Excitotoxicity revisited: mitochondria on the verge of a nervous breakdown

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Abstract

Excitotoxicity is likely to occur in pathological scenarios in which mitochondrial function is already compromised, shaping neuronal responses to glutamate. In fact, mitochondria sustain cell bioenergetics, tune intracellular Ca^{2+} dynamics and regulate glutamate availability by using it as metabolic substrate. In this Opinion paper, we suggest the need to explore glutamate toxicity in the context of specific disease models in which it may occur, re-evaluating the impact of mitochondrial dysfunction on glutamate excitotoxicity. Our ambition is to signpost new approaches, perhaps combining glutamate and pathways to rescue mitochondrial function as therapeutic targets in neurological disorders.

Glutamate excitotoxicity from the beginning

Since the seminal observations of John Olney in the 1960's [1], it has been known that exposure of neurons to high concentrations of the excitatory neurotransmitter glutamate can precipitate neuronal cell death, giving rise to the term 'excitotoxicity'. The discovery that extracellular glutamate concentrations can rise into the tens of μM in ischaemic stroke lent support to the idea that excitotoxicity might account for the evolving **penumbra** (see Glossary), while it seemed plausible that more insidious, low-grade chronic excitotoxicity might underlie chronic neuronal loss in neurodegenerative disease (reviewed in [2]). These discoveries fostered enormous optimism, and suggested that antagonists to glutamate receptors might represent a panacea, protecting central nervous system (CNS) function in ischaemic injury and limiting neurodegeneration in diseases such as amyotrophic lateral sclerosis (ALS), Huntington's Disease (HD), Alzheimer's disease (AD) and Parkinson's disease (PD) [3].

Over many years of work, and a huge number of publications (a PubMed search of the term 'excitotoxicity' yields over 13500 papers as of December 2020), this therapeutic promise has largely failed to materialize, and while it still seems likely that excitotoxicity plays a role in neuronal death, this has failed to translate into tangible therapeutic benefit.

In this short essay, we wish to share some thoughts about why this might be and how we might refocus on excitotoxicity as a fundamental driver in neurological disease.

Extracellular glutamate levels in health and disease

The extra-synaptic glutamate concentration has been experimentally measured by using microelectrode arrays or microdialysis in diverse models. Measurements vary over a remarkable range - from about 1 to 30 μM in healthy conditions depending on the brain region evaluated, on the applied stimulus and on the method used for the measurement [4][5][6][7][8][9][10]. Despite this variability, which may also be due to differences in spatial and temporal resolution across measurements, to our knowledge the other available methodologies to measure glutamate are not helpful in this context. While proton magnetic resonance spectroscopy (MRS) allows quantification of the level of glutamate in the brain *in vivo* [11], it measures the total glutamate concentration in both intra- and extra-cellular compartments. This limited spatial resolution precludes quantification of the extracellular glutamate concentration, which is the relevant quantity in excitotoxic mechanisms. Another approach involves the use of fluorescent sensors for glutamate, which have been widely used to investigate glutamate release with high speed [12][13]. However, these sensors

need to be expressed in cells and, in any case, quantification is extremely difficult and so they tend to be used to provide relative changes in signal only [12].

The extracellular concentration of glutamate is determined by a balance between its synthesis, release and clearance. Increases in extra-synaptic glutamate have been related to ageing, brain trauma or diseases which cause synaptic spillover or uncontrolled astrocyte and microglia release of the neurotransmitter. Other factors contributing to the neuronal responses to impaired glutamate homeostasis are the expression and localization of glutamate receptors in cells, which vary among different neuronal types [2].

As a simplified model, the cellular pathophysiology of glutamate toxicity has been extensively explored in neuronal cell cultures by exposing otherwise normal cells to very high concentrations of exogenous glutamate (100 μM or more) or by modulating glutamate synthesis and/or clearance [1][14][15]. However, in the pathophysiological contexts in which glutamate toxicity may be important, the tissue is not normal: it may be hypoxic in stroke, where it is likely also experiencing substantial ionic imbalance; the cells may harbor multiple pathologies associated with the disease in ALS, HD, AD, PD, and others. Therefore, it may be crucial to consider the intersection of the underlying pathophysiological mechanisms with the responses to glutamate if we aim to identify key pathways and potential therapeutic targets.

