos, The mitochondrial uncoupler 2,4-dinitrophenol attenuates tissue damage and improves mitochondrial homeostasis following transient focal cerebral ischemia, J. Neurochem. 94 (2005) 1676–1684.
[11] S.L. Mehta, P.A. Li, Neuroprotective role of mitochondrial uncoupling protein 2 in cerebral stroke, J. Cereb. Blood Flow Metab. 29 (2009) 1069–1078.
[12] S. Diano, R.T. Matthews, P. Patrylo, L. Yang, M.F. Beal, C.J. Barnstable, T.L. Horvath, Uncoupling protein 2 prevents neuronal death including that occurring during seizures: a mechanism for preconditioning, Endocrinology 144 (2003) 5014–5021.
[13] Z.B. Andrews, S. Diano, T.L. Horvath, Mitochondrial uncoupling proteins in the CNS: in support of function and survival, Nat. Rev. Neurosci. 6 (2005) 829–840.
[14] J.P. Brennan, R. Southworth, R.A. Medina, S.M. Davidson, M.R. Duchen, M.J. Shattock, Mitochondrial uncoupling, with low concentration FCCP, induces ROS-dependent cardioprotection independent of KATP channel activation, Cardiovasc. Res. 72 (2006) 313–321.
[15] D. Liu, S.L. Chan, N.C. de Souza-Pinto, J.R. Slevin, R.P. Wersto, M. Zhan, K. Mustafa, R. de Cabo, M.P. Mattson, Mitochondrial UCP4 mediates an adaptive shift in energy metabolism and increases the resistance of neurons to metabolic and oxidative stress, Neuromolecular Med. 8 (2006) 389–414.
[16] A.S. Marsin, L. Bertrand, M.H. Rider, J. Deprez, C. Beauloye, M.F. Vincent, G. Van den Berghe, D. Carling, L. Hue, Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia, Curr. Biol. 10 (2000) 1247–1255.
[17] D. Liu, M. Pitta, M.P. Mattson, Preventing NAD(+) depletion protects neurons against excitotoxicity: bioenergetic effects of mild mitochondrial uncoupling and caloric restriction, Ann. N. Y. Acad. Sci. 1147 (2008) 275–282.
[18] C. Peralta, R. Bartrons, A. Serafin, C. Blazquez, M. Guzman, N. Prats, C. Xaus, B. Cutillas, E. Gelpi, J. Rosello-Catafau, Adenosine monophosphate-activated protein kinase mediates the protective effects of ischemic preconditioning on hepatic ischemia-reperfusion injury in the rat, Hepatology 34 (2001) 1164–1173.
[19] C. Culmsee, J. Monnig, B.E. Kemp, M.P. Mattson, AMP-activated protein kinase is highly expressed in neurons in the developing rat brain and promotes neuronal survival following glucose deprivation, J. Mol. Neurosci. 17 (2001) 45–58.
[20] P. Weisova, C.G. Concannon, M. Devocelle, J.H. Prehn, M.W. Ward, Regulation of glucose transporter 3 surface expression by the AMP-activated protein kinase mediates tolerance to glutamate excitation in neurons, J. Neurosci. 29 (2009) 2997–3008.
[21] C.G. Concannon, L.P. Tuffy, P. Weisova, H.P. Bonner, D. Davila, C. Bonner, M.C. Devocelle, A. Strasser, M.W. Ward, J.H. Prehn, AMP kinase-mediated activation of the BH3-only protein Bim couples energy depletion to stress-induced apoptosis, J. Cell Biol. 189 (2010) 83–94.
[22] J. Li, L.D. McCullough, Effects of AMP-activated protein kinase in cerebral ischemia, J. Cereb. Blood Flow Metab. 30 (2010) 480–492.
[23] P. Weisova, D. Davila, L.P. Tuffy, M.W. Ward, C.G. Concannon, J.H. Prehn, Role of 5′-adenosine monophosphate-activated protein kinase in cell survival and death responses in neurons, Antioxid Redox Signal 14 (10) (2011) 1863–1876.
[24] M.W. Ward, A.C. Rego, B.G. Frenguelli, D.G. Nicholls, Mitochondrial membrane potential and glutamate excitotoxicity in cultured cerebellar granule cells, J. Neurosci. 20 (2000) 7208–7219.
[25] D.G. Nicholls, M.W. Ward, Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts, Trends Neurosci. 23 (2000) 166–174.
[26] M.W. Ward, H.J. Huber, P. Weisova, H. Dussmann, D.G. Nicholls, J.H. Prehn, Mitochondrial and plasma membrane potential of cultured cerebellar neurons during glutamate-induced necrosis, apoptosis, and tolerance, J. Neurosci. 27 (2007) 8238–8249.
[27] N. Yadava, D.G. Nicholls, Spare respiratory capacity rather than oxidative stress regulates glutamate excitotoxicity after partial respiratory inhibition of mitochondrial complex I with rotenone, J. Neurosci. 27 (2007) 7310–7317.
[28] T. Hayashi, M.F. Hirshman, N. Fujii, S.A. Habinowski, L.A. Witters, L.J. Goodyear, Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism, Diabetes 49 (2000) 527–531.
