



## Review

# Neuroprotective hypothermia – Why keep your head cool during ischemia and reperfusion



N. Talma<sup>1</sup>, W.F. Kok<sup>\*,1</sup>, C.F. de Veij Mestdagh, N.C. Shanbhag, H.R. Bouma, R.H. Henning

Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

## ARTICLE INFO

## Article history:

Received 20 April 2016

Received in revised form 28 June 2016

Accepted 25 July 2016

Available online 28 July 2016

## Keywords:

Therapeutic hypothermia

Neuroprotection

Ischemia and reperfusion injury

Sumoylation

Unfolded protein response

Cold shock proteins

## ABSTRACT

**Background:** Targeted temperature management (TTM) is the induced cooling of the entire body or specific organs to help prevent ischemia and reperfusion (I/R) injury, as may occur during major surgery, cardiac resuscitation, traumatic brain injury and stroke. Ischemia and reperfusion induce neuronal damage by mitochondrial dysfunction and oxidative injury, ER stress, neuronal excitotoxicity, and a neuroinflammatory response, which may lead to activation of apoptosis pathways.

**Scope of review:** The aim of the current review is to discuss TTM targets that convey neuroprotection and to identify potential novel pharmacological intervention strategies for the prevention of cerebral ischemia and reperfusion injury.

**Major conclusions:** TTM precludes I/R injury by reducing glutamate release and oxidative stress and inhibiting release of pro-inflammatory factors and thereby counteracts mitochondrial induced apoptosis, neuronal excitotoxicity, and neuroinflammation. Moreover, TTM promotes regulation of the unfolded protein response and induces SUMOylation and the production of cold shock proteins. These advantageous effects of TTM seem to depend on the clinical setting, as well as type and extent of the injury. Therefore, future aims should be to refine hypothermia management in order to optimize TTM utilization and to search for pharmacological agents mimicking the cellular effects of TTM.

**General significance:** Bundling knowledge about TTM in the experimental, translational and clinical setting may result in better approaches for diminishing I/R damage. While application of TTM in the clinical setting has some disadvantages, targeting its putative protective pathways may be useful to prevent I/R injury and reduce neurological complications.

© 2016 Elsevier B.V. All rights reserved.

## 1. Introduction

Ischemia and reperfusion (I/R) of the brain results in extensive neuronal injury and forms a substantial medical burden because of high morbidity and mortality. In adults, cerebral ischemic insults typically result from ischemic stroke or cardiac arrest, while in infants cerebral ischemia generally ensues from complications during labor and delivery or surgery for congenital heart disease, resulting in neonatal hypoxic-ischemic encephalopathy [26]. The high oxygen and glucose demands of the brain compared to other organs require continuous blood supply, which is guaranteed under physiological conditions by the autoregulation of brain circulation. However, the relatively high oxygen and glucose demand makes the brain extremely vulnerable to hypoxia and ischemia. Disrupted blood flow causes an imbalance

between the energy generated by glucose oxidation, the main source for energy in the brain, resulting in loss of cellular homeostasis. Restoring blood flow and thus re-establishing nutrient and oxygen delivery to the ischemic brain is essential to salvage neurons, although reperfusion itself causes additional, substantial brain damage [56]. Reperfusion injury occurs due to enhanced production of reactive oxygen species in mitochondria, disruption of calcium ( $\text{Ca}^{2+}$ ) homeostasis through glutamate-induced excitotoxicity, an exaggerated neuroinflammatory response by stimulation of the TNF-receptor, and a cellular stress response in the endoplasmic reticulum (ER), which may further damage neuronal cells [50].

Targeted temperature management (TTM) is thus far generally applied in aforementioned conditions as a neuroprotective strategy to prevent acute ischemia-reperfusion injury (I/R injury) of the central nervous system (CNS). TTM is the induced cooling of the body or a specific organ to prevent or treat injuries. The impact of TTM seems to depend on the target temperature [66]. Randomized studies directly comparing different target temperatures are often performed by comparing just two temperature targets [13,45]. However, protection by TTM is highly dependent on the clinical setting, as well as type and

\* Corresponding author at: Department of Clinical Pharmacy and Pharmacology (EB71), University Medical Center Groningen, Antonius Deusinglaan 1, 9713 AV, Groningen, The Netherlands.

E-mail address: [w.f.kok@umcg.nl](mailto:w.f.kok@umcg.nl) (W.F. Kok).

