The plasma membrane channel ORAI1 mediates detrimental calcium influx caused by endogenous oxidative stress

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The mouse hippocampal cell line HT22 is an excellent model for studying the consequences of endogenous oxidative stress. Addition of extracellular glutamate depletes the cells of glutathione (GSH) by blocking the glutamate–cystine antiporter system x̄CT. GSH is the main antioxidant in neurons and its depletion induces a well-defined program of cell death called oxytosis, which is probably synonymous with the iron-dependent form of non-apoptotic cell death termed ferroptosis. Oxytosis is characterized by an increase of reactive oxygen species and a strong calcium influx preceding cell death. We found a significant reduction in store-operated calcium entry (SOCE) in glutamate-resistant HT22 cells caused by downregulation of the Ca²⁺ channel ORAI1, but not the Ca²⁺ sensors STIM1 or STIM2. Pharmacological inhibition of SOCE mimicked this protection similarly to knockdown of ORAI1 by small interfering RNAs. Long-term calcium live-cell imaging after induction of the cell death program showed a specific reduction in Ca²⁺-positive cells by ORAI1 knockdown. These results suggest that dysregulated Ca²⁺ entry through ORAI1 mediates the detrimental Ca²⁺ entry in programmed cell death induced by GSH depletion. As this detrimental Ca²⁺ influx occurs late in the course of the cell death intervention, it might be amenable to therapeutic intervention in diseases caused by oxidative stress.

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Oxidative stress is a phenomenon involved in a broad range of pathological conditions of the nervous system such as Alzheimer’s and Parkinson’s disease, stroke and trauma. It is provoked by an imbalance between superoxide production in the mitochondria and insufficient clearance of reactive oxygen species (ROS) through antioxidative defense mechanisms of the cell. One major defense pathway of nerve cells against ROS depends on the production of glutathione (GSH), a tripeptide composed of glutamate, cysteine and glycine, via the enzymes glutamate–cysteine ligase and GSH synthetase. In its reduced state, GSH serves as electron donor to detoxify ROS and is thereby oxidized to form GSH disulfide. The recovery of GSH from GSH disulfide is mediated by GSH reductase under NADPH consumption.¹

GSH depletion serves to study the effects of endogenous oxidative stress and can be excellently studied in vitro by treating cells with glutamate, which inhibits cystine uptake through the glutamate/cystine antiporter system x̄CT. Within the cell, cystine is rapidly converted to cysteine, the rate-limiting amino acid for GSH synthesis. Cystine deprivation then causes secondary GSH depletion and a programmed cell death by oxytosis or oxidative glutamate toxicity, which is clearly distinct from apoptosis, necrosis, and cell death associated with autophagy, but probably synonymous with the recently described iron-dependent form of non-apoptotic cell death termed ferroptosis, which seems to be involved in the selective elimination of some tumor cells and protection from neurodegeneration.²

A well-established model system for oxytosis/ferroptosis is glutamate-induced cell death in the hippocampal cell line HT22, which has been used extensively to clarify the cascade leading to cell death and to identify antioxidant pathways and

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Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; ANOVA, Analysis of variance; BSO, L-buthionine-(S,R) sulfoximine; cGMP, Cyclic guanosine monophosphate; CTB, Cell titer blue; EGTA, Ethylene glycol tetraacetic acid; ER, Endoplasmic reticulum; FCS, Fetal calf serum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GSH, Glutathione; HBSS, Hank’s balanced salt solution; HPRT, Hypoxanthine-phosphoribosyltransferase; IP3, Inositol trisphosphate; IP3R, Inositol trisphosphate receptor; iPLA2, Independent phospholipase A2; LOX, Lipoxygenase; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-l-cysteine; NADPH, Nicotinamide adenine dinucleotide phosphate; PBS, Phosphate-buffered saline; pCPT-cGMP, 8-(4-Chlorophenylthio)-guanosine 3',5'-cyclic monophosphate; RFP, Red fluorescent protein; ROS, Reactive oxygen species; SEM, Standard error of the mean; SERCA, Sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase; SOCE, Store-operated calcium entry; STIM1, Stromal interaction molecule 1; STIM2, Stromal interaction molecule 2; TRPM7, Transient receptor potential melastatin 7

