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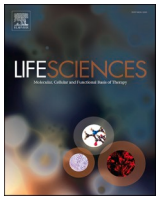

Review article

Molecular mechanisms of ischemia and glutamate excitotoxicity

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ABSTRACT

Excitotoxicity is classically defined as the neuronal damage caused by the excessive release of glutamate, and subsequent activation of excitatory plasma membrane receptors. In the mammalian brain, this phenomenon is mainly driven by excessive activation of glutamate receptors (GRs). Excitotoxicity is common to several chronic disorders of the Central Nervous System (CNS) and is considered the primary mechanism of neuronal loss of function and cell death in acute CNS diseases (e.g. ischemic stroke). Multiple mechanisms and pathways lead to excitotoxic cell damage including pro-death signaling cascade events downstream of glutamate receptors, calcium (Ca^{2+}) overload, oxidative stress, mitochondrial impairment, excessive glutamate in the synaptic cleft as well as altered energy metabolism. Here, we review the current knowledge on the molecular mechanisms that underlie excitotoxicity, emphasizing the role of Nicotinamide Adenine Dinucleotide (NAD) metabolism. We also discuss novel and promising therapeutic strategies to treat excitotoxicity, highlighting recent clinical trials. Finally, we will shed light on the ongoing search for stroke biomarkers, an exciting and promising field of research, which may improve stroke diagnosis, prognosis and allow better treatment options.

1. Introduction

Excitotoxicity is characterized by the extracellular accumulation of high concentrations of glutamate or other excitatory amino acids, leading to excessive stimulation of glutamate receptors. It is a pathological process that occurs in neurological diseases such as ischemia, traumatic brain injury and epileptic seizures [1]. It has also been linked to several neurodegenerative diseases such as Huntington's, Alzheimer's, Parkinson's, Multiple Sclerosis and Amyotrophic Lateral Sclerosis (ALS). Classically, glutamate excitotoxicity is defined as a massive influx of extracellular Ca^{2+} through *N*-methyl-D-Aspartate receptors (NMDAR), which can be followed by Ca^{2+} release from intracellular stores, further increasing the cytosolic free Ca^{2+} concentration [2]. The overstimulation of other families of glutamate receptors (GRs) also triggers the influx of Na^{+} and Cl^{-} ions, which is accompanied by the diffusion of water to counterbalance the osmotic pressure, thus resulting in cellular swelling [3].

In this review, the molecular pathways leading to excitotoxicity will be discussed. In particular, we will focus on the contribution of each class of GRs in glutamate-induced excitotoxicity. We also go beyond the classical view that focuses exclusively on the postsynaptic side and discuss alternative contributions from the presynaptic neuron, astrocytes and endothelial cells from the blood-brain barrier in regulating several aspects of brain ischemia. We also examine the contribution of energy metabolism, in particular the role of NAD metabolism, in the protection against excitotoxicity. Additionally, ongoing therapeutic strategies and exciting new clinical trials, which target distinct players in the excitotoxicity cascade that may provide novel therapeutical approaches, will be reviewed as well. Finally, we address the search for biomarkers to aid the clinical management of stroke.

Abbreviations: AHS, Acute Hemorrhagic Stroke; AMPAR, α-amino acid-3-hydroxy-5-methyl-4-isoxazole Receptors; CPIPs, Cell Penetrating Interfering Peptides; CNS, Central Nervous System; CSF, Cerebrospinal Fluid; GRs, Glutamate Receptors; iGluRs, Ionotropic Glutamate Receptors; IS, Ischemic Stroke; KAR, Kainic Acid Receptor; LTD, Long-Term Depression; LTP, Long-Term Potentiation; mGluRs, Metabotropic Glutamate Receptors; NMDAR, *N*-methyl-D-Aspartate Receptor; OGD, Oxygen-Glucose Deprivation; TBI, Traumatic Brain Injury; tPA, Tissue Plasminogen Activator.

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2. Overview of glutamate receptors

L-Glutamate, the main excitatory neurotransmitter of the Central Nervous System (CNS), acts on two major classes of receptors: ionotropic receptors (iGluRs) and G protein-coupled metabotropic receptors (mGluRs). Under physiological conditions, activation of subsets of these receptors results in excitatory synaptic transmission and in processes related to synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), the molecular mechanisms underlying learning and memory paradigm [4].

Upon glutamate binding, ionotropic glutamate receptors mediate fast excitatory neurotransmission through their function as ion channels, leading to the flow of Na^+ and K^+ cations across the plasma membrane to generate an action potential. After exerting its effect, glutamate is then removed from the synaptic cleft by Excitatory Amino Acid Transporters (EAATs) to terminate synaptic transmission and allow subsequent action potentials [5]. Three groups of iGluRs are known to date and are classified according to the affinity of their specific agonists: NMDA, α-amino acid-3-hydroxy-5-methyl-4-isoxazole (AMPA) and kainic acid (KA) receptors [6].

The NMDAR possesses a higher permeability to cations, most notably Ca^{2+}. Under resting conditions, the channel is blocked by Mg^{2+}. AMPA receptor (AMPAR) activation and membrane depolarization removes the Mg^{2+}-mediated channel inhibition by decreasing its binding site affinity [7]. Three subsets of NMDARs with multiple subtype composition have been identified so far, GluN1, GluN2 (GluN2A-D) and GluN3 (GluN3A-B). Obligatory GluN1 and usually GluN2 dimers form the traditional heterotetrameric NMDAR. Both subunits show different biochemical and biophysical properties. While the GluN2 subunit binds glutamate, GluN1 contains the binding site for the co-agonist glycine, both of which are necessary for full activation of NMDARs [8]. GluN3 subunits can also assemble with GluN1 subunits to form functional receptors. Recent data have shown that NMDARs containing GluN3 are more permeable to Ca^{2+} than those with the GluN1/GluN2 combination [9], and that the GluN3 subunit acts as a negative regulator of synapse maturation [10].

AMPAR are tetrameric complexes formed by the combination of four GluA subunits (GluA1–4) in a homomeric or heteromeric fashion [11]. Under physiological conditions the GluA2 gene undergoes post transcriptional RNA editing, which converts a codon for glutamine to a codon for arginine at position 607 in the channel pore. This process is catalyzed by adenosine deaminase acting on RNA 2 (ADAR2), which results in the generation of GluA2-containing AMPAR impermeable to Ca^{2+} [11,12].

Kainic acid receptors (KARs) are tetrameric cation channels made up of 5 GluK subunits (GluK1–5). Different KAR homomeric and heteromeric complexes can be formed; however, GluK4 and GluK5 require the association of at least one GluK1–3 to become functional [13].

Finally, metabotropic GR (mGluRs) combine an extracellular domain responsible for binding of glutamate with an intracellular domain that is coupled to G-proteins. Glutamate binding results in G-protein activation, dissociation from the receptor and modulation of various effectors including enzymes, transcription factors and several other ion channels. These receptors are monomeric and comprise eight different isoforms (mGluR1–8) divided into three groups (I, II and III), that have differences in sequence similarity, pharmacology and intracellular effects [14].

2.1. GR composition, localization and function in excitotoxicity

Intense investigation has revealed that NMDAR plasma membrane localization and subunit composition have distinct roles in neuronal death and survival. Regardless of their biochemical properties, studies have shown that their inhibition is protective after ischemic damage [15]. The *subunit composition hypothesis* is one of the existing theories that explain NMDAR activation. According to this hypothesis, NMDARs containing GluN2B are excitotoxic while NMDARs containing GluN2A are neuroprotective [16]. GluN2A is commonly associated with pro survival cascades including the cAMP response element-binding protein and phosphatidylinositol 3-kinase (PI3K) pathways, whereas GluN2B is often associated with pro-death mechanisms such as PTEN-mediated AKT and BAD dephosphorylation [17]. The toxicity of the GluN2B subtype was confirmed in a *knock-in* mouse model, where the C-terminal domain of Glu2NA and GuN2B were swapped [18]. One of the pitfalls of this theory is the fact that it does not include the contribution of NMDARs that do not contain GluN2A or GluN2B. For instance, GluN3A and GluN2D have been shown to mediate neuroprotection and excitotoxic cell death, respectively [19,20]. Regarding GluN2C, whether this subtype promotes neuroprotection or excitotoxic cell death is still controversial [21,22]. Specific modulators of these subtypes should be designed to better understand their contribution to excitotoxicity. Another theory, the *localization hypothesis*, states that the consequences of NMDAR activation are strictly dependent on localization. Hence, if NMDARs have a synaptic or extrasynaptic localization, pro-survival or pro-death pathways will be elicited, respectively [23]. Selective synaptic NMDAR activation promotes the induction of survival genes and suppression of death-associated genes, while activation of extrasynaptic NMDAR promotes the opposite effect [24]. However, in certain cell types that only express extrasynaptic NMDARs (e.g. retinal ganglion cells), exposure to high concentrations of glutamate and NMDA (condition that activate both type of populations), failed to induce cell death [25]. Since synaptic and extrasynaptic NMDARs can be activated by different endogenous co-agonists [26], we may speculate that this protocol is not able to induce sufficient cell death by the following reasons; i) insufficient glutamate concentration in the media; ii) absence of co-agonist for full receptor activation; iii) combination of both. Yet, all of these hypotheses were not followed-up in subsequent studies.

