

## Review

# Loss of endoplasmic reticulum Ca<sup>2+</sup> homeostasis: contribution to neuronal cell death during cerebral ischemia

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The loss of Ca<sup>2+</sup> homeostasis during cerebral ischemia is a hallmark of impending neuronal demise. Accordingly, considerable cellular resources are expended in maintaining low resting cytosolic levels of Ca<sup>2+</sup>. These include contributions by a host of proteins involved in the sequestration and transport of Ca<sup>2+</sup>, many of which are expressed within intracellular organelles, including lysosomes, mitochondria as well as the endoplasmic reticulum (ER). Ca<sup>2+</sup> sequestration by the ER contributes to cytosolic Ca<sup>2+</sup> dynamics and homeostasis. Furthermore, within the ER Ca<sup>2+</sup> plays a central role in regulating a host of physiological processes. Conversely, impaired ER Ca<sup>2+</sup> homeostasis is an important trigger of pathological processes. Here we review a growing body of evidence suggesting that ER dysfunction is an important factor contributing to neuronal injury and loss post-ischemia. Specifically, the contribution of the ER to cytosolic Ca<sup>2+</sup> elevations during ischemia will be considered, as will the signalling cascades recruited as a consequence of disrupting ER homeostasis and function.

**Keywords:** Ca<sup>2+</sup> homeostasis; ischemia; ER stress; IP3R; RyR; SERCA; unfolded protein response(UPR); neuronal cell death

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The endoplasmic reticulum (ER), an important organelle present in all eukaryotic cells, consists of a continuous network of tubules, cisterns and vesicles. The ER contributes to the synthesis of membrane lipids and proteins. It also contributes to the regulation of intracellular calcium dynamics. Nowhere is this more evident than in neurons where the ER has been proposed to function as a “neuron-within-a-neuron”<sup>[1]</sup> due to its ability to rapidly integrate and respond to Ca<sup>2+</sup> signals initiated at the plasma membrane (PM). Extending from dendritic spines, through the cell body, axon, and into presynaptic terminals, the ER contributes to all aspects of neuronal function including transmitter release, synaptic plasticity and gene transcription<sup>[1, 2]</sup>.

The ability of the ER to integrate and contribute to rapid Ca<sup>2+</sup> signalling is predicated upon its capacity to store, buffer and release Ca<sup>2+</sup> to and from the cytosol. The intraluminal ER Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>ER</sub>) is primarily determined through the concerted activities of resident Ca<sup>2+</sup> channels, transporters and Ca<sup>2+</sup> binding proteins. An extensive review of all

mechanisms contributing to the regulation of Ca<sup>2+</sup> within the ER is beyond the scope of this review, but has been covered elsewhere<sup>[1, 3]</sup>. Rather, in the following sections we will focus on ER-based mechanisms that have been proposed to contribute to neuronal cell injury and death in ischemic models of stroke.

## Loss of ER Ca<sup>2+</sup> homeostasis during ischemia

Obstructed blood flow during a stroke initiates a cascade of events that culminate in neuronal cell death. Deprived of oxygen and glucose, cellular energy stores (*ie* ATP levels) are rapidly depleted and ionic homeostasis is no longer possible; neurons begin to depolarize and release their transmitter stores. The resulting massive release of the excitatory transmitter glutamate provokes further depolarization due to activation of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-*D*-aspartate (NMDAR) glutamate receptor, as well as voltage-gated Ca<sup>2+</sup> channels. Importantly, as NMDARs are permeable to Ca<sup>2+</sup>, neurons begin to accumulate toxic levels of this cation. The resulting pathological rise in Ca<sup>2+</sup> triggers numerous downstream effectors and contributes to the generation of reactive oxygen and nitrogen species (ROS/RNS). As a result of the conditions prevailing during

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ischemia (*ie* hypoglycemia, elevated cytosolic  $\text{Ca}^{2+}$ , ROS/RNS, *etc*) ER homeostasis is disrupted, resulting in  $\text{Ca}^{2+}$  store depletion<sup>[4-6]</sup>. Mechanistically, depletion of  $\text{Ca}^{2+}$  stores can be achieved through facilitated release and/or reduced re-uptake to and from the cytosol, respectively. Evidence suggests that  $\text{Ca}^{2+}$ -store depletion during ischemia occurs as a result of both aberrant release, primarily achieved through ryanodine and  $\text{IP}_3$  receptor channels (RyRs and  $\text{IP}_3$ Rs, respectively), as well as impaired re-uptake, mediated by the sarcoplasmic/endoplasmic reticulum ATPase (SERCA).

