The metabolic response to excitotoxicity - lessons from single-cell imaging.

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Abstract

Excitotoxicity is a pathological process implicated in neuronal death during ischaemia, traumatic brain injuries and neurodegenerative diseases. Excitotoxicity is caused by excess levels of glutamate and over-activation of NMDA or calcium-permeable AMPA receptors on neuronal membranes, leading to ionic influx, energetic stress and potential neuronal death. The metabolic response of neurons to excitotoxicity is complex and plays a key role in the ability of the neuron to adapt and recover from such an insult. Single-cell imaging is a powerful experimental technique that can be used to study the neuronal metabolic response to excitotoxicity *in vitro* and, increasingly, *in vivo*. Here, we review some of the knowledge of the neuronal metabolic response to excitotoxicity gained from *in vitro* single-cell imaging, including calcium and ATP dynamics and their effects on mitochondrial function, along with the contribution of glucose metabolism, oxidative stress and additional neuroprotective signalling mechanisms. Future work will combine knowledge gained from single-cell imaging with data from biochemical and computational techniques to garner holistic information about the metabolic response to excitotoxicity at the whole brain level and transfer this knowledge to a clinical setting.

Keywords: Excitotoxicity, single-cell imaging, neuronal metabolism, bioenergetics, fluorescence microscopy

Abbreviations: AMPK: AMP-activated protein kinase; ANLS: astrocyte-neuron lactate shuttle; ATP: adenosine triphosphate; Ca^{2+}: calcium; CGN: cerebellar granule neuron; DCD: delayed Ca^{2+} deregulation; G6P: glucose-6-phosphate; GLUT3: glucose transporter isoform 3; FRET: Förster resonance energy transfer; GFP/CFP: green/cyan fluorescent protein; GSH: reduced glutathione; HK: hexokinase; Na^{+}: sodium; NAD(P)H: nicotinamide adenine dinucleotide; NMDA: N-methyl-D-aspartate; NOX: NAD(P)H oxidase; O_2^{-}: superoxide anion; OxPhos: oxidative phosphorylation; OGD: oxygen and glucose deprivation; pmf: proton-motive force; PPP: pentose phosphate pathway; ROS: reactive oxygen species; TCA Cycle: tricarboxylic acid cycle; TMRM: tetramethylrhodamine methyl ester; $\Delta\psi_{m/p}$: mitochondrial/plasma membrane potential

Single-cell fluorescence time-lapse imaging as a tool to monitor neuronal metabolism

All biological processes require energy. The bioenergetic requirements of neurons, however, differ vastly from those of other cells, as neurons must propagate action potentials and maintain tightly regulated electrochemical gradients, such as across the plasma, mitochondrial and vesicular membranes [1]. These intensive energy demands, however, are not met by an enhanced energy supply compared to other cells [2], and neurons do not generally utilise fatty acid or glycogen for energy metabolism [3]. Neurons are therefore particularly sensitive to energetic stress [1,2].

Neuronal metabolism is similar to that of other mammalian cells, details of which can be found in most biochemistry textbooks. Neurons are capable of ATP production through both glycolysis and mitochondrial oxidative phosphorylation (OxPhos; Figure 1). Indeed, in cultured neurons at rest, mitochondrial or glycolytic ATP production alone is sufficient to maintain ATP levels [4-7], although OxPhos is a more efficient source of ATP production [8]. Within the mitochondria, the respiratory chain (Complex I, III, IV) pumps protons (H+) out of the mitochondrial matrix to generate a proton concentration and electrochemical gradient across the mitochondrial membrane. Maintenance of this proton-motive force (primarily contributed to by the mitochondrial membrane potential $\Delta\psi_m$) critically regulates the ability of the mitochondria to generate ATP through the action of the F_0F_1-ATP synthase (Complex V).

Glucose is widely accepted as the major energy substrate for the resting and activated brain, although it has been hypothesised that neurons preferentially utilise the astrocyte-neuron lactate shuttle (ANLS) to supply pyruvate directly to the mitochondria for ATP production [9,10]. This hypothesis has been contested however [11-13], and it is likely that neurons utilise both glucose and astrocyte-derived lactate for energy production, along with other energy substrates, especially under stress or in glucose-deprived conditions [14]. The primary source of ATP production may even change during neuronal development [15], or may vary according to location within the neuron [16].

In addition to bioenergetics, glucose is also involved in anti-oxidant mechanisms through the pentose phosphate pathway (PPP; reviewed in [17]). In physiological conditions, neurons may prioritise anti-oxidant defence mechanisms by metabolising glucose primarily through the PPP [18] (although in a different study, the percentage of neuronal glucose metabolised through the PPP was calculated at only 5% [19]). The PPP produces ribose-5-phosphate (R5P), and reduces nicotinamide adenine dinucleotide phosphate ($NAD(P)^+$) to NAD(P)H. NAD(P)H is also generated in the mitochondria by transhydrogenase. NAD(P)H facilitates the reduction of reactive oxygen species (ROS) through the regeneration of reduced glutathione (GSH). Glucose can also be stored in the form of glycogen. Although brain glycogen stores are generally assumed to be present only in astrocytes, evidence is now emerging that neurons may in fact generate and mobilise glycogen stores [3,20]. Needless to say, the precise fate of neuronal glucose, as well as other aspects of neuronal metabolism, are still disputed [21]. For an excellent recent review on several aspects of metabolism, see [22].

Biochemical measurements in cell populations can be skewed by intrinsic cell-to-cell heterogeneity and unsynchronised responses [23], and intracellular processes can be irreversibly altered by cell lysis or fixation, preventing a thorough investigation of temporal dynamics. Neuronal population measurements can also be confounded by differential expression of glutamate receptors, dying neurons or contributions from non-neuronal cells. In contrast to population-level measurements, single-cell time-lapse imaging can track individual living cells, facilitating the direct correlation of specific events with individual cell outcome [23]. The development of confocal fluorescence microscopy, utilising fluorescent dyes, sensors and genetically engineered proteins, has further enabled the real-time visualisation of intracellular compartments and protein localisation, and the measurement of molecule/protein concentration dynamics and protein-protein interactions. Although these measurement techniques are not without flaws (e.g. interference with physiological processes, protein over-expression photo-toxicity, or measurement artefacts [24,25]), they are well suited to the study of the rapidly changing, dynamic process of neuronal metabolism. Some of the tools and concepts of fluorescence single-cell microscopy and their applicability to the study of brain cell metabolism are dealt with in further detail in many excellent references [25-28].

In vitro investigation of excitotoxicity demonstrates a critical role for metabolism

Neuronal excitotoxicity is a pathological process implicated in neuronal death following ischaemic stroke, traumatic brain injuries and numerous neurodegenerative diseases such as Alzheimer's and Parkinson's Disease [29-32]. Excess levels of the excitatory neurotransmitter glutamate in the synaptic cleft over-activate glutamate receptors [N-methyl-D-aspartate (NMDA), AMPA, Kainate] on the membranes of glutamatergic neurons, leading to a massive influx of ions such as sodium (Na^+) and calcium (Ca^{2+}) (Figure 2) [33,34]. Loss of ionic homeostasis depolarises the plasma membrane potential [35,36] and decreases intracellular pH [37,38]. Ca^{2+} sequestration in the mitochondria leads to depolarisation of the mitochondrial membrane potential ($\Delta\psi_m$) [32,39-41] and perturbation of mitochondrial respiration [35,42,43]. The over-activation of ATP-dependent ion pumps, in an attempt to restore ionic homeostasis, leads to ATP depletion and energetic stress [35,39,44-47].

As detailed earlier, neurons are highly sensitive to energetic stress. Although much is known about the neuronal response to excitotoxicity, no successful therapies have yet been developed. Numerous clinical trials of NMDA receptor antagonists, for example, have failed due to cytotoxic side-effects [48]. A more detailed understanding of excitotoxicity and its molecular mechanisms is therefore pivotal to the development of improved therapies.

In vitro experimental models of excitotoxicity involve over-stimulating glutamate receptors on primary neurons (usually cortical, cerebellar or hippocampal) by direct exposure to excitotoxic stimuli such as glutamate or pharmacological compounds such as NMDA [35,44,49,50]. These studies have established that severe or prolonged excitotoxicity leads almost exclusively to necrosis, characterised by sustained (biphasic) Ca^{2+} deregulation, plasma and mitochondrial

membrane depolarisation, NAD(P)H depletion, and a failure to restore bioenergetics [38,40,45,51,52]. In contrast, following mild or transient excitotoxicity neurons re-establish Ca^{2+} homeostasis and restore ATP, NAD(P)H and their plasma and mitochondrial membrane potentials [40,46,52]. Following this recovery, however, some neurons nevertheless undergo delayed apoptotic death in the hours following the insult, exhibiting delayed calcium deregulation (DCD), nuclear condensation and cell shrinkage [36,45,51,52]. A collapse of $\Delta\psi_m$ and ATP and an increase in ROS levels are also observed at this time [53,54].

Evidently, the metabolic health and bioenergetic response of the neuron to excitotoxic insults are critical in determining neuronal fate [36,45,47,51,55,56]. These responses have been investigated via single-cell fluorescence imaging in a number of elegant ways. Below, we review some of these studies, many of which were performed in combination with population-level analysis. Indeed, single-cell imaging is at its most powerful in conjunction with alternative techniques.

Single-cell imaging of the metabolic response to excitotoxicity

Calcium

Ca^{2+} dynamics during and following excitotoxicity have been intensely studied at the single-cell level, utilising fluorescent calcium-sensitive indicators such as Fluo-4 and the ratiometric Fura-2 [57,58]. For an in-depth review of neuronal calcium imaging, see [59]. Briefly, excitotoxic stressors induce an immediate and acute increase in intracellular Ca^{2+}, primarily through the action of NMDA receptors [60]. The influx of Ca^{2+} through NMDA receptors is considered to be the principal cause of excitotoxic injury [32,35,44,60,61]. Sequestration of Ca^{2+} in the mitochondria also affects mitochondrial function (discussed later).