Despite some contradictory evidence [27,28], a mixture of the above-mentioned theories has been the most accepted view. Nowadays, it is widely accepted that the synaptic localization of GluN2A mediates the neuroprotective effect of NMDAR, whereas the extrasynaptic localization of GluN2B mediates its toxic effect [29]. This rationale gave rise to Neu2000, a molecule that specifically targets GluN2B and that also scavenges reactive oxygen species (ROS) generated following excitotoxic damage. Neu2000 was shown to prevent excitotoxicity and oxidative stress both in *in vitro* and *in vivo* models of brain ischemia [30], and its large therapeutic time window encouraged clinical trials in acute ischemic stroke patients within 6 h of disease onset (NCT04453800 and NCT04486430).

NMDAR has functions apart from ion flux through the receptor that mediate both physiological and pathophysiological processes [31,32]. Recently, the ion flux independent non-ionotropic NMDAR has been associated with excitotoxicity. The non-ionotropic functions of NMDAR involve conformational changes in its C-terminal domains induced by ligand binding to the receptor and require basal levels of Ca^{2+} [33]. These non-canonical functions of NMDARs greatly increase the complexity of mechanisms involved in excitotoxicity. Some of them will be further discussed.

Interestingly, populations of neurons expressing Ca^{2+}-permeable AMPARs (CP-AMPAR) are also prone to excitotoxicity that is associated with lacking or unedited GluA2 subunits [11]. The administration of 1-naphthylacetyl spermine, a blocker of CP-AMPARs, revealed a significant neuroprotective effect for post-ischemic injury in CA1 hippocampal neurons [34]. Although it is difficult to distinguish between GluA2-lacking and GluA2-unedited AMPAR, single cell PCR combined with editing assays suggested that unedited GluA2 is a player in excitotoxicity [35]. Despite only approximately 1 % of GluA2 RNA encodes unedited forms, GluA2 editing deficiencies have a major role in the process of excitotoxicity observed in ischemia as well as in motor neurons degeneration in the context of ALS [11,36]. Additionally, both ADAR2 cleavage and subsequent accumulation of Glu2A-unedited CP-AMPAR trafficking to the synapse are important features in excitotoxicity.

Excessive glutamate stimulation of cortical neurons *in vitro* induces the calpain-dependent cleavage of ADAR2 leading to a decrease or loss of GluA2 editing [12]. In agreement, oxygen-glucose deprivation (OGD), an *in vitro* model that mimics transient global ischemia, was shown to direct GluA2-lacking AMPAR to synaptic sites in hippocampal neurons [37]. The GluA2-unedited CP-AMPAR is more rapidly trafficked to the synapse when compared to their counterparts, and still allow ion influx when desensitized [38]. Inhibiting ADAR2 cleavage and blocking peptide trafficking of the unedited form of GluA2 might be two strategies to tackle excitotoxicity in conditions where editing failure is detrimental [39,40].

The lack of structural evidence has limited the search for drugs that are able to target CP-AMPAR. These receptors can be blocked by endogenous polyamines; however, repetitive activation of these receptors results in removal of this blockade thereby facilitating ion flux. Recently, Twomey et al. provided structural evidence for the mechanism of channel blockers and their effect on CP-AMPARs, by using exogenously applied polyamine-based channel blockers [41]. However, the relevance to excitotoxic neuronal damage remains to be seen.

The contribution of metabotropic receptors during neuronal excitotoxicity is also reported but mainly restricted to group I. Group I mGluRs (mGluR1 and mGluR5) are found at the post-synapse and modulate currents mediated by iGluRs. This process occurs *via* activation of phospholipase C leading to the formation of diacylglycerol and inositol 1,4,5-triphosphate (IP3), followed by the activation of protein kinase C (PKC) that results in Ca^{2+} release from neuronal stores thus resulting in the potentiation of NMDAR-mediated Ca^{2+} influx [42]. In particular, mGluR5 is responsible for relieving Mg^{2+} blockade of NMDAR, and continuous NMDAR signaling amplifies mGluR5 activity and prevents NMDAR desensitization [43]. Although the development of negative allosteric modulators of mGluR5 has been proposed to protect against excitotoxicity, the resulting clinical trials involving this receptor have failed [44].

2.2. GR-associated pro-death complexes and signaling

Unsuccessful efforts to find NMDAR antagonists capable of being translated into clinical practice have increased research efforts to the study NMDAR complexes, as well as the pro-death signals elicited by these complexes. This alternative strategy could reduce the severe side effects observed with NMDAR antagonists, maximizing cell survival signaling and providing a wider therapeutic time window [16]. Therefore, cell penetrating interfering peptides (CPIPs) and small molecules capable of crossing the blood-brain barrier have been developed. In the context of excitotoxicity, CPIPs encompass peptides that target calpain-cleavage sequences in proteins and peptides that interfere with protein-protein interactions.

Following activation, the NMDAR is included in early endosomes to be differentially sorted. They can either be recycled back to the plasma membrane, or alternatively, enter the late endosome for degradation in the lysosome [45]. When overactivated, NMDAR endocytosis is enhanced, an event that precedes the onset of neuronal death [46]. This turning point is somehow achieved by disrupting Extracellular-signal-regulated kinase (ERK) survival pathways *via* Kinase D-interacting substrate of 220 kDa (Kidins220), a component of the NMDAR complex that is essential for neuronal survival through the activation of the GTPase Ras-associated protein 1 (Rap1), which in turn is involved in ERK stimulation [47]. During late excitotoxicity, Kidins220 is internalized in early endosomes, translocated to the Golgi Apparatus, and degraded by calpain together with Rap1 activation complexes [47]. Using this information, a peptide that interferes with calpain cleavage of Kidins220 (Tat—K) was synthesized. By preventing the cleavage of Kidins220 under conditions of excessive NMDA incubation in cultured cerebrocortical neurons, ERK activity was preserved, and neuronal survival under the latter conditions was enhanced. [48]. Disrupting endocytosis *per se* would be harmful for neuronal cells due to the involvement of NMDAR trafficking in conditions of elicited synaptic activity. The association of GluN2B but not GluN2A endocytosis in excitotoxicity indicates that the NMDAR subunit composition may interfere with these processes, and suggests that further attention should be given to the endocytic signaling associated with GluN2B [46].

Other NMDAR complexes have been associated with excitotoxic cell death, namely the GluN2B-PSD95-nNOS complex. The interaction between the GluN2B subunit of NMDAR complexed with PSD95 and neuronal nitric oxide synthase (nNOS), an enzyme that catalyses the formation of nitric oxide (NO) downstream of NMDAR activation, ultimately leads to oxidative stress and cell death [49]. A peptide that interferes with the calpain cleavage site of PSD-95 has been developed ($TP95_{414}$), and positive results were obtained in a mouse model of stroke [50]. Additionally, the peptide NA-1 and the small molecules ZL006 and AVLX-144, which dissociates nNOS from NMDAR signaling while maintaining intact the NMDAR ionic currents also demonstrated neuroprotective properties against excitotoxicity in *in vitro* and *in vivo* models [51–53]. NA-1 was used in phase III clinical trials for stroke (ESCAPE-NA.1, ClinicalTrials.gov number, NCT02930018), however, a drug-drug interaction between NA-1 and alteplase, one of the thrombolytic drugs considered to be the gold standard of care in ischemic type stroke patients (almost 90 % of stroke cases), was identified [54]. Hence, a phase III clinical trial is now considering patients that did not receive alteplase (ESCAPE-NEXT, ClinicalTrials.gov NCT04462536). Although the interaction of alteplase with NA-1 affects the effectiveness of NA-1, administration of the peptide just a few minutes prior the administration of the thrombolytic drug was shown to avoid drug-drug interactions of any type, conferring overt neuroprotection capabilities [55]. Indeed, a clinical trial is also ongoing aiming to confirm the absence of interactions by administrating NA-1 before thrombolytic drugs in patients with suspected ischemic stroke (FRONTIER, ClinicalTrials.gov NCT02315443). Also, the wider therapeutic time window of AVLX-144 over NA-1 in pre-clinic models [56] encouraged the initiation of a phase I clinical trial for this compound (ClinicalTrials.gov, NCT04689035).