### Ryanodine receptor channels (RyRs)

The RyR family consists of three members: RyR1, RyR2, and RyR3. While all of the isoforms are expressed within the CNS, RyR<sub>2</sub> is considered the predominant neuronal isoform<sup>[7]</sup>. Within neurons, immunocytochemical evidence suggests a broad subcellular distribution of RyRs extending from the perikaryon to dendrites and even spines, where they have been shown to contribute to the generation of highly localized  $\text{Ca}^{2+}$  signals<sup>[8]</sup> important for synaptic plasticity<sup>[9-12]</sup>. RyRs are predominantly gated by elevations in intracellular  $\text{Ca}^{2+}$ , a process referred to as  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR)<sup>[13]</sup>. The activity of RyRs is further regulated by a number of intracellular modulators, including  $\text{Mg}^{2+}$ , ATP, and cyclic ADP ribose (cADPR). In post-ischemic neurons, intracellular  $\text{Ca}^{2+}$  levels are elevated as a result of the excitotoxic activation of  $\text{Ca}^{2+}$  permeable NMDARs and voltage-gated  $\text{Ca}^{2+}$  channels. However, additional  $\text{Ca}^{2+}$  permeable channels, including TRP, ASIC, and pannexin channels<sup>[14-17]</sup>, are also likely to make important contributions in this respect. Consequently, elevated intracellular  $\text{Ca}^{2+}$  promotes RyR activation, resulting in  $\text{Ca}^{2+}$  release from the ER and a further rise in intracellular  $\text{Ca}^{2+}$  levels.

Aberrant RyR activation during ischemia is exacerbated through the post-translational modification of RyRs by ROS/RNS. This is proposed to occur through redox modification of cysteine residues, as first shown for RyRs expressed in cardiac muscle<sup>[18]</sup>. The relevance of such a mechanism to brain ischemia was demonstrated by Bull<sup>[19]</sup> who demonstrated that S-glutathionylation of RyR2 augments CICR and proposed that such a mechanism might contribute to cortical neuronal death. Mechanistically, augmented RyR channel activity during ischemia may be due to an increased sensitivity of the oxidized channels to activation by  $\text{Ca}^{2+}$  and a reduced sensitivity to inhibition by  $\text{Mg}^{2+}$ <sup>[20]</sup>. Moreover, maximal activation can be attained at lower concentrations of ATP for oxidized RyR channels<sup>[21]</sup>. Alternatively, Kakizawa<sup>[22]</sup> recently showed that nitric oxide (NO) can induce RyR1 activation through S-nitrosylation at a specific cysteine residue (C3635) and evoke  $\text{Ca}^{2+}$  release from the ER. Using cultured neurons derived from RyR1<sup>-/-</sup> mice, in which NO-induced  $\text{Ca}^{2+}$  release is absent, they demonstrated that NO-induced neuronal cell death was reduced. Given the important contribution of NO to neuronal injury following ischemia<sup>[23]</sup>, the authors propose that NO-induced RyR1 activation could contribute to ischemic cell death. Regardless of the underlying mechanism, the contribution of RyR activation to cell death during ischemia is

underscored by results demonstrating neuroprotection following treatment with dantrolene<sup>[24-26]</sup>, a blocker of this particular family of channels.

### Inositol triphosphate receptor channels ( $\text{IP}_3$ Rs)

In addition to RyRs, ER  $\text{Ca}^{2+}$  can also be released during ischemia through  $\text{IP}_3$ R-dependent mechanisms. Like RyRs, three isoforms have been identified ( $\text{IP}_3$ R1-3), with all isoforms being present within the CNS. While  $\text{IP}_3$ R2 is strictly expressed within glial cells, both  $\text{IP}_3$ R1 and  $\text{IP}_3$ R3 are expressed neuronally. Each neuronal isoform is differentially expressed during development,  $\text{IP}_3$ R1 being the predominant adult form<sup>[27]</sup>. As their names imply, the primary means of activating these receptors is through the intracellular production of  $\text{IP}_3$  downstream of phospholipase C (PLC) activation. Oxygen-glucose deprivation (OGD) followed by reoxygenation (REOX) has been shown to cause a dramatic increase in  $\text{IP}_3$  levels in cultured cortical neurons<sup>[28]</sup> associated with  $\text{IP}_3$ R-dependent  $\text{Ca}^{2+}$  release from the ER. Consistent with this, in a separate study, inhibition of PLC has been shown to protect cultured neurons from mild excitotoxic insult<sup>[29]</sup>. OGD/REOX-induced ER  $\text{Ca}^{2+}$  release was contingent on the PLC-coupled metabotropic glutamate receptor, mGluR1. Moreover, inhibition of mGluR1 receptors protected neurons from cell death, consistent with previous reports of neuroprotection by group I, mGluR antagonists<sup>[30]</sup>.