Prolonged excitotoxicity leads to a biphasic Ca^{2+} response, characterised by an initial peak, recovery to a plateau and a subsequent collapse of Ca^{2+} homeostasis, termed delayed Ca^{2+} deregulation (DCD) [39,62]. In contrast, short-term excitotoxicity induces a transient elevation of Ca^{2+} and recovery to baseline (Figure 3 A, B). Nevertheless, these neurons can also subsequently undergo DCD [36]. DCD is closely correlated with necrotic and delayed apoptotic death and may be triggered by enhanced calcium influx to the cell, dumping of mitochondrial calcium into the cytoplasm, collapse of calcium extrusion mechanisms, or a combination of these processes [39]. Ca^{2+} dynamics during excitotoxicity are not reviewed in further detail here - interested readers are referred to [36,40,62,63].

ATP

Loss of ionic homeostasis during an excitotoxic event leads to over-activation of ATP-dependent ion pumps, such as the Na^+/K^+ or Ca^{2+} ATPases, and depletes ATP stores in single neurons [7,38]. Indeed, DCD and neuronal death following prolonged excitotoxicity may be directly due to a failure of bioenergetics [64], caused at least in part by deregulation of the plasma membrane Na^+/Ca^{2+}

exchanger [38]. The ability to restore and maintain ATP levels after an excitotoxic insult is therefore vital to neuronal survival. Following a transient excitotoxic event, surviving neurons retain the ability to restore ATP [6,7], as measured at the single-cell level utilising a recently developed FRET-based reporter of ATP concentration (Figure 4 A, B) [65]. ATP depletion and recovery following transient excitotoxicity have also been measured at population level [45-47].

As a cell requires sufficient ATP even to undergo apoptosis (in apoptosome formation, for example), the recovery of ATP levels may be prioritised in excitotoxic neurons at the expense of other processes. The acute ATP depletion measured during excitotoxicity may therefore not correlate with subsequent neuronal death [7,33,44].

As excitotoxicity is commonly considered to impair mitochondrial function (discussed in further detail below), the question arises as to what provides ATP during this period of increased energetic demand, and, afterwards, what contributes to its recovery. Following excitotoxicity, ATP production may be prioritised by diverting glucose away from the pentose phosphate pathway towards glycolysis [66], suggesting that glycolysis can provide ATP in this phase. Excitotoxic neurons in glucose-free media, however, recovered and maintained stable ATP levels when supplemented with pyruvate or lactate [7,49]. This suggested that extracellular glucose is not required for ATP recovery, and that mitochondrial ATP production also contributes. Indeed, mitochondria continue to generate ATP following glutamate exposure [40], and inhibition of the mitochondrial ATP synthase with oligomycin prevented ATP recovery in single cells exposed to glutamate [7]. This further indicates that mitochondria do indeed retain an active role in ATP recovery following excitotoxicity. Of further interest would be an investigation of localised ATP production in this recovery phase, utilising targeted ATP-sensitive probes (e.g. mitochondria-targeted [65]) to differentiate glycolytic and mitochondrial ATP production, especially in situations of oxygen deprivation.

Mitochondrial function

It has long been established that mitochondria play a vital and complex role in excitotoxicity [36,40,50], a role that has been reviewed in significant detail many times [61-63,67]. The precise impact of excitotoxicity on mitochondrial function (and its contribution to ATP recovery), however, has yet to be fully elucidated. The mitochondrial membrane potential ($\Delta\psi_m$) is used as an indicator of mitochondrial function and can be measured in single cells using charged fluorescent dyes such as tetramethylrhodamine methyl ester (TMRM), TMRE or Rhodamine-123, which accumulate in the negatively charged mitochondrial matrix [39,68]. These probes also respond to changes in the plasma membrane potential ($\Delta\psi_p$), with the extent of the sensitivity depending on the rate of probe equilibration across the plasma and mitochondrial membranes [39,40,68]. Changes in $\Delta\psi_m$ can indicate alterations in mitochondrial respiration, ATP production or ionic flux [36,39,69].

Mitochondria sequester Ca^{2+} during excitotoxicity, in a process controlled by the mitochondrial Ca^{2+} uniporter (Mcu), as demonstrated in single neurons with a

GFP-tagged Mcu protein [70]. Whilst potentially helping the cell to maintain cytoplasmic Ca^{2+} homeostasis, this ionic uptake depolarises $\Delta\psi_m$, potentially impairing mitochondrial function [39,70,71]. Indeed, prolonged decreases in mitochondrial activity after brain ischaemia have been described [72]. Evidence suggests, however, that mitochondria still retain some neuroprotective functionality. Pharmacological impairment of mitochondria during excitotoxicity exacerbates neuronal death [42], and pyruvate or lactate supplementation (providing fuel for mitochondrial respiration and OxPhos) allows cytoplasmic Ca^{2+} homeostasis to be maintained for longer [61] and protects cerebellar granule neuron populations from excitotoxic injury [43,50]. Interestingly, this protection was not seen when pyruvate was added during glutamate exposure [6]. As detailed in the previous section, mitochondria indeed remain capable of ATP production following excitotoxicity. During times of increased ATP requirements such as excitotoxicity, glycolytic substrate supply to the mitochondria may become rate-limiting and pyruvate supplementation, by providing fuel directly to the mitochondria, would therefore be neuroprotective.

The activity of the mitochondrial respiratory chain (respiration) may also be increased during and following transient excitotoxicity or oxygen and glucose deprivation (OGD), as determined by oxygen consumption at both single-cell and population level [35,42] or by sustained $\Delta\psi_m$ hyperpolarisation, as measured using TMRM (Figure 3 A, B) [36,73]. (Previous analysis demonstrated that the observed increase in TMRM fluorescence intensity after glutamate excitation corresponded to a hyperpolarisation of $\Delta\psi_m$, with almost no contribution from changes in $\Delta\psi_p$ [36]). $\Delta\psi_m$ hyperpolarisation occurs when the electrochemical potential between the mitochondrial matrix and the inter-membrane space becomes more negative, and could indicate ionic imbalance, reduced ATP production (decreasing proton influx to the matrix), or elevated respiration (increasing proton efflux) [36,39].

It has been shown that mitochondrial Ca^{2+} can increase respiration by elevating enzyme activity in the TCA cycle [74,75]. In excitotoxic conditions, increased respiration may be associated with survival, to drive mitochondrial ATP production to meet increased energetic demands and to prevent the collapse of $\Delta\psi_m$ and neuronal death [36,42,43]. Increased activity of the mitochondrial respiratory chain, however, may increase the production of toxic mitochondrial ROS (discussed further below) [76]. Interestingly, Jekabsons *et al*, after correcting for dead cells in the culture, suggested that respiration may not be altered significantly during glutamate exposure, highlighting the difficulties in determining the dynamics of cellular behaviour from population-level measurements [43].

Sustained $\Delta\psi_m$ hyperpolarisation nevertheless suggests a reprogramming of the neuronal metabolic state. However, if the proton-motive force (pmf) is maintained, a decrease in the proton gradient (ΔpH) may also occur, possibly via proton leaks ($pmf = \Delta\psi_m - 61.5*\Delta pH$). This interaction may not always hold true [68], however, and measurement of the mitochondrial ΔpH in excitotoxic neurons would provide valuable information on the metabolic state [77].

Indeed, the precise dynamics of mitochondrial respiration and ATP production following excitotoxicity likely depend on the extent of the insult and the prior

metabolic health of the cell. To this end, low levels of excitotoxicity, not normally toxic in a healthy brain, can be pathological in neurodegenerative diseases such as Huntington's or Alzheimer's Disease, where mitochondrial dysfunction and oxidative stress are often evident [42,78].

Glucose metabolism

Increased mitochondrial respiration may indicate a higher availability of metabolic substrates such as glucose [36]. We recently measured an accumulation of intracellular glucose in single neurons after exposure to glutamate [7], using a FRET-based reporter of glucose concentration [79]. Correspondingly, an excitotoxicity-mediated increase in glucose import has been measured in CGN populations [36,74,80] and in single cortical neurons using 2-NBDG [81]. 2-NBDG is a fluorescent analog of glucose, although it is metabolised differently [82]. In contrast, Porras *et al* measured an inhibition of 2-NBDG import in hippocampal neurons exposed to glutamate [83], although they used high glutamate concentrations (500 μM). Glutamate has also been shown to induce glucose import (and lactate release) in cultured astrocyte populations [9].

Glucose import is primarily facilitated by a family of glucose transporters (GLUTs), with GLUT3 being the predominant neuronal isoform [84]. Enhanced glucose import during excitotoxicity may be mediated by an increase in GLUT3 expression on neuronal membranes [46]. Interestingly, GLUT3 has higher transport activity than the astrocytic GLUT1, suggesting that neurons would have preferential access to glucose, especially under conditions of metabolic stress [85,86].

Increased glucose uptake may be a neuroprotective mechanism. Indeed, depriving CGNs of glucose (or inhibiting glucose metabolism with 2-DG) reduced their ability to recover Ca^{2+} homeostasis and $\Delta\psi_m$ following a glutamate-induced excitotoxic event [6,50]. Likewise, Delgado *et al.* showed that increasing media glucose levels following transient excitotoxicity (from 5 to 20 mM) protected neuronal populations against excitotoxicity [87]. Indeed, the higher sensitivity of older neurons to energetic stress or glutamate-induced toxicity may be due to an inability to rapidly increase glucose import [88,89].

Increased intracellular glucose would provide substrate directly for ATP production through glycolysis and downstream mitochondrial OxPhos. As mentioned earlier, the restoration of ATP is critical following excitotoxicity, and may be prioritised over other processes in the cell. Work in the Bolaños lab has investigated the role of a key glycolytic promoting enzyme PFKFB3, utilising a GFP-PFKFB3 reporter. They demonstrated that NMDA receptor activation stabilised PFKFB3 in the hours following glutamate exposure, shifting glucose from metabolism via the PPP towards glycolytic metabolism [66]. They hypothesised that this long-term neuroprotective signal prioritised ATP production following periods of energetic stress. This "metabolic re-programming", however, reduced the flux through the PPP, and may ultimately lead to NAD(P)H deficiency, hampering the anti-oxidant glutathione cycle and increasing oxidative stress [66].

Increased intracellular glucose could also indicate reduced glucose consumption, pointing to downstream metabolic dysfunction. Indeed, glucose phosphorylation levels were lower in tissue that died following an ischaemic event, compared to tissue that survived the insult [90].

The inability to properly regulate these elevated glucose levels could therefore be toxic. Hyperglycaemia (excessive glucose levels) has been shown to increase the generation of ROS [91,92]. Increased glycolysis can also produce more lactate and induce acidosis. Acidosis has been measured in single hippocampal neurons during glutamate exposure [37] and is commonly observed in ischaemia [33]. Although mild acidity may be protective, severe acidosis is harmful to neurons [93,94]. The recent development of a FRET-based reporter of intracellular lactate should contribute to future investigations [27,95].

Finally, in a further twist, neurons could mobilise glycogen stores in times of excitotoxicity-induced energetic stress. Although controversial, neurons actively store low levels of glycogen, as measured utilising a GFP-tagged muscle glycogen synthase protein [3]. These glycogen stores may be protective in hypoxic neurons [20]. The first step of glycogen mobilisation does not require ATP, and may occur more rapidly than glycolysis, making it a valuable resource during times of energetic stress [8]. Glycogen metabolism may contribute to the excitotoxicity-induced accumulation of intracellular glucose measured in single CGNs [7]. Glutamate itself can also impact glycogen metabolism [96]. Excessive glycogen metabolism, however, may be harmful [3,97].

It is clear that glucose metabolism in excitotoxic neurons is a complex and tightly regulated balance between energetic and anti-oxidant mechanisms that may critically impact neuronal survival. Interestingly, we recently showed that prolonged intracellular glucose accumulation following transient excitotoxicity correlated with earlier neuronal death, indicating that neurons unable to maintain this tight regulation may be more susceptible to excitotoxicity-induced death [7].

Reactive oxygen species (ROS)

A further implication of metabolic dysregulation following excitotoxicity may involve increased levels of reactive oxygen species (ROS). Although ROS are important intracellular signalling molecules, at high concentrations they can be cytotoxic, leading to oxidative stress. Specific forms of neuronal ROS include the superoxide anion (O_2^-), nitric oxide (NO^-), the hydroxyl radical (OH) and hydrogen peroxide (H_2O_2). ROS are generated in the mitochondria by proton and electron leaks and by the respiratory chain (particularly complexes I and III), and in the cytosol by nitric oxide synthase (NOS), NAD(P)H oxidase (NOX) and other pro-oxidant enzymes. ROS production can be monitored in single cells and within mitochondria using fluorescent dyes such as dihydroethidium (HEt) or dichlorofluorescein (DCF) derivatives, the emission spectra of which are altered by irreversible oxidation [76,98].

Increased mitochondrial ROS formation has been measured during excitotoxicity. NMDA-receptor activity and Ca^{2+} influx may induce O_2^- production within the mitochondria [76,99]. ROS levels are also linked to $\Delta\psi_m$ maintenance, with

increased $\Delta\psi_m$ associated with increased ROS formation [39,54]. As 1-2% of mitochondrial electron flow can leak from the respiratory chain to generate O_2^-, enhanced activity of the mitochondrial respiratory chain following excitotoxicity could contribute to increased ROS formation [42,53,76]. Indeed, inhibition of the respiratory chain reduced O_2^- generation in hippocampal neurons exposed to NMDA [76,99], and Bindokas *et al* implicated the complex I – complex III electron donor (ubi)semiquinone as the major leak site [76]. ROS may also be generated by increased proton leaks. Nevertheless, these conclusions are not without controversy, as redox-sensitive dyes may accumulate in the mitochondria, where they themselves may be affected by changes in $\Delta\psi_m$ [94,100]. Indeed, in other studies, NMDA receptor activation was not found to increase ROS formation prior to DCD [54].

The primary source of ROS production during excitotoxicity may in fact be cytoplasmic [54], specifically due to increased activity of NOX2 induced by calcium influx through NMDA receptors [5,101,102] (reviewed in [94]). NOX2-mediated extracellular ROS production may also affect neighbouring cells, propagating excitotoxicity-induced oxidative stress [103]. NOX2, as its name implies, is fuelled by NAD(P)H. Diversion of glucose away from the PPP, as mentioned in the previous section, may therefore serve as an additional pro-survival mechanism, by reducing glucose-mediated NAD(P)H generation [94]. Indeed, NMDA and OGD-induced ROS generation and neuronal death were reduced by inhibition of the PPP or removal of glucose from the extracellular media (supplemented with pyruvate) [5,102]. In addition, the activity of NOX2 is inhibited in reduced pH, possibly contributing to the protective effects of mild acidosis [94]. Due to some of the issues associated with the commonly utilised redox-sensitive fluorescent dyes [100], analysis of redox-sensitive fluorescent proteins during excitotoxicity would be of interest [104].

In addition to increased ROS production, anti-oxidant defence mechanisms may be hampered following transient excitotoxicity, by glutathione (GSH) or NAD(P)H depletion [42,66]. GSH depletion (glutathione oxidation) occurs following NMDA receptor activity and increased mitochondrial respiration [42,101]. NAD(P)H levels are also decreased in single neurons undergoing excitotoxicity [36]. NAD(P)H auto-fluoresces in its reduced, but not in its oxidised state, and can therefore be monitored via fluorescence microscopy methods (primarily mitochondrial NAD(P)H) [105]. Both GSH and NAD(P)H depletion can be reduced by glucose supplementation [87]. As mentioned above, glucose-derived NAD(P)H, while providing fuel for the anti-oxidant glutathione cycle, may also increase substrate supply to the pro-oxidant NOX2, and tight regulation of this balance may be required. Interestingly, NAD(P)H levels are restored and even increase above baseline following transient excitotoxicity, providing further evidence of metabolic re-programming after excitotoxic stress [36].

The resultant overall increase in intracellular ROS may be cytotoxic [66], leading to oxidative stress, Ca^{2+} deregulation, DNA mutations and neuronal death [5,40,53,101,106]. The deleterious effect of reperfusion *in vitro* is likely due, at least in part, to increased ROS production following the restoration of oxygen and glucose supplies, and may be primarily due to NOX activity [101,102]. To this end, ROS scavengers and anti-oxidants protect against DCD and neuronal death

following transient excitotoxicity [5,53,61,106] and OGD [101]. Anti-oxidants, however, did not prevent the necrotic neuronal death seen following prolonged glutamate exposure [54], confirming that different mechanisms contribute to the immediate necrotic death following prolonged excitotoxicity and the delayed apoptotic death after transient excitotoxicity [36,45,52]. During prolonged excitotoxicity, multiple aspects of cellular physiology are severely impaired, and ROS formation may therefore be a less critical determinant of neuronal death. Indeed, any increase in ROS levels observed around the time of neuronal death may be a consequence of DCD rather than a cause, occurring downstream of mitochondrial cytochrome *c* release and disruption of the electron transport chain [40,53,54]. Following transient excitotoxicity, however, the survival-oriented re-programming of metabolism, as described throughout this review, may irreversibly alter the balance between pro- and anti-oxidant mechanisms, and critically contribute to the demise of the cell.

Neuroprotective cell signalling following excitotoxicity

AMPK activity

The AMP-activated protein kinase (AMPK) is a serine/threonine kinase activated during energetic stress that regulates energy homeostasis and is therefore intricately linked with metabolism [107,108]. AMPK is activated in single neurons undergoing excitotoxicity [7]. Interestingly, AMPK activation in excitotoxicity can be both neuroprotective and deleterious [109,110]. AMPK mediated the increased surface expression of GLUT3 measured in excitotoxic neurons, facilitating increased glucose uptake [46], and may also promote metabolic adaptation, by upregulating glycolysis through PFKFB activation, though this has yet to be demonstrated in neurons [111]. In contrast, AMPK also mediates apoptotic pathways following excitotoxicity [47], inducing the nuclear translocation of the FoxO3 transcription factor (Figure 5 A, B) and transcription of the pro-apoptotic protein Bim [112]. Crucially, the duration and location of AMPK activity may be critical in determining neuronal fate during excitotoxicity or neurodegeneration [112,113]. The use of fluorescently-tagged AMPK constructs (incorporating localisation sequences) will be useful in determining the precise role of AMPK in the metabolic response to excitotoxicity [113,114].

Autophagy

Autophagy degrades and recycles cellular content to generate energy. Although autophagy has been associated with pro-death mechanisms, it provides a pro-survival function during metabolic stress [109]. ATP depletion or increased ROS levels during excitotoxicity may induce autophagy, through AMPK-mTOR-ULK signalling [115,116]. Whether autophagy activation during excitotoxicity is pro-survival or pro-apoptotic remains to be fully elucidated [109,117].

Hexokinase

Hexokinase (HK) catalyses the first step in glucose metabolism, the phosphorylation of glucose to glucose-6-phosphate (G6P). The isoform, activity and location of HK may determine whether G6P is further metabolised via glycolysis or the PPP, or in other pathways [118]. HKI, the predominant isoform in the brain, is thought to promote the metabolism of G6P through glycolysis and reduce mitochondrial oxidative stress [118,119]. HKI is located primarily on the outer mitochondrial membrane, where it is believed to have preferential access to intra-mitochondrial generated ATP [118]. Detachment from the mitochondrial membrane decreases the activity of HKI, and may occur in neurodegenerative disorders, reducing energetics and increasing oxidative stress [78]. Mitochondrial localisation of HKII may also be protective in models of neurodegeneration [120], although intriguingly, inhibition of HK activity has been shown to protect against kainic acid induced excitotoxicity [121].