Uncoupling the Ca^{2+}/calmodulin dependent protein kinase DAPK1 and GluN2B is another neuroprotective approach that has been explored [57]. Following excitotoxic Ca^{2+} influx, DAPK1 is primarily dephosphorylated by the Ca^{2+}-dependent protein calcineurin thereby promoting its recruitment and subsequent phosphorylation of GluN2B subunits at Ser1303, which in turn increases activity [58]. The interfering peptide Tat-NR2B-CT was developed to abolish this interaction and subsequent post-translational modification, thus promoting neuronal survival against stroke damage *in vivo* [58]. However, some authors questioned the involvement of DAPK1-GluN2B signaling in excitotoxicity. McQueen and co-workers have shown that excitotoxicity takes place independently of DAPK1 absence, and increased GluN2B phosphorylation, both in *in vitro* and *in vivo* models of ischemia is observed regardless of the background genotype (using Dapk1 KO cultures/animals) [59]. These authors also demonstrated that Tat-NR2B-CT, due its high positive charge, acts as direct antagonist of the NMDAR [59], which may provide an explanation for its neuroprotective effects.

Additionally, proteins that are indirectly recruited to the NMDAR can be associated with excitotoxic neuronal damage. Peptidyl-prolyl isomerase 1 (Pin1) is one such protein that binds to, and isomerizes, specific phosphorylated Ser/Thr-Pro residues. This interaction induces conformational changes in PSD-95 that ultimately affects its ability to bind NMDAR [60]. Inhibition of Pin1 increases NMDAR-mediated excitotoxic effects [51], but contradicting results indicate a detrimental role for Pin1 in excitotoxicity *via* the CAST/Calpain2 pathway in rat retinal neurons [61]. Interestingly, Pin1 was also shown to interact with ADAR2 [62]. This interaction is required for the nuclear localization, stability and function of ADAR2 thus pointing Pin1 as an important player in both NMDAR- and AMPAR-mediated functions. Another protein that binds PSD-95 and modulates NMDAR function is PSD-95-

interacting regulator of spine morphogenesis (Preso). Preso modulates NO and Ca^{2+} responses through PSD-95 and CDK5-mediated NMDAR phosphorylation, respectively, in models of TBI [63]. Overall, exploring Pin1 and Preso functions in excitotoxicity may drive the development of new peptides or small molecules to tackle excitotoxic-related disease conditions.

Perhaps the two most cited pro-death pathways elicited by the excitotoxic NMDAR complexes are the p38 and c-Jun N-terminal kinase (JNK) signaling cascades. STEP is a tyrosine protein phosphatase that antagonizes the duration of the signal produced by the NMDAR through dephosphorylation of GluN2B-containing NMDAR and p38 [64]. The activation of p38 occurs during excitotoxicity due to the extrasynaptic NMDAR stimulation and induces calpain-mediated cleavage and consequent inactivation of STEP [65]. To overcome this, the CPIP Tat-STEP was developed and significant neuroprotection in *in vitro* ischemic models by reducing p38 activation was shown [66]. The p38 inhibitor SB239063 showed protection against excitotoxicity both in *in vitro* and *in vivo* ischemic models of brain ischemia [67], but its short therapeutic time window compared to the NA-1 peptide possibly limits its clinical use. In contrast, inhibiting JNK seems to be a promising therapeutic strategy. The CPIP Tat-JBD20, designed to block the interaction between JNK and its downstream effector c-Jun, protected against cell death both in *in vitro* and *in vivo* models of ischemic stroke [68]. More importantly, this peptide offers a larger therapeutic time window in stroke models [68,69].

All the afore-mentioned complexes have the requirement of Ca^{2+} influx across the NMDAR in common; however, non-ionotropic signaling has also been linked with excitotoxicity which include the NMDAR-Src kinase-Pannexin-1 complex (NMDAR-Src-Panx1). Src kinase-dependent phosphorylation and subsequent activation of Panx1 mediate excitotoxic secondary currents following the initial current through NMDAR that activates Src kinase [70]. These secondary currents are initiated by ligand binding, and are independent of Ca^{2+} influx, as the NMDAR channel blocker antagonist MK-801 failed to block this event, but antagonists of both glutamate and glycine binding sites block these secondary currents [71]. To block Panx1 activation, the CPIP Tat-$Panx_{308}$ that inhibits the phosphorylation of Panx1 by Src kinase and consequently the dissociation of the complex, revealed significant neuroprotection capabilities in *in vivo* stroke models [71].

The overactivation of NMDAR also leads to calpain-mediated cleavage of mGluR1. This cleavage is associated with changes from mGluR1-associated survival pathways to a pro-death signaling in ischemia. As expected, the CPIP Tat-mGluR1 was developed and neuronal cell death in *in vitro* and *in vivo* models of ischemia was prevented. [72].

Apart from the NMDAR, understanding the contribution of CP-AMPAR to excitotoxicity must also consider the pro-death signaling elicited by the receptor. In this context, JNK and AP-1 transcription factor activation have been related to the excitotoxic effects triggered by CP GluA4-containing receptors [73]. Future experiments may explore CP-AMPAR complexes in the context of excitotoxicity.

3. Ca^{2+} deregulation, oxidative stress and the mitochondria

In excitotoxicity, Ca^{2+} influx into the postsynaptic neuron is characterized by two peaks; one fast transient increase peak followed by a secondary peak called Delayed Ca^{2+} Deregulation, when the mitochondria buffering capacity is saturated [74]. Taking into consideration the abnormal influx of Ca^{2+} into the cell and its massive efflux from intracellular stores during excitotoxicity, one of the strategies to counteract this is to block Ca^{2+} channels, transporters and exchangers associated with these mechanisms. An initial approach focused on inhibiting voltage-gated Ca^{2+} channels (VGCC) localized at the plasma membrane which, alongside NMDAR and CP-AMPAR, is the primary source of Ca^{2+} entry in neuronal cells. Taurine inhibits VGCC leading to the attenuation of glutamate excitotoxicity, and its wider regulatory roles, including the modulation of Endoplasmic Reticulum (ER) stress, suggests its potential role in ameliorating excitotoxic-related neurologic disorders [75]. Blocking acid-sensing channels and transient receptor potential channels (TRPCs) is also a promising therapeutic strategy, due to the role of these channels in mediating late stage Ca^{2+} entry during excitotoxicity [76]. In the case of TRPCs, the data suggest that the interaction between extrasynaptic NMDAR and TRPM4 is important for excitotoxicity and disruption of this interaction not only provides robust neuroprotection but also renders extrasynaptic NMDAR nontoxic [77]. Recently, the interaction between TRPM2 and extrasynaptic NMDAR has also been associated with enhanced excitotoxicity in *in vitro* and *in vivo* ischemic conditions [78].

A multi target method to inhibit intracellular Ca^{2+} influx and release from intracellular stores was proposed and inspired by the mitochondrial Na^{+}/Ca^{2+} exchanger inhibitor CGP37157. This molecule could prevent cell death by negatively modulating both Ca^{2+} release from mitochondria as well as Ca^{2+} overload through VGCC [79], and could also modulate Store-operated Ca^{2+} entry [79]. This occurs through Store-operated Ca^{2+} channels present at the plasma membrane, which are sensitive to decreased levels of Ca^{2+} in the ER. When these levels are diminished, Stromal-interacting molecule (STIM) proteins located in the ER oligomerize and form complexes with Ca^{2+}-selective and non-selective channels in the ER membrane. These interactions further convey Ca^{2+} entry and activation of the ER Ca^{2+} ATPase (SERCA) pump, which induces Ca^{2+} influx into the ER [80]. STIM proteins were shown to inhibit VGCC [81] and negatively modulate NMDAR-mediated Ca^{2+} influx in cortical neurons [82]. Therefore, upregulation of STIMs may be useful to protect against NMDA-induced Ca^{2+} overload and important in maintaining ER-associated Ca^{2+} homeostasis.

Sustained NMDAR overactivation and Ca^{2+} influx leads to the overproduction of free radicals subsequently leading to oxidative stress. Preserving natural antioxidant signaling defences, such as the constitutive PKD1-IKK/NF-κB/SOD2 detoxification pathway, is a more recent strategy. During early excitotoxicity, protein kinase D1 (PKD1) is inactivated through phosphatase-dependent mechanisms. The generation of a dephosphorylation-resistant PKD1 mutant preserved and potentiated the IKK/NF-κB/SOD2 cascade and protected against excitotoxicity both *in vitro* and *in vivo* by increasing the threshold of ROS that neurons are able to cope with without inducing significant cell death [83].

As mentioned above, NO has an early role in NMDAR-mediated excitotoxicity through the GluN2B-PSD-nNOS pathway. Superoxide is also produced under these conditions, and excessive NMDAR stimulation contributes to oxidative stress in the cytosol and in neighbouring neurons and astrocytes through extracellular release of superoxide [84]. In this case, multiple lines of evidence point to NAPDH oxidase 2 (NOX2) as the first source of excitotoxic superoxide production [85,86]. The induction of NMDAR-induced superoxide production through NOX2 involves not only Ca^{2+} influx through the NMDAR, but also non-ionotropic NMDAR cascades. The non-ionotropic NMDAR signaling through NMDAR-containing GluN2B also accounts for superoxide production and depends on ligand binding independently of Ca^{2+} influx [84]. This event further enables the association of PI3K-containing p85 regulatory subunits with GluN2B and NOX2 activation. Although the association of p85 with Glu2NB does not involve GluN2B phosphorylation, other post-translational modifications cannot be disregarded as being the major culprits involved in this process, as well as the GluN2B conformational changes triggered by agonist binding.

The combination of NO and superoxide may form peroxynitrite, which in turn can generate other radicals including hydroxyl and carboxyl radicals. These react with proteins, lipids and nucleic acids and inhibit mitochondrial respiratory chain enzymes leading to neuronal death [87]. The free radical scavenger *Edaravone* was shown to attenuate excitotoxicity in both *in vitro* and *in vivo* brain ischemia and ALS models, by interacting with both hydroxyl and peroxyl radicals and forming oxidized compounds [88,89]. It was approved by the U.S. Food and Drug Administration in 2017 for the management of ALS [90].

Another free radical scavenger is the glutathione peroxidase mimetic *Ebselen* (2-phenyl-1,2-benzisoselenazol-3(2H)-one). Despite significant protection against ischemic damage shown in pre-clinical studies, the borderline efficacy in phase III clinical trials halted the use of *Ebselen* as a marketed drug [91]. On the other hand, treatment with *Ebselen* showed significant protection in Huntington disease (HD) model organisms, which indicates a strong clinical potential of *Ebselen* in HD therapy and possibly other neurodegenerative disorders. [92].

The effects of S-nitrosylation are also a matter of debate in excitotoxicity. NO can induce the S-nitrosylation of a huge plethora of substrates, including GAPDH and Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2). The S-nitrosylation of GAPDH in excitotoxicity promotes its interaction with the E3 ubiquitin ligase Siah1. This newly formed complex migrates to the nucleus where it promotes the p300/CBP-associated acetylation of nuclear proteins and apoptosis [93]. In the nucleus, GAPDH can also interact with p53 and leads to the activation of p53-mediated apoptotic cell death pathway [94]. The S-nitrosylation of SHP-2 is increased after NMDA exposure *in vitro* and in an *in vivo* model of ischemia. This mechanism inhibited SHP-2 phosphatase activity and blocked the associated ERK1/2 neuroprotective pathways [95].

Among other deleterious effects, Ca^{2+} overload and oxidative stress are also associated with mitochondrial dysfunction. Following NMDAR activation, Ca^{2+} accumulation in this organelle occurs through the mitochondrial Ca^{2+} uniporter (MCU). The *mcu* gene is transcriptionally repressed by synaptic activity *via* nuclear Ca^{2+} and Cam Kinase-mediated induction of Neuronal PAS domain-containing protein 4 (Npas4) [96]. This process points to Npas4 as a potential regulator of MCU activity. In fact, knockdown of Npas4 in mouse cortical neurons resulted in increased necrosis, inflammation and brain lesion size following OGD-induced ischemia [97]. Mitochondrial morphology and complexity, as well as biogenesis are also affected by excitotoxicity. Downregulation of the mitochondrial fusion protein Mfn2 is a late event in excitotoxicity and involves excitotoxicity-dependent degradation of MEF2 transcription factors [98]. Enhancement of mitochondrial biogenesis through the mitochondrial division inhibitor 1 (mdivi-1) induced neuroprotection against NMDA-induced excitotoxicity by modulating mitochondrial function and Ca^{2+} signaling [99]. Phenoxythiophene sulfonamide B355252, a compound that acts on attenuating mitochondria fission, also conferred neuroprotective effects following glutamate-induced excitotoxicity in HT22 hippocampal cells [100]. On the other hand, enhancement of mitophagy revealed significant neuroprotection following an excitotoxic insult *in vitro*. This important process that maintains the normal function of mitochondria involved the PINK1/Parkin signaling pathway [101].

Additionally, recent work emphasised the importance of low rate Ca^{2+} entry in the mitochondria rather than full inhibition of uptake by MCU inhibitors that result in the loss of some of its physiological roles. The compound TG-2112x inhibited low mitochondrial Ca^{2+} uptake, mitochondrial depolarization and cell death during excitotoxicity [74], but it remains to be elucidated whether TG-2112x partially inhibits MCU or acts by blocking MCU-independent Ca^{2+} uptake mechanisms.

4. Glutamate spillover

The homeostasis of glutamate in the brain is a highly regulated process, carried out *via* glutamate EAATs, mainly EAAT2 that exist in neurons, astrocytes and in the parenchymal side of brain capillary endothelial cells [102]. In astrocytes, glutamate enters into the Tricarboxylic acid (TCA) cycle or is converted to the non-excitatory amino acid glutamine, which is then released from the astrocyte back to the presynaptic neuron. In the neuron, glutamine is converted to glutamate (or GABA), that is in turn accumulated in synaptic vesicles through specialized transporters. Upon filling, loaded synaptic vesicles can be used for synaptic transmissions as necessary [5,103].

The anti-luminal endothelial cells of the blood-brain barrier also accumulate glutamate through the EAAT2 and mediate brain-to-blood glutamate efflux by X_{G^-} transporters that only exist in the luminal membrane [102]. During excitotoxicity, this balance is severely affected. Glutamate accumulates in endothelial cells, and a disturbance in the gradient between the blood and the brain is observed [102,104]. Therefore, several procedures have been studied to restore this gradient and therefore favour the normal diffusion of glutamate into the blood for proper clearing [105]. One of these systems for clearing glutamate that is being currently tested in human trials for stroke is the peritoneal dialysis of glutamate (EudraCt Number: 2012-000791-42), after being successfully applied in animal models [106]. Glutamate scavengers such as glutamate-oxaloacetate transaminase (GOT) are also able to restore the gradient by metabolizing glutamate present in the blood. The blood-resident enzyme GOT transforms glutamate and oxaloacetate into α-ketoglutarate and aspartate. The administration of recombinant GOT (rGOT) and/or oxaloacetate in animal models of stroke is neuroprotective by reducing both brain and plasma glutamate levels [104,107]. Among all glutamate scavengers tested so far in clinical settings, riboflavin stands out as the most promising. Riboflavin was identified as the main hit compound based on its ability to interact with GOT in an *in vitro* high throughput screening of 1120 compounds [108]. Its neuroprotective effect was confirmed in an ischemic animal model and in a clinical trial involving 50 stroke patients (EudraCT Number: 2014-003123-22) [108]. This was the first human trial showing the efficacy of glutamate scavengers in an excitotoxic-related pathology thus opening the window for testing rGOT in clinical trials, due to its increased stability when compared with riboflavin.

A new glutamate-scavenging therapy has emerged recently and couples the glutamate uptake transporter EEAT2 with mesenchymal stem cells (MSCs). MSCs are a very versatile cell type due to their ability to secrete molecules that mediate immunomodulatory and trophic activities, and are one of the most promising therapies for various excitotoxic-related pathologies [109,110]. MSCs themselves protect against excitotoxicity by reducing NMDAR subunits and Ca^{2+} responses, as well as GluA1 AMPAR surface expression in mouse cortical neurons. These cells also secrete TNF that may mediate the neuroprotective effect [111]. Expressing EAAT2 in MSCs showed greater glutamate scavenging activity when compared to the controls in *in vitro* and *in vivo* models of ischemia. In short, this new strategy may drive future cell therapies to tackle excitotoxicity, and improve neurorehabilitation of stroke patients [102].

Another way to prevent glutamate spillover is by specifically targeting the presynaptic NMDAR and metabotropic signaling cascades. Although NMDAR functions have been canonically associated with the postsynaptic neuron, it has been shown that presynaptic NMDAR can also have a role in models of excitotoxicity [112]. *Marcelli* et al. demonstrated that the interaction between c-Jun N-terminal kinase 2 and Syntaxin-1 is essential for NMDAR-dependent glutamate overflow [113]. The disruption of this interaction by CPIP JGRi1 showed a significant reduction in presynaptic NMDAR-dependent glutamate release *in vitro*, but additional *in vivo* experiments in models of excitotoxicity are needed. In the case of metabotropic receptors, it was suggested that the spillover of glutamate activates presynaptic group III metabotropic receptors in order to inhibit presynaptic glutamate release in hippocampal neurons *in vivo* [114].

Additionally, studying the mechanisms involving astrocytic glutamate uptake and release may uncover novel targets to be tackled. Astrocytic EAAT2 plays a major role in glutamate clearance [115], however other studies report an important contribution for neuronal EAAT2 in glutamate uptake and synaptic glutamate homeostasis in axon terminals of many brain regions [116–118]. Regardless, the levels of this transporter are often decreased in excitotoxic-related pathologies [119,120]. A plethora of factors that regulate expression of EAAT2 at the transcriptional level, including growth factors, hormones and histone deacetylase (HDAC) inhibitors, were shown to increase EAAT2 levels [5]. Unbiased screening of about 1000 FDA-approved drugs identified

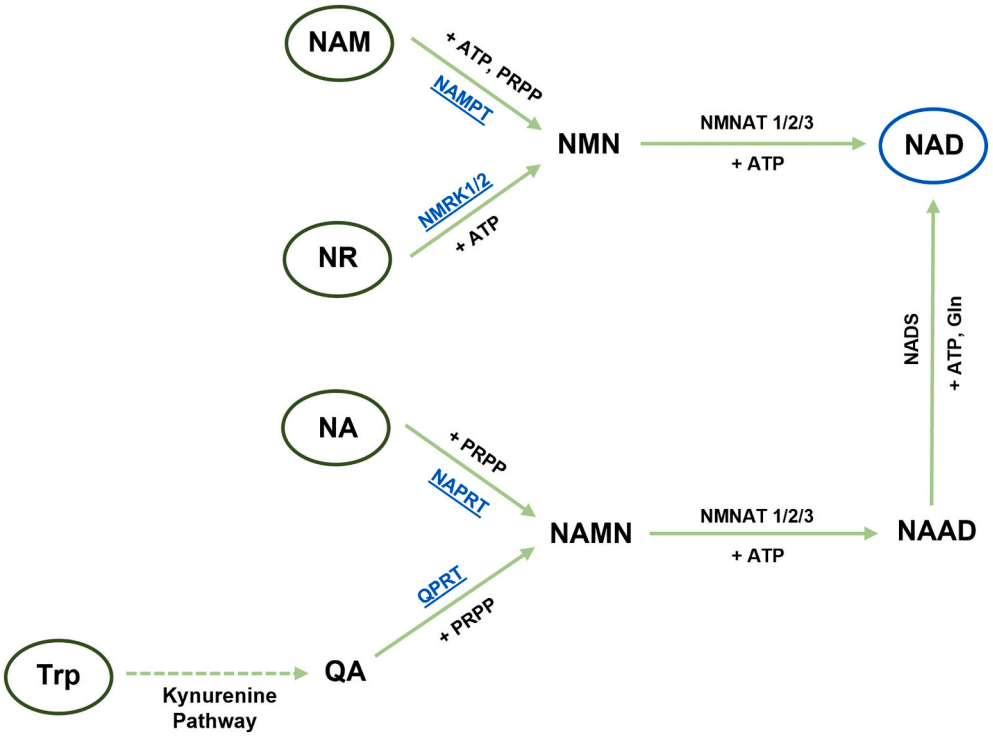

Fig. 1. Metabolic pathways of NAD biosynthesis in mammals. The main precursors of the pyridine moiety for NAD synthesis are circled and include nicotinic acid (NA), nicotinamide (NAM), nicotinamide riboside (NR) and tryptophan (Trp). The rate limiting enzymes involved in the salvage pathway are in blue and include NAMPT, NAPRT and NMRK1/2. The *de novo* pathway starts with the kynurenine pathway and after the step involving the quinolinic acid precursor (QA) is similar to the route of NA (Preiss-Handler pathway). ATP: adenosine triphosphate; Gln: glutamine; NAAD: nicotinic acid adenine dinucleotide; NAD: nicotinamide adenine dinucleotide; NADS: NAD synthetase; NAMN: nicotinic acid mononucleotide; NAMPT: nicotinamide phosphoribosyltransferase; NAPRT: nicotinate phosphoribosyltransferase; NMN: nicotinamide mononucleotide; NMNAT1/2/3: nicotinamide mononucleotide adenyltransferases 1/2/3; NMRK1/2: nicotinamide riboside kinase 1/2; QPRT: quinolinate phosphoribosyltransferase; PRPP: phosphoribosyl pyrophosphate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

β-lactam antibiotics as inducers of EAAT2 gene expression. Among those, ceftriaxone, was capable of upregulating EAAT2 levels through the NF-kB signaling pathway. Despite decreasing extracellular glutamate content, as well as reducing neuronal cell death in cellular and animal models of excitotoxic-related disorders [5], translational attempts to reproduce these results into the clinic setting have failed [121]. Recent data showed that the effect of ceftriaxone-induced increase in EAAT2 expression was not necessarily related with accelerated glutamate uptake in the cortex, further suggesting additional mechanisms for ceftriaxone in preventing excitotoxic damage [122].

Given the fact that the expression and function of EAAT2 is dysregulated at the translational level during excitotoxicity, restoring its levels is also a very promising therapeutic strategy. LDN/OSU-0212320 is a pyridazine derivative that exerts its function by activating PKC and Y-box-binding protein 1, which in turn activate EAAT2 translation [123]. This small molecule showed significant protection against excitotoxicity *in vitro* [123], as well as in *in vivo* Alzheimer's disease (AD) and ALS animal models [123,124]. However, other studies showed that despite increased EAAT2 levels, LDN/OSU-0212320 did not equally affect glutamate uptake in all brain regions [122].

In addition to the above-mentioned modes of action, astrocytic metabotropic receptors namely those belonging to the group II (mGluR2 and mGluR3) have been widely studied in the context of excitotoxicity. These receptors were shown to positively modulate EAAT2 (and also EAAT1) levels and consequently control glutamate uptake through ERK and PI3K signaling pathways [125]. It should be noted, however, that the same mGluR can be either neuroprotective or neurotoxic depending on which cell type is being expressed. For instance, mGluR5 has been associated with excitotoxicity in both neurons and astrocytes, despite having anti-inflammatory role in the microglia [126–128]. How to reconcile these antagonistic processes is an open and unsolved question.

The release of glutamate from astrocytes results from a process known as "gliotransmitter release". It has been shown that this process is responsible for regulating firing frequency and synaptic transmission [129]. The disruption of this process has been associated with defective neural information processing in the CNS as well as abnormal behaviours in animal models [129,130]. Apart from few contradicting results, this process is thought to be mostly mediated by Ca^{2+}-dependent exocytosis of vesicles, in a mechanism that highly resembles neurons [131]. In astrocytes, most of the Ca^{2+} necessary for glutamate release arises from ER stores through the action of metabotropic receptors present at the ER surface [132]. In response to intracellular Ca^{2+} elevation, vesicular fusion proteins VAMP2 and VAMP3 bind to astrocytic membrane fusion proteins syntaxin and SNAP23, which ultimately cause glutamate release from vesicles into the extracellular space [133]. Other ways of glutamate release from astrocytes include ion channels and pumps, already reviewed elsewhere [134]. Some of them have revealed major contributions to glutamate release in ischemia following metabotropic receptor activation, including the volume-regulated anion channels, Bestrophin 1 and potassium channel subfamily K member 2 [52,135]. Others, such as xCT need to be further explored in the context of excitotoxicity. xCT is an amino acid antiporter that exports glutamate in exchange with cystine. Cystine is involved in glutathione synthesis in astrocytes and xCT-mediated glutathione supply is one of the major sources of glutathione to neurons [136]. Under oxidative stress conditions, the expression of xCT increases, which is thought to be beneficial, but it also can lead to glutamate excitotoxicity through its mechanistic action.

The reverse efflux through EAATs may also contribute to glutamate efflux in several pathological conditions [137] and appears to involve the inflammatory molecules cyclooxygenase-2 and prostaglandin E2 [138]. In addition, the mitochondrial NOD-like receptor NLRX1 has been associated with enhanced glutamate uptake and inhibition of glutamate release by blocking Ca^{2+}-dependent exocytosis [133] and this mechanism has a tremendous potential as a regulator of glutamate homeostasis in the brain. Despite these recent advances in understanding the mechanisms of glutamate release from astrocytes, information about which have more impact in excitotoxicity, is lacking.