Mitochondrial-related molecular mechanisms of excitotoxic cell death

Since the first descriptions of glutamate excitotoxicity, it has been clear that the key driver of this mechanism is a massive increase in intracellular calcium (Ca^{2+}) concentration, $[Ca^{2+}]_i$, which then initiates downstream processes that drive cell death. Exposure of normal neurons to high concentrations of glutamate cause neuronal death through a cascade of pathways. We will focus on those that have a greater impact on mitochondrial bioenergetics, or which are more affected by defects in mitochondrial function

These include activation of **neuronal nitric oxide (NO) synthase (nNOS)**, **NADPH oxidase**, **poly(ADP-ribose) polymerase (PARP)**, mitochondrial Ca^{2+}-overload and opening of the **mitochondrial permeability transition pore (mPTP)** (Figure 1, Key figure). However, there have been surprisingly few studies in which the responses to glutamate (and the involvement of these pathways) have been explored in the context of a disease models.

The contribution of nNOS to excitotoxic cell death was established to be a consequence of the stimulation of ionotropic **NMDA glutamate receptors** (**NMDAR**)

(Figure 1). This is facilitated by the close spatial juxtaposition of the NMDARs with nNOS through the post-synaptic density protein PSD-95, so that nNOS is exposed to microdomains of high [Ca^{2+}] and activated. Accordingly, inhibition or knock out (KO) of nNOS, or disruption of the NMDAR/nNOS connection, are protective [16][17][18].

Mechanisms of NO-mediated damage may include the reversible inhibition of complex IV due to the competition of NO with oxygen [19], which may be particularly important when mitochondrial function is already compromised, and the non-enzymatic reaction of NO with superoxide to form peroxynitrite, which may further damage mitochondria [20]. Interestingly, NO may also stimulate neuronal glutamate release (Figure 1), further suggesting a mechanism that might exacerbate a situation in which mitochondrial function is already compromised [21].

One of the other contributors to excitotoxic cell death is the enzyme NADPH oxidase, whose activation is coupled to NMDAR stimulation by glutamate via a phosphoinositide 3-kinase (PI3K)-mediated signaling pathway, in which PI3K is activated by the influx of Ca^{2+} and whose impact on neuronal NADPH oxidase is mediated by protein kinase C (PKC) [22] (Figure 1). NADPH oxidase catalyzes the production of superoxide free radicals [23] and the inhibition of upstream PI3K prevents its activation and consequent cell death, without affecting the rise in $[Ca^{2+}]_i$ and mitochondrial depolarization [22]. Similarly to nNOS-mediated toxicity mechanisms, the synergy between the NADPH oxidase-produced superoxide, the increased superoxide production due to mitochondrial dysfunction and defective antioxidant defenses is still understudied; however, these may play a crucial role in those neurodegenerative diseases in which mitochondrial function is already compromised, or in which there is already a measure of oxidative stress and depletion of antioxidant defenses.

PARP-1 is a DNA repair enzyme, activated by single strand DNA breaks usually caused by superoxide (Figure 1). Studies using PARP-1 KO mice showed resistance to glutamate induced toxicity *in vitro* and reduced infarct volume in stroke models *in vivo* in the absence of PARP-1 [24][25][26]. The mechanism by which PARP-1 hyperactivation leads to cell death remains controversial: PAR polymer formation consumes intracellular NAD^+ stores, and depletion of cytosolic NAD^+ causes impaired glycolysis, effectively starving neuronal mitochondria of substrate [27][28], causing mitochondrial depolarization and energetic collapse (Figure 1). Interestingly, PARP can also be activated by NO, a mechanism that establishes a link between these two excitotoxicity pathways [29].

It has also been suggested that PAR polymers cause release of **Apoptosis-inducing factor (AIF)** [30], contributing further to precipitate cell death, but there is some controversy about how much AIF contributes to cell death, which is likely driven primarily by bioenergetic collapse due to mitochondrial dysfunction. Again, this mechanism may have a particularly major impact on neurons that are already bioenergetically compromised [28][30].

Thus, it seems important to reconsider the synergistic roles of each of these toxicity mechanisms in neurons in which cellular bioenergetics are altered, such as in neuronal models for neurodegenerative diseases or ischemia, and within an experimental paradigm that considers physiological concentrations of glutamate.