Interestingly, the conformation of HKI is controlled by the mitochondrial membrane potential [122], and HKI activity may feedback to $\Delta\psi_m$, affecting ROS formation [119]. This suggests that the inner mitochondrial membrane (across which $\Delta\psi_m$ is measured) and the outer mitochondrial membrane (where HKI binds) communicate with each other [122]. This could be facilitated by the binding of HKI to the voltage-dependent anion channel (VDAC) on the outer mitochondrial membrane, potentially in regions where the inner and outer membranes are in close proximity [118]. Of note, cytosolic proteins, such as Parkin, can also be recruited to the mitochondria upon mitochondrial inner membrane depolarisation, suggesting the activation of specific signalling events induced by $\Delta\psi$m depolarisation [123]. Changes in its conformation or intracellular localisation would allow HKI to rapidly and selectively utilise intra-mitochondrial generated ATP depending on the mitochondria's metabolic state. Thus, HK provides an additional regulatory mechanism by which glucose metabolism and oxidative stress may be further controlled during the excitotoxic response. The numerous GFP-tagged HK constructs available will be useful in determining the precise role of HK in excitotoxicity [124].

Pre-conditioning

Pre-conditioning occurs when a sub-lethal stressor applied to cells renders them more resistant to subsequent injury, and has been well studied in the neuronal response to excitotoxicity. Neurons can increase their tolerance to subsequent excitotoxic insults following exposure to numerous stimuli, including bacterial toxins [125], activators of NMDA receptors or AMPK [126,127] or mild mitochondrial depolarisation [49,89]. Neuroprotective mechanisms induced by pre-conditioning stimuli include altered protein expression, enhanced anti-oxidant defence mechanisms and adaptation of metabolic reserves [128]. Mitochondrial biogenesis may alter calcium buffering capabilities, rendering neurons more resistant to toxic calcium transients [125]. Indeed, the highly dynamic nature of mitochondria enables them to rapidly respond to changes in the intracellular environment, enhancing the capability of the neuron to withstand future bioenergetic insults. Pre-conditioning induced by mild mitochondrial depolarisation also involves AMPK activation and associated energetic

adaptations [89]. Some of these mechanisms are discussed in a review of pre-conditioning at the whole brain level [128]. Therapeutic pre-conditioning could enhance endogenous neuroprotective mechanisms in scenarios where excitotoxicity is anticipated, although the intensity and duration of the pre-conditioning stimulus, required to induce resistance without causing damage, is likely critical.

Conclusion

Neuronal metabolism is a vital yet complex process and the precise roles of metabolic mechanisms in energetic crises such as excitotoxicity are still being elucidated. Single-cell studies have contributed greatly to our understanding of these mechanisms, some of which are discussed in this review. Recent studies investigating the intricate role of neuronal glucose and the glycogen shunt highlight the level of uncertainty in our knowledge of neuronal metabolism, and many questions remained unanswered. To what extent are mitochondria impaired during and following an excitotoxic insult? What role does the metabolism of glucose play in the excitotoxic response? What contributes to the ultimate demise of the neuron following excitotoxicity and can any of these processes be realistic therapeutic targets? And finally, could there be a critical 'tipping-point', beyond which neuronal fate is sealed? Future studies using intravital microscopy and molecular imaging *in vivo* will contribute further important insights into the regulation of neuronal bioenergetics both during physiological neuronal excitation and during pathophysiological processes such as excitotoxicity. The importance of precise and consistent reporting of experimental design cannot be understated and is vital to fully elucidate the dynamics and complexities of the neuronal metabolic response to excitotoxicity.

Neurons, although more sensitive to energetic and oxidative stress than other cell types, remain remarkably resilient - the vast majority of our neurons survive an entire lifetime. It is likely that neuronal, glial (astrocytes and oligodendrocytes), and indeed whole brain metabolism is highly adaptable to environmental alterations, and retains sufficient plasticity to utilise multiple energy sources for numerous purposes. Nevertheless, tight metabolic regulation is required and a 'goldilocks region' of energetics and metabolism likely exists – too low and the energetic and anti-oxidant requirements are not met, too high and toxic side-products lead to the death of the cell. In times of excitotoxic stress, restoration of energetic homeostasis may be prioritised, but not without cost. If the delicate metabolic balance is perturbed too far, the resultant failure of mitochondrial bioenergetics and anti-oxidant mechanisms may lead to cellular demise.

Acknowledgements

We thank Dr. Heiko Düssmann for critical reading of the manuscript. This work was supported by grants from Science Foundation Ireland (08/IN.1/B1949; 13/IA/1881). The authors declare that they have no conflict of interest.

Reference List

1. Attwell, D., & Laughlin, S. B. (2001). An energy budget for signaling in the grey matter of the brain. *J Cereb Blood Flow Metab, 21*(10), 1133-1145.
2. Ridge, J. W. (1972). Hypoxia and the energy charge of the cerebral adenylate pool. *Biochem J, 127*(2), 351-355.
3. Vilchez, D., Ros, S., Cifuentes, D., Pujadas, L., Valles, J., Garcia-Fojeda, B., et al. (2007). Mechanism suppressing glycogen synthesis in neurons and its demise in progressive myoclonus epilepsy. *Nat Neurosci, 10*(11), 1407-1413.
4. Budd, S. L., & Nicholls, D. G. (1996). A reevaluation of the role of mitochondria in neuronal Ca2+ homeostasis. *J Neurochem, 66*(1), 403-411.
5. Brennan, A. M., Suh, S. W., Won, S. J., Narasimhan, P., Kauppinen, T. M., Lee, H., et al. (2009). NADPH oxidase is the primary source of superoxide induced by NMDA receptor activation. *Nat Neurosci, 12*(7), 857-863.
6. Khodorov, B. I., Mikhailova, M. M., Bolshakov, A. P., Surin, A. M., Sorokina, E. G., A, R. S., et al. (2012). Dramatic Effect of Glycolysis Inhibition on the Cerebellar Granule Cells Bioenergetics. *Biochemistry (Moscow), 6*(2), 186-197.
7. Connolly, N. M., Anilkumar, U., Dussmann, H., Huber, H. J., & Prehn, J. H. (2014). Single-cell imaging of bioenergetic responses to neuronal excitotoxicity and oxygen and glucose deprivation. *J Neurosci., 34*(31), 10192-205.
8. Hertz, L. (2008). Bioenergetics of cerebral ischemia: a cellular perspective. [Review]. *Neuropharmacology, 55*(3), 289-309.
9. Pellerin, L., & Magistretti, P. J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci U S A, 91*(22), 10625-10629.
10. Pellerin, L., & Magistretti, P. J. (2012). Sweet sixteen for ANLS. *J Cereb Blood Flow Metab, 32*(7), 1152-1166.
11. Chih, C. P., & Roberts Jr, E. L. (2003). Energy substrates for neurons during neural activity: a critical review of the astrocyte-neuron lactate shuttle hypothesis. [Review]. *J Cereb Blood Flow Metab, 23*(11), 1263-1281.
12. Simpson, I. A., Carruthers, A., & Vannucci, S. J. (2007). Supply and demand in cerebral energy metabolism: the role of nutrient transporters. *J Cereb Blood Flow Metab, 27*(11), 1766-1791.
13. Mangia, S., DiNuzzo, M., Giove, F., Carruthers, A., Simpson, I. A., & Vannucci, S. J. (2011). Response to 'comment on recent modeling studies of astrocyte-neuron metabolic interactions': much ado about nothing. *J Cereb Blood Flow Metab, 31*(6), 1346-1353.
14. Genc, S., Kurnaz, I. A., & Ozilgen, M. (2011). Astrocyte-neuron lactate shuttle may boost more ATP supply to the neuron under hypoxic conditions--in silico study supported by in vitro expression data. *BMC Syst Biol, 5*, 162.
15. Surin, A. M., Khiroug, S., Gorbacheva, L. R., Khodorov, B. I., Pinelis, V. G., & Khiroug, L. (2012). Comparative analysis of cytosolic and mitochondrial ATP synthesis in embryonic and postnatal hippocampal neuronal cultures. *Frontiers in molecular neuroscience, 5*, 102.
16. Wu, K., Aoki, C., Elste, A., Rogalski-Wilk, A. A., & Siekevitz, P. (1997). The synthesis of ATP by glycolytic enzymes in the postsynaptic density and the effect of endogenously generated nitric oxide. *Proc Natl Acad Sci U S A, 94*(24), 13273-13278.
17. Wamelink, M. M., Struys, E. A., & Jakobs, C. (2008). The biochemistry, metabolism and inherited defects of the pentose phosphate pathway: a review. [Review]. *Journal of inherited metabolic disease, 31*(6), 703-717.
18. Herrero-Mendez, A., Almeida, A., Fernandez, E., Maestre, C., Moncada, S., & Bolanos, J. P. (2009). The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. *Nat Cell Biol, 11*(6), 747-752.
19. Brekke, E. M., Walls, A. B., Schousboe, A., Waagepetersen, H. S., & Sonnewald, U. (2012). Quantitative importance of the pentose phosphate pathway determined by incorporation of 13C from [2-13C]- and [3-13C]glucose into TCA cycle intermediates and neurotransmitter amino acids in functionally intact neurons. *J Cereb Blood Flow Metab, 32*(9), 1788-1799.

