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Review article

Beyond the critical point: An overview of excitotoxicity, calcium overload and the downstream consequences

Daniele Bano[a,*], Maria Ankarcrona[b]

[a] German Center for Neurodegenerative Diseases (DZNE), Bonn, Germany
[b] Center for Alzheimer Research, Division of Neurogeriatrics, Department of Neurobiology, Care Sciences and Society, Karolinska Institutet, Stockholm, Sweden

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ABSTRACT

Over the past several decades, an overwhelming body of research has greatly expanded our understanding of the mechanisms underlying excitotoxicity in brain ischemia as well as in many chronic neurodegenerative diseases. The identification of an array of molecular targets has opened avenues for neuroprotective strategies and, consequently, has sparked considerable interest for their attractive therapeutic means as pharmacological options. The purpose of this work is to provide a general overview of neuronal excitotoxicity and the inevitable downstream consequences of Ca^{2+} overload. We also discuss the contribution of Ca^{2+} transporters in excitotoxicity. This article is part of a Special Issue entitled "Calcium Pumps and Exchangers in Neuronal Injury and Neurodegeneration".

1. Introduction

Neurological and psychiatric disorders have a significant impact on public health systems both in industrialized as well as in low-income countries. Based on the current projections and statistics, as well as taking the measures of disability-adjusted life year (DALY) into account, brain disorders are responsible for almost 30% of the global burden of disease, with stroke representing a common cause of long-term disability and one of the degenerative conditions with a high mortality rate in individuals over 60 [1]. Haemorrhagic strokes result from a ruptured vessel and the subsequent blood release into the surrounding brain tissue, while ischemic strokes occur due to a vascular occlusion and thus a lack of blood supply in the affected area of the brain. At the core of the ischemic stroke, the constrained blood flow causes a shortage of oxygen and glucose, profoundly affecting energy metabolism and, consequently, the proper maintenance of neuronal activity. With time, the irreversible functional damage spreads to more distal brain regions, generally referred to as ischemic penumbra, where collateral microcirculation allows a sufficient gradient of oxygen and metabolites that support the survival of those regions surrounding the infarcted tissue. One of the critical aspects for successful clinical outcomes is to reduce the extent of cerebral infarction in these vulnerable areas adjacent to the hypoperfused core. With a therapeutic time window of a few hours from the ischemic attack, neurovascular specialists focus to relieve the vessel occlusion and to restore normal blood flow using complementary methods, such as mechanical endovascular thrombectomy or pharmacological clot lysis. In general, later interventions may result in severe brain lesions that spread from the infarcted core to the ischemic penumbra and beyond, frequently leading to long-term hospitalization, permanent disability and eventually death [2,3]. Given the complexity and the narrow timing for effective treatments, there is a growing interest in novel strategies that can delay neuronal loss in the penumbra and effectively protect the still viable tissue. In this regard, several lines of evidence have identified key molecular processes that contribute to neuronal demise and play critical roles during ischemia in experimental models of stroke, with potential implication for the development of neuroprotective drugs and novel clinical approaches [2,3]. Compared to other organs, the remarkable vulnerability of the adult brain to transient ischemia seems mainly due the amino acid glutamate. As the primary excitatory neurotransmitter in the central nervous system, glutamate controls physiological neuronal activity and a wide range of associated downstream signaling cascades. However, during ischemia glutamate accumulates to neurotoxic concentrations in the extracellular space as a result of multiple factors, including energy failure of neurons and glial cells. In support of this model, pioneering studies in mammals demonstrated the damaging effect of excessive glutamate on neuronal survival *in vivo* in sensitive tissues, like the retina and the brain [4–7]. These proof-of-principle findings, along with later independent works, established the importance of glutamate-mediated neurotoxicity in ischemia and shaped the concept of excitotoxicity (reviewed in [3,8–11]). Since the original hypothesis of glutamate-dependent cell death, an impressive body of

* Corresponding author at: Deutsches Zentrum für Neurodegenerative Erkrankungen (DZNE), Sigmund-Freud-Str. 27, 53127, Bonn, Germany.
E-mail address: daniele.bano@dzne.de (D. Bano).