In addition to being gated by  $\text{IP}_3$ ,  $\text{IP}_3$ R activation is regulated by  $\text{Ca}^{2+}$  thus allowing  $\text{IP}_3$ Rs to contribute to CICR<sup>[31-34]</sup>. In this way,  $\text{Ca}^{2+}$  influx contributed by NMDARs, for example, can promote  $\text{IP}_3$ R activation. The importance of such a mechanism to excitotoxic cell death was highlighted in a study by Ruiz<sup>[29]</sup> in which they demonstrated that the inhibition of  $\text{IP}_3$ Rs was especially effective in reducing  $\text{Ca}^{2+}$  overload and cell death during excitotoxicity. Interestingly, inhibition of  $\text{IP}_3$ Rs may provide a neuroprotective effect by attenuating mitochondrial damage. Indeed, recent evidence has shown that  $\text{IP}_3$ Rs are enriched at regions of close contact between the ER and mitochondria, called MAMs (mitochondria-associated membranes, for review see Decuypere<sup>[35]</sup>). Here  $\text{IP}_3$ Rs play a critical role in initiating  $\text{Ca}^{2+}$  exchange between these two organelles under both physiological and pathological conditions. Ruiz *et al* demonstrated that the inhibition of  $\text{IP}_3$ Rs prevented the loss of mitochondrial membrane potential induced by NMDA treatment of cultured neurons. Furthermore, inhibition of  $\text{IP}_3$ Rs largely prevented NMDA-induced caspase-3 activation, whereas inhibition of RyRs was ineffective. This model may have important implications as recruitment of mitochondrial-mediated cell death pathways contribute to ischemic neuronal cell loss<sup>[36]</sup>.

### Sarcoplasmic/endoplasmic $\text{Ca}^{2+}$ -ATPase (SERCA)

$\text{Ca}^{2+}$  homeostasis within the ER, and indeed more broadly within the cytosol, is further compromised during ischemia as a result of the impairment of the SERCA. The primary transport mechanism responsible for the uptake of  $\text{Ca}^{2+}$  from the cytosol to the ER, SERCA pumps are encoded by a family of 3

highly homologous genes, with alternative splicing of SERCA2 generating further diversity (SERCA2a and SERCA2b). Of the two splice forms identified, SERCA2b is the dominant neuronal form<sup>[37]</sup>. Ischemia has been shown to cause inhibition of Ca<sup>2+</sup> sequestration within the ER as a result of decreased SERCA activity<sup>[38]</sup>. As ATP is required for transport, inhibition of Ca<sup>2+</sup> uptake by SERCA is likely a consequence of ischemia-induced ATP-depletion. However, recent evidence suggests that additional factors contribute to the associated inhibition of SERCA activity. Indeed, ATPase activity has been shown to be uncoupled from Ca<sup>2+</sup> as a result of ischemia<sup>[39]</sup>. Mechanistically, inhibition of SERCA activity may be caused by the associated rise in ROS/RNS as several reports have shown reduced SERCA activity under conditions of oxidative/nitrosative stress<sup>[40-42]</sup>, including more specifically for SERCA2b<sup>[43]</sup>, the predominant neuronal isoform. Modifications of reactive tyrosine (protein nitration) and cysteine (S-glutathionylation) residues are thought to underlie the inhibition of pump activity. More specifically, hydroxyl radicals have been shown to disrupt the Ca<sup>2+</sup>-ATPase activity by attacking the ATP binding site, presumably through modification of cysteine residues localized within the active site of the enzyme<sup>[44]</sup>. Additionally, tyrosine nitration in response to peroxynitrate application has been proposed to affect SERCA activity<sup>[45, 46]</sup>. The resulting protein modification targeted tyrosine residues in proximity to sites essential for Ca<sup>2+</sup> translocation. Irrespective of the mechanistic basis, it is worth noting that inhibition of SERCA activity, by application of specific inhibitors (eg thapsigargin), is sufficient to disrupt ER function, leading to ER stress and the activation of downstream signalling cascades capable of initiating cell death.