<sup>1</sup> Both authors contributed equally to this manuscript.

extent of the injury. Clinical application of TTM encompasses a wide spectrum of neurological conditions and complications. Mild (32–35 °C) and moderate hypothermia (28–32 °C) prevent further injury during or following cardiac arrest [23], hypoxic-ischemic encephalopathy in neonates [58], traumatic brain injury [72], and may be used to prevent brain injury during or after stroke. Deep hypothermia (<28 °C) on the other hand is used during circulatory arrest in surgical procedures on congenital heart disease in neonates, aortic arch in adults, and intracranial aneurysms [37]. Although current data suggest that TTM protects against I/R neuronal injury, the precise underlying mechanisms remain to be elucidated. Revealing the molecular mechanisms of TTM in neuronal I/R (Fig. 1) may allow to identify potential targets and assist the development of novel pharmacological intervention strategies against cerebral I/R injury. In this review, we give an overview of the molecular mechanisms underlying neuronal I/R-induced cell death, neuroprotective effects of TTM in I/R injury and possible pharmacological approaches to mimic TTM.

## 2. Cerebral ischemia leads to mitochondrial dysfunction and oxidative injury

I/R injury is characterized by mitochondrial dysfunction and oxidative stress in the brain. The acute decrease in cerebral oxygen and glucose levels during ischemia lead to an imbalance in energy homeostasis, which disrupts mitochondrial function. Disruption of mitochondrial function leads to reduced adenosine triphosphate (ATP) production, impaired  $\text{Ca}^{2+}$  buffering by opening of the mitochondrial permeability transition pore (mPTP) and, in particular, the overproduction of reactive oxygen species (ROS) as found in in vitro cardiomyocytes [32] and in the brain [14]. In certain situations, such as mitochondrial  $\text{Ca}^{2+}$  overload induced by *N*-methyl-D-aspartate (NMDA) receptor overstimulation, cellular stability relies primarily upon energy production, i.e. mitochondrial function [57]. High intracellular  $\text{Ca}^{2+}$  levels damage the mitochondria by activating the  $\text{Ca}^{2+}$  sensitive protease calpain, which then cleaves mitofusin2 (MFN2),

leading to mitochondrial fragmentation [64]. As fragmentation of the mitochondrial network proceeds, it results in further neuronal damage because of progressive ATP depletion. The enhanced  $\text{Ca}^{2+}$  uptake into the mitochondria, combined with the increase in metabolic rate provoked by increased intracellular  $\text{Ca}^{2+}$ , results in the formation of ROS [52,57].

Increased intracellular  $\text{Ca}^{2+}$  levels ultimately also increase the  $\text{Ca}^{2+}$  in mitochondria, which triggers ROS production. ROS radicals will react with virtually any cellular component, such as carbohydrates, amino acids, DNA and phospholipids. Free radicals furthermore trigger a vicious cycle in the mitochondria, with inhibition of electron transport mechanisms leading to excess superoxide production and activation of apoptotic mechanisms. During cerebral ischemia complexes I, II, and III of the mitochondrial respiratory chain are damaged, leading to impaired electron transport and excess superoxide production [43]. ROS production is closely linked to excitotoxicity, energy loss and ionic imbalances. The CNS is a particularly vulnerable to ROS-mediated injury because it only holds moderate levels of endogenous antioxidants and antioxidant enzymes and these levels decrease rapidly following I/R injury. ROS induce a pro-apoptotic state in which generation of the Bcl-2 family members Bax/Bak permeabilize the mitochondrial membranes by creating large pores. Mitochondrial membrane permeabilization is a critical factor in determining the survival of neuronal cells. The permeabilization is initialized to counteract the effect of high intracellular  $\text{Ca}^{2+}$  levels have on mitochondrial  $\text{Ca}^{2+}$  homeostasis. Permeabilization of the mitochondrial outer membrane (MOM) results in the release of pro-apoptotic proteins from the intermembrane space to the cytoplasm, including cytochrome c, which can lead to apoptotic cell death [25]. Also, MOM permeabilization decreases mitochondrial ATP generation by disturbing the mitochondrial membrane potential ( $\Delta\Psi$ ) and thereby uncoupling the process of respiration from ATP synthase. The decrease in  $\Delta\Psi$  and subsequent uncoupling results from the opening of the mitochondrial permeability transition pore (mPTP) in response to elevated levels of mPTP activators ( $\text{Ca}^{2+}$ , ROS, inorganic phosphate from used ATP) and decreased levels of mPTP inhibitors (ATP/