20. Saez, I., Duran, J., Sinadinos, C., Beltran, A., Yanes, O., Tevy, M. F., et al. (2014). Neurons have an active glycogen metabolism that contributes to tolerance to hypoxia. *J Cereb Blood Flow Metab, 34*, 945-955.
21. Rodriguez-Rodriguez, P., Almeida, A., & Bolanos, J. P. (2013). Brain energy metabolism in glutamate-receptor activation and excitotoxicity: role for APC/C-Cdh1 in the balance glycolysis/pentose phosphate pathway. *Neurochem Int, 62*(5), 750-756.
22. Metallo, C. M., & Vander Heiden, M. G. (2013). Understanding metabolic regulation and its influence on cell physiology. *Mol Cell, 49*(3), 388-398.
23. Loewer, A., & Lahav, G. (2011). We are all individuals: causes and consequences of non-genetic heterogeneity in mammalian cells. *Curr Opin Genet Dev, 21*(6), 753-758.
24. Baens, M., Noels, H., Broeckx, V., Hagens, S., Fevery, S., Billiau, A. D., et al. (2006). The dark side of EGFP: defective polyubiquitination. *PLoS One, 1*(1), e54.
25. Mullassery, D., Horton, C. A., Wood, C. D., & White, M. R. (2008). Single live-cell imaging for systems biology. *Essays Biochem, 45*, 121-133.
26. Taraska, J. W., & Zagotta, W. N. (2010). Fluorescence applications in molecular neurobiology. *Neuron, 66*(2), 170-189.
27. Barros, L. F., San Martin, A., Sotelo-Hitschfeld, T., Lerchundi, R., Fernandez-Moncada, I., Ruminot, I., et al. (2013). Small is fast: astrocytic glucose and lactate metabolism at cellular resolution. *Frontiers in cellular neuroscience, 7*, 27.
28. Tantama, M., Hung, Y. P., & Yellen, G. (2012). Optogenetic reporters: Fluorescent protein-based genetically encoded indicators of signaling and metabolism in the brain. *Prog Brain Res, 196*, 235-263.
29. Beal, M. F. (1992). Mechanisms of excitotoxicity in neurologic diseases. *FASEB J, 6*(15), 3338-3344.
30. Lee, J. M., Zipfel, G. J., & Choi, D. W. (1999). The changing landscape of ischaemic brain injury mechanisms. *Nature, 399*, A7-14.
31. Sattler, R., & Tymianski, M. (2001). Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol Neurobiol, 24*(1-3), 107-129.
32. Wang, Y., & Qin, Z. H. (2010). Molecular and cellular mechanisms of excitotoxic neuronal death. *Apoptosis, 15*(11), 1382-1402.
33. Raichle, M. E. (1983). The pathophysiology of brain ischemia. *Ann Neurol, 13*(1), 2-10.
34. Choi, D. W. (1994). Calcium and excitotoxic neuronal injury. *Ann N Y Acad Sci, 747*, 162-171.
35. Gleichmann, M., Collis, L. P., Smith, P. J., & Mattson, M. P. (2009). Simultaneous single neuron recording of O2 consumption, [Ca2+]i and mitochondrial membrane potential in glutamate toxicity. *J Neurochem, 109*(2), 644-655.
36. Ward, M. W., Huber, H. J., Weisova, P., Dussmann, H., Nicholls, D. G., & Prehn, J. H. (2007). Mitochondrial and plasma membrane potential of cultured cerebellar neurons during glutamate-induced necrosis, apoptosis, and tolerance. *J Neurosci, 27*(31), 8238-8249.
37. Wang, G. J., Randall, R. D., & Thayer, S. A. (1994). Glutamate-induced intracellular acidification of cultured hippocampal neurons demonstrates altered energy metabolism resulting from Ca2+ loads. *J Neurophys, 72*(6), 2563-2569.
38. Surin, A. M., Gorbacheva, L. R., Savinkova, I. G., Sharipov, R. R., Khodorov, B. I., & Pinelis, V. G. (2014). Study on ATP concentration changes in cytosol of individual cultured neurons during glutamate-induced deregulation of calcium homeostasis. *Biochemistry (Moscow), 79*(2), 146-157.
39. Nicholls, D. G., & Budd, S. L. (2000). Mitochondria and neuronal survival. *Physiol Rev, 80*(1), 315-360.
40. Ward, M. W., Rego, A. C., Frenguelli, B. G., & Nicholls, D. G. (2000). Mitochondrial membrane potential and glutamate excitotoxicity in cultured cerebellar granule cells. *J Neurosci, 20*(19), 7208-7219.
41. Nicholls, D. G. (2008). Oxidative stress and energy crises in neuronal dysfunction. *Ann N Y Acad Sci, 1147*, 53-60.
42. Garcia, O., Almeida, A., Massieu, L., & Bolanos, J. P. (2005). Increased mitochondrial respiration maintains the mitochondrial membrane potential and promotes survival of cerebellar neurons in an endogenous model of glutamate receptor activation. *J Neurochem, 92*(1), 183-190.
43. Jekabsons, M. B., & Nicholls, D. G. (2004). In situ respiration and bioenergetic status of mitochondria in primary cerebellar granule neuronal cultures exposed continuously to glutamate. *J Biol Chem, 279*(31), 32989-33000.
44. Marcaida, G., Minana, M. D., Grisolia, S., & Felipo, V. (1995). Lack of correlation between glutamate-induced depletion of ATP and neuronal death in primary cultures of cerebellum. *Brain Res, 695*(2), 146-150.

45. Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., et al. (1995). Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron, 15*(4), 961-973.
46. Weisova, P., Concannon, C. G., Devocelle, M., Prehn, J. H., & Ward, M. W. (2009). Regulation of glucose transporter 3 surface expression by the AMP-activated protein kinase mediates tolerance to glutamate excitation in neurons. *J Neurosci, 29*(9), 2997-3008.
47. Concannon, C. G., Tuffy, L. P., Weisova, P., Bonner, H. P., Davila, D., Bonner, C., et al. (2010). AMP kinase-mediated activation of the BH3-only protein Bim couples energy depletion to stress-induced apoptosis. *J Cell Biol, 189*(1), 83-94.
48. Muir, K. W. (2006). Glutamate-based therapeutic approaches: clinical trials with NMDA antagonists. *Curr Opin Pharmacol, 6*(1), 53-60.
49. Castilho, R. F., Hansson, O., Ward, M. W., Budd, S. L., & Nicholls, D. G. (1998). Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells. *J Neurosci, 18*(24), 10277-10286.
50. Vergun, O., Han, Y. Y., & Reynolds, I. J. (2003). Glucose deprivation produces a prolonged increase in sensitivity to glutamate in cultured rat cortical neurons. *Exp Neurol, 183*(2), 682-694.
51. Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., & Lipton, S. A. (1995). Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci U S A, 92*(16), 7162-7166.
52. D'Orsi, B., Bonner, H., Tuffy, L. P., Dussmann, H., Woods, I., Courtney, M. J., et al. (2012). Calpains are downstream effectors of bax-dependent excitotoxic apoptosis. *J Neurosci, 32*(5), 1847-1858.
53. Luetjens, C. M., Bui, N. T., Sengpiel, B., Munstermann, G., Poppe, M., Krohn, A. J., et al. (2000). Delayed mitochondrial dysfunction in excitotoxic neuron death: cytochrome c release and a secondary increase in superoxide production. *J Neurosci, 20*(15), 5715-5723.
54. Vesce, S., Kirk, L., & Nicholls, D. G. (2004). Relationships between superoxide levels and delayed calcium deregulation in cultured cerebellar granule cells exposed continuously to glutamate. *J Neurochem, 90*(3), 683-693.
55. Novelli, A., Reilly, J. A., Lysko, P. G., & Henneberry, R. C. (1988). Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced. *Brain Res, 451*(1-2), 205-212.
56. Lipton, S. A., & Nicotera, P. (1998). Calcium, free radicals and excitotoxins in neuronal apoptosis. *Cell Calcium, 23*(2-3), 165-171.
57. Grynkiewicz, G., Poenie, M., & Tsien, R. Y. (1985). A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem, 260*(6), 3440-3450.
58. Gee, K. R., Brown, K. A., Chen, W. N., Bishop-Stewart, J., Gray, D., & Johnson, I. (2000). Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. *Cell Calcium, 27*(2), 97-106.
59. Grienberger, C., & Konnerth, A. (2012). Imaging calcium in neurons. *Neuron, 73*(5), 862-885.
60. Garthwaite, G., & Garthwaite, J. (1986). Neurotoxicity of excitatory amino acid receptor agonists in rat cerebellar slices: dependence on calcium concentration. *Neurosci Lett, 66*(2), 193-198.
61. Nicholls, D. G., Budd, S. L., Castilho, R. F., & Ward, M. W. (1999). Glutamate excitotoxicity and neuronal energy metabolism. *Ann N Y Acad Sci, 893*, 1-12.
62. Khodorov, B. (2004). Glutamate-induced deregulation of calcium homeostasis and mitochondrial dysfunction in mammalian central neurones. *Prog Biophys Mol Bio, 86*(2), 279-351.
63. Nicholls, D. G., Vesce, S., Kirk, L., & Chalmers, S. (2003). Interactions between mitochondrial bioenergetics and cytoplasmic calcium in cultured cerebellar granule cells. *Cell Calcium, 34*(4-5), 407-424.
64. Abramov, A. Y., & Duchen, M. R. (2010). Impaired mitochondrial bioenergetics determines glutamate-induced delayed calcium deregulation in neurons. *Biochim Biophys Acta, 1800*(3), 297-304.
65. Imamura, H., Nhat, K. P., Togawa, H., Saito, K., Iino, R., Kato-Yamada, Y., et al. (2009). Visualization of ATP levels inside single living cells with fluorescence resonance energy transfer-based genetically encoded indicators. *Proc Natl Acad Sci U S A, 106*(37), 15651-15656.