Another mechanism that can participate in excitotoxic cell death is related to Ca^{2+} mishandling (Figure 1). The influx of Ca^{2+} specifically through NMDARs is essential in excitotoxicity [31] because similar increases in $[Ca^{2+}]_i$ induced by different routes do not trigger cell death [32][33][34]. Recently, however, different "non-ionotropic" NMDAR signaling pathways, possibly involving pannexin-1 activation [35], have been suggested to play a role in glutamate-induced neuronal death [33][34][35]. These signals are induced by agonist binding to NMDAR but are independent from Ca^{2+} influx through this channel, although they proceed in parallel, coupled with elevated $[Ca^{2+}]_i$ [33].

Mitochondria actively participate in Ca^{2+} signaling by taking up the cation into the matrix and extruding it back to the cytosol. The process of mitochondrial Ca^{2+} uptake through the inner mitochondrial membrane (IMM) is controlled by the **mitochondrial calcium uniporter** (MCU) complex (MCUC), composed of channel forming subunits (MCU/MCUb), by the essential component EMRE and by different regulators (MICU1/2/3) [36][37]. Of note, while MICU1 loss-of-function mutations have been associated with a brain and muscle disorder, characterized by proximal myopathy and learning difficulties [38], MICU3 is specifically expressed in the nervous system and sensitizes MCUC to lower $[Ca^{2+}]_i$ [39], favoring mitochondrial Ca^{2+} uptake and thus promoting ATP synthesis in relation to synaptic activity [40]. While this feature could help neurons to achieve metabolic flexibility, it might represent a double-edged sword, sensitizing these cells to mitochondrial Ca^{2+} overload, which has been suggested to be a key event in excitotoxic neuronal death [41][42]. Thus, reducing mitochondrial Ca^{2+} uptake by down-regulation of the MCU seems to protect mouse neurons exposed to excitotoxic stimuli [43] (Figure 1), whereas MCU-overexpression triggers neuronal loss, astrocyte activation and **gliosis** [44]. Surprisingly, however, global or acute neuron-specific MCU ablation has been reported to induce

distinct effects in mouse models. In both cases, a reduced mitochondrial Ca^{2+} uptake and decreased Ca^{2+}-induced mPTP opening were observed. However, the constitutive, gene-trap generated whole body MCU-KO [45] did not significantly affect the extent of hypoxic-ischemic brain injury [46], whereas the conditional, forebrain neuron-restricted MCU-deletion at 8-12 weeks of age reduced damage [47]. It is tempting to speculate that the impaired mitochondrial bioenergetics and the profound metabolic adaptations observed upon global MCU deletion [46] might trigger an energetic defect promoting ischemia/reperfusion-associated damage. Conversely, acute MCU-depletion (or inhibition), by reducing mitochondrial Ca^{2+} uptake while preserving mitochondrial functionality [47], could be an efficient therapeutic strategy to be pursued. Similar compensatory mechanisms have been suggested to occur upon ablation of the **mitochondrial Na^{+}/Ca^{2+} exchanger** in the heart (NCLX, the main mitochondrial Ca^{2+} efflux protein in excitable cells; [48]), which is lethal when performed in adult mouse, whereas it is well tolerated when induced immediately after birth [49]. Interestingly, a reduced NCLX expression/activity has been reported in some neurodegenerative disorders, including AD [50] and PD [51][52], supporting the importance of mitochondrial Ca^{2+} signalling for neuronal health.

Importantly, a rise in mitochondrial matrix [Ca^{2+}] is the most potent mPTP inducer [53], thereby providing a direct link between sustained Ca^{2+} rises and mitochondrial dysfunction during excitotoxicity [15][54][55]. In addition, upon toxic glutamate exposure, changes in the relative ATP/ADP levels have been suggested to further modulate mPTP opening [56] (Figure 1). The prolonged mitochondrial depolarization can reverse **ATP-synthase** activity [57], further contributing to ATP hydrolysis and accelerating a catastrophic energetic crisis [14]. However, while mPTP induction has been proven to be a key event underlying heart damage upon ischaemia-reperfusion [58], whether it is necessary to trigger neuronal death during excitotoxicity is contentious. Indeed, prevention of mPTP opening by cyclosporine-A or by deletion of the mPTP regulator, cyclophilin D, did not significantly reduce excitotoxic death [15], although the mPTP might be involved in glutamate-induced axonal degeneration, a process recently suggested to depend on a compartmentalized, neurite-specific activation of necroptosis [59].

Other key determinants of mitochondrial Ca^{2+} fluxes are the members of the Bcl-2 family. In fact, Bcl-2 proteins can regulate Ca^{2+} influx at both the IMM and outer mitochondrial membrane (OMM) [60][61]. In particular, the Bcl-2 family member Bax is known to regulate Ca^{2+} response via NMDAR activation in neurons: *bax*-/- mice are

protected against "**delayed-Ca^{2+}-deregulation**" (**DCD**) when stimulated with 100 μM NMDA [62]. However, further studies are needed to determine to what extent the role of Bcl-2 proteins is relevant for neuronal toxicity in conditions involving physiological glutamate concentrations.

Impaired mitochondrial metabolism sensitises cells to excitotoxicity

During physiological glutamate-induced excitation, cytosolic [Na^+] and [Ca^{2+}] rapidly rise, thereby increasing neuronal metabolic workload to recover ion homeostasis. The response to this increased metabolic demand is met by the potentiation of ATP supply through increased mitochondrial respiration and ATP synthesis, mediated by the increase in intramitochondrial [Ca^{2+}] [63][64]. The regulation of mitochondrial activity relies on cytosolic ATP consumption, which stimulates oxidative phosphorylation, and mitochondrial Ca^{2+} uptake, which stimulates key metabolite transporters in the IMM, three rate-limiting matrix-located dehydrogenases and possibly the ATP synthase (reviewed in [65]). Therefore, it is not surprising that impaired mitochondrial activity may sensitize neurons to glutamate exposure, lowering the excitotoxic threshold [66][67][68]. For instance, mitochondrial function is severely compromised in cortical neurons cultured from a mouse model of neuropathic Gaucher's Disease. In this model, the KO of the lysosomal enzyme glucocerebrosidase (**GBA1)** causes a severe neurodegenerative phenotype. This is associated with decreased $\Delta\Psi_m$, an increased ROS production and reduced mitochondrial Ca^{2+} uptake. Neurons from the KO animals, and even from heterozygotes, respond to physiological concentrations of glutamate (10 μM) with DCD and $\Delta\Psi_m$ collapse, typical features of glutamate excitotoxicity [69]. Measurements of the ATP/ADP ratio using the fluorescent reporter 'PercevalHR' [70] showed no change at rest in the different genotypes, but a collapse in the KO cells in response to the glutamate that failed to recover, while glutamate caused only a transient decrease in ATP/ADP in wild type neurons. Thus, the mitochondrial defects may be adequately compensated until overwhelmed by an increased energy demand, which unveils their potential to trigger a rapid pathological cascade. Similarly, in primary cortical neurons from an AD mouse model (expressing a presenilin 2 mutation) displaying defective mitochondrial pyruvate uptake and lower mitochondrial Ca^{2+} signal, ATP levels at rest were almost normal, whereas a fast decline in ATP was observed upon cell exposure to specific stimuli inducing high energy-demand [71]. In this model, neuronal stimulation with just 0.5 μM glutamate caused DCD, a response rescued by restoring mitochondrial pyruvate flux. These results would

need to be further validated in other neurodegenerative models but suggest a mechanism of general relevance for different neurological pathologies, and for many mitochondrial diseases that present defective mitochondrial metabolism.

The central role of glutamate in metabolism

In addition to its action as neurotransmitter, glutamate is a key intermediate metabolite. Indeed, not only it is an essential building block for protein synthesis, but it can also fuel the tricarboxylic acid (TCA) cycle (as an anaplerotic substrate, see Box 1 and Figure 2). Although the brain relies primarily on pyruvate oxidation for energy homeostasis, glutamate influx into the TCA cycle might confer a degree of metabolic flexibility [72][73]. In particular, in primary cortical neurons, glutamate has been reported to be used as an alternative substrate of the TCA cycle, upon chemical inhibition of mitochondrial pyruvate uptake [72]. This metabolic rewiring depletes the intracellular glutamate pool, limiting its loading into synaptic vesicles and its release in the extracellular space, thereby protecting neurons from the positive-feedback cascade underlying excitotoxicity [72].