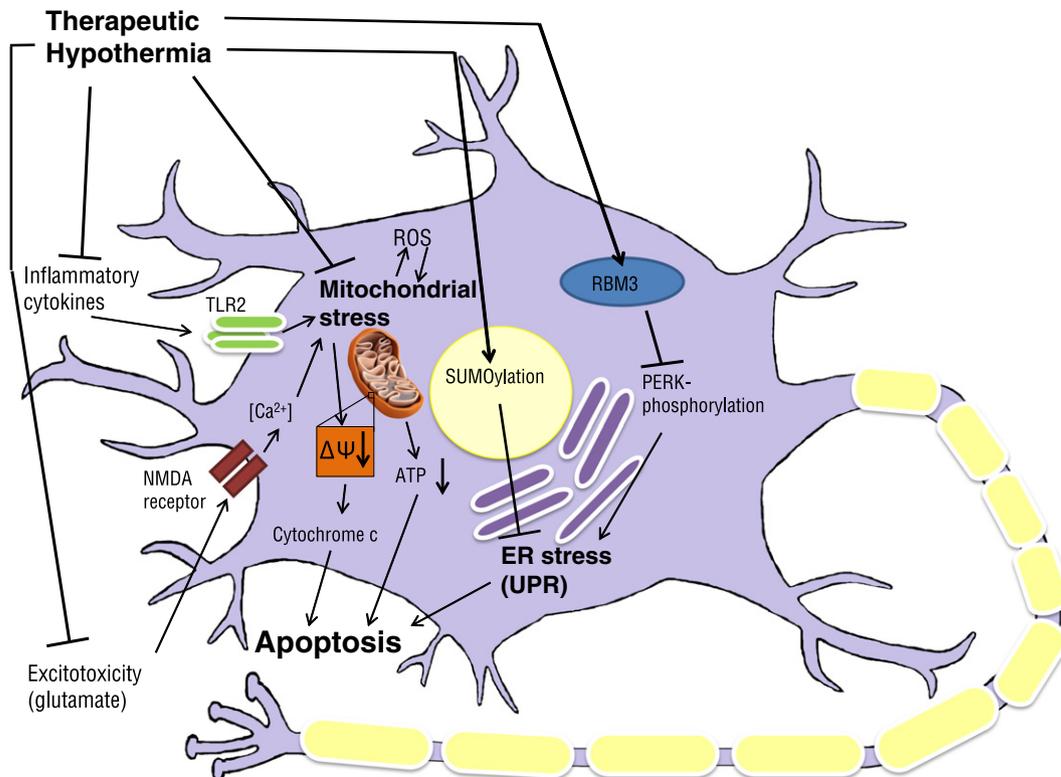


Fig. 1. Overview of protective properties of therapeutic hypothermia on molecular pathways in the neuron.

ADP). Long-lasting mPTP opening is a point-of-no-return in apoptosis [15]. Hereby, mitochondrial membrane permeabilization is a critical factor in determining the survival of a cell.

In response to mitochondrial damage, mitophagy is activated. Mitophagy constitutes a subtype of autophagy, a bulk degradation system sequestering and eliminating large cytosolic proteins, protein aggregates and organelles via the lysosome. Mitophagy removes impaired mitochondria in order to promote cellular survival and maintain mitochondrial integrity and function [41]. However, the I/R associated decrease in ATP production may lead to insufficient mitophagy and removal of damaged mitochondria, which can cause cell injury and may eventually lead to apoptosis or necrosis [71]. In conclusion, mitochondrial dysfunction is a main effector pathway of cellular injury in cerebral I/R injury related processes, such as excitotoxicity and activation of cell death.