5. The role of NAD metabolism and NAD-dependent signaling in neuroprotection

Neurons are particularly sensitive to metabolic stress. Under physiological conditions, about 20 % of circulating glucose enters the brain.

There is a strong controversy about the main energy substrate for neurons. Accordingly, it is suggested that these cells alternate between glucose and lactate from astrocytic glycolysis as source of energy but may prefer lactate during increased energy demand. Following astrocytic glycolysis, lactate goes to the neuron through an astrocyte-neuron lactate shuttle, allowing the generation of pyruvate [139]. Such alternative source of energy emerges from the use of glucose to support the pentose phosphate pathway (PPP) in neurons. NADPH is produced in the PPP and is essential for antioxidant defence mechanisms involving glutathione regeneration. The activation of this pathway in neurons is possible *via* the degradation of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) by the anaphase-promoting complex/cyclosome-Cdh1 (APC/C-Cdh1) complex. PFKFB3 activity generates fructose-2,6-bisphosphate, a positive allosteric effector of 6-phosphofructo-1-kinase involved in glycolysis [43,140]. During excitotoxicity, this bioenergetic process is severely disturbed. The activity of the APC/C-Cdh1 complex is inhibited by Cdk5-mediated phosphorylation, which stabilizes PFKFB3 and induces a PPP to glycolysis pathway switch [141]. Inhibition of PFKFB3 was shown to confer neuroprotection in excitotoxic-related injuries [142]. Also, by sharing partial structural similarity with the PFKFB family, TP53-induced glycolysis and apoptosis regulator (TIGAR) protects against ischemic brain injury *via* increased PPP flux [143]. L-Lactate has also been associated with neuroprotection against excitotoxicity *via* several mechanisms. This requires a functional malate-aspartate shuttle [144], and involves the activation of P2Y receptors that lead to intracellular signaling cascades involving PI3K and K_{ATP} channels [145].

In order to meet all cell energetic needs, either by glycolysis or oxidative phosphorylation, the NAD^+/NADH (NAD) coenzyme is required. In fact, NAD depletion is an early event following excitotoxicity in many *in vitro* and *in vivo* excitotoxic-related models and a major contributor to deregulation in energy homeostasis. NAD biosynthesis in mammals is supported by four main pathways: the *de novo* pathway that uses tryptophan as a precursor, and salvage pathways that rely on nicotinamide (NAM), nicotinic acid (NA) and nicotinamide riboside (NR) as precursors (Fig. 1). NAM and NA are both converted to their respective mononucleotides by nicotinamide phosphoribosyltransferase (NAMPT) and nicotinic acid phosphoribosyltransferase (NAPRT), respectively. NMN and NAMN are then transformed into NAD or its NAAD precursor by NMN adenyltransferases (NMNAT1–3). NMNATs have distinct subcellular localization. NMNAT1 is nuclear, NMNAT2 localizes in the Golgi and cytoplasm and NMNAT3 varies its localization between the mitochondria and the cytoplasm depending on the cell type [146]. NAAD can be subsequently converted to NAD by NAD synthetase 1 (NADS). NMN is also produced through phosphorylation of NamR by nicotinamide riboside kinases 1 and 2 (NMRK1/2). Although different tissues have different main precursors for NAD generation, NAD salvage from NAM ensures almost all NAD production across the human body [147].

To counteract reduced NAD levels in excitotoxicity, NAD supplementation and NAD biosynthetic enzyme overexpression have shown to be neuroprotective. NAD confers neuroprotection through an improvement in mitochondrial biogenesis and function in a neuronal primary culture bath with glutamate [148]. In addition to NAD, NR was particularly associated with protection against excitotoxicity-induced axon degeneration and conferred better protection than NAD supplementation [149].

Overexpression of NAMPT in the glutamate-induced excitotoxic model has revealed a significant increase in neuronal survival, decreased translocation of the apoptosis inducing factor (AIF) from the mitochondria to the nucleus, as well as inhibition of the activation of effector caspase-3, thus culminating in apoptosis. Furthermore, mitochondrial fragmentation and loss of mitochondrial DNA are also suppressed by NAMPT overexpression [150]. Accumulating evidence suggests that Nampt activation can be a therapeutic strategy in excitotoxic-related diseases such as stroke [151]. Interestingly, in certain stress conditions NAMPT is translocated to the nucleus in GAPDH-NAMPT complexes [152]. This scenario could also be observed in excitotoxicity since GAPDH has a role in mediating cell death in this condition.

NMNAT-1 has shown to be protective against NMDAR-induced excitotoxicity in neonatal cerebral hypoxia-ischemia (H—I) by influencing the necrosis pathway [153]. In addition to NMNAT-1, NMNAT-3 has been associated with neuroprotection in H—I since knockdown of NMNAT-3 caused an increase in NMDAR-associated excitotoxic cell death in immature neurons. The molecular mechanisms associated with a NMNAT-3-related neuroprotective phenotype in the injured neonatal brain involve a decrease in calpastatin degradation, a known endogenous calpain inhibitor, that consequently diminishes its activation as well as caspase-3 [154].

NAD is also a substrate for NAD-consuming enzymes, namely Sirtuins (SIRTs), Poly-ADP-ribosyl transferases (PARPs) and the ADP-ribosyl cyclase CD38. Sirtuins are a family of enzymes (SIRT1–7) that remove acetyl groups from specific protein substrates, regulating gene expression responses following bioenergetic stresses [155]. SIRT1, the most intensively studied sirtuin, is particularly relevant in conferring neuroprotection in mouse cortical primary neurons, partly by inhibiting p53 acetylation following NMDA-induced excitotoxicity [156]. Another study showed that elevated Ca^{2+} levels in excitotoxicity activate PKC, which in turn phosphorylates HuR and decreases SIRT1 expression at the mRNA level [157]. SIRT1 downregulation in excitotoxic conditions can be surpassed by the inhibition of the Ca^{2+}-PKC-HuR-SIRT1 pathway. SIRT1 was also shown to reduce excitotoxicity in cortical neurons by deacetylating peroxisome proliferator-activated receptor-coactivator 1α (PGC1α), a key factor involved in mitochondrial biogenesis [158]. The overexpression of the mitochondrial SIRT-3 was also shown to protect cortical neurons against NMDA excitotoxicity [155,159]. This increased neuroprotection is possibly achieved by balanced mitochondrial protein acetylation homeostasis [155]. Furthermore, the mitochondrial SIRT4 was found upregulated in excitotoxic conditions and SIRT4 KO reduced EAAT2 expression and therefore glutamate uptake in hippocampal neurons [160].

PARPs have a well-known function in mediating DNA repair. PARP1 accounts for approximately 90 % of PARP activity in the cell and its massive activation under excitotoxic conditions results in a form of cell death known as Parthanatos [161]. Depending on the strength of the insult, PARP1 activation is an early event during excitotoxic cell death [161]. Following massive PARP1 activation, NAD and ATP levels drop significantly and PAR polymers are formed [162,163]. PAR polymers are generated in the nucleus and translocated to the mitochondria. Once in the mitochondria, PAR polymers bind to the AIF, which result in its release from the mitochondria. AIF binds to the macrophage migration inhibitory factor (MIF) and this complex migrates to the nucleus where MIF cleaves genomic DNA [163].

Although NAD depletion, defects in mitochondrial function and PAR accumulation are the most important biochemical features of parthanatos, the primary mechanism following PARP1 activation is a matter of debate. Since PARP1 activity seems not to be affected by oscillations in NAD levels [147], the identification of PAR-binding proteins such as the endogenous inhibitor of parthanatos Iduna [164], along with NAD or NAD precursor(s) supplementation, could be a strategy to tackle this caspase-independent mechanism of cell death.

CD38, is less explored in the context of excitotoxicity. However, its major role as a NADase in mammalian cells [165] and in the mobilization of intracellular Ca^{2+} stores suggest that CD38 may be a promising target to tackle excitotoxicity.