### ER response to ischemia

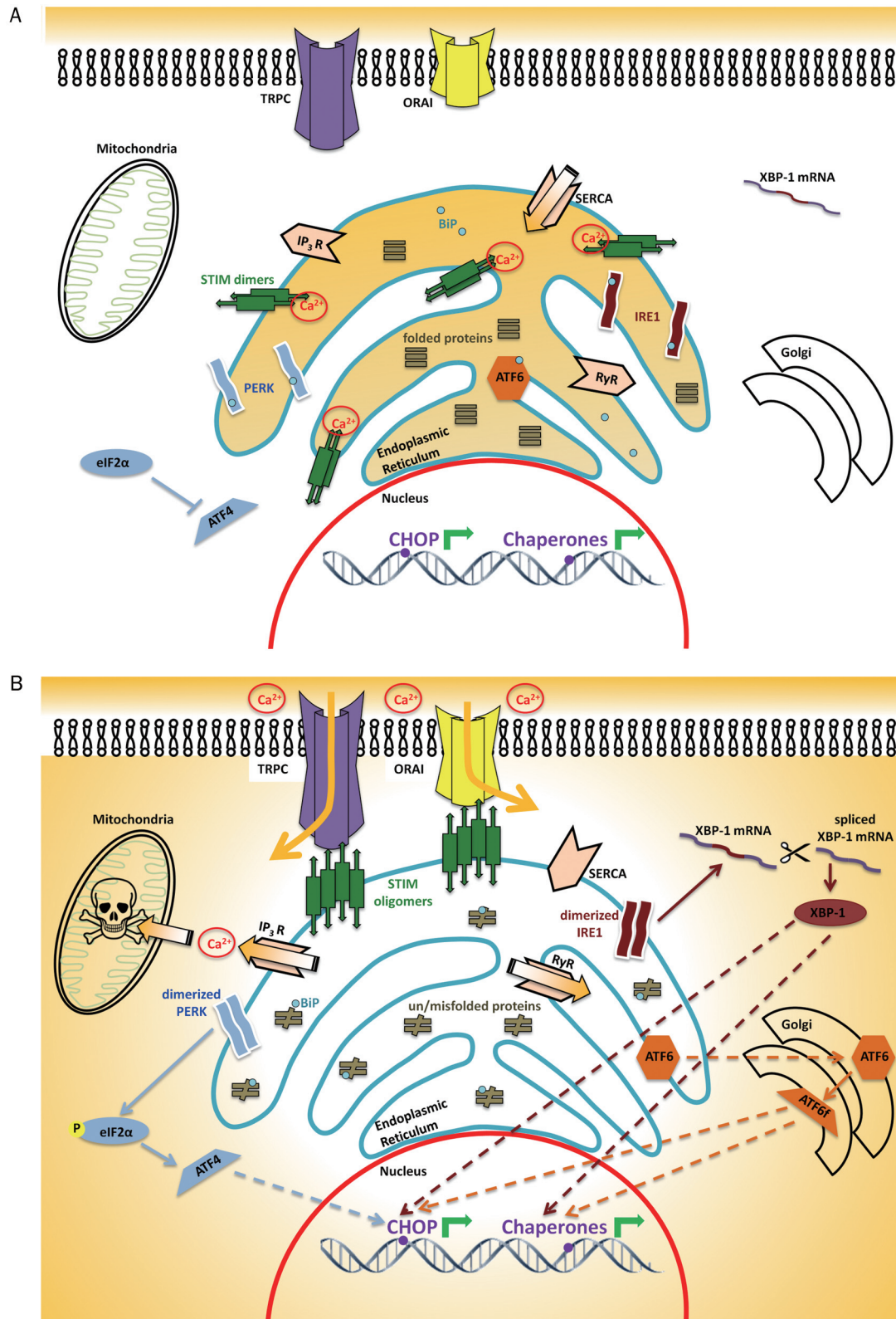
The evidence summarized in the preceding sections highlights mechanisms through which ER Ca<sup>2+</sup> stores are depleted during ischemia. The release of Ca<sup>2+</sup> from stores passively contributes to neuronal injury through the resulting rise of cytosolic Ca<sup>2+</sup>; however, the loss of ER Ca<sup>2+</sup> homeostasis and resulting disruption of ER function may be equally meaningful in this respect. In addition to Ca<sup>2+</sup> signalling, the ER contributes to the post-translational processing, folding and export of proteins<sup>[47, 48]</sup>. This essential function of the ER is mediated by a complex multi-protein network of molecular chaperones and foldases, most commonly protein-disulfide-isomerase, binding immunoglobulin protein (BiP), calnexin and calreticulin. Critically, many of the proteins that assist with protein folding are reliant on [Ca<sup>2+</sup>]<sub>ER</sub><sup>[47, 48]</sup>. Moreover, in binding Ca<sup>2+</sup> these same proteins contribute to ER Ca<sup>2+</sup> homeostasis. For example, it is estimated that BiP, an Hsp70 family member, accounts for around 25% of Ca<sup>2+</sup> storage within the ER<sup>[49]</sup>. Accordingly, protein folding and Ca<sup>2+</sup> homeostasis within the ER are tightly coupled<sup>[47, 48, 50]</sup>. Consequently, disruption of luminal Ca<sup>2+</sup> homeostasis leads to the accumulation of unfolded/misfolded proteins in the ER lumen, thereby causing ER stress. Interestingly, protein aggregates have been shown to accumulate following transient cerebral ischemia<sup>[51-53]</sup>. Severe protein aggre-

gate formation was observed in vulnerable CA1 pyramidal neurons destined to die, but not in surviving neurons of the dentate gyrus, CA3 or cortex. Moreover, aggregate formation coincided with the time course of cell death. Further support for some intimate relation between protein aggregation and cell death comes from the finding that ischemic preconditioning, in which brief sublethal ischemic episodes confer resistance to subsequent ischemic insult, reduces protein aggregate formation and cell death in a model of transient ischemia<sup>[54]</sup>. Preconditioning is known to induce an array of stress response genes, including molecular chaperones, which are expected to counter the accumulation of misfolded proteins observed following ischemia.

The accumulation of misfolded proteins within the ER (ER stress) triggers a pro-survival adaptation, the unfolded protein response (UPR)<sup>[55, 56]</sup>. Three ER resident proteins are responsible for initiating UPR; 1) PERK (double-stranded RNA-activated protein kinase-like ER kinase), 2) IRE1 (inositol requiring enzyme 1) and 3) ATF6 (activating transcription factor-6). Each of these single-pass transmembrane proteins functions as transducers relaying protein folding status within the ER lumen to the nucleus and cytosol through phosphorylation events as well as the generation and translocation of transcription factors.

The accumulation of misfolded proteins is thought to trigger the UPR by disrupting the association of PERK, IRE1, and ATF6 with BiP (binding immunoglobulin protein), a member of the HSP70 chaperone family and one of the most highly expressed proteins within the ER (Figure 1). A multifunctional protein, BiP possesses an N-terminal ATPase activity, but can also bind to the hydrophobic moiety of nascent unfolded proteins through its C-terminal peptide binding domain. Through its interaction with the luminal domains of UPR transducers, BiP is thought to constrain their signalling. The accumulation of unfolded proteins competitively displaces BiP, thereby initiating signalling downstream of three main arms of the UPR (however, note recent evidence suggesting that IRE1 may signal independently of BiP<sup>[57]</sup>). IRE1 is a serine/threonine protein kinase and endoribonuclease. Its activation is contingent on oligomerization and trans-autophosphorylation<sup>[55, 56]</sup>. Signalling by IRE1 proceeds through a non-conventional splicing of mRNA transcripts for XBP1, a transcription factor. Like IRE1, PERK possesses kinase activity and is activated through self-assembly and autophosphorylation. Activated PERK primarily mediates translation attenuation through the phosphorylation of eIF2 $\alpha$  (eukaryotic translation initiation factor 2 $\alpha$ ), an initiation factor required for protein translation. Lastly, ATF6 is a membrane-anchored transcription factor whose signalling is initiated following translocation to the Golgi, where it is subjected to regulated proteolysis. As a result of which, the cytoplasmic transcriptional domain (ATF6f) is released and translocates to the nucleus to affect changes in the expression of genes involved with chaperone activity and the degradation of misfolded proteins<sup>[58]</sup>. By reducing *de novo* protein synthesis and increasing the expression of ER resident chaperones, the UPR seeks to re-establish ER homeostasis by





**Figure 1.** (A) At physiological levels of luminal Ca<sup>2+</sup>, BiP remains bound to PERK, IRE1 and ATF6, suppressing their signalling activity. (B) Ischemia induced depletion of ER luminal calcium. ER protein folding capacity is exceeded causing competitive displacement of BiP from PERK, IRE1 and ATF6. Signalling pathways of the unfolded protein response are triggered when BiP dissociates from PERK, IRE1, and ATF6 allowing their dimerization and activation. In turn, transcription of CHOP and ER chaperones is upregulated. Release of ER Ca<sup>2+</sup>, particularly through IP<sub>3</sub>Rs, promotes uptake into mitochondria leading to mitochondrial injury and apoptosis. Store depletion further exacerbates the loss of cytosolic Ca<sup>2+</sup> homeostasis through STIM-dependent signalling, possibly involving surface expressed Ca<sup>2+</sup> permeable channels.

increasing misfolded protein handling capacity. However, if homeostasis cannot be restored, the main signalling pathways of the UPR are subverted from pro-survival to pro-apoptotic processes<sup>[50, 59]</sup>. Accordingly, in their active form, UPR transducers serve as ER stress markers. Critically, cumulative evidence suggests that all three signalling arms of the UPR are activated following ischemia<sup>[28, 60-71]</sup>.

### UPR activation during ischemia

Although the underlying mechanisms was not recognized at the time, one of the earliest evidence of UPR activation came from studies demonstrating a long lasting inhibition of protein synthesis following cerebral ischemia. We now know that increased eIF2 $\alpha$  phosphorylation contributes to the observed suppression of protein synthesis. In this respect increased PERK activity has been well documented in several studies following ischemia<sup>[72-76]</sup> as well as in cellular models of ischemia<sup>[77]</sup>. Increased PERK activity following ischemia can be inferred from its decreased association with BiP<sup>[72]</sup>, increased autophosphorylation as well as through increased phosphorylation of its target substrate eIF2 $\alpha$ . Definitive evidence that PERK is responsible for eIF2 $\alpha$  phosphorylation comes from a study by Owen<sup>[76]</sup> in which they examined the consequence of ischemia/reperfusion in transgenic mice with targeted disruption of the PERK gene. They noted that in the absence of PERK expression, basal phosphorylation of eIF2 $\alpha$  was reduced. More importantly, the increase in eIF2 $\alpha$  phosphorylation following transient ischemia was completely prevented and a substantial rescue of protein synthesis was observed. An important consideration arising from these studies is whether protein synthesis inhibition is neuroprotective or cytotoxic. Circumstantially, increased eIF2 $\alpha$  phosphorylation and transient inhibition of protein synthesis occurs in all post-ischemic neurons and while protein synthesis eventually recovers in regions resistant to ischemia-induced cell death, it remains depressed in vulnerable regions, suggesting that transient inhibition may serve as a protective mechanism. Consistent with this, a broad-based inactivation of eIF2 $\alpha$  and protein synthesis inhibition is observed in neurons following ischemic preconditioning<sup>[78]</sup>. Moreover, salubrinal, an inhibitor of GADD34/PP1X, the phosphatase responsible for eIF2 $\alpha$  dephosphorylation, has similarly been shown to be neuroprotective following acute ischemia<sup>[29]</sup>. Results from these and other studies suggest that transient inhibition of protein synthesis *per se* is not cytotoxic; in stark contrast, prolonged protein inhibition may serve as an indicator of impending neuronal demise<sup>[78]</sup>. It is however important to point out that mechanisms distinct from those involving PERK/eIF2 $\alpha$  have been proposed to underlie long lasting inhibition of protein synthesis. Accordingly, eIF2 $\alpha$  may be appropriate as a marker of ER stress, just not as a marker of impending neuronal demise. As will be discussed below, ER stress markers activated on a more protracted time course are more appropriate in this respect.

One of the most consistently reported markers of ER stress following ischemia is the up-regulation of BiP<sup>[28, 60-71]</sup>. As

outlined previously, BiP functions as the master regulator of the UPR. Yet BiP is also subject to transcriptional regulation downstream of UPR activation. Although the precise mechanisms contributing to BiP upregulation following ischemia have yet to be examined, previous work has proposed that BiP expression can be upregulated by ATF6 as well as by IRE1/XBP1<sup>[58]</sup>. Recent evidence suggests that ATF6 signalling is not engaged following ischemia<sup>[74, 75]</sup>, suggesting IRE1/XBP1 pathways may predominate in inducing BiP expression during ischemia-induced ER stress<sup>[79]</sup>. Note however, that ATF6 activation has been observed in cultured neurons following treatment with kainic acid (excitotoxic model of neuronal cell death)<sup>[68]</sup>, suggesting that ATF6 signalling may participate under some circumstances. BiP upregulation serves to protect the ER through several distinct mechanisms; 1) re-establishment of Ca<sup>2+</sup> homeostasis, 2) protein folding and 3) suppression of ER stress signalling. The importance of BiP is underscored by several studies examining the effects of altered BiP expression on cell fate. Downregulation of BiP exacerbates cell death in response to excitotoxic insult<sup>[71]</sup>, conversely, its upregulation has been shown to have a neuroprotective effect<sup>[62, 63, 71, 80]</sup>. With this knowledge in mind, Kudo *et al*<sup>[62]</sup> used high throughput screening and identified Bix (BiP inducer X), a compound capable of inducing BiP expression. They demonstrated that Bix treatment could induce a 3-fold increase in BiP expression in a neuroblastoma cell line. More importantly, they went on to show that Bix treatment reduced infarct area, brain swelling and apoptosis in the ischemic penumbral regions following focal cerebral ischemia. Critically, the neuroprotective effect of Bix is preserved even if treatment is delayed for up to 3 h<sup>[81]</sup>, suggesting Bix may have potential therapeutic applications in stroke.

### ER-initiated cell death cascades

The evidence summarized so far highlights early ER signalling events (*eg* increased phosphorylation of eIF2 $\alpha$  by PERK, induction of BiP expression)<sup>[82, 83]</sup> that are triggered following ischemia and play a protective role attempting to restore cellular homeostasis. However, if balance cannot be restored, then elements of these same signalling pathways trigger pro-apoptotic processes. One of the best characterized cascades in this respect is through the induction of the transcription factor CHOP (C/EBP homologous protein, also known as GADD153). Indeed, in numerous cell types, overexpression of CHOP has been shown to induce apoptosis and, conversely, knockout of CHOP renders cells more resistant to cell death<sup>[69, 84-86]</sup>. The promoter region for CHOP contains binding sites for ATF6 as well XBP1, allowing activation by each of these transcription factors. In addition, CHOP can be activated by activating transcription factor 4 (ATF4), itself regulated downstream of PERK/eIF2 $\alpha$ . Accordingly, CHOP expression can be promoted through all 3 main arms of the UPR<sup>[87]</sup>. Increased neuronal CHOP expression has been demonstrated in response to transient forebrain ischemia<sup>[61, 63, 69, 88-92]</sup> as well as kainic acid treatment of cultured hippocampal neurons<sup>[91]</sup>. Increased CHOP expression is also observed in astrocytes OGD<sup>[60]</sup>. More

recently, clarification of the mechanisms through which CHOP mediates cell death has emerged. CHOP has been reported to sensitize cells to ER stress through downregulation of the anti-apoptotic protein Bcl-2<sup>[93]</sup>. Moreover, expression of the pro-apoptotic mediator Bim is augmented by CHOP<sup>[94]</sup>. Of direct relevance to mechanisms contributing to neuronal apoptosis, CHOP was recently shown to bind to the promoter region of Puma (p53 upregulated modulator of apoptosis) and induce its expression<sup>[84]</sup>. CHOP-induced expression of Puma was shown to be critical for ER stress-mediated neuronal cell death. Accordingly, through transcriptional regulation, CHOP has been proposed to facilitate cell death by altering the balance between pro- and anti-apoptotic Bcl-2 family members<sup>[95]</sup>. The importance of CHOP as a contributor to ischemic neuronal cell death was demonstrated through the use of reverse genetic approaches<sup>[69, 96]</sup>.