[29] L.M. Rohas, J. St-Pierre, M. Uldry, S. Jager, C. Handschin, B.M. Spiegelman, A fundamental system of cellular energy homeostasis regulated by PGC-1alpha, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 7933–7938.

[30] G. Zhou, R. Myers, Y. Li, Y. Chen, X. Shen, J. Fenyk-Melody, M. Wu, J. Ventre, T. Doebber, N. Fujii, N. Musi, M.F. Hirshman, L.J. Goodyear, D.E. Moller, Role of AMP-activated protein kinase in mechanism of metformin action, J. Clin. Invest. 108 (2001) 1167–1174.
[31] J. Kim, M. Kundu, B. Viollet, K.L. Guan, AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1, Nat. Cell Biol. 13 (2011) 132–141.
[32] T.L. Horvath, S. Diano, C. Barnstable, Mitochondrial uncoupling protein 2 in the central nervous system: neuromodulator and neuroprotector, Biochem. Pharmacol. 65 (2003) 1917–1921.
[33] G. Mattiasson, M. Shamloo, G. Gido, K. Mathi, G. Tomasevic, S. Yi, C.H. Warden, R.F. Castilho, T. Melcher, M. Gonzalez-Zulueta, K. Nikolich, T. Wieloch, Uncoupling protein-2 prevents neuronal death and diminishes brain dysfunction after stroke and brain trauma, Nat. Med. 9 (2003) 1062–1068.
[34] S.L. Budd, D.G. Nicholls, Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells, J. Neurochem. 67 (1996) 2282–2291.
[35] R.F. Castilho, M.W. Ward, D.G. Nicholls, Oxidative stress, mitochondrial function, and acute glutamate excitotoxicity in cultured cerebellar granule cells, J. Neurochem. 72 (1999) 1394–1401.
[36] B. Billups, I.D. Forsythe, Presynaptic mitochondrial calcium sequestration influences transmission at mammalian central synapses, J. Neurosci. 22 (2002) 5840–5847.
[37] M.D. Brand, C. Affourtit, T.C. Esteves, K. Green, A.J. Lambert, S. Miwa, J.L. Pakay, N. Parker, Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins, Free Radic. Biol. Med. 37 (2004) 755–767.
[38] S. Vesce, L. Kirk, D.G. Nicholls, Relationships between superoxide levels and delayed calcium deregulation in cultured cerebellar granule cells exposed continuously to glutamate, J. Neurochem. 90 (2004) 683–693.
[39] L.I. Johnson-Cadwell, M.B. Jekabsons, A. Wang, B.M. Polster, D.G. Nicholls, 'Mild Uncoupling' does not decrease mitochondrial superoxide levels in cultured cerebellar granule neurons but decreases spare respiratory capacity and increases toxicity to glutamate and oxidative stress, J. Neurochem. 101 (2007) 1619–1631.
[40] J. Poels, M.R. Spasic, P. Callaerts, K.K. Norga, Expanding roles for AMP-activated protein kinase in neuronal survival and autophagy, BioEssays 31 (2009) 944–952.
[41] M.R. Spasic, P. Callaerts, K.K. Norga, AMP-activated protein kinase (AMPK) molecular crossroad for metabolic control and survival of neurons, Neuroscientist 15 (2009) 309–316.
[42] G.V. Ronnett, S. Ramamurthy, A.M. Kleman, L.E. Landree, S. Aja, AMPK in the brain: its roles in energy balance and neuroprotection, J. Neurochem. 109 (Suppl 1) (2009) 17–23.
[43] L.G. Fryer, E. Hajduch, F. Rencurel, I.P. Salt, H.S. Hundal, D.G. Hardie, D. Carling, Activation of glucose transport by AMP-activated protein kinase via stimulation of nitric oxide synthase, Diabetes 49 (2000) 1978–1985.
[44] T. Hayashi, M.F. Hirshman, E.J. Kurth, W.W. Winder, L.J. Goodyear, Evidence for 5′ AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport, Diabetes 47 (1998) 1369–1373.
[45] P. Cidad, A. Almeida, J.P. Bolanos, Inhibition of mitochondrial respiration by nitric oxide rapidly stimulates cytoprotective GLUT3-mediated glucose uptake through 5′-AMP-activated protein kinase, Biochem. J. 384 (2004) 629–636.
[46] B.S. McEwen, L.P. Reagan, Glucose transporter expression in the central nervous system: relationship to synaptic function, Eur. J. Pharmacol. 490 (2004) 13–24.
[47] A. Almeida, S. Moncada, J.P. Bolanos, Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway, Nat. Cell Biol. 6 (2004) 45–51.
[48] M.O. Dietrich, T.L. Horvath, The role of mitochondrial uncoupling proteins in lifespan, Pflugers Arch 459 (2010) 269–275.
[49] C.C. Caldeira da Silva, F.M. Cerqueira, L.F. Barbosa, M.H. Medeiros, A.J. Kowaltowski, Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity, Aging Cell 7 (2008) 552–560.
[50] B.D. Lemire, M. Behrendt, A. DeCorby, D. Gaskova, *C. elegans* longevity pathways converge to decrease mitochondrial membrane potential, Mech. Ageing Dev. 130 (2009) 461–465.