However, whether this effect is relevant *in vivo*, where multiple cell types shape glutamate homeostasis at the synaptic cleft (Figure 2), is not known. Nevertheless, profound perturbations in metabolism of glutamate and related neurotransmitters have been observed in brain samples from **mitochondrial pyruvate carrier (MPC1)** KO mouse embryos, suggesting that glutamate homeostasis might be substantially affected by changes in mitochondrial activity [74]. Therefore, conditions in which mitochondrial function is altered might hamper the capacity of neurons to oxidize glutamate. For instance, excitotoxicity is frequently observed during ischemia, whereby hypoxia impairs mitochondrial respiration and metabolism. In this context, mitochondria cannot switch to glutamate oxidation, because of O_2 shortage. Furthermore, the Ca^{2+} signal represents an integral component of the glutamate response. The relay of Ca^{2+} to the mitochondrial matrix stimulates some rate-limiting enzymes of the TCA cycle, including oxoglutarate dehydrogenase, whose activity is critical for glutamate metabolism (Figure 2). In addition, mitochondrial glutamate uptake, mostly mediated by the glutamate/aspartate antiporter **Aralar**, is directly upregulated by the $[Ca^{2+}]$ in the mitochondrial intermembrane space (reviewed in [65]). Interestingly, Aralar expression has been reported to modulate the susceptibility to kainic acid-induced excitotoxicity *in vivo* [56][75]. In general, alterations of mitochondrial Ca^{2+} signaling can deeply affect the extent and the significance of metabolic flexibility.

Overall, the complex integration of the specific cellular context (hypoxia, nutrient availability, interplay with different cells, etc.) with mitochondrial signals (Ca^{2+} levels, respiratory chain and transporter activity, etc.) likely determines the role of glutamate as a neurotransmitter and a fundamental intermediate metabolite.

Excitotoxicity gone wrong

As discussed in the previous sections, glutamate excitotoxicity is thought to play a major role in the pathophysiology of ischemic stroke and to contribute to many neurodegenerative diseases, including AD, PD and ALS (recently reviewed by [2]). However, pharmacological intervention targeting excitotoxicity has been largely ineffective, despite the wealth of data pointing toward this mechanism as a promising target in these diseases. The early approaches used selective antagonists to prevent NMDAR activation, whereas alternative methods were based on the induction of the expression of excitatory amino acid transporters to increase glutamate clearance [3].

Despite expectations, these approaches generally failed to provide therapies which slow the progression of these pathological conditions. Some exceptions are the only FDA-approved drug which prolongs survival in ALS, albeit only by a few months, *i.e.*, **riluzole**, which inhibits synaptic glutamate release and NMDAR activation, [76]. In the context of AD, the FDA-approved molecule **memantine**, a non-competitive NMDAR inhibitor, is among the few drugs providing modest symptomatic benefits in patients (reviewed in [77]). Their beneficial effects notwithstanding, the relatively limited impact of these drugs on disease progression raises questions about the actual efficacy of targeting excitotoxicity mechanisms in neurodegenerative disorders more broadly.

It is conceivable that designing novel strategies to target excitotoxicity in neurodegenerative diseases may lead to more effective treatments, but the considerations discussed earlier underscore the need to better understand the specific cellular responses to glutamate in disease models. For instance, the recent observations of “non-ionotropic” signals induced by ligand binding to NMDAR (see above) might in part explain the insufficient efficacy of specific receptor inhibitors. Ca^{2+} elevations are essential in excitotoxicity, but when non-ionotropic NMDAR signaling is activated, alternative routes of Ca^{2+} influx can still trigger cell death [33][35]. Moreover, the imbalance of mitochondrial bioenergetics that is frequently observed in neurological diseases should also be considered, because it contributes to the failure of cells to recover ionic homeostasis. Therefore, one possibility might be to couple drugs that impact the glutamate responses

with interventions that support mitochondrial bioenergetics, for example by promoting mitochondrial biogenesis or supporting intermediary metabolism, impacting both the mechanisms that may contribute to neurodegeneration in these diseases.