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proteins (reviewed in3). In this system, GSH depletion leads to an exponential increase in ROS that mostly originates from mitochondrial complex I activity.4 After ~6 h of glutamate exposure, the lipid-oxidizing enzyme 12/15-lipoxygenase (12/15-LOX; EC 1.13.11.33) is activated and generates 12- and 15- hydroxyeicosatetraenoic acids5 that directly damage mitochondria, and cause mitochondrial depolarization and increased ROS production.6 The eicosanoids produced by 12-LOX are, however, also activators of soluble guanylate cyclases and thereby increase the concentration of intracellular cyclic guanosine monophosphate (cGMP), resulting in a detrimental influx of calcium at the end of the cell death cascade through a yet uncharacterized cGMP-dependent calcium channel.7 This Ca2+ 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 Ca2+ influx is blocked by CoCl2 or in Ca2+-free medium,7-9 but the molecular identity of the contributing Ca2+ channels is still unknown.

To identify the mechanism of Ca2+ entry in the final phase of oxidative glutamate toxicity, we compared the cellular calcium state of glutamate-sensitive and resistant HT22 cells, which are resistant due to the increased expression of various proteins with antioxidant properties,10-13 and found an isolated attenuation of store-operated calcium entry (SOCE) in the resistant cells. SOCE is activated when the endoplasmic reticulum (ER), the main cellular calcium store, is depleted, for example, during inositol trisphosphate (IP3)-mediated signaling events. When a membrane receptor is activated by its ligand, IP3 is generated near the plasma membrane and quickly diffuses through the cytoplasm to reach its receptor (Inositol trisphosphate receptor, IP3R) at the ER membrane. Binding of IP3 to IP3R releases Ca2+ stored in the ER lumen and generates a cytosolic Ca2+ signal, resulting in ER Ca2+-store depletion. To refill the ER, plasma membrane Ca2+ channels need to be activated to permit Ca2+ entry from the extracellular space, so-called SOCE. The molecule that transmits the information of [Ca2+]ER to plasma membrane Ca2+ channels is stromal interaction molecule 1 (STIM1).14,15 STIM1 clusters into punctae close to the plasma membrane upon store depletion and binds and activates ORAI1, a plasma membrane calcium channel,16-18

In this study, we provide evidence that dysregulated SOCE through ORAI1 is the main calcium entry mechanism during oxidative glutamate toxicity, suggesting that SOCE inhibition might be a valuable tool in the treatment of diseases associated with increased oxidative stress.

**Results**

**Reduced store-operated Ca2+ entry in hippocampal cells resistant to oxidative stress.** Glutamate-resistant HT22 cells are protected against oxidative glutamate toxicity (Figure 1a) mainly via an elevated GSH content due to an increased expression of the glutamate/cystine antiporter subunit xCT.19 They also show an increased expression of other proteins with antioxidant properties.10,11,13 We therefore reasoned that the function and expression of the sought-after detrimental Ca2+ channel might be altered in these cells and studied the content of the main cellular Ca2+ store, the ER, by staining the cells with the ratiometric Ca2+-sensitive dye Fura2 followed by treatment with the irreversible sarcoplasmic/ER Ca2+-ATPase (SERCA) inhibitor thapsigargin, which resulted in the expected biphasic cytosolic Ca2+ rise. Although HT22S and R cells behaved similarly during the first phase of Ca2+ rise, which corresponds to Ca2+ passively leaking out of the ER, the second phase of cytosolic Ca2+ rise was remarkably reduced in HT22R compared with HT22S cells (Figure 1b).