66. Rodriguez-Rodriguez, P., Fernandez, E., Almeida, A., & Bolanos, J. P. (2012). Excitotoxic stimulus stabilizes PFKFB3 causing pentose-phosphate pathway to glycolysis switch and neurodegeneration. *Cell Death Differ, 19*(10), 1582-1589.
67. Fiskum, G., Murphy, A. N., & Beal, M. F. (1999). Mitochondria in neurodegeneration: acute ischemia and chronic neurodegenerative diseases. *J Cereb Blood Flow Metab, 19*(4), 351-369.
68. Perry, S. W., Norman, J. P., Barbieri, J., Brown, E. B., & Gelbard, H. A. (2011). Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *BioTechniques, 50*(2), 98-115.
69. Nicholls, D. G. (2006). Simultaneous monitoring of ionophore- and inhibitor-mediated plasma and mitochondrial membrane potential changes in cultured neurons. *J Biol Chem, 281*(21), 14864-14874.
70. Qiu, J., Tan, Y. W., Hagenston, A. M., Martel, M. A., Kneisel, N., Skehel, P. A., et al. (2013). Mitochondrial calcium uniporter Mcu controls excitotoxicity and is transcriptionally repressed by neuroprotective nuclear calcium signals. *Nat Comms, 4*, 2034.
71. Kushnareva, Y. E., Wiley, S. E., Ward, M. W., Andreyev, A. Y., & Murphy, A. N. (2005). Excitotoxic injury to mitochondria isolated from cultured neurons. *J Biol Chem, 280*(32), 28894-28902.
72. Anderson, M. F., & Sims, N. R. (1999). Mitochondrial respiratory function and cell death in focal cerebral ischemia. *J Neurochem, 73*(3), 1189-1199.
73. Iijima, T., Mishima, T., Tohyama, M., Akagawa, K., & Iwao, Y. (2003). Mitochondrial membrane potential and intracellular ATP content after transient experimental ischemia in the cultured hippocampal neuron. *Neurochem Int, 43*(3), 263-269.
74. Bak, L. K., Walls, A. B., Schousboe, A., Ring, A., Sonnewald, U., & Waagepetersen, H. S. (2009). Neuronal glucose but not lactate utilization is positively correlated with NMDA-induced neurotransmission and fluctuations in cytosolic Ca2+ levels. *J Neurochem, 109*, 87-93.
75. Llorente-Folch, I., Rueda, C. B., Amigo, I., del Arco, A., Saheki, T., Pardo, B., et al. (2013). Calcium-regulation of mitochondrial respiration maintains ATP homeostasis and requires ARALAR/AGC1-malate aspartate shuttle in intact cortical neurons. *J Neurosci, 33*(35), 13957-13971.
76. Bindokas, V. P., Jordan, J., Lee, C. C., & Miller, R. J. (1996). Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. *J Neurosci, 16*(4), 1324-1336.
77. Poburko, D., Santo-Domingo, J., & Demaurex, N. (2011). Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations. *The Journal of Biological Chemistry, 286*(13), 11672-11684.
78. Saraiva, L. M., Seixas da Silva, G. S., Galina, A., da-Silva, W. S., Klein, W. L., Ferreira, S. T., et al. (2010). Amyloid-beta triggers the release of neuronal hexokinase 1 from mitochondria. *PLoS One, 5*(12), e15230.
79. Takanaga, H., Chaudhuri, B., & Frommer, W. B. (2008). GLUT1 and GLUT9 as major contributors to glucose influx in HepG2 cells identified by a high sensitivity intramolecular FRET glucose sensor. *Biochim Biophys Acta, 1778*(4), 1091-1099.
80. Minervini, M., Atlante, A., Gagliardi, S., Ciotti, M. T., Marra, E., & Calissano, P. (1997). Glutamate stimulates 2-deoxyglucose uptake in rat cerebellar granule cells. *Brain Res, 768*(1-2), 57-62.
81. Ferreira, J. M., Burnett, A. L., & Rameau, G. A. (2011). Activity-dependent regulation of surface glucose transporter-3. *J Neurosci, 31*(6), 1991-1999.
82. Loaiza, A., Porras, O. H., & Barros, L. F. (2003). Glutamate triggers rapid glucose transport stimulation in astrocytes as evidenced by real-time confocal microscopy. *J Neurosci, 23*(19), 7337-7342.
83. Porras, O. H., Loaiza, A., & Barros, L. F. (2004). Glutamate mediates acute glucose transport inhibition in hippocampal neurons. *J Neurosci, 24*(43), 9669-9673.
84. Maher, F., & Simpson, I. A. (1994). The GLUT3 glucose transporter is the predominant isoform in primary cultured neurons: assessment by biosynthetic and photoaffinity labelling. *Biochem J, 301 (Pt 2)*, 379-384.
85. Maher, F., Davies-Hill, T. M., & Simpson, I. A. (1996). Substrate specificity and kinetic parameters of GLUT3 in rat cerebellar granule neurons. *Biochem J, 315 (Pt 3)*, 827-831.
86. Simpson, I. A., Dwyer, D., Malide, D., Moley, K. H., Travis, A., & Vannucci, S. J. (2008). The facilitative glucose transporter GLUT3: 20 years of distinction. *Am J Physiol Endocrinol Metab, 295*(2), E242-253.

87. Delgado-Esteban, M., Almeida, A., & Bolanos, J. P. (2000). D-Glucose prevents glutathione oxidation and mitochondrial damage after glutamate receptor stimulation in rat cortical primary neurons. *J Neurochem, 75*(4), 1618-1624.
88. Patel, J. R., & Brewer, G. J. (2003). Age-related changes in neuronal glucose uptake in response to glutamate and beta-amyloid. *J Neurosci Res, 72*(4), 527-536.
89. Weisova, P., Anilkumar, U., Ryan, C., Concannon, C. G., Prehn, J. H., & Ward, M. W. (2012). 'Mild mitochondrial uncoupling' induced protection against neuronal excitotoxicity requires AMPK activity. *Biochim Biophys Acta, 1817*(5), 744-753.
90. Belayev, L., Zhao, W., Busto, R., & Ginsberg, M. D. (1997). Transient middle cerebral artery occlusion by intraluminal suture: I. Three-dimensional autoradiographic image-analysis of local cerebral glucose metabolism-blood flow interrelationships during ischemia and early recirculation. *J Cereb Blood Flow Metab, 17*(12), 1266-1280.
91. Vincent, A. M., McLean, L. L., Backus, C., & Feldman, E. L. (2005). Short-term hyperglycemia produces oxidative damage and apoptosis in neurons. *FASEB J, 19*(6), 638-640.
92. Tomlinson, D. R., & Gardiner, N. J. (2008). Glucose neurotoxicity. *Nat Rev Neurosci, 9*(1), 36-45.
93. Tombaugh, G. C., & Sapolsky, R. M. (1990). Mild acidosis protects hippocampal neurons from injury induced by oxygen and glucose deprivation. *Brain Res, 506*(2), 343-345.
94. Brennan-Minnella, A. M., Won, S. J., & Swanson, R. A. (2014). NADPH Oxidase-2: Linking Glucose, Acidosis, and Excitotoxicity in Stroke. *Antioxidants & Redox Signaling*, doi:10.1089/ars.2013.5767.
95. San Martin, A., Ceballo, S., Ruminot, I., Lerchundi, R., Frommer, W. B., & Barros, L. F. (2013). A genetically encoded FRET lactate sensor and its use to detect the Warburg effect in single cancer cells. *PLoS One, 8*(2), e57712.
96. Brown, A. M. (2004). Brain glycogen re-awakened. *J Neurochem, 89*(3), 537-552.
97. Duran, J., Tevy, M. F., Garcia-Rocha, M., Calbo, J., Milan, M., & Guinovart, J. J. (2012). Deleterious effects of neuronal accumulation of glycogen in flies and mice. *EMBO molecular medicine, 4*(8), 719-729.
98. Hempel, S. L., Buettner, G. R., O'Malley, Y. Q., Wessels, D. A., & Flaherty, D. M. (1999). Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2',7'-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123. *Free Radic Biol Med, 27*(1-2), 146-159.
99. Sengpiel, B., Preis, E., Krieglstein, J., & Prehn, J. H. (1998). NMDA-induced superoxide production and neurotoxicity in cultured rat hippocampal neurons: role of mitochondria. *The European journal of neuroscience, 10*(5), 1903-1910.
100. Zielonka, J., & Kalyanaraman, B. (2010). Hydroethidine- and MitoSOX-derived red fluorescence is not a reliable indicator of intracellular superoxide formation: another inconvenient truth. *Free Radic Biol Med, 48*(8), 983-1001.
101. Abramov, A. Y., Scorziello, A., & Duchen, M. R. (2007). Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. *J Neurosci, 27*(5), 1129-1138.
102. Suh, S. W., Shin, B. S., Ma, H., Van Hoecke, M., Brennan, A. M., Yenari, M. A., et al. (2008). Glucose and NADPH oxidase drive neuronal superoxide formation in stroke. *Annals of neurology, 64*(6), 654-663.
103. Reyes, R. C., Brennan, A. M., Shen, Y., Baldwin, Y., & Swanson, R. A. (2012). Activation of neuronal NMDA receptors induces superoxide-mediated oxidative stress in neighboring neurons and astrocytes. *J Neurosci, 32*(37), 12973-12978.
104. Hanson, G. T., Aggeler, R., Oglesbee, D., Cannon, M., Capaldi, R. A., Tsien, R. Y., et al. (2004). Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. *J Biol Chem, 279*(13), 13044-13053.
105. Shuttleworth, C. W. (2010). Use of NAD(P)H and flavoprotein autofluorescence transients to probe neuron and astrocyte responses to synaptic activation. *Neurochem Int, 56*(3), 379-386.
106. Patel, M., Day, B. J., Crapo, J. D., Fridovich, I., & McNamara, J. O. (1996). Requirement for superoxide in excitotoxic cell death. *Neuron, 16*(2), 345-355.
107. Hardie, D. G., Ross, F. A., & Hawley, S. A. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol, 13*(4), 251-262.
108. Ronnett, G. V., Ramamurthy, S., Kleman, A. M., Landree, L. E., & Aja, S. (2009). AMPK in the brain: its roles in energy balance and neuroprotection. *J Neurochem, 109 Suppl 1*, 17-23.