### 2.1. Effect of targeted temperature management on mitochondrial dysfunction

TTM can prevent neuronal damage by diminishing mitochondrial dysfunction as demonstrated in a resuscitation model in Chinese minipigs [16], in a post-cardiac arrest model in rats [33] and in a neuronal cell model [22]. In a resuscitation model after 8 min of ventricular fibrillation, mild hypothermia reduces mitochondrial oxidative stress at 24 h following I/R by limiting mitochondrial membrane permeabilization, resulting in inhibition of opening the mPTP. Consequently, TTM limits the release of pro-apoptotic substances, caspase 3 cleavage and apoptosis, and maintains  $\Delta\Psi$  and mitochondrial respiration [15]. Similar effects were found in primary cultures of neuronal cells from 18-day-old Wistar rat cortex, in which moderate hypothermia at 30 °C was found to inhibit hypoxic neuronal cell death by reducing mitochondrial injury, maintaining  $\Delta\Psi$  and inhibiting apoptosis [22]. Moreover, hypothermic conditions displayed an anti-oxidant effect by upregulating manganese superoxide dismutase (MnSOD), which can detoxify free radical superoxide generated by mitochondrial respiration [16]. Additionally, TTM limits the impairment of mitochondrial respiratory chain enzymes complex I and III, thus precluding excess production of ROS by mitochondrial respiration [16]. Furthermore, hypothermia (32 °C) precluded the excessive activation of intracellular autophagy, including mitophagy, and limited neuronal injury when 4 h of hypothermia was applied immediately after resuscitation from cardiac arrest in rats [33]. Thus, TTM protects the structural integrity and function of mitochondria after I/R injury by reducing oxidative stress, inhibiting the opening of mPTP and precluding excessive mitophagy, and by inducing anti-oxidative properties.

### 3. Endoplasmic reticulum stress and SUMOylation

In addition to mitochondrial stress, I/R injury induces a cellular stress response in the endoplasmic reticulum (ER). In the ER of neuronal cells I/R injury induces an unfolded protein response (UPR), which is highly dependent on intensity and duration of the ischemic period. When the UPR is activated for an extended time, apoptosis will be induced via post-translational modification of phosphorylation of protein kinase RNA-like endoplasmic reticulum kinase (PERK), which enhances the transcription of CCAAT-enhancer-binding protein homologous protein (CHOP) as a part of the apoptosis pathway. CHOP is involved in ER stress-induced apoptosis by reducing the expression of Bcl-2 and inducing intracellular cleavage of caspase 12 and subsequent cleavage of caspase 3 as an executive apoptotic protein [19].

ER stress is subject to regulation via protein SUMOylation, i.e. a post-translational modification involving the conjugation of Small Ubiquitin-like Modifier (SUMO) proteins to target proteins, thus influencing protein stability, stress response, and cell proliferation and apoptosis. Similar to ubiquitination, SUMOylation comprises an enzymatic cascade, involving the action of activating enzyme E1 and conjugation

through enzyme E2 and E3 protein ligase. As the main target proteins for the SUMO conjugation are transcription factors, SUMOylation represents a form of transcriptional reprogramming, in which SUMOylation generally suppresses the transcription of target genes. However, SUMOylation of the active form of X-box-binding protein 1 (XBP1), a key transcription factor of the UPR, downregulates the transcriptional activity of XBP1 towards UPR target genes [3]. In addition, global SUMOylation has neuroprotective capacities after ischemic brain injury [28]. Moreover, SUMO conjugation increases in response to stress inducing stimuli, such as heat shock and high ROS production [54]. SUMOylation may increase via activation of the Ubc9-catalyzed conjugation process or by inhibition of SUMO-specific proteases, belonging to the sentrin-specific proteases (SENPs) family, of which there are six known in mammals (SEN1–3 and SEN5–7). Interestingly, acute ischemic injury of the spinal cord increases SENP3 expression, lasting for several days after the injury and coinciding with neuronal apoptosis [65].

### 3.1. Effects of hypothermia on UPR and SUMOylation

When the UPR fails and ER homeostasis is disturbed, it often leads to cellular dysfunction and cell death. Prolonged ER stress is part of the pathogenesis of cerebral ischemic damage and progression into neuronal death, and may be effectively inhibited by TTM. Mild hypothermia for 3 h after global ischemia was found to protect hippocampal neurons against I/R injury-induced ER stress [30]. Further, mild hypothermia (31 °C) limits the upregulation of CHOP I/R injury in rat hippocampus during a state of hypoxia after two-vessel occlusion and systemic hypotension [48].

Support for TTM conveying beneficial effects via increased SUMOylation was found in Ubiquitin conjugating enzyme 9 transgenic (Ubc9 Tg) mice, which overexpress the sole E2 SUMO conjugating enzyme and thereby display increased levels of SUMO-conjugation. Hypothermia had no additional beneficial effect in Ubc9 Tg mice, which have constitutively elevated levels of global SUMOylation even under normothermic conditions, in contrast to the WