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The role of Ca^{2+} in cell death caused by oxidative glutamate toxicity and ferroptosis

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Abstract

Ca^{2+} ions play a fundamental role in cell death mediated by oxidative glutamate toxicity or oxytosis, a form of programmed cell death similar and possibly identical to other forms of cell death like ferroptosis. Ca^{2+} influx from the extracellular space occurs late in a cascade characterized by depletion of the intracellular antioxidant glutathione, increases in cytosolic reactive oxygen species and mitochondrial dysfunction. Here, we aim to compare oxidative glutamate toxicity with ferroptosis, address the signaling pathways that culminate in Ca^{2+} influx and cell death and discuss the proteins that mediate this. Recent evidence hints towards a role of the machinery responsible for store-operated Ca^{2+} entry (SOCE), which refills the endoplasmic reticulum (ER) after receptor-mediated ER Ca^{2+} release or other forms of store depletion. Pharmacological inhibition of SOCE or transcriptional downregulation of proteins involved in SOCE like the ER Ca^{2+} sensor STIM1, the plasma membrane Ca^{2+} channels Orai1 and TRPC1 and the linking protein Homer protects against oxidative glutamate toxicity and direct oxidative stress caused by hydrogen peroxide or 1-methyl-4-phenylpyridinium (MPP+) injury, a cellular model of Parkinson's disease. This suggests that SOCE inhibition might have some potential therapeutic effects in human disease associated with oxidative stress like neurodegenerative disorders.

1 Oxidative glutamate toxicity

In 1989, Murphy and colleagues reported that in the N18-RE-105 neuroblastoma X retina cell line, glutamate induced Ca^{2+} -dependent cell death by inhibition of cystine import via the cystine/glutamate antiporter system x_c^- [1], resulting in depletion of the important

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antioxidant glutathione (GSH) and oxidative stress [2]. This type of cytotoxicity has been named oxidative glutamate toxicity [3] and has been extensively studied in the hippocampal cell line HT22, a glutamate-sensitive subclone of the hippocampal cell line HT4 [4]. Oxidative glutamate toxicity is distinct from excitotoxicity where increased extracellular glutamate over-stimulates ionotropic glutamate receptors thereby leading to a massive Ca^{2+} influx and rather rapid nerve cell death [5]. The series of events leading to cell death by oxidative glutamate toxicity have been quite well characterized although some questions and controversies remain. Following the inhibition of system x_c^- by glutamate, GSH levels drop in a time-dependent manner. When the GSH levels fall below ~20% (6–8 hours after glutamate treatment), reactive oxygen species (ROS) levels as measured using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCF) [6], MitoSOX red [7] or Bodipy (unpublished) start to increase exponentially [6] which then gives rise to the activation of signaling pathways that culminate in the influx of Ca^{2+} and cell death. This Ca^{2+} influx is essential for the completion of the cell death program as proven by the fact that glutamate-treated HT22 cells do not die when Ca^{2+} influx is blocked by CoCl_2 or in Ca^{2+} -free medium [2,4,8]. In this review, we will compare oxidative glutamate toxicity to similar and possibly identical pathways of programmed cell death such as ferroptosis, address the signaling pathways that culminate in Ca^{2+} influx and cell death and discuss the possible Ca^{2+} channels that mediate this.

1.1 Signaling pathways involved in oxidative glutamate toxicity

As indicated above, inhibition of system x_c^- by glutamate inhibits cystine import resulting in depletion of intracellular cysteine, the rate-limiting building block of the tripeptide GSH which is composed of the amino acids γ -glutamate, cysteine, and glycine. When GSH levels drop, ROS levels start to increase exponentially. Interestingly, it is not the accumulation of large amounts of intracellular ROS that causes death, but this is just a necessary step in the cell death process. Consistent with this idea, compounds (e.g. LY83583, flavonol, 6-hydroxyflavonol, 7-hydroxyflavonol) that block signaling pathways downstream of ROS accumulation can be protective even in the presence of elevated levels of ROS (e.g. [9]). The major source of these ROS appears to be complex I of the mitochondrial electron transport chain [6]. The importance of mitochondrial ROS production is supported by the observation that the mitochondrial uncoupler cyanide p-trifluoromethoxyphenylhydrazine (FCCP) and other mitochondrial inhibitors (e.g. DPI) protect nerve cells from oxidative glutamate toxicity [6,7]. In addition, ruthenium red, an inhibitor of mitochondrial Ca^{2+} uptake, also blocks high rates of ROS formation suggesting that mitochondrial Ca^{2+} contributes to ROS production [6]. Other sources of ROS include the NADPH oxidase Nox4 [10] and lysosomes [11].

GSH depletion also results in the inhibition of glutathione peroxidase 4 (GPx4), which depends on an adequate supply of GSH for activity [12]. Both GPx4 inhibition and GSH depletion lead to the activation of 12/15-lipoxygenase (12/15-LOX, the protein product of the ALOX15 gene) [12,13]. GPx4 is unique in its ability to reduce membrane-embedded lipid hydroperoxides, the products of 12/15-LOX [14]. Activation of 12/15-LOX generates multiple metabolites including 12- and 15-HETE (hydroxyeicosatetraenoic acid) which are involved in the increase in Ca^{2+} influx that is required for the final phase of oxidative

glutamate toxicity [1,6] (to be discussed in full detail below). The increase in cytosolic Ca^{2+} also increases 12/15-LOX membrane binding via low-affinity binding sites and enzyme activity via high affinity binding sites resulting in a vicious circle [15]. Activated 12/15-LOX integrates into the membranes of various organelles including the endoplasmic reticulum (ER) and mitochondria [16] and also has direct effects on mitochondria thereby further increasing ROS production [17]. Approximately 10–12 hours after the induction of oxidative glutamate toxicity, when both ROS and intracellular Ca^{2+} levels have reached their maximum, the pro-apoptotic Bcl-2 family member Bid (BH3-interacting domain death agonist) translocates to the mitochondria and Bid-loaded mitochondria accumulate around the nucleus and lose their membrane integrity [18]. At the same time, apoptosis-inducing factor (AIF) translocates from the mitochondria to the nucleus, where it rapidly induces caspase-independent cell death [18]. The cascade of events and the time frame of cell death caused by oxidative glutamate toxicity are depicted in Figure 1.

Whereas the Maher and Schubert labs have observed a transient hyperpolarization of the mitochondria during the exponential increases in ROS and intracellular Ca^{2+} [3,7], others reported depolarization upon the release of AIF from the mitochondria in the final phase of oxidative glutamate toxicity [18]. A recent study [19] provides further support for the idea that hyperpolarization actually precedes the final depolarization which occurs near to the time of cell death. Although caspase-3 is activated during oxidative glutamate toxicity in HT22 cells [18], caspase inhibitors do not inhibit cell death [18,20]. However, several protease inhibitors do protect against oxidative glutamate toxicity indicating a role for serine proteases and calpains in the cell death pathway [20,21]. Since macromolecular synthesis inhibitors (actinomycin D, cycloheximide) inhibit oxidative glutamate toxicity in HT22 cells [21], synthesis of RNA and protein seem to be required for the execution of the cell death pathway. A recent study provided further insight into that requirement by demonstrating a critical role of p53-mediated GADD45 α synthesis in glutamate-mediated cell death in HT22 cells [22] but the precise relationships between GSH depletion, ROS production and p53 activation still remain to be determined. However, others have argued that inhibition of protein synthesis simply reduces the consumption of cysteine which is essential for GSH synthesis [23]. This argument is contradicted by the observation that macromolecular synthesis inhibitors also protect HT22 cells from cell death induced by treatment with buthionine sulfoximine, an inhibitor of GSH synthesis [21].

2 Differences between oxidative glutamate toxicity and other forms of programmed cell death

While oxidative glutamate toxicity has some of the features of apoptosis such as the requirement for protein synthesis, it is missing many others such as nuclear and DNA fragmentation and chromatin condensation [21]. HT22 cells exposed to glutamate for 10 hours show severe damage to their ER, Golgi apparatus and mitochondria [21]. Another, recently described, form of cell death called ferroptosis has some of these same characteristics. Ferroptosis was originally described as a non-apoptotic, iron-dependent form of cell death whose defining initiator was the anti-tumor compound erastin [24]. Identical to glutamate, erastin was found to inhibit cystine import via the cystine/glutamate antiporter

system x_c^- [24]. Further studies showed that ferroptosis could also be induced by inhibitors of GPx4 [25] which is consistent with GPx4 activation being downstream of GSH depletion. Although it has been argued that ferroptosis is distinct from oxidative glutamate toxicity because ferroptosis is independent of mitochondria [26,27], this observation has not been fully explored [24]. Similar to ferroptosis, oxidative glutamate toxicity can be inhibited by iron chelators (e.g. [28]). In addition, the ferroptosis inhibitor ferrostatin-1 can also inhibit oxidative glutamate toxicity [19]. Moreover, both cell death pathways require macromolecule synthesis [21,24]. Importantly, the role of Ca^{2+} in ferroptosis has not been fully explored. Recent work in the Maher lab (Table 1) has found that compounds that are known to inhibit oxidative glutamate toxicity by blocking mitochondrial ROS production (FCCP [6]), inhibiting 12/15 LOX (NDGA, PD146176 [13]) or reducing Ca^{2+} influx (cobalt chloride [6], apomorphine [29], LY83583 [8]) all protect cells from treatment with either erastin, the classical inducer of ferroptosis, or sulfasalazine, another system x_c^- inhibitor and ferroptosis inducer [24]). Thus, it appears that ferroptosis and oxidative glutamate toxicity are very similar, if not identical, and should both be classified under the more general term oxytosis, coined in 2001[3].

3 Relevance of oxytosis in vivo

While most mechanistic studies of oxytosis have been carried out in HT22 cells or related cell types, there is now ample evidence that elements of the pathway are activated and relevant in human disease in vivo. However, glutamate may not always be the best trigger as other triggers can also initiate the pathway; conversely, glutamate can also elicit additional injury pathways. Because glutamate release can also lead to excitotoxicity, which in cultured neurons occurs at lower concentrations as compared with oxidative glutamate toxicity, it may again be better to use the more general term oxytosis when referring to the oxidative glutamate toxicity pathway in vivo. Likewise, other events including mitochondrial damage and nitric oxide signaling are too non-specific to serve as hallmarks of oxytosis, unless additional components of the pathway can also be detected. It is thus perhaps more useful to look at the individual downstream components of oxytosis.

Importantly, loss of glutathione and consequent oxidative stress is a feature of many diseases, especially those involving ischemia. This is particularly relevant in brain pathologies, notably stroke. In neurons, glutathione represents the major antioxidant defense, so maintaining adequate amounts of reduced glutathione is critical. Following a stroke, intracerebral glutathione levels drop within the first several hours [30] and Ca^{2+} becomes dysregulated. Supplementing glutathione from exogenous sources, which at first glance would appear to be the most straightforward way to counter glutathione loss, has to date not been very successful. Glutathione itself is highly water soluble, but does not readily cross the blood – brain barrier. Glutathione precursors including N-acetylcysteine (NAC) and γ -glutamylcysteine ethyl ester (GCEE) require enzymatic conversion to glutathione, which may be disrupted following a stroke. Nonetheless, these approaches are still being considered, and may be successful if the aforementioned limitations can be overcome. In traumatic brain injury, another pathology in which glutathione levels drop, GCEE has been found to protect the blood – brain barrier [31]. Interestingly, the dual role of system x_c^- as a cystine importer and glutamate antiporter seems to obscure the effects of its deficiency. Mice

lacking xCT, the major component of system x_c^- are viable [32] and are even protected against 6-hydroxydopamine-induced toxicity [33] probably by a significant decrease in striatal extracellular glutamate levels [34]. The specific cell type that upregulates xCT also seems to be important; while a complete knockout of xCT had no effect on the severity of experimental autoimmune encephalomyelitis, cell-type specific knockout only in immune cells alleviated disease severity [35].

Further down the pathway 12/15-LOX, activated following glutathione depletion and one of the central actors in oxytosis, is up-regulated via the STAT family of transcriptional regulators under ischemic conditions [36]. With some time delay, AIF immunoreactivity in these cells is also increased, again mirroring the events in oxytosis [37]. Supporting this idea, 12/15-LOX gene knockout confers protection against experimental stroke [38,39] and against global cerebral ischemia [40], a model for cardiac arrest-induced brain injury. Similarly, a variety of 12/15-LOX inhibitors are protective in rodent and rabbit models of stroke; 12/15-LOX is thus an attractive target for the development of novel drugs to treat stroke. Harlequin mice, which have reduced levels of AIF, also show reduced infarct sizes following focal ischemia [41], indicating that multiple steps along the oxytosis pathway may be amenable to protective intervention.

Stroke is an important case study of oxytosis in vivo for another reason. 12/15-LOX is increased in neurons and endothelial cells after stroke, where it contributes to neuronal cell death and blood - brain barrier leakage [39,42]. Many reagents that block oxytosis in HT22 cells are also successful neuroprotectants in rodent models of stroke. This goes beyond a number of 12/15-LOX inhibitors (NDGA, PD146176 [13], tocotrienol [38,43], baicalein [39,44], LOXBlock-1 [45,46], and ML351 [47]) to also include proteasome inhibitors [48,49]. Furthermore, a number of redox-active compounds including the tyrphostins protect against oxytosis [50], although their lack of specificity makes a correlation with in vivo protective effects difficult to pin down to one mechanism. HT22 cells have been introduced as a highly efficient screening tool for 12/15-LOX inhibitors [47], as well as neuroprotective antioxidants in general [51]. Due to their rapid growth and easy maintenance, they have been successfully adapted to 96-well and even 384-well plate formats. This is also favored by the high rate of cell death (60–100%) following glutamate treatment coupled with almost complete protection in the presence of a 12/15-LOX inhibitor, which provides an excellent readout with a low rate of false positives.

Similar to stroke but on a much different time scale, Parkinson's and Alzheimer's diseases also involve oxidative stress, and there may be a role for oxytosis in these pathologies as well. Especially in Alzheimer's, 12/15-LOX has been reported to be increased in early phases of the disease [52], although this is somewhat controversial [53]. While these findings are intriguing, demonstrating oxytosis as a dominant mechanism in early Alzheimer's would require far more study. Similarly, oxidative stress is involved in Parkinson's disease pathology [54], but again, more research is needed. In general, for any disease involving oxidative stress and lipid peroxidation, the oxytosis pathway should at least be considered.

In most in vivo models, controlling the activity of 12/15-LOX appears to be essential for preventing oxytosis. The role of GPx4 in regulating 12/15-LOX activity in vivo was demonstrated in GPx4 knockout mice, where the absence of GPx4 elicits a 12/15-LOX-dependent cell death program that is also characterized by AIF translocation to the nucleus, and thus shows the hallmarks of oxytosis [12]. Here again, neurons are specifically vulnerable to the loss of GPx4. In a possibly similar mechanism, injection of GSSG, the oxidized form of glutathione, into the brain also leads to widespread cell death through 12/15-LOX activation [55]. However, in this case, the molecular details need to be further characterized before the conclusion can be reached that this is a clear example of oxytosis. Interestingly, whether or not the superoxide dismutases are involved in modulating 12/15-LOX activity has not been reported to date.

4 The crosstalk between oxidative stress and the cellular and mitochondrial Ca^{2+} machinery

Oxidative stress and more generally redox processes impinge on the cellular Ca^{2+} machinery in many ways. Intracellularly, Ca^{2+} is mainly stored in the ER from which it is released by PLC-induced generation of the second messenger inositol 1,4,5-trisphosphate (IP₃). IP₃ binds and opens IP₃ receptors 1–3 (IP₃R1–3) at the ER membrane triggering Ca^{2+} -release from the ER lumen. This Ca^{2+} release then activates Ca^{2+} -induced Ca^{2+} release via ryanodine receptors 1–3 (RyR1–3) at the ER membrane. The subsequent decrease in $[\text{Ca}^{2+}]_{\text{ER}}$ induces a strong Ca^{2+} influx from the extracellular space, named store-operated Ca^{2+} entry (SOCE) [56], which is followed by removal of cytosolic Ca^{2+} and replenishment of luminal Ca^{2+} through sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPases (SERCA). The Ca^{2+} sensor that conveys information about the Ca^{2+} load of the ER lumen to store-operated Ca^{2+} channels (SOCs) is stromal interaction molecule 1 (STIM1) [57,58], a single transmembrane protein located in the ER membrane with a luminal EF hand and a cytosolic domain, which activates Ca^{2+} release-activated Ca^{2+} (CRAC) channels [57,58]. Upon store depletion, STIM1 clusters into punctae near the plasma membrane [59] and activates CRAC channels within the plasma membrane, mainly Orai1 [60,61] but also Orai2 and 3 as well as transient receptor potential (TRP) channels.

Interestingly, oxidative stress can influence most of these key players in intracellular Ca^{2+} homeostasis. Oxidative stress increases PLC-dependent production of IP₃ and subsequent release of Ca^{2+} from the ER [62], whereas SERCA pumps are inhibited [63]. Oxidative stress also influences Orai1 activity, but here the effects are less clearly understood. One study demonstrated that H_2O_2 enhanced ICRAC-mediated Ca^{2+} influx (the electrophysiological equivalent of SOCE) through activation of IP₃ receptors and subsequent store depletion without a direct effect on Orai1 [64], whereas others suggested a direct inhibitory effect of H_2O_2 on Orai1-mediated Ca^{2+} influx induced by store depletion with thapsigargin, which was explained by the oxidation of cysteine residues located in an extracellular loop of Orai1 [65]. This was recently reproduced and the mechanism refined. Oxidation of Orai1 at C195 apparently locks the channel in a closed conformation by eliminating intramolecular interaction with S239 [66]. ROS also directly activate Ca^{2+} entry via S-glutathionylation of STIM1 at cysteine 56, which induces store-independent Ca^{2+}

entry and persistent CRAC channel activation [67]. This cysteine and the similarly conserved cysteine C49 were shown by others to be connected via an intramolecular disulfide bond and are also involved in the binding of the ER oxidoreductase ERp57. SOCE is accelerated in the absence of ERp57 and inhibited in the double-mutant C49A and C56A but in an unresolved conundrum apparently constitutively active in C56A cells [67]. Thus, during profound oxidative stress, SERCA is inhibited, IP3 receptors activated, and SOCE turned on by S-glutathionylation of the ER signaling molecule STIM1; this eventually results in an increase in the cytosolic Ca^{2+} concentration and ER- Ca^{2+} store depletion potentially leading to enhanced mitochondrial dysfunction, ER stress and cell death [68].

4.1 Oxytosis, ER Ca^{2+} release and store-operated Ca^{2+} entry

In line with this, the Methner lab has recently shown that dysregulated SOCE through Orai1 mediates (part of) the detrimental Ca^{2+} entry in oxytosis [69]. By comparing different key aspects of intracellular Ca^{2+} handling like cytosolic $[\text{Ca}^{2+}]$, store content and SOCE, we found a significant reduction in SOCE in glutamate-resistant HT22 (HT22R) cells. HT22R cells were generated by repeated exposure to large, detrimental concentrations of glutamate [70] and are resistant to diverse cell stressors such as oxidative stress and also ER stress [71]. Part of this protection is caused by metabolic remodeling leading to reduced mitochondrial respiration and increased glutathione recovery through the pentose phosphate pathway [72] but also through transcriptional upregulation of various proteins with antioxidant properties [71,73–75] mainly activated by upregulation of the transcription factor ATF4 [76].

Because of these changes at the transcriptional level, we hypothesized that the reduced SOCE observed in HT22R cells might be mediated via downregulation of potentially detrimental components of the cellular SOCE machinery and analyzed the expression levels of the SOCE-contributing proteins STIM1, STIM2 and Orai1 as well the transient receptor potential melastatin 7 (TRPM7) channel, a Ca^{2+} permeable channel that is also blocked by the SOCE inhibitor 2-APB [77] and has been previously implicated in cell death (reviewed in [78]). Only Orai1 was significantly downregulated and its knockdown by small interfering RNAs protected against oxytosis similar to pharmacological inhibition of SOCE. In addition, long-term Ca^{2+} live-cell imaging after induction of the cell death program also demonstrated a specific reduction in Ca^{2+} -positive cells by Orai1 knockdown. We therefore concluded that dysregulated Ca^{2+} entry through Orai1 mediates the detrimental Ca^{2+} entry in oxytosis induced by GSH depletion [69]. It is possible that the reported inhibition of Orai1 by oxidation of C195 represents a compensatory or protective mechanism which inhibits this dysregulated Ca^{2+} entry [65,66]. Here it is of interest that Orai1 channels can also mediate store-independent Ca^{2+} entry via arachidonic acid-activated ARC channels by interacting with a pool of plasma membrane-resident STIM1 [79]. It remains to be investigated whether products of 12/15-LOX like 12- and 15-HETE, which are actually downstream of arachidonic acid, can also open these channels in addition to arachidonic acid itself.

Others later reproduced the protective effect of pharmacological inhibition of SOCE in HT22 cells exposed to H_2O_2 . The protective effects included an attenuated intracellular Ca^{2+}

overload, restored mitochondrial membrane potential and decreased cytochrome c release [80]. SOCE inhibition also protected neuronal PC12 cells against 1-methyl-4-phenylpyridinium (MPP⁺) injury, a cellular model of Parkinson's disease. Pharmacological inhibition significantly increased cell viability, decreased apoptotic cell death and reduced intracellular ROS production and lipid peroxidation in MPP⁺ injured cells. SOCE inhibition also prevented MPP⁺-induced mitochondrial dysfunction and activation of mitochondrial related apoptotic factors but had no effect on mitochondrial biogenesis [81]. In these studies [80,81], downregulation of the Orai1-activating protein STIM1 was as protective as pharmacological inhibition of SOCE. Others, in contrast, found that over-expressing STIM1 in a neuronal cell line protected against cell death caused by glutathione depletion [82] which corresponds to our findings that STIM1 ^{-/-} mouse embryonic fibroblasts are more susceptible to oxidative stress [83]. Perhaps these differences are caused by the presence or absence of Homer proteins which interact with the cellular Ca²⁺ machinery and are upregulated by pharmacological inhibition of SOCE or STIM1 knockdown [81]. Homer proteins connect extracellular receptors - such as metabotropic or ionotropic glutamate receptors and the transient receptor potential canonical (TRPC) family of cation channels - with intracellular receptors such as inositol triphosphate and ryanodine receptors. Homer1 also interacts with STIM1 and Orai1 in a Ca²⁺-dependent manner [84]. Interestingly, knockdown of Homer1a partly reversed the protective effects induced by SOCE inhibition in PC12 cells [81]. In a follow-up study, the same authors reported that Homer1a protected HT22 cells from oxytosis by inhibiting the final Ca²⁺ deathblow [85]. In line with our results [69], the authors found increased STIM1 punctae after glutamate treatment suggesting that the lethal Ca²⁺ entry occurs through STIM1-gated SOC channels. Homer1a inhibited SOCE and decreased the association of STIM1-Orai1 triggered by glutamate [85]. Homer1 also interacts with TRPC channels (reviewed in [86]), which were also shown to be involved in Ca²⁺ influx caused by oxytosis [87]. In these studies, knockdown of TRPC1 mRNA by shRNA or blocking of TRPC channels with 2-APB (which blocks SOCE in general) robustly attenuated glutamate-induced cell death in HT22 cells [87]