109. Poels, J., Spasic, M. R., Callaerts, P., & Norga, K. K. (2009). Expanding roles for AMP-activated protein kinase in neuronal survival and autophagy. *Bioessays, 31*(9), 944-952.
110. Li, J., & McCullough, L. D. (2010). Effects of AMP-activated protein kinase in cerebral ischemia. *J Cereb Blood Flow Metab, 30*(3), 480-492.
111. Bolanos, J. P., Almeida, A., & Moncada, S. (2009). Glycolysis: a bioenergetic or a survival pathway? *Trends Biochem Sci, 35*(3), 145-149.
112. Davila, D., Connolly, N. M., Bonner, H., Weisova, P., Dussmann, H., Concannon, C. G., et al. (2012). Two-step activation of FOXO3 by AMPK generates a coherent feed-forward loop determining excitotoxic cell fate. *Cell Death Differ, 19*, 1677-1688.
113. Ju, T. C., Chen, H. M., Lin, J. T., Chang, C. P., Chang, W. C., Kang, J. J., et al. (2011). Nuclear translocation of AMPK-{alpha}1 potentiates striatal neurodegeneration in Huntington's disease. *J Cell Biol, 194*(2), 209-227.
114. Tsou, P., Zheng, B., Hsu, C. H., Sasaki, A. T., & Cantley, L. C. (2011). A fluorescent reporter of AMPK activity and cellular energy stress. *Cell Metab, 13*(4), 476-486.
115. Egan, D., Kim, J., Shaw, R. J., & Guan, K. L. (2011). The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR. *Autophagy, 7*(6), 643-644.
116. Kim, J., Kundu, M., Viollet, B., & Guan, K. L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol, 13*(2), 132-141.
117. Ginet, V., Spiehlmann, A., Rummel, C., Rudinskiy, N., Grishchuk, Y., Luthi-Carter, R., et al. (2014). Involvement of autophagy in hypoxic-excitotoxic neuronal death. *Autophagy, 10*(5), 846-860.
118. Wilson, J. E. (2003). Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. *J Exp Biol, 206*(Pt 12), 2049-2057.
119. da-Silva, W. S., Gomez-Puyou, A., de Gomez-Puyou, M. T., Moreno-Sanchez, R., De Felice, F. G., de Meis, L., et al. (2004). Mitochondrial bound hexokinase activity as a preventive antioxidant defense: steady-state ADP formation as a regulatory mechanism of membrane potential and reactive oxygen species generation in mitochondria. *J Biol Chem, 279*(38), 39846-39855.
120. Gimenez-Cassina, A., Lim, F., Cerrato, T., Palomo, G. M., & Diaz-Nido, J. (2009). Mitochondrial hexokinase II promotes neuronal survival and acts downstream of glycogen synthase kinase-3. *J Biol Chem, 284*(5), 3001-3011.
121. Lee, K. H., Park, J. H., Won, R., Lee, H., Nam, T. S., & Lee, B. H. (2011). Inhibition of hexokinase leads to neuroprotection against excitotoxicity in organotypic hippocampal slice culture. *J Neurosci Res, 89*(1), 96-107.
122. Hashimoto, M., & Wilson, J. E. (2000). Membrane potential-dependent conformational changes in mitochondrially bound hexokinase of brain. *Archives of biochemistry and biophysics, 384*(1), 163-173.
123. Narendra, D., Tanaka, A., Suen, D. F., & Youle, R. J. (2008). Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *The Journal of Cell Biology, 183*(5), 795-803.
124. Sun, L., Shukair, S., Naik, T. J., Moazed, F., & Ardehali, H. (2008). Glucose phosphorylation and mitochondrial binding are required for the protective effects of hexokinases I and II. *Mol Cell Biol, 28*(3), 1007-1017.
125. Stetler, R. A., Leak, R. K., Yin, W., Zhang, L., Wang, S., Gao, Y., et al. (2012). Mitochondrial biogenesis contributes to ischemic neuroprotection afforded by LPS pre-conditioning. *J Neurochem, 123 Suppl 2*, 125-137.
126. Navon, H., Bromberg, Y., Sperling, O., & Shani, E. (2012). Neuroprotection by NMDA preconditioning against glutamate cytotoxicity is mediated through activation of ERK 1/2, inactivation of JNK, and by prevention of glutamate-induced CREB inactivation. *J Mol Neurosci, 46*(1), 100-108.
127. Anilkumar, U., Weisova, P., Dussmann, H., Concannon, C. G., Konig, H. G., & Prehn, J. H. (2013). AMP-activated protein kinase (AMPK)-induced preconditioning in primary cortical neurons involves activation of MCL-1. *J Neurochem, 124*(5), 721-734.
128. Obrenovitch, T. P. (2008). Molecular physiology of preconditioning-induced brain tolerance to ischemia. *Physiol Rev, 88*(1), 211-247.

Figure Legends

Fig. 1 A vastly simplified illustration of some of the metabolic pathways addressed in this review, including glycolysis, the pentose phosphate pathway (PPP) and NAD(P)H production, the mitochondrial respiratory chain and ATP production, the generation of mitochondrial H_2O_2 (reactive oxygen species), and the oxidation of reduced glutathione (GSH) to glutathione disulfide (GSSG). Many of the reactions shown are reversible, and consist of numerous intermediate steps. NAD(P)H can also be produced in the mitochondria. Comp I, III and IV are the complexes involved in the mitochondrial respiratory chain. H^+: protons, e-; electrons. Other abbreviations and further details in text

Fig. 2 Simplified illustration of some of the excitotoxicity-related signalling pathways discussed throughout this review, including glutamate-mediated activation of NMDA receptors, Ca^{2+} sequestration in the mitochondria, ROS generation by mitochondria and NOX2, ROS removal by pentose phosphate pathway (PPP)-produced NAD(P)H, glycolytic and mitochondrial ATP production and ATP consumption via the Ca^{2+} and Na^+/K^+ ATPases. K^+: potassium. The flat line arrowhead between NAD(P)H and ROS indicates the reduction of ROS. Other abbreviations and further details as per text

Fig. 3 (A) Image sequence of a single cerebellar granule neuron before, during and after transient glutamate exposure and mitochondrial membrane depolarisation. The neurons were stained with the calcium-sensitive indicator Fluo-4 (green) and the reporter of mitochondrial membrane potential, TMRM (red). Cells were equilibrated in 10 nM TMRM (non-quench mode). (B) Such dynamic behaviour can be analysed by plotting the changes in single-cell fluorescence intensity over time. During glutamate exposure, intracellular Ca^{2+} increases and the mitochondrial membrane depolarises. Although neurons can recover ionic and potential homeostasis following a transient insult, delayed neuronal death is evidenced by a secondary delayed Ca^{2+} deregulation and collapse of the hyperpolarised mitochondrial membrane potential. Labelled areas (i-ix) correspond to the time at which the images in (A) were obtained

Fig. 4 (A) Fluorescence intensity images of a rat cerebellar granule neuron transfected with a FRET-based reporter of ATP concentrations [65], stained with TMRM (red), and transiently exposed to glutamate. The blue-red ratiometric FRET/CFP signal equates to low - high relative ATP levels. (B) Plotting the temporal fluorescence signals demonstrates that ATP levels are depleted during glutamate exposure, but recover following termination of the excitotoxic stimulus. Subsequent neuronal death is characterised by depolarisation of the mitochondrial membrane potential (loss of TMRM signal) and a collapse of ATP. Labelled areas (i-ix) correspond to the time at which the images in (A) were obtained

Fig. 5 (A) Rat cerebellar granule neurons transfected with a GFP-tagged siRNA targeting AMPK or a control sequence (green). Nuclei were stained with Hoechst 33258 (blue) and immunofluorescence labelling of an antibody against FoxO3 (red) was used to determine the localisation of FoxO3 (nuclear or cytoplasmic). Scale bar = 5 μm. (B) Combining analysis of multiple cells demonstrated that the silencing of AMPK expression blocked the glutamate-induced nuclear translocation of FoxO3 at the population level (* $p < 0.05$). Figures reproduced from [112]

FIGURE 1

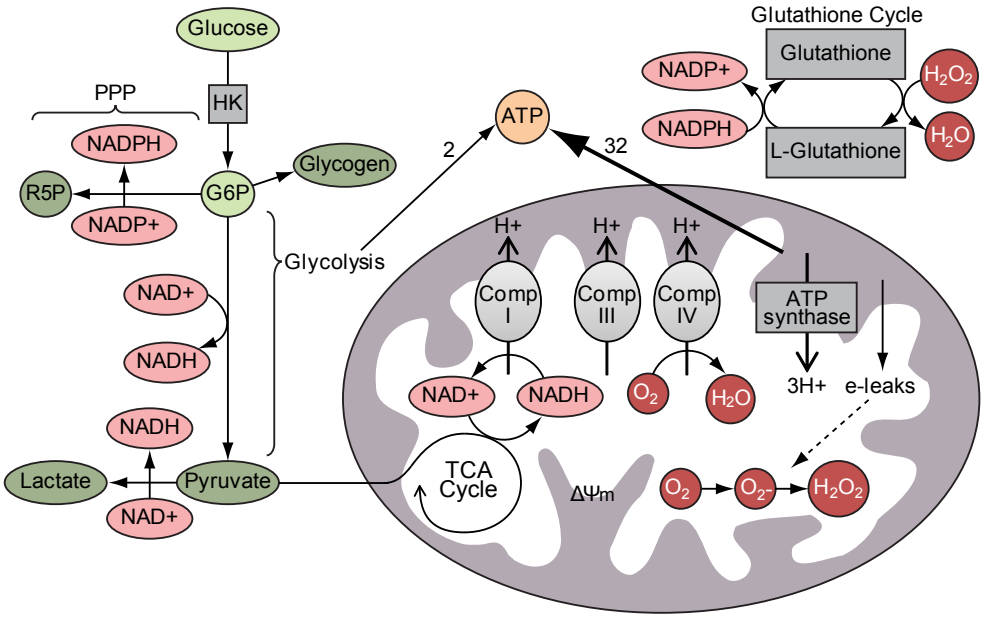

Glucose
PPP
HK
NADPH
R5P
G6P
Glycogen
NADP+
Glycolysis
NAD+
NADH
NADH
Lactate
Pyruvate
NAD+
2
ATP
32
Glutathione Cycle
Glutathione
NADP+
NADPH
L-Glutathione
H2O2
H2O
H+
H+
H+
Comp I
Comp III
Comp IV
ATP synthase
3H+
e-leaks
NAD+
NADH
O2
H2O
TCA Cycle
ΔΨm
O2
O2-
H2O2