Concluding Remarks and Future Perspectives

Emerging evidence supports the notion that the bioenergetic state of neurons is a crucial determinant of their response to glutamate at physiological concentrations, suggesting that any pharmacological attempt to counteract excitotoxicity should consider mitochondrial function and the pathways that couple glutamate exposure to mitochondrial collapse. The increased energy demand imposed by normal glutamate stimulation at synapses may be sufficient to overwhelm an already limited bioenergetic reserve in diseased neurons, whereby defective mitochondria may additionally exacerbate oxidative stress and Ca^{2+} mishandling. Therefore, while targeting glutamate receptors, or glutamate clearance, can still be an option for developing new treatments, specific strategies aimed at simultaneously supporting mitochondrial performance might be of help.

Re-evaluation of past work and new experimental outcomes (see Outstanding questions) will be needed to determine whether combined strategies could offer an effective path for counteracting disease progression in different neurological disorders.

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Declaration of Interests

The authors declare no competing interests.

Glossary

Penumbra: area of a damaged ischemic tissue but not yet irreversibly injured, thus representing a target for acute therapies.

Neuronal nitric oxide synthase (nNOS): the enzyme that catalyses the production of nitric oxide (NO) from L-arginine. nNOS activity is regulated by Ca^{2+}/calmodulin-dependent pathways and controls the level of NO in the brain, which is a key signaling molecule in mammals.

NADPH oxidase: the enzyme responsible for the production of a superoxide radical by transferring one electron from NADPH to O_2.

Poly(ADP-ribose) polymerase (PARP): the multidomain protein localized in the nucleus involved in several cellular processes including DNA-damage repair, whose catalytic activity is the polymerization of poly(ADP-ribose).

Mitochondrial permeability transition pore (mPTP): the high-conductance channel of the IMM whose opening underlies mitochondrial swelling and cell death-induction in response to elevated [Ca^{2+}] in the mitochondrial matrix.

N-methyl-D-aspartate receptor (NMDAR): the ionotropic glutamate receptor mostly expressed in neuronal cells. Upon glutamate and glycine (or D-serine) binding, it allows the influx of Na^+ and Ca^{2+}. NMDARs at synapses modulate synaptic plasticity, whereas extra-synaptic NMDARs have been associated with excitotoxicity.

Apoptosis-inducing factor (AIF): the mitochondrial protein that functions as NADH oxidoreductase and proapoptotic factor in a caspase-independent manner.

Mitochondrial calcium uniporter (MCU): the channel-forming subunit of the mitochondrial calcium uniporter complex (MCUC), an IMM multi-protein-complex mediating mitochondrial Ca^{2+} uptake.

Mitochondrial Na^+/Ca^{2+} exchanger (NCLX): the sodium/calcium antiporter located in the IMM that mediates Na^+-dependent Ca^{2+} efflux from the mitochondrial matrix.

Gliosis: the process associated with the proliferation/activation of glial cells (astrocytes, microglia, oligodendrocytes) upon an injury in the CNS.

ATP synthase: the enzymatic complex of the IMM which synthesizes ATP by using the proton gradient generated across the IMM by the electron transport chain. In certain conditions, it can work in reverse, hydrolyzing ATP to sustain the proton gradient.

Glucocerebrosidase (GBA1): the enzyme whose mutations in homozygosity cause Gaucher disease, and in heterozygosity represent the major genetic risk for Parkinson's disease.

Riluzole: an FDA-approved drug used in ALS treatment since 1995 and known to directly inhibit kainate and NMDARs.

Memantine: a reversible NMDAR-antagonist which binds to the receptors and inhibits Ca^{2+} influx. It has been suggested to be mostly effective on extra-synaptic NMDARs.

Mitochondrial Pyruvate carrier (MPC): the IMM carrier formed by the assembly of MPC1 and MPC2 subunits, which allows pyruvate uptake into the mitochondrial matrix.

Aralar: the mitochondrial carrier that mediates the Ca^{2+}-dependent exchange of glutamate with aspartate across the IMM. It is endowed with a regulatory EF-hand domain sensing Ca^{2+} in the intermembrane space.

Delayed Ca^{2+} deregulation (DCD): the irreversible rise in $[Ca^{2+}]_i$ observed upon exposure of neurons to excitotoxic glutamate levels, occurring minutes-to-hours after an initial transient increase in $[Ca^{2+}]_i$. DCD parallels a collapse of mitochondrial $\Delta\psi_m$.