4.2 Oxytosis and guanylate cyclase-mediated Ca²⁺ entry

As mentioned above, inhibitors of 12/15 LOX prevent the rise in intracellular Ca²⁺ levels following GSH depletion [13]. Among the targets of LOX metabolites is soluble guanylate cyclase (sGC) [8]. Interestingly, the sGC inhibitor LY83583 inhibited cell death but not GSH loss or ROS accumulation followed by glutamate treatment but rather prevented Ca²⁺ influx suggesting that sGC-responsive Ca²⁺ channels also contribute to the detrimental Ca²⁺ influx caused by oxytosis. This is supported by the fact that the cell permeable and phosphodiesterases-resistant cGMP analogue, CPT-cGMP potentiates oxytosis even after 12 hours of glutamate treatment [8]. Elevated cGMP levels eventually contribute to the opening of an as yet unidentified Ca²⁺ channel that contributes to the detrimental influx of extracellular Ca²⁺ and cell death [8]. This detrimental effect of CPT-cGMP was later reproduced by the Methner lab [88]. Others, however, reported that CPT-cGMP blocks SOCE in airway smooth muscle cells [89,90] but this was, at least in the second publication, attributed to a reduced expression of TRPC1 and TRPC6 [90] which could increase susceptibility to oxidative stress by other means. In our hands, intracellular cGMP was detrimental (CPT-cGMP) as reported [8] but extracellular cGMP and its metabolites GMP

and guanosine were protective [88]. Inhibition of the conversion of GMP to guanosine attenuated this effect, suggesting that GMP offers protection through its conversion to guanosine. While guanosine clearly had an effect via transcriptional upregulation of xCT, the functional subunit of system x_c⁻, we also observed that it provided protection when added late in the cell death cascade. It also significantly reduced the number of Ca²⁺-peaking cells suggesting non-transcriptional mechanisms [88] through direct inhibition of the yet undiscovered detrimental Ca²⁺ channel from the outside. It remains to be clarified whether guanosine blocks SOCE through TRPC or Orai channels.

4.3 Oxytosis and mitochondrial Ca²⁺

The increase in intracellular [Ca²⁺] and excessive ROS production during oxytosis causes mitochondrial Ca²⁺ ([Ca²⁺]_m) overload, extensive mitochondrial fragmentation and breakdown of the mitochondrial membrane potential [91–93].

It is well known that under physiological conditions, [Ca²⁺]_m uptake drives mitochondrial metabolism, as several dehydrogenases participating in the citric acid cycle are Ca²⁺-dependent [94–96]. [Ca²⁺]_m uptake enhances oxidative phosphorylation, the conversion of pyruvate into substrates that are used for mitochondrial respiration in order to generate ATP. [Ca²⁺]_m uptake is also essential to buffer changes in cytosolic Ca²⁺ ([Ca²⁺]_c). Transient oscillations in [Ca²⁺]_c are accompanied by an increase in [Ca²⁺]_m uptake that leads to short-term enhancement of the mitochondrial metabolism, a process termed mitochondrial buffering of [Ca²⁺]_c [97]. In addition, IP₃R-dependent stimulation of ER-Ca²⁺ release leads to the transfer of small Ca²⁺ microdomains at the ER-mitochondrial interface which also enhances mitochondrial respiration [98,99].