**Figure 1** Reduced store-operated Ca2+ in hippocampal cells resistant to oxidative stress. (a) 5000 glutamate-sensitive and -resistant HT22S and R cells were seeded into 96-well plates and subjected to the indicated concentrations of glutamate 24 h later; viability was quantitated again 16 h later with the cell-titer blue reagent. Mean ± S.E.M. fluorescence values of three replicates obtained in three independent experiments were plotted against the glutamate concentration. (b–d) Fura2-live-cell imaging of glutamate-sensitive and -resistant HT22S and R cells. (b) After 1 min of baseline recording in HBSS, 2 μM of thapsigargin was added and the fura2 signal recorded for an additional 4 min. (c) After 30 s of baseline recording in HBSS with Ca2+, EGTA was added. After a further 30 s, 5 μM of ionomycin was applied and the fura2 signal recorded for the following 4 min. (d) For measurement of SOCE, ER Ca2+ stores of HT22S and R cells were depleted by incubation in EGTA buffer supplemented with 2 μM thapsigargin. After 6 min of store depletion, the buffer was exchanged with HBSS supplemented with 1.26 mM Ca2+ and SOCE was recorded. Graph shows only Ca2+ rise, which corresponds to Ca2+ passively leaking out of the ER, *P<0.05
We hypothesized that this second plateau corresponds to reduced SOCE and decided to delve further into this. To distinguish between Ca\(^{2+}\) leaking from the ER and Ca\(^{2+}\) entering from outside the cell, we measured the whole-cellular Ca\(^{2+}\) store content and SOCE separately. Fura2-based Ca\(^{2+}\) live-cell imaging yielded no significant difference between HT22S and HT22R in the cytosolic Ca\(^{2+}\) rise induced by treatment with the Ca\(^{2+}\) ionophore ionomycin, which releases the whole-cellular Ca\(^{2+}\) content from the intracellular stores (Figure 1c). For measurement of SOCE activity, we depleted the ER Ca\(^{2+}\) store by addition of thapsigargin in EGTA buffer. After 6 min of store depletion, Ca\(^{2+}\) was readded and Ca\(^{2+}\) influx corresponding to SOCE was measured. In line with the data shown in Figure 1b, SOCE activity was significantly reduced in HT22R cells (Figure 1d).

To verify the reduced SOCE capacity of HT22R cells at the single cell level, we assessed SOCE activity by electrophysiological recordings using the whole-cell patch-clamp technique. Currents were evoked by application of 1 \(\mu\)M thapsigargin to achieve ER store depletion. Consistent with our results obtained in fura2 life – cell imaging experiments, HT22S and HT22R cells demonstrated different SOCE current – voltage (I/V) relation (Figure 2a). SOCE currents in HT22S cells revealed an inward rectification as expected for \(\text{I}_{\text{CRAC}}\) currents provided by ORAI1 channels, whereas SOCE currents in HT22R cells demonstrated a linear I/V relation with a reversal potential around +40 mV. There was no difference in amplitude or speed of SOCE currents development in time in HT22S and HT22R cells (Figure 2b). These data are in line with an altered composition or abundance of \(\text{I}_{\text{CRAC}}\) channels in HT22R cells. We therefore set out to investigate the role of SOCE during oxidative glutamate toxicity.

**Prolonged oxidative stress reduces the ER Ca\(^{2+}\) content and SOCE activity.** Glutamate-resistant HT22R cells were continuously cultivated in medium containing 10 \(\mu\)M glutamate to preserve their resistant phenotype. To test whether glutamate directly or indirectly influences SOCE capacity, we aimed to measure SOCE activity in glutamate-treated HT22S cell. We also hypothesized that cytosolic cGMP might influence the SOCE capacity similarly to glutamate because the Ca\(^{2+}\) influx leading to cell death by oxytosis is preceded by a cytosolic increase of cGMP. Application of 8-(4-chlorophenylthio)-guanosine 3’5’-cyclic monophosphate (pCPT-cGMP), a cell-permeable cGMP analog, indeed induced death of HT22 cells in a concentration-dependent manner (Figure 3a) and opened plasma membrane Ca\(^{2+}\) channels resulting in a slow but pronounced cytosolic Ca\(^{2+}\) rise, which we measured with the genetically-encoded Ca\(^{2+}\) sensor GCaMP5, expressed from a bicistronic transcript together with the red fluorescent protein (RFP), to normalize the GCaMP5 signal to the level of transfection (Figure 3b).

We elicited SOCE by store depletion through thapsigargin-induced SERCA inhibition under Ca\(^{2+}\)-free conditions and subsequent restitution of Ca\(^{2+}\). After 8 h of incubation with either glutamate or pCPT-cGMP, HT22S cells showed a reduced \([\text{Ca}^{2+}]_{\text{ER}}\) and reduced SOCE (Figure 3c). Addition of pCPT-cGMP or glutamate directly at the onset of measurement had no effect (Figure 3d). We therefore hypothesized that this reduction in \([\text{Ca}^{2+}]_{\text{ER}}\) and SOCE after induction of the cell death program might be a consequence of the endogenous oxidative stress that eventually kills the cells. And indeed, preventing oxidative stress with the antioxidant N-acetyl-L-cysteine (NAC) protected glutamate-treated cells from cell death (Figure 3e), and partially rescued the ER Ca\(^{2+}\) content as well as the SOCE capacity after 8 h of treatment (Figure 3f).