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knowledge gained over several decades has shed light on the complex cascades that ultimately cause neurodegeneration. Under normal physiological conditions, glutamate controls activity-dependent neuronal depolarization and contributes to differentiation and survival of neurons. In a very simplified model, presynaptic release of glutamate promotes the opening of AMPA/kainate receptors and the consequent Na^+ influx, which depolarizes the postsynaptic membrane and allows the activity of the ionotropic glutamate NMDA receptor (NMDAR) upon the release of the Mg^{2+} block at the mouth of the channel. Along with the voltage-dependent channels, Ca^{2+}-permeable NMDARs cause the influx of large amount of Ca^{2+} following its natural electrochemical gradient. Its sequestration within intracellular organelles, its binding to buffering proteins and its extrusion outside the cell efficiently prevent the rise of an intracellular Ca^{2+} concentration above a critical toxic threshold. In ischemic tissue, on the other hand, the build-up of extracellular glutamate triggers the activation of synaptic and extrasynaptic ionotropic glutamate receptors, with consequent dissipation of the membrane potential and prolonged depolarization of neurons [12–14]. It is also worth noting that the anoxic condition of the injured tissue triggers the opening of other plasma membrane channels that further contribute to the Ca^{2+} overload. Among them, the increased oxidative and nitrosative stress in the cell enhances the Ca^{2+} permeability of the transient receptor potential cation channel TRPM7, while the decreased extracellular pH activates the Ca^{2+}-permeable acid-sensing ion channel ASIC1a [15–18]. The constant influx of large amount of Ca^{2+} ions, along with the energetic crisis due to the lack of oxygen and metabolites, cause the failure of the homeostatic machinery that tightly regulate intracellular Ca^{2+} levels. Ultimately, the resulting Ca^{2+} overload leads to the excitotoxic cascade that contributes considerably to neuronal loss during brain ischemia [10,19,20]. In the next section, we will discuss the importance of Ca^{2+} overload in glutamate-mediated death processes.

2. Excitotoxic Ca^{2+} overload

Ca^{2+} signaling is a simple and versatile mechanism that, in spatially and temporally defined manners, regulates virtually every single biological process. Throughout evolution, eukaryotes developed an array of molecular machineries that tightly regulate the intracellular Ca^{2+} level and promptly restore the physiological concentration upon transient Ca^{2+} rises above the resting values (reviewed in [21–24]). In light of these considerations, it is not surprising that massive Ca^{2+} entry irreversibly alters a vast range of cellular functions and inevitably compromises neuronal survival. From the original idea of excitatory amino acids as key mediators of neuronal death in brain ischemia [5–7], later remarkable studies determined the cascade of events that mechanistically underlie excitotoxicity. In cultured neurons, prolonged glutamate exposure causes a rapid swelling that is primarily dependent on Na^+ and water influx from the extracellular medium [25]. However, it is the subsequent entry of Ca^{2+}, rather than Na^+, through the permeable plasma membrane channels that determines the cytotoxic effects of glutamate. This critical distinction was further supported by a series of *in vitro* studies, where removal of extracellular Ca^{2+} from the saline solution was sufficient to delay the death of cultured primary neurons [26–29]. Equally important, single-cell analyses from independent groups described the time course of glutamate-induced Ca^{2+}-dependent neuronal death. In primary dissociated neurons, glutamate application evokes an initial Ca^{2+} rise that recovers its basal levels within a few minutes [30,31]. However, while brief glutamate exposure does not lead to cell death, longer challenges lead to a secondary phase of sustained Ca^{2+} rise that reaches a critical threshold from which cells rarely recover [32–35]. Ultimately, this prolonged elevation of intracellular Ca^{2+} concentration induces an aberrant signaling cascade, which inevitably compromises cell survival in Ca^{2+}-dependent fashion (Fig. 1). As shown in primary dissociated neurons exposed to glutamate, part of the challenged cells acutely die from