6. Stroke biomarkers

A biomarker is classically defined as a characteristic that is objectively measured and evaluated as an indicator of normal and pathogenic processes, or pharmacological response to a therapeutic intervention [166]. Hence, any clinical and/or imaging measurements, as well as

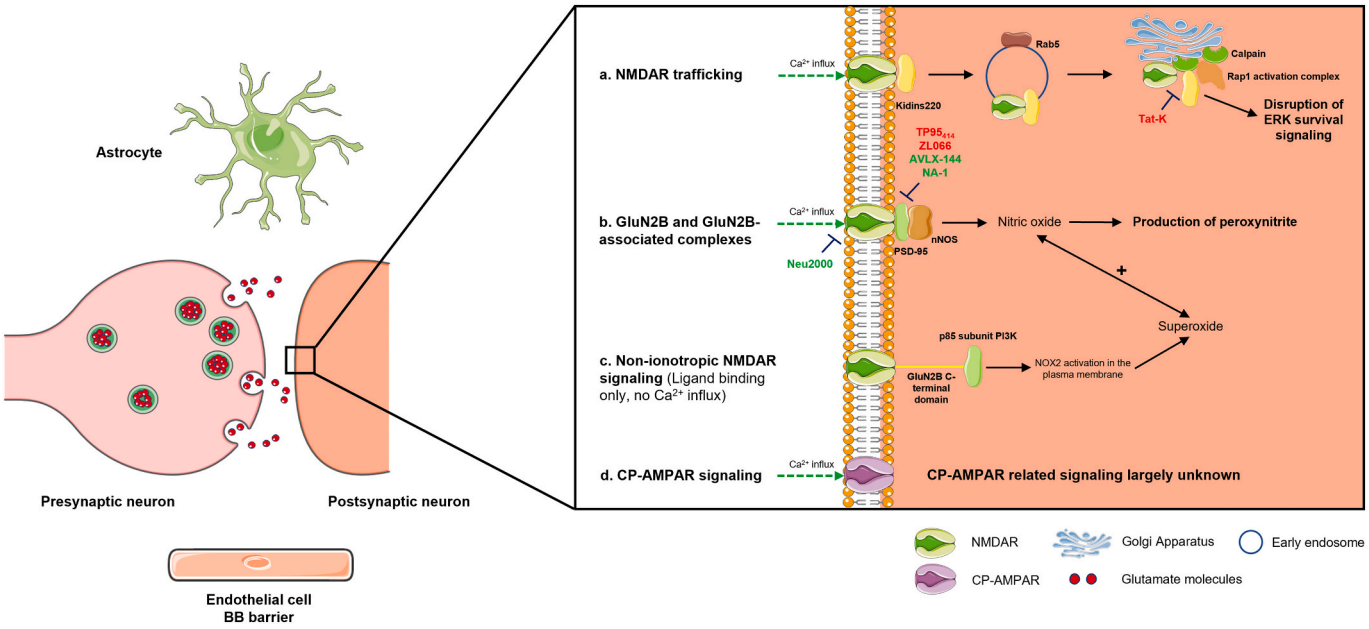

Fig. 2. Targeting postsynaptic GRs in excitotoxicity. Protection against postsynaptic GRs in excitotoxicity involves the excessive activation of a. NMDAR trafficking through kidins220. Kidins220 forms complexes at the neuronal surface with NMDAR and is normally trafficked through Rab5 -positive endosomes to the Golgi apparatus (GA) to be involved in neuronal survival through Rap1-mediated ERK signaling activation. When NMDAR is overactivated by excessive Ca^{2+} entry, kidins220 first and then other components of the Rap1 activation complex are degraded by GA-residing calpain which disrupts ERK survival signaling. The Tat-k peptide interferes with the calpain cleavage site of kidins220 thereby promoting Rap1/ERK cascades and neuronal survival; b. GluN2B and GluN2B-associated complexes. Neu2000 is in clinical trials for acute ischemic stroke and targets excitotoxic Glu2NB subunits of the NMDAR. In addition, targeting GluN2B-associated complexes including the GluNB-PSD95-nNOS complex is also promising. By dissociating NMDAR and nNOS but maintaining its functions intact, avoids excessive production of nitric oxide, AVLX-144 and NA-1are neuroprotective against excitotoxicity *in vitro* and *in vivo* and are in clinical trials in patients with stroke; c. Non-ionotropic NMDAR signaling. NMDAR also have functions that do not involve Ca^{2+} flow, ligand binding to the NMDAR promotes the association of the p85 regulatory subunits of PI3K and the C-terminal of GluN2B leading to the production of superoxide through NOX2 activation. This further enables the association of superoxide and nitric oxide which generates harmful peroxynitrite; d. CP-AMPAR signaling. Ca^{2+} entry through the CP-AMPAR, a type of AMPAR that arises from having unedited GluA2 subunits or its complete absence, also accounts for excitotoxicity however its related signaling events are largely unknown. Neuroprotective molecules in clinical trials are indicated in bold green while the ones that have revealed neuroprotection in pre-clinical models are indicated in bold red. CP-AMPAR: Ca^{2+}-permeable α-amino acid-3-hydroxy-5-methyl-4-isoxazole receptors; ERK: extracellular-signal-regulated kinase; Kidins220: Kinase D-interacting substrate of 220 kDa; NMDAR: *N*-methyl-D-aspartate receptor; nNOS: neuronal nitric oxide synthase; PSD-95: postsynaptic density protein 95; Rap1: Ras-associated protein 1. Cartoon made using Servier Medical Art (https://smart.servier.com/). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

circulating molecules in body fluids fits this definition, and may be useful to characterize any disease state.

Currently, imaging techniques are considered the gold standard in stroke diagnosis, which will be particularly relevant for therapeutic options. Non-contrast Computed Tomography scan is crucial in discriminating patients with acute hemorrhagic (AHS) and ischemic stroke (IS), the latter eligible for intravenous tissue plasminogen activator treatment [167]. This fast diagnosis is of utmost importance due to its relatively short time-window (3 to 4.5 h) for therapeutic intervention after the onset of symptoms [168]. Yet, discussion of imaging techniques goes beyond the scope of this review, and current progress, limitations and pitfalls are discussed thoroughly elsewhere [169–171]. Herein, we will discuss the major findings of proteins present in several body fluids that show great potential as stroke type diagnosis indicators.

Omics approaches have revolutionized the biomarker field, revealing tremendous potential to diagnose, and help predict the outcome of almost every single disease. Although the analysis of specific molecular signatures is widely used in the context of several brain disorders [172–174], its application in stroke is still in its infancy. Previous work has suggested a blood test using the biomarker panel matrix metalloproteinase 9 (MMP-9), brain natriuretic factor, d-dimer, and S-100β as diagnostic to distinguish AHS from all different strokes and its mimics [175]. The results showed a modest sensitivity of 86 % in terms of detecting all strokes, and 94 % for AHS, when patients were admitted to the hospital up to 6 h after the onset of symptoms [175]. A subsequent study, evaluating the blood levels of C-reactive protein, D-dimer, soluble receptor for advanced glycation end products, MMP-9, S100B, brain natriuretic peptide, caspase-3, neurotrophin-3, chimerin and secretagogin, from patients admitted to the hospital up to 3 h, revealed a sensitivity of 87 % [176]. However, the reported levels fall below what is required for the clinical practice. Two main reasons might explain why stroke biomarkers consistently fail when translation to the clinic was attempted; i) the multifactorial nature of stroke injury, ii) the origin of the molecules that are being tested in these assays. Therefore, more efforts should be devoted to tackle these two issues.

Another potential candidate stroke biomarker emerged almost two decades ago. Glial Fibrillary Acidic Protein (GFAP) protein was found increased in the serum of patients with AHS in comparison to IS, when evaluated 24 h after the onset of symptoms [177]. Indeed, even when evaluated at 6 h after admission, GFAP protein levels were detected in only 5 % of the admitted IS patients (median values of 0 ng/l), but greatly increased in 84 % of the AHS patients (median values of 11 ng/l) [178]. Using a large cohort of patients, the same increase in GFAP levels in the blood of AHS patients was also reported [179]. In patients with acute IS, higher levels of serum GFAP are associated with a poor outcome when evaluated 1 year after [180]. Prehospital monitoring of GFAP release rate using an ultra-sensitive single-molecule array was able to rule out AHS with high certainty in 68 % of patients with IS (sensitivity for AHS 96.6 %) [181]. A major caveat of this prehospital assessment becomes evident if the patient presents smaller hemorrhages. Under these circumstances, GFAP protein levels can no longer be used as a surrogate biomarker to discriminate ASH from IS affected

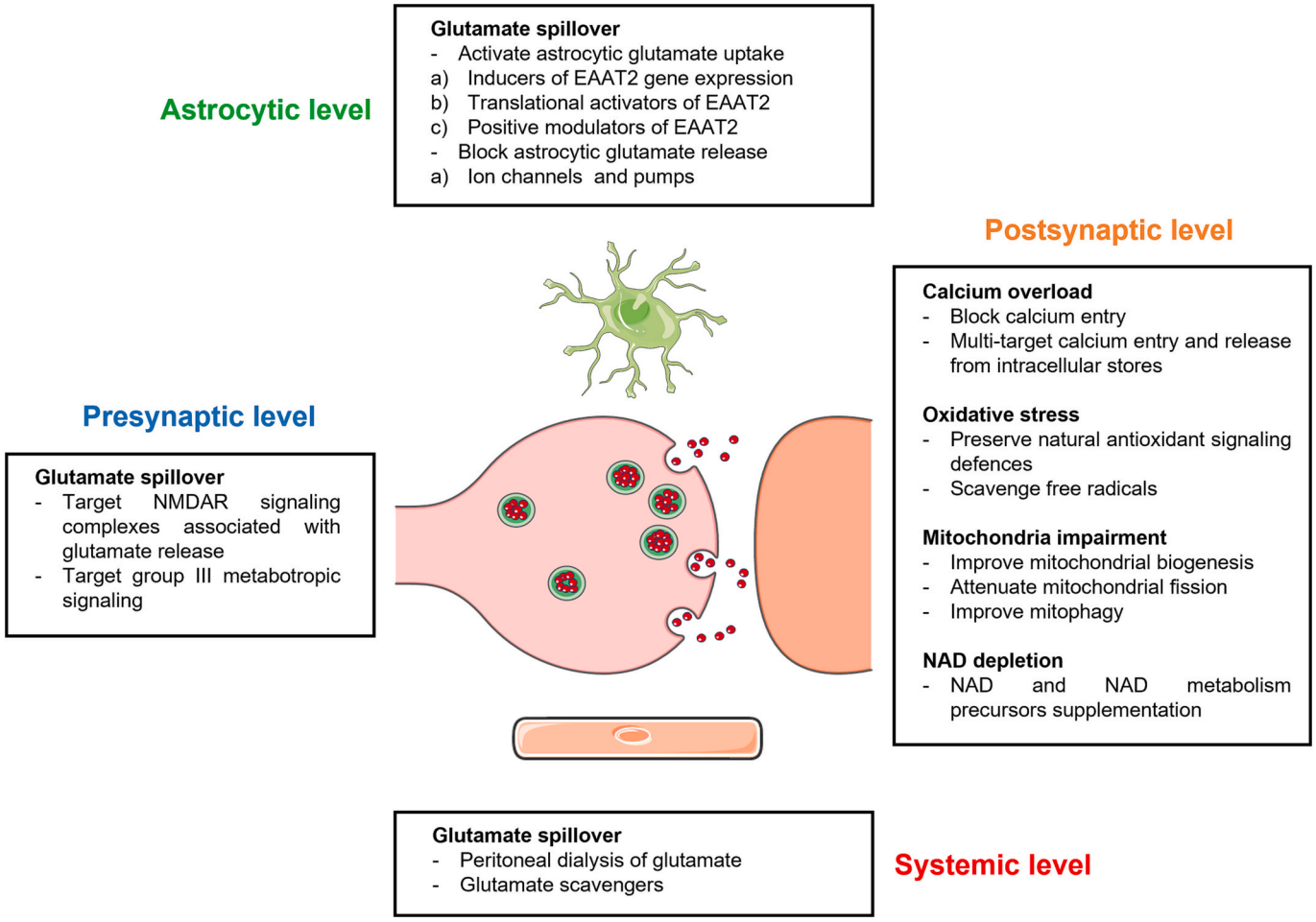

Fig. 3. Targeting excitotoxic mechanisms that do not directly involve the postsynaptic GRs. Representation of the main levels of action and these include presynaptic, postsynaptic, astrocytic and systemic levels. At the postsynaptic level, targeting Ca^{2+} overload, oxidative stress, mitochondria impairment and NAD depletion are the main topics of research in excitotoxicity. On the other hand, at presynaptic, astrocytic and systemic levels, targeting of glutamate spillover is the main area of investigation. Each area contains the major mechanisms and processes described in the review. EAAT2: excitatory amino acid transporter 2. Cartoon made using Servier Medical Art (https://smart.servier.com/).

patients since their levels were below to the minimum threshold defined by the authors (>0.29 ng/mL) [182]. Therefore, GFAP levels in the blood can function both as diagnostic and prognostic factor of cerebral ischemia, but there is a lack of standardization of sample collection and processing, and specific concentration cut-off criteria. More work should be performed along these lines.

The analysis of human extracellular fluids through microdialysis of the infarct core and the penumbra revealed increased levels of 53 proteins when compared with the contralateral unaffected region [183]. They include, Glutathione S-transferase P, peroxiredoxin-1, and S100B (further confirmed through an ELISA assay), where 8 to 20 fold increases were found. [183]. Strikingly, all three proteins show some degree of association with stroke type diagnosis, as well as with functional outcome when evaluated in the blood [184–186].

Cerebrospinal fluid (CSF) is a highly valuable biofluid in the search for novel molecular biomarkers of brain disorders, since it represents the repertoire of neuro-secreted, biosynthesized and metabolized molecular products of the CNS [172]. Yet, its collection is considered invasive, and contraindicated when anticoagulant medication is used due to its severe side effects [187]. In addition to this, due to brain edema, the volume of CSF changes in the rodent [188], and human brain [189]. Hence, any change in concentration of molecules found in this body fluid may not be direct result of their release from the brain, but because of water influx to the brain. Two seminal works using post-mortem CSF samples as a model of massive brain injury, shed light on the identification of novel biomarkers relevant for neurodegeneration. Among the identified peptides, both works showed alterations in proteins involved in the fatty acid metabolism, heart-fatty acid binding protein [190], and fatty acid-binding proteins 4,5, among 11 other proteins [191]. Strikingly, the later also showed alterations in components of the Ubiquitin Pathway (UCH-L1 and UFD1), a system whose function is severely impaired after cerebral ischemia (for reviews see [192–194]). Moreover, proteomic analysis of the CSF of rodents subjected to middle cerebral artery occlusion allowed the identification of proteins that are augmented in this mice model of focal cerebral ischemia [195]. Further analysis of plasma levels in human samples revealed the potential of creatine kinase B-type and UMP-CMP kinase to diagnose stroke, and calcium-calmodulin-dependent kinase 2 subunit β and CMPK for functional stroke outcome [196].

7. Concluding remarks

Research in excitotoxicity covers many different areas. The most explored is excitotoxic postsynaptic GR complexes and downstream effectors through peptides and inhibitors without compromising the survival signaling associated with the receptors (Fig. 2). Targeting NMDAR pro-death complexes has already provided longer therapeutic time windows not only in animal stroke models but also in clinical trials [54,58]. Future investigation regarding the GRs may also explore synapse-type specific CP-AMPAR as well as non-ionotropic NMDAR

signaling. A striking example where non-ionotropic NMDAR signaling is crucial to understand excitotoxicity is related to the extracellular release of superoxide mediated by NOX2 residing in the plasma membrane. This process involves, in part, a non-classical view of the NMDAR, expanding our knowledge of the complexity of the mechanisms involved in this process.

Tackling excitotoxicity also includes strategies that do not directly involve postsynaptic GRs (Fig. 3). One emerges by blocking different routes for Ca^{2+} overload in the cell, namely, the mitochondrial uptake and ER release. A multi-target approach would possibly avoid pro-death events such as Ca^{2+}-derived oxidative stress and mitochondrial impairment. Glutamate scavenging is another promising approach to fight excitotoxicity *via* the ability to metabolize glutamate in the blood. Given the fact that they do not interfere with normal brain neurophysiology, glutamate scavengers have revealed neuroprotective effects in animal stroke models and more recently in clinical trials for stroke, which is the case for Riboflavin.

Excitotoxicity is also coupled with failure in energy metabolism. The supplementation of NAD metabolism precursors showed improvements in several processes related to synaptic plasticity and neuronal morphology in neurodegenerative diseases [197,198], and multiple clinical trials with NAD metabolism precursors are under way for many neurological disorders [197]. Since NAD decline is pivotal in excitotoxicity, NAD supplementation or NAD metabolism enzyme activators may be the target for future clinical trials in excitotoxic-related pathologies.

The understanding of the diverse processes involved in excitotoxicity and its interplay may give rise to combined therapies to tackle excitotoxicity, and similar disorders. At least, the number of combination drugs has increased since the middle of the past century [199], which indicates that the future of treatments against excitotoxic-related diseases may not be monotherapy.

Finally, stroke biomarkers discovery is a growing field bringing diagnosis and prognosis closer and allowing better choice of treatment options. However, it is hampered by the multifactorial aspects of the injury, brain area affected, the enormous cell types that are affected and by assuming that the molecules have a brain origin. Moreover, there is a lack of standardization of procedures when identification and validation take place. Multicentre validation of the identified molecules should also be performed by scientists and physicians.

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Declaration of competing interest

The authors declare no competing interests.

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