Apoptotic cell death induced by ER stress is ultimately effected through recruitment of caspases that contribute to the morphological and biochemical changes that are characteristic of this form of cell death<sup>[97]</sup>. Caspase-12 localizes to the ER and a number of studies have shown that it is subject to proteolytic processing as a consequence of extended ER stress<sup>[98]</sup>. Caspase-12 is proposed to function as an inducer caspase initiating the sequential recruitment of effector caspases-9 and -3<sup>[99]</sup>. That being said, the importance of caspase-12 has been questioned by reports suggesting it is not always strictly necessary for ER stress-induced apoptosis<sup>[100]</sup>. Nevertheless, caspase-12 has been implicated in cell death associated with Alzheimer's disease<sup>[101]</sup>, prion disease<sup>[102]</sup> as well as cerebral ischemia<sup>[28, 66-68, 77, 89, 90, 103]</sup>. Several pathways leading to the recruitment of caspase-12 have been proposed. Like other caspase family members, caspase-12 is synthesized as a proenzyme and requires proteolytic processing to become active. In contrast to apoptotic pathways involving mitochondria, activation of caspase-12 is not reliant on cytochrome *c*. ER stress-induced mechanisms responsible for activation include proteolytic processing following translocation of cytosolic caspase-7<sup>[104]</sup> to the ER surface as well as by Ca<sup>2+</sup>-dependent recruitment of calpain<sup>[105]</sup>. In addition, caspase-12 is capable of autolytic processing following its homodimerization, an activity promoted downstream of IRE1 activation<sup>[106, 107]</sup>. Recent evidence has shown that proteolytic activation of procaspase-12 following OGD of cultured cortical neurons can be blocked by inhibitors of calpains, but not caspases, suggesting Ca<sup>2+</sup>-dependent calpain processing may predominate under these conditions<sup>[77]</sup>.

### Store-operated Ca<sup>2+</sup> entry, stromal-interacting molecules and ischemic neuronal cell death

The preceding section highlights an important and growing body of evidence implicating ER stress pathways as important contributors to the cascades of signalling events underlying neuronal injury and cell death following ischemia. A recurring theme in many of these studies is that depletion of ER Ca<sup>2+</sup> stores, contributed through activation of RyRs and IP<sub>3</sub>Rs as well as impaired SERCA function, is an important trigger

for ER stress. Consistent with this, depletion of ER Ca<sup>2+</sup> stores through inhibition of SERCA activity is sufficient to initiate ER stress and cell death in a variety of cell types<sup>[108]</sup>. Typically, ER stress is operationally defined on the basis of UPR induction. However, canonical UPR signalling is unlikely to represent the earliest response of the ER to cellular stressors, which may include depletion of ATP, loss of Ca<sup>2+</sup> homeostasis and ROS/RNS. Interestingly, emerging evidence suggests that additional non-canonical stress sensors may also contribute to neuronal cell death during ischemia. Specifically, STIM (stromal-interacting molecules) proteins, which function as ER resident Ca<sup>2+</sup> sensors, have recently been identified as important contributors to neuronal cell death post-ischemia.

In the mid-1980s, it was proposed that when ER stores are depleted of Ca<sup>2+</sup>, a refilling process via Ca<sup>2+</sup> influx involving ER proteins and plasma membrane channels is utilized<sup>[109]</sup>. This process has been termed store-operated Ca<sup>2+</sup> entry (SOCE), sometimes referred to as capacitive calcium entry (CCE). The Ca<sup>2+</sup> dependence of ER chaperones and foldases makes correct protein folding in the ER contingent on luminal Ca<sup>2+</sup> levels being maintained by steady refilling processes thus avoiding activation of ER stress pathways. Recently identified ER resident proteins, stromal interaction molecules 1 and 2 (STIM1 and STIM2), act as Ca<sup>2+</sup> sensors and relay messages of alterations in luminal Ca<sup>2+</sup> to the plasma membrane<sup>[110]</sup> where they interact directly with Ca<sup>2+</sup> influx channels<sup>[111]</sup>. This interaction is a constituent of the aforementioned SOCE.