Text Box 1: The role of pyruvate and glutamate in the TCA cycle

The tricarboxylic acid (TCA) cycle, also known as the Krebs cycle, consists of a series of reactions occurring in the mitochondrial matrix, capable of extracting the energy stored within acetyl-CoA molecules. The energy yield for each cycle is 1 molecule of $FADH_2$ and 3 of NADH (in turn fueling the electron transport chain), as well as 1 molecule of GTP (or ATP). Various substrates can fuel the TCA cycle (Figure 2), with pyruvate being the predominant one in the brain. Pyruvate, the end-product of glycolysis, enters into the mitochondrial matrix through the IMM-located mitochondrial pyruvate carrier (MPC, formed by hetero-oligomers of the MPC1 and MPC2 subunits; [78][79]): its oxidative decarboxylation by pyruvate dehydrogenase (PDH) leads to the formation of acetyl-CoA, whose condensation with oxaloacetate forms citrate and represents the first step of the cycle. Alternatively, different enzymes can convert ketone bodies and short-chain fatty acids into acetyl-CoA. These pathways are observed during prolonged starvation or ketosis, but they can sustain only partially cerebral energy demand. In certain conditions, such as hypoglycemia or defective mitochondrial pyruvate flux, a truncated TCA cycle (utilizing glutamate as an alternative substrate and comprising the steps from α-ketoglutarate to oxaloacetate) might operate and yield ~75% of the energy produced by the entire cycle [80][72]. In neurons, Aralar allows mitochondrial glutamate uptake by importing one molecule of glutamate (plus a H^+) and exporting one aspartate [81]. Glutamate and the TCA cycle intermediate α-ketoglutarate can be interconverted by the combined activity of aspartate-aminotransferase (AAT, comprising both cytosolic and mitochondrial isoforms) and glutamate-dehydrogenase (GDH), whereas α-ketoglutarate can be exported to the cytosol through the malate/α-ketoglutarate carrier [80] (Figure 2). Therefore, the dynamic equilibrium between formation of glutamate, from TCA cycle intermediates, and its oxidation, in the cycle, depends on the availability of the different substrates and, ultimately, on the intrinsic TCA cycle activity. Importantly, glutamate can be taken up and transformed into glutamine in astrocytes, whereas glutamine can be taken up and converted into glutamate in neurons. A critical interplay between astrocytes and neurons, involving both anaplerotic and cataplerotic reactions and known as the glutamate-glutamine cycle [82][83][84], ensures a precise balance between the key signaling and metabolic functions of glutamate (Figure 2).

Figure Legends

Figure 1, Key Figure: Mitochondrial-related mechanisms of excitotoxicity. Most of the excitotoxicity mechanisms are tightly connected to mitochondrial function, including activation of neuronal nitric oxide (NO) synthase (nNOS), NADPH oxidase, poly(ADP-ribose) polymerase (PARP), mitochondrial Ca^{2+}-overload and opening of the mitochondrial permeability transition pore (mPTP). When mitochondria are bioenergetically compromised, as in neurodegenerative diseases, in stroke or brain injuries, the activation of these mechanisms can occur at physiological glutamate concentrations and trigger the pathological cascade causing neuronal death.

Figure 2: Synaptic glutamate turnover and mitochondrial metabolism. After release from synaptic vesicles into the synaptic cleft, glutamate binds to postsynaptic NMDARs, triggering Na^{+} and Ca^{2+} influx. Ca^{2+} taken up by mitochondria modulates mitochondrial metabolism and, in certain conditions, is a key determinant of excitotoxic cell death. After neurotransmission, glutamate clearance is guaranteed by its cellular re-uptake through the excitatory amino acid transporters (EAATs), mostly expressed in astrocytes, whereby glutamate can be converted into glutamine and released through glutamine transporters. In turn, glutamine can be taken up by neurons and converted back into glutamate, which can be used to replenish synaptic vesicles or taken up by mitochondria and used as a TCA cycle substrate. Ca^{2+} signals modulate key steps of the TCA cycle, thus affecting glutamate availability (see also Text Box 1 for details).

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