FIGURE 2

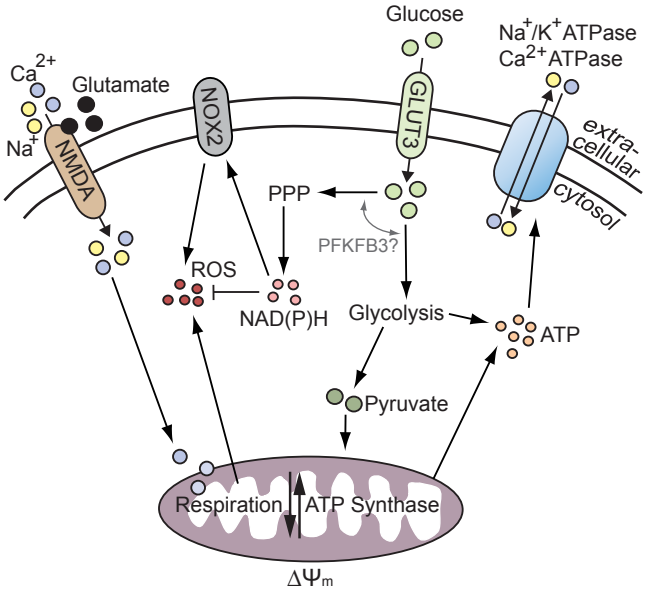

Glucose
Na+/K+ATPase
Ca2+ATPase
Ca2+
Glutamate
Na+
NMDA
NOX2
GLUT3
extra-cellular
cytosol
PPP
PFKFB3?
ROS
NAD(P)H
Glycolysis
ATP
Pyruvate
Respiration
ATP Synthase
ΔΨm

FIGURE 3

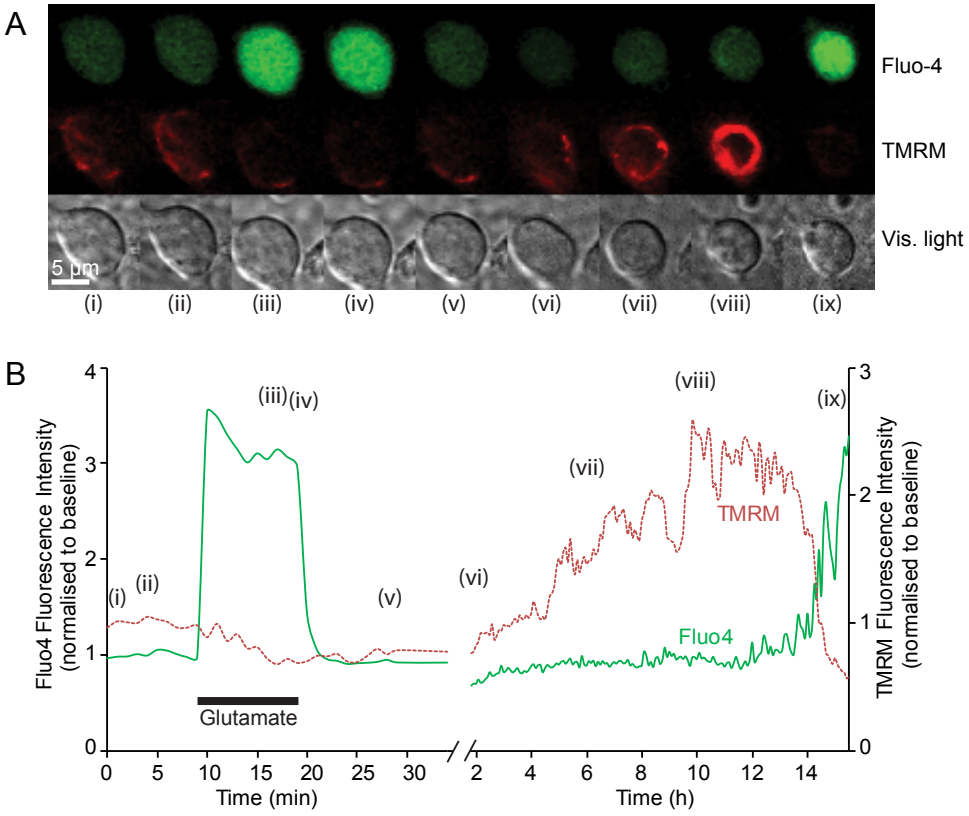

A
Fluo-4
TMRM
Vis. light
5 µm
(i)
(ii)
(iii)
(iv)
(v)
(vi)
(vii)
(viii)
(ix)
B
Fluo4 Fluorescence Intensity (normalised to baseline)
TMRM Fluorescence Intensity (normalised to baseline)
Glutamate
TMRM
Fluo4
Time (min)
Time (h)

FIGURE 4

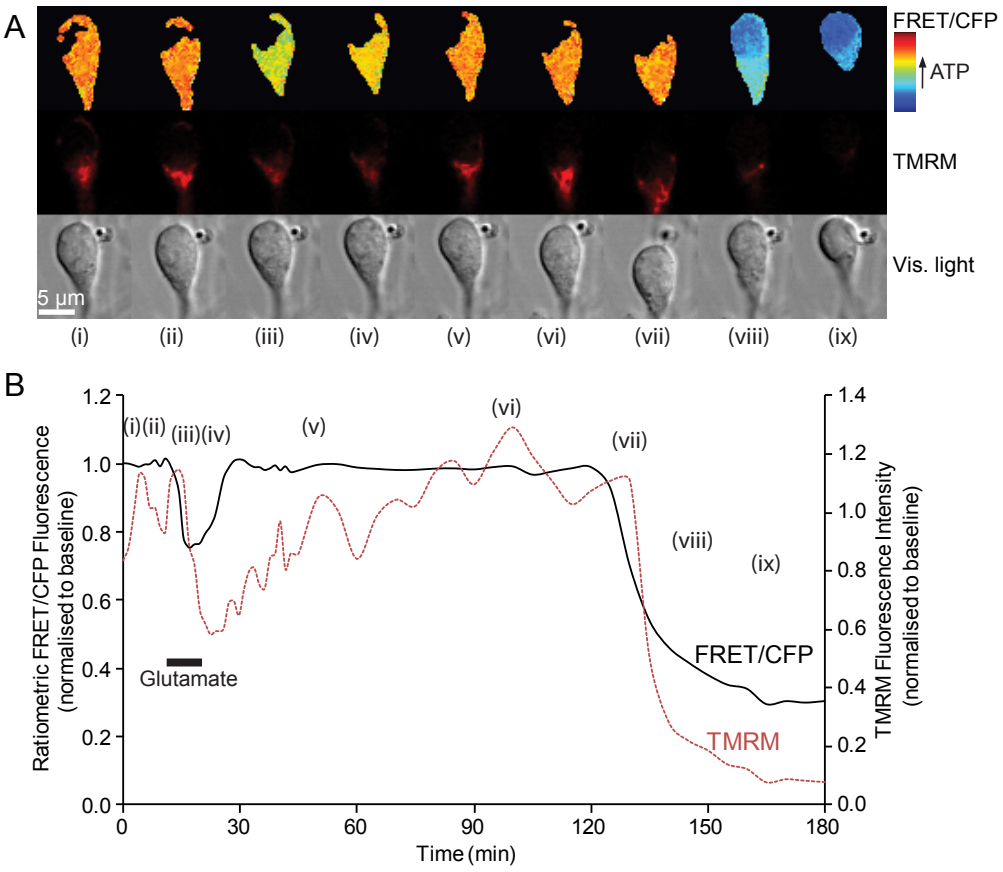
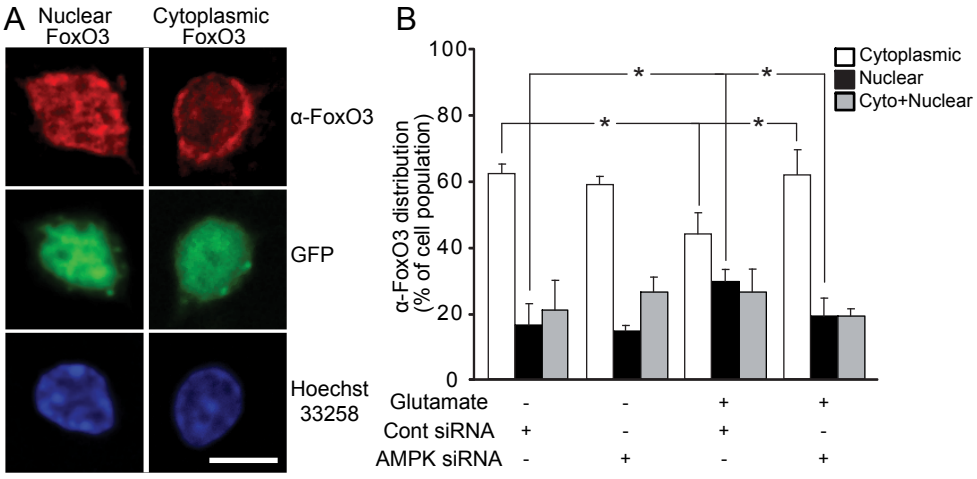

A
FRET/CFP
ATP
TMRM
Vis. light
5 µm
(i)
(ii)
(iii)
(iv)
(v)
(vi)
(vii)
(viii)
(ix)
B
Ratiometric FRET/CFP Fluorescence (normalised to baseline)
TMRM Fluorescence Intensity (normalised to baseline)
Glutamate
FRET/CFP
TMRM
Time (min)
0
30
60
90
120
150
180
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4

FIGURE 5


A
Nuclear FoxO3
Cytoplasmic FoxO3
α-FoxO3
GFP
Hoechst 33258
B
Cytoplasmic
Nuclear
Cyto+Nuclear
α-FoxO3 distribution (% of cell population)
100
80
60
40
20
0
*
*
*
*
Glutamate - - + +
Cont siRNA + - + -
AMPK siRNA - + - +