[Ca²⁺]_m overload, irrespective of the signaling pathway that induces it, is detrimental to the cell and induces cell death. Excessive Ca²⁺ influx into mitochondria leads to dissipation of the mitochondrial membrane potential, dysfunction of mitochondrial metabolism and opening of the mitochondrial permeability transition pore (mPTP) to induce cytochrome c-dependent cell death [100–102]. Furthermore, it was demonstrated that [Ca²⁺]_m dysregulation is preceded by enhanced mitochondrial ROS formation, thereby revealing an important interplay between oxidative stress and [Ca²⁺]_m signaling [103].

[Ca²⁺]_m homeostasis is regulated by different channel proteins that control Ca²⁺ influx and efflux. The voltage-dependent anion channel 1 (VDAC1) in the outer mitochondrial membrane as well as the multiprotein mitochondrial Ca²⁺ uniporter (MCU), the reverse mode Na⁺/Ca²⁺ exchanger (mNCX), the H⁺/Ca²⁺ exchanger (HCX) and Ca²⁺-activated K⁺ channels at the inner mitochondrial membrane are key players in the regulation of [Ca²⁺]_m homeostasis [92,104–106]. Preventing [Ca²⁺]_m overload in conditions of oxidative stress overall restores mitochondrial respiration and blocks subsequent [Ca²⁺]_c dysregulation as induced by SOCE [107]. Inhibition of [Ca²⁺]_m uptake by Nelfinavir or the MCU inhibitor Ruthenium Red successfully preserved mitochondrial integrity and protected against cell death in HT22 cells exposed to oxygen-glucose deprivation *in vitro*, and in a model of hypoxia ischemia/reperfusion in neonatal mice *in vivo* [108].

Recently, the Dolga lab has shown that pharmacological activation of small conductance Ca^{2+} -activated K^+ (SK) channels in HT22 cells protects against oxytosis by reducing mitochondrial respiration and restoring $[\text{Ca}^{2+}]_m$ homeostasis [92]. Oxytosis increased the uptake of $[\text{Ca}^{2+}]_m$, while SK channel activation attenuated $[\text{Ca}^{2+}]_m$ influx in conditions of increased oxidative stress, as assessed by the fluorescent dye rhodamine-2 and subsequent flow cytometric analysis. The advantage of flow cytometric analysis of rhodamine-2 fluorescence is that it allows for analyzing $[\text{Ca}^{2+}]_m$ changes as an endpoint in response to different stress stimuli which involve $[\text{Ca}^{2+}]_m$ overload as a critical factor to execute cell death. Therefore, it enables screening long-term effects of stress stimuli on their ability to induce $[\text{Ca}^{2+}]_m$ uptake. The disadvantage of this method for HT22 cells, however, is that it is not suitable to assess small changes in $[\text{Ca}^{2+}]_m$ levels due to a lack of sensitivity of the fluorescent dye. We have identified a substantial role for $[\text{Ca}^{2+}]_m$ influx in HT22 cells during oxytosis induced by glutamate or erastin, and during oxidative stress induced by H_2O_2 . In contrast, $[\text{Ca}^{2+}]_m$ influx is dispensable for ER stress in these cells, as detected by the ratiometric dye Fura-2AM [109].

Luminescence-based methods offer higher sensitivity to assess changes in $[\text{Ca}^{2+}]_m$ uptake in HT22 cells by monitoring real-time changes in $[\text{Ca}^{2+}]_m$ in response to stimuli that trigger $[\text{Ca}^{2+}]_m$ uptake without affecting the cell viability. This luminescence-based measurement system is performed in HT22 cells stably overexpressing a mitochondria-targeted variant of the photoprotein aequorin (HT22-mtAEQ) [110] allowing $[\text{Ca}^{2+}]_m$ measurements in a population-based 96-well plate format using a luminescence plate reader. Notably, assessment of $[\text{Ca}^{2+}]_m$ uptake in HT22-mtAEQ cells showed stable responses to 500 μM carbachol and 100 μM ATP (Honrath:2017gs). Inhibition of either MCU by KB-R7943 or of mitochondrial complex III by antimycin A has been shown to attenuate the uptake of $[\text{Ca}^{2+}]_m$ in response to ATP and carbachol-dependent IP_3R stimulation (Honrath:2017gs). Another advantage of this method is that changes in Ca^{2+} can be quantified into an actual Ca^{2+} concentration using defined parameters and calculations, if this is desired in the experimental design. Recently, GFP-aequorin fusion proteins have been developed to measure Ca^{2+} changes in the cytosol or the ER *in vitro* and *in vivo* [111,112]. Notably, these chimeric proteins might overcome the issue of signal interference if proteins with a fluorescent tag are overexpressed. Therefore, further experiments are required to test these constructs in HT22 cells and to compare the consistency of the results to those obtained by the mitochondrial aequorin measurements.