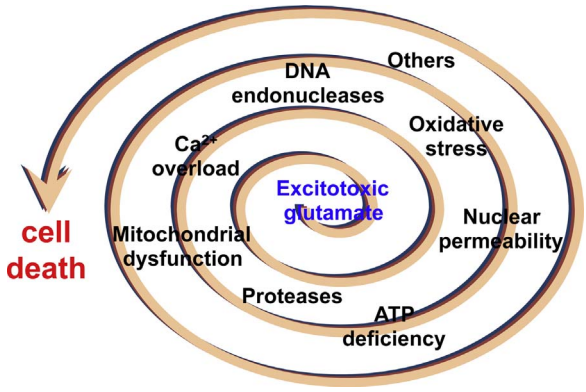

Fig. 1. Prolonged glutamate stimulation causes a sustained intracellular Ca^{2+} rise that affects a large number of cellular processes. The consequent impairment of mitochondrial function and the activation of Ca^{2+}-dependent proteases ultimately lead to cell death via apoptosis or necrosis.

necrosis while others undergo apoptosis [36]. Based on this and other studies, it seems that the same excitatory insult can lead to either necrotic or apoptotic cell death depending on mitochondrial function. In this regard, glutamate exposure causes a transient loss of mitochondrial membrane potential and neuronal energy capacity [36–38]. Those neurons that survive the first wave of Ca^{2+} rise usually recover mitochondrial function and later undergo apoptotic cell death [36,39–43]. Based on this line of evidence, it seems that one of the downstream consequences of neuronal Ca^{2+} overload is the impairment of mitochondrial function, which ultimately compromises the recovery and survival of injured cells (Fig. 1).

3. Mitochondrial Ca^{2+} uptake and the role of mitochondria-associated membranes (MAM)

Apart from the well-established role in the apoptotic cascade and its regulation [19,20], mitochondria can also contribute to neuronal demise through the regulation of cytosolic and mitochondrial Ca^{2+} levels [44]. A set of Ca^{2+} transporters and ion exchangers regulate mitochondrial Ca^{2+} uptake and release [45]. In the outer mitochondrial membrane (OMM), Ca^{2+} is transported *via* the voltage dependent anion-selective channel protein 1 (VDAC1) [46,47]. VDAC1 is also a transporter of Cl^-, K^+, Na^+, glutamate, ADP, ATP, NADH, acetylcholine and dopamine. The transport of ions, metabolites and nucleotides through VDAC1 is bi-directional [48]. Subsequently, Ca^{2+} enters the mitochondrial matrix *via* the mitochondrial calcium uniporter (MCU) located in the inner mitochondrial membrane (IMM) [49,50]. The structure of the pore domain of MCU was recently published, and it was reported that the second transmembrane helix of the MCU homo-oligomer forms a hydrophobic pore across the membrane [51]. Ca^{2+} is removed from the mitochondrial matrix by the mitochondrial $Na^+/Li^+/Ca^{2+}$ exchanger (NCLX) [52,53]. NCLX is located in IMM and exchanges 3 Na^+ (or 3 Li^+) for 1 Ca^{2+}. Under conditions of high Ca^{2+}-levels in the matrix, opening of the permeability transition pore (PTP) is triggered and results in the depolarisation of the mitochondrial membrane and, potentially, cell death [45,54]. Ca^{2+} is also directly transferred from the endoplasmic reticulum (ER) into mitochondria at specialized contact sites between them, generally referred to as mitochondria-associated membranes (MAM). At MAM, inositol trisphosphate receptors (IP3Rs), glucose regulated protein 75 (Grp75) and VDAC1 form functional scaffolding complexes [55]. In a highly regulated manner, Ca^{2+} is released from the ER *via* IP3Rs and taken up by mitochondria *via* VDAC1 in the OMM and MCU in the IMM. The importance and contribution of ER-mitochondria interplay in neurodegeneration (Alzheimer's disease (AD), Parkinson's disease and FTD/ALS) has been recently addressed [56]. In experimental models of these diseases, such as patient-derived cells and postmortem samples, it has