These results were of course not in line with our hypothesis that increased SOCE mediates the cytotoxic Ca\(^{2+}\) influx at the end of the cascade leading to cell death. We reasoned that the cell death program, which is already irreversible after 8 h of glutamate or 8-pCPT-cGMP treatments, alters many aspects of the intracellular Ca\(^{2+}\) homeostasis, which might obscure the effects on SOCE. We therefore decided to study the effect of SOCE on glutamate- and cGMP-induced cell death pharmacologically.

**Pharmacological inhibition of SOCE protects against oxidative stress.** We treated HT22 cells with a pharmacological inhibitor of SOCE, 2-aminoethoxydiphenyl borate (2-APB), and induced oxidative stress by addition of glutamate. 2-APB concentration-dependently protected against glutamate toxicity (Figure 4a) even when added up to 8 h after glutamate addition (Figure 4b). We also tested the ability of 2-APB to protect from pCPT-cGMP-induced cell death and observed a remarkable protective effect of 2-APB under these conditions, which was even stronger than that of the general Ca\(^{2+}\)-channel blocker CoCl\(_2\) (Figure 4c). As 2-APB is a well-known inhibitor of SOCE and protects HT22 cells from oxidative glutamate toxicity and pCPT-cGMP even when applied several hours after induction of oxidative stress, these observations support the hypothesis that the detrimental, late-onset Ca\(^{2+}\) influx induced by endogenous oxidative stress is mediated via components of the SOCE machinery.

**The SOCE Ca\(^{2+}\) influx channel ORAI1 is downregulated in glutamate-resistant HT22R cells.** After clarifying that the observed reduction of SOCE in HT22R cells (Figures 1d...
and 2a) is relevant for the resistance against oxidative stress, we decided to identify the involved proteins. Resistance against oxidative stress in HT22R cells is mediated in part via transcriptional regulation of protective proteins.\textsuperscript{10,11,13} We therefore hypothesized that the reduced SOCE observed in HT22R cells might be mediated via downregulation of components of the cellular SOCE machinery and analyzed the expression pattern of the SOCE-contributing proteins STIM1, STIM2 and ORAI1, as well the transient receptor potential melastatin 7 (TRPM7) channel, a Ca\textsuperscript{2+} permeable channel that is also blocked by 2-APB.\textsuperscript{20} The protein levels of STIM1 and STIM2 were equal in HT22S and R (Figure 5a and b). TRPM7 expression was analyzed by quantitative real-time-PCR (qRT-PCR) and also found to be the similar in both cell types (Figure 5c). Immunoblotting against ORAI1 and b). TRPM7 expression was analyzed by quantitative real-time-PCR (qRT-PCR) and also found to be the similar in both cell types (Figure 5c). Immunoblotting against ORAI1 and the more prominent 80 kDa band. Both bands were significantly downregulated in HT22R cells (Figure 5d), consistent with the reduced SOCE activity in these cells. Downregulation of these bands by small inhibitory RNA (siRNA) transfection, which correlated with mRNA quantification by RT-PCR (Figure 6h and i), also suggested specificity of the antibody used.

These results pointed to ORAI1 as being the protein that mediated the reduced SOCE observed in HT22R cells. To...
complement this and to show that this downregulation is indeed involved in the resistance against oxidative stress observed in HT22R cells, we decided to knockdown the components of the SOCE machinery, STIM1, STIM2, ORAI1 and TRPM7 by siRNA and determine the susceptibility of the cells to oxidative glutamate toxicity.

Knockdown of ORAI1 protects from GSH depletion. We transfected HT22 cells with two different siRNAs against STIM1, STIM2, ORAI1 or TRPM7 and treated these cells with glutamate or L-buthionine-(S,R) sulfoximine (BSO), an inhibitor of the rate-limiting enzyme glutamate-cysteine ligase, 48 h after transfection. Successful knockdown was verified by RT-PCR analysis (Figure 6a, d, f and h) and immunoblotting (Figure 6b and i). In line with the protein expression pattern observed in glutamate-resistant cells (Figure 5), knockdown of STIM1 or STIM2 induced no significant protective effect against GSH depletion elicited either by glutamate or BSO (Figure 6c and e), whereas reduced ORAI1 levels strongly protected HT22 cells from oxidative stress (Figure 6j). Interestingly, siRNA-mediated knockdown of TRPM7 in HT22S cells significantly increased susceptibility to oxidative glutamate toxicity (Figure 6g).

We conclude from these data that the protective effect of 2-APB is unlikely to involve STIM1, STIM2 or TRPM7 inhibition, as reduction of these Ca$^{2+}$ entry modulating proteins has either no or even the opposite effect of 2-APB. Rather, two lines of evidence suggest that ORAI1 is involved in oxidative stress-induced Ca$^{2+}$ influx: ORAI1 is downregulated in glutamate-resistant cells and its knockdown protects glutamate-sensitive cells from cell death mediated by GSH depletion. We conclude that the protective effect of 2-APB is mediated by the inhibition of ORAI1-mediated Ca$^{2+}$ influx and that reduced expression of ORAI1 is involved in the resistance of HT22R cells against oxidative stress.

Knockdown of ORAI1 inhibits cytosolic calcium elevation during oxytosis. To directly investigate the contribution of ORAI1 to the detrimental Ca$^{2+}$ influx induced by glutamate, we continued with long-term Ca$^{2+}$ live-cell imaging experiments to visualize the cytosolic Ca$^{2+}$ rise during oxytosis. We transfected HT22 cells with the two different siRNAs against ORAI1 (Figure 6h, i) or non-targeting control-siRNA together with the Ca$^{2+}$-sensor GCaMP5. Knockdown efficiency was controlled by RT-PCR (data not shown). 48 h after transfection, the cells were exposed to glutamate overnight and the cytosolic calcium signal was monitored in a high-content microscope equipped with an environmental chamber to keep the temperature and CO$_2$ concentration constant. Two hours after glutamate addition, we started to acquire the GCaMP and the control RFP signal every 15 min for the next 14 h and calculated the GCaMP/RFP ratio. This showed that cells peak in a seemingly random manner between 5–16 h of glutamate exposure. After a sharp increase in GCaMP signal, the cells quickly lyse, which was visible through vigorous quenching of GCaMP and RFP fluorescence. A typical sequence of pictures is shown in Figure 7a. The appearance of these Ca$^{2+}$ spikes was highly asynchronously spread over the whole-time frame (Figure 7a lower panel) and significantly less frequent in ORAI1-siRNA-transfected cells compared...
Ca\textsuperscript{2+} as described above. Interestingly, pCPT-cGMP did not evoke Ca\textsuperscript{2+} occurring with a steady increase, we calculated the slope of SOCE using the well-established inhibitor 2-APB similarly blocking cystine uptake with glutamate and inhibiting protected against cell death induced by GSH depletion, but amounts of ORAI1. Knockdown of ORAI1 protected against two different causes of GSH depletion; STIM1 had no effect, speaks against a dysregulation of the whole SOCE machinery and points instead to ORAI1 as the major culprit in these cells. When we investigated the effects of STIM1 and ORAI1 knockdown using the same siRNAs in

\begin{figure}
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\caption{Knockdown of ORAI1 protects from GSH depletion. HT22S cells were transfected with two independent siRNAs against STIM1, STIM2, ORAI1 and TRPM7 or with non-targeting control siRNA and 48 h later subjected to cell survival experiments. (a, d, f and h) RT-PCR analyses of successful knockdown of STIM1 (a), STIM2 (d), ORAI1 (h) and TRPM7 (f). Signals of genes of interest were normalized to hprt and plotted as expression in percentage relative to control/siRNA transfected samples. (b and i) Verification of successful knockdown by immunoblot. The membranes were simultaneously probed with antibody against either STIM1 (b) or ORAI1 (i) and GAPDH respectively acting as loading control and analyzed with the Licor infrared imaging system. (c, e, g and j) Survival experiments of siRNA-transfected cells. The indicated amounts of glutamate or BSO were added 48 h after transfection and viability was quantified with the CTB reagent 16 h after glutamate or BSO addition. The graphs show the mean fluorescence ± S.E.M. of 15 replicates obtained in three independent experiments plotted against glutamate or BSO concentration.}
\end{figure}

Discussion

We conclude that ORAI1 channels has a major role in the detrimental Ca\textsuperscript{2+} influx caused by oxidative stress. We found that glutamate-resistant HT22R cells exhibit a reduction in SOCE and reduced amounts of ORAI1. Knockdown of ORAI1 protected against two different causes of GSH depletion; blocking cystine uptake with glutamate and inhibiting GSH synthesis with BSO. Pharmacological inhibition of SOCE using the well-established inhibitor 2-APB similarly protected against cell death induced by GSH depletion, but not against ER stress elicited with the N-glycosylation inhibitor tunicamycin (not shown), which suggests that dysregulation of ORAI1 function is not a general aspect of cell death, despite the fact that glutamate-resistant cells are also resistant against ER stress as well as direct activation of the caspase cascade caused by overexpression of the apoptotic protein p53. As 2-APB is a known inhibitor of Ca\textsuperscript{2+} influx, its cell survival-promoting action is most probably caused by its inhibitory action on the detrimental Ca\textsuperscript{2+} influx during oxidative stress. This hypothesis is strengthened by our time course experiments, which still showed a beneficial effect of 2-APB when applied up to 8 h after glutamate addition, further pointing to a mechanism of action that interferes with late events in the cell death program such as Ca\textsuperscript{2+} influx. In addition, pCPT-cGMP-induced cell death was prevented by 2-APB, and the observed effect was even stronger than that of the general Ca\textsuperscript{2+} channel blocker CoCl\textsubscript{2}. Cytosolic elevation of cGMP through increased activity of soluble guanylate cyclases occurs very late in the cell death program induced by glutamate and is responsible for the opening of plasma membrane Ca\textsuperscript{2+} channels allowing detrimental Ca\textsuperscript{2+} influx.7 The fact that knockdown of the upstream initiator of SOCE STIM1 had no effect, speaks against a dysregulation of the whole SOCE machinery and points instead to ORAI1 as the major culprit in these cells. When we investigated the effects of STIM1 and ORAI1 knockdown using the same siRNAs in
Knockdown of ORAI1 inhibits cytosolic calcium elevation during oxytosis. HT22S cells were transfected with two different siRNAs against ORAI1 or non-targeting siRNA as control together with a plasmid encoding the GCaMP5-IRES-RFP Ca2+ sensor. The direct effect of ORAI1 knockdown in the survival assays. Direct knockdown of ORAI1 inhibits cytosolic Ca2+ influx in the late phase of oxidative glutamate toxicity. (a) Typical GCaMP pictures of a control siRNA transfected cell under glutamate exposure. The cytosolic Ca2+ concentration stays low for several hours, but eventually rises quickly, leading to cell lysis assessed by quenching of the fluorescence signal. The lower panel shows sample traces from glutamate-treated cells transfected with control siRNA, which illustrate the asymptotic appearance of Ca2+-spikes. For reasons of clarity, only Ca2+-peak positive cells from one well are shown. (b) Analysis of long-term live-cell imaging of GCaMP-IRES-RFP and siRNA-transfected cells under glutamate exposure in comparison to vehicle treatment. For each picture, a GCaMP5/RFP ratio was calculated. To identify peaking cells, the maximal GCaMP/RFP ratio was divided by the average GCaMP/RFP ratio calculated over the whole measurement period. Each cell that reached a value above the average value of control siRNA transfected cells was counted as Ca2+-peak positive. The amount of Ca2+-peaking cells was normalized to vehicle control and plotted as a bar graph. The bars represent mean \( \pm S.E.M. \) of 15 replicate wells measured in three independent experiments. (c) Analysis of the increase in the GCaMP/RFP ratio in pCPT-cGMP treated HT22 cells transfected with ORAI1 or control siRNA. The slope was normalized to vehicle and plotted as mean \( \pm S.E.M. \) in bar graphs. Two independent experiments were performed containing >150 cells for each condition. N.S., not significant, *P<0.05 by ANOVA with Tukey’s post hoc test.
thapsigargin-releasable Ca$^{2+}$ pool, implying reduced SERCA activity or increased ER Ca$^{2+}$ efflux. Others, however, suggested a direct inhibitory effect of H$_2$O$_2$ 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 ORAI1.28 This could be interpreted as an attempt to withstand the cytotoxic Ca$^{2+}$mediated by ORAI1.

We conclude from the presented data that dysregulated ORAI1-mediated Ca$^{2+}$ influx contributes in a significant manner to programmed cell death induced by GSH depletion in neuronal cells. As this detrimental Ca$^{2+}$ influx occurs late in the course of the cell death program, it might be amenable to therapeutic intervention in acute diseases where oxidative stress have a role.

Materials and Methods

Cell culture. The glutamate-resistant HT22R cell line was generated from the parental mouse hippocampal cell HT22 as previously described.19 Both cell lines, HT22S and HT22R, were cultured in DMEM high glucose (PAA Laboratories, Pasching, Austria) supplemented with 5% fetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA) and 100 U per ml penicillin and 100 µg per ml streptomycin (Gibco, Darmstadt, Germany) in a humidified incubator with 5% CO$_2$ and 95% air.

Cell survival assays. HT22 cells were seeded in 96-well plates at a density of 5000 cells/well and 24 h later subjected to cell death experiments. siRNA-transfected cells were seeded 24 h post transfection. Pharmacological agents were added together with, or at specified time periods after, induction of cell death by the indicated drugs. Cell viability was quantified 16–24 h after stress onset with the cell titer blue (CTB) reagent (Promega, Madison, WI, USA). Alternatively, in some experiments, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro- mide (MTT) assay was used.19 For CTB fluorescence, emission was measured at 500 nm after excitation at 562 nm using a GENios Pro microplate reader (Tecan, Männedorf, Switzerland); for the MTT-assay, absorption was measured at 570 nm.

Calcium live-cell imaging. Fura2 Ca$^{2+}$ imaging experiments were performed on a BD Pathway 855 High Content Imaging System (BD Biosciences, Heidelberg, Germany). The cells were seeded in 96-well imaging plate (BD Bioscience) the day before the experiment in a density of 5000 cells/well and loaded with 5 µM Fura2-AM (Molecular Probes, Darmstadt, Germany) in HBSS for 30 min before the experiment. Measurement was performed with excitation at 390 and 340 nm for ratiometric analysis and pictures were taken with a 20× objective. For comparison of SOCE in HT22S and HT22R cells, seeded on 12 mm coverslips at a density of 100,000/well, loaded with Fura2-AM and placed into a flow chamber. The measurement was performed using an Olympus IX81 fluorescence microscope with the cell^R imaging software. Images were acquired at 340 and 380 nm excitation and the ratio was calculated every 5 s for every single cell. After 1 min of baseline recording, ER-calcium stores were depleted in EGTA buffer (Ca$^{2+}$-free HBSS supplemented with 0.5 µM EGTA, 20 µM HEPES, 1µM MgCl$_2$ and 1 g per l Glucose) with 2 µM thapsigargin for 6 min and subsequently Ca$^{2+}$ was readded by changing the buffer to HBSS.

Electrophysiology. We used whole-cell patch clamping to measure currents through ion channels of single cells. Whole-cell recordings were performed using an Axopatch 200B patch clamp amplifier (Axon Instruments, Sunnyvale, CA, USA) and digitized with Digidata 1332 (Axon Instruments). For SOC channels recordings the pipette solution contained (in mM) 120 CsCl, 5 Bapta-Na, 30 Cs-HEPES pH 7.3, 1.5 MgCl$_2$, 0.4 Na$_2$ATP, 0.4 Na$_2$GTP and 1.6 CaCl$_2$ (pCa 7.0). Extracellular solution contained (in mM) 140 NMDG-Asp, 10 BaCl$_2$, 10 Cs-HEPES, pH 7.3. During recording the currents were sampled at 5 kHz and filtered digitally at 500 Hz, the pClamp9 software (Axon Instruments) was used for data acquisition and analysis. In all whole-cell experiments the holding potential was −40 mV, periodically (once every 5 s) the membrane potential was stepped to −100 mV (for 30 ms) and a 200 ms voltage ramp to −100 mV was applied. Currents were evoked by application of 1 µM thapsigargin (Sigma, Munich, Germany) in external solution, which was added to the bath perfusion. The traces recorded before current activation were used as templates for leak subtraction. Whole-cell currents were normalized to the cell capacitance. The mean value of cell capacitance was 19±5 (total number of experiments n = 20).

Quantitative RT-PCR. Whole-cell RNA was isolated using ZR RNA MiniPrep Kit (Zymo, Irvine, CA, USA) and transcribed into cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). RT-PCR analysis was performed on a 7500 Fast cycler (Applied Biosystems) with FAST BLUE qPCR MasterMix (Eurogentec, Cologne, Germany) running the 7500 standard program. Primers and Probes for STIM1 and 2, ORAI1 and TRPM7 were designed by the universal probe library assay design center (Roche, Mannheim, Germany) and the hypoxamine-phosphoribosyltransferase gene (HPRT) served as an endogenous control with individually designed primers and probe purchased from MWG (Ebersberg, Germany).

siRNA transfections. FlexiTube siRNAs against ORAI1, STIM1, STIM2 and TRPM7 were purchased from Qiagen (Hilden, Germany) (no.SI00972272, no.SI01435623, no.SI01435685, no.SI01435672, no.SI02694727 and no.SI02742695) and transfected into cells with LiPawns Maxin (In vitrogen, Darmstadt, Germany) when cells were grown to 70–80% confluence in six-well plates. Successful knockdown was verified by RT-PCR or immunoblot.

Immunoblotting. Cells were lysed in ice-cold RIPA buffer (Thermo Fisher Scientific) containing mini complete protease inhibitor cocktail (Roche) and centrifuged for 30 min at 16,000 g. The supernatants were separated on 6–18% polyacrylamide gels (Thermo Fisher Scientific), transferred onto nitrocellulose membranes with the Blot System (In vitrogen, Darmstadt, Germany) and blocked in 5% nonfat dry milk in phosphate-buffered saline containing 0.5% Tween-20 (PBS-T) for 1 h at room temperature before overnight incubation with primary antibodies against STIM1 (BD Biosciences, no.610955 1:250), STIM2 (Cell Signaling, Danvers, MA, USA, no.49171 : 1000), ORAI1, Alomone Jerusalem, Israel, no.ACC-062 1:500), Actin (Millipore, Billerica, MA, USA, no.MAB1501 1:5000) or GAPDH (Cell Signaling, no.2118 1:5000) followed by secondary antibody (Licor, Lincoln, NE, USA, 1:30000). The membranes were scanned for infrared fluorescence at 680 or 800 nm using the Odyssey system (Licer) and the signal was analyzed quantitatively with the image-processing software ImageJ (http://rsweb.nih.gov/ij/).

Long-term calcium live-cell imaging. GCaMP5 was a kind gift of Douglas S. Kim (Howard Hughes Medical Institute, Ashburn) and subcloned with EcoR I and NotI into the IRES-RFP-containing vector PB331A1 (System Biosciences, Mountain View, CA, USA). Cells were transfected with GCaMP5-IRES-RFP together with two different siRNAs against ORAI1 or non-targeting control-siRNA with Lipofectamine 2000 (Invitrogen) in six-well plates and 24 h later transferred to 96-well imaging plates (BD Biosciences) at a density of 5000 cells/ well. For calcium imaging, phenol red containing medium was replaced by colorless DMEM (PAA Laboratories, Pasching, Austria). Two hours before the onset of measurement, 25 µl glutamate was added to the cells, control wells were supplemented with vehicle and the plate was incubated in the BD Pathway 855 at 37 °C with 5% CO2 and 95% air. For pCPT-cGMP-induced Ca$^{2+}$ influx, measurement was started immediately after addition of 2.5 µM pCPT-cGMP. Cytosolic calcium was monitored by GCaMP5 and normalized to RFP fluorescence; pictures were taken every 15 min. For analysis of glutamate-induced Ca$^{2+}$ peaks, the maximal ratio was divided by the average ratio and a threshold was defined by the mean of control-siRNA transfected cells. Every cell above that threshold was counted as Ca$^{2+}$ peak positive. For analysis of pCPT-cGMP-induced Ca$^{2+}$ influx, the slope of Ca$^{2+}$ increase was calculated over the first 2 h of the measurement.

Statistical analysis. Data were analyzed as mean ± S.E.M. and the statistical significance assessed using two-tailed Student’s t-test or analysis of variance (ANOVA) with Tukey’s Multiple Comparison Test as indicated.

Conflict of Interest

The authors declare no conflict of interest.