5 Conclusions and perspectives

In this review article, we described the signaling pathways that culminate in Ca^{2+} influx and cell death during oxidative glutamate toxicity or oxytosis, a form of programmed cell death caused by glutathione depletion most probably identical to ferroptosis and very similar to necroptosis. During oxytosis, Ca^{2+} is directly released from organelles like mitochondria and the ER by activated 12/15-LOX and enters the cytosol from the extracellular space through Ca^{2+} channels that are otherwise involved in store-operated Ca^{2+} entry like Orai and TRPC channels. An open question is whether these channels are activated via intracellular metabolites of 12/15-LOX, the ER Ca^{2+} release or by direct activation through ROS. Interestingly, ARC channels, which are also composed of STIM1 and Orai1, are activated by

arachidonic acid, a substrate of 12/15-LOX. It is also still unclear whether and how cytoprotective extracellular guanosine, which inhibits oxytosis-induced Ca^{2+} entry, blocks these channels. After the detrimental surge in cytosolic Ca^{2+} and the collapse of mitochondrial function caused by mitochondrial Ca^{2+} overload, Ca^{2+} -dependent proteases like calpains are activated and AIF translocates from the mitochondria to the nucleus which seals the fate of the cell (summarized in Figure 2). Finally, as oxytosis also plays an important role in neuronal cell death caused by ischemia and reperfusion injury and probably also neurodegeneration in Alzheimer's and Parkinson's disease it is possible that interventions that prevent dysregulated Ca^{2+} entry caused by oxytosis might have some potential therapeutic effect in these diseases.

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7 Literature

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Highlights

- Oxytosis/ferroptosis causes cell death by glutathione depletion and Ca^{2+} influx
- Ca^{2+} influx occurs through channels involved in store-operated Ca^{2+} entry
- Ca^{2+} influx by oxytosis causes mitochondrial Ca^{2+} overload

Figure 1. Time frame and cascade of events leading to cell death by oxytosis

Glutamate blocks cystine import which leads to a decrease of intracellular GSH. Once GSH levels drop below 20%, reactive oxygen species (ROS) rise and glutathione peroxidase 4 (GPx4) is inhibited leading to activation of 12/15-lipoxygenase (12/15-LOX). Activation of 12/15-LOX generates multiple metabolites including 12- and 15-HETE which are probably involved in the increase in Ca²⁺ influx that is required for the final phase of oxidative glutamate toxicity. 12/15-LOX also directly integrates into the membranes of various organelles like the ER and mitochondria further increasing lipid peroxidation and ROS production. Approximately 10–12 hours after the induction of oxidative glutamate toxicity, truncated pro-apoptotic Bcl-2 family member Bid translocates to the mitochondria and mitochondrial apoptosis-inducing factor AIF translocates to the nucleus, where it rapidly induces caspase-independent cell death. Time scale is only approximate. In the early phase *de novo* synthesis of RNA and proteins is necessary.

Figure 2. Ca^{2+} channels involved in cell death by oxytosis

Increased ROS and reduced GSH lead to activation of 12/15-LOX and production of 12- and 15-HETE which coincides with dysregulated Ca^{2+} entry from the extracellular space via Ca^{2+} channels involved in store-operated Ca^{2+} entry (SOCE) like Orai and TRPC1 channels. 12/15-LOX also integrates in the ER and mitochondrial membranes causing additional ROS production and possibly Ca^{2+} release.

Table 1

Compound	glutamate	sulfosalazine	erastin
No treatment	13.5%	15%	10%
FCCP (1 μ M)	83.3 $\pm$ 3.4%	81.7 $\pm$ 1.5%	75.1 $\pm$ 17%
Baicalein (10 μ M)	82.5 $\pm$ 7.7%	89.8 $\pm$ 2.5%	89.3 $\pm$ 1.4%
NDGA (10 μ M)	67.1 $\pm$ 2.0%	69.7 $\pm$ 4.5%	67.8 $\pm$ 4.4%
PD146176 (5 μ M)	94.1 $\pm$ 1.3%	88.7 $\pm$ 1.0%	88.4 $\pm$ 3.2%
LY83583 (1 μ M)	76.0 $\pm$ 2.6%	95.7 $\pm$ 1.0%	81.1 $\pm$ 2.6%
Apomorphine (5 μ M)	86.9 $\pm$ 1.0%	75.9 $\pm$ 1.5%	78.6 $\pm$ 7.2%
CoCl ₂ (100 μ M)	77.8 $\pm$ 1.1%	86.4 $\pm$ 1.9%	72.9 $\pm$ 4.0%