The single-pass transmembrane proteins STIM1 and STIM2, originally named GOK and proposed to be involved in tumor suppression and modifications of cell morphology<sup>[112]</sup>, detect decreases in ER-luminal Ca<sup>2+</sup> through their N-terminal Ca<sup>2+</sup> binding EF-hand domains. Also at the N-terminal are dense clusters of sterile  $\alpha$  motif (SAM) domains that function to stabilize STIM in a dimeric form when EF-hands are Ca<sup>2+</sup> bound<sup>[113]</sup>. In the case of depleted Ca<sup>2+</sup> stores, as seen in ischemia induced ER stress, Ca<sup>2+</sup> dissociates from the EF-hand domains of STIM, unfolding and destabilizing the EF-SAM clusters. This promotes activation wherein STIM dimers aggregate into oligomeric STIM complexes<sup>[114]</sup>. Effectively, STIM behaves as a Ca<sup>2+</sup> sensor and is responsible for relaying the status of ER Ca<sup>2+</sup> stores to the plasma membrane. Translocation of STIM along the ER membrane to ER-PM junctions enables STIM interaction with Ca<sup>2+</sup> influx channels expressed at the cell surface to facilitate SOCE. So far, members of the Orai, TRPC and L-type voltage-gated Ca<sup>2+</sup> channel families have been identified as coupling targets for activated STIM<sup>[111, 115-117]</sup>. Aside from sensing decreases in ER Ca<sup>2+</sup> levels, STIM has recently been regarded as a general cellular stress sensor because it is also activated by hypoxia-induced acidosis<sup>[118]</sup>, ER stress in dopamine neurons<sup>[119]</sup>, oxidative stress<sup>[120]</sup> and transient temperature changes<sup>[121]</sup>. Hypoxia itself can cause STIM1 activation likely as a result of lowering ATP levels, reducing the activity of SERCA pump and depletion of Ca<sup>2+</sup> stores. In response to supraphysiological ROS levels, S-glutathionylation of Cys56 on STIM, adjacent to its EF-SAM domains, causes a dissociation of Ca<sup>2+</sup> from STIM and subsequent SOCE without ER



Ca<sup>2+</sup> depletion<sup>[120]</sup>. Additionally, STIM1 has been shown to oligomerize and translocate to ER-PM junctions when cells are heated from 37–40°C. Subsequent cooling back to 37°C triggers Ca<sup>2+</sup> influx, independent of ER Ca<sup>2+</sup> levels, thus implicating STIM as a sensor to transient temperature change as well<sup>[122]</sup>.

The two isoforms, STIM1 and STIM2, possess homologous functional domains with the only differences between the two isoforms being slight variations in the amino acid sequences of their N and C termini. Functional consequences of these variations are in their affinities for Ca<sup>2+</sup> binding, thus affecting each isoform's sensitivity of ER Ca<sup>2+</sup> detection as well as activation kinetics and contribution to SOCE signalling<sup>[123]</sup>. The higher affinity isoform, STIM1, is rapidly activated under ER Ca<sup>2+</sup> depletion making STIM1 the principal modulator of SOCE<sup>[124]</sup>. Contrastingly, the lower affinity STIM2 isoform, despite being more sensitive to ER Ca<sup>2+</sup> depletion, demonstrates slower activation kinetics and is considered to be responsible for the maintenance of basal cytosolic and ER Ca<sup>2+</sup> concentrations within tight limits<sup>[125]</sup>. This view has recently been called into question by Berna-erro *et al*<sup>[126]</sup> as they implicated SOCE in ischemic neuronal cell death and label STIM2, rather than STIM1, as the critical mediator of SOCE. In their study, which asserted STIM2 as the predominant isoform in the brain, calcium-imaging experiments showed reduced SOCE in STIM2<sup>-/-</sup> but not in STIM1<sup>-/-</sup> or Orai1<sup>-/-</sup> mice. Furthermore, neurons from their STIM2<sup>-/-</sup> mice showed increased survival under hypoxic conditions and *in vivo* STIM2<sup>-/-</sup> mice under the middle-cerebral artery occlusion model of ischemic stroke did not demonstrate neurological damage. From this evidence, it was proposed that STIM2<sup>-/-</sup> mice are protected from ischemic stroke. In contrast to the findings of Berna-erro, several other groups have detected neuronal STIM1 expression<sup>[127–129]</sup>. However, the precise contribution of STIM1 to neuronal function remains to be elucidated. In addition, a major unresolved question remains as to the identity of the plasma membrane Ca<sup>2+</sup> channel responsible for STIM2-initiated Ca<sup>2+</sup> influx during ischemia. Candidate channels include TRPC as well as Orai channel family members (Figure 1). This should represent an important focus for future studies.

## Conclusions

Mounting evidence implicates the loss of ER homeostasis and function to the pathology associated with cerebral ischemia. ER function can be perturbed under numerous circumstances including, but not limited to, oxidative stress, Ca<sup>2+</sup> dysregulation and the accumulation of misfolded proteins<sup>[130, 131]</sup>. If sustained, these conditions lead to ER stress, increased Ca<sup>2+</sup> influx, increased membrane permeability, and, eventually, cell death by apoptosis<sup>[50, 132]</sup>. Neuroprotection has been demonstrated for several agents targeting various elements involved in regulating ER-dependent Ca<sup>2+</sup> signalling (*eg* IP<sub>3</sub>R and RyR antagonists) and protein synthesis and folding (*eg* eIF2α phosphatase inhibitors, BiP inducers). However, numerous additional candidates targets have been identified that could be exploited in the development of new neuroprotective agents.

Given recent evidence that STIM2 is a critical mediator of ischemic neuronal cell death, future studies elucidating the mechanisms through which STIM2 couples to cell death may identify novel therapeutic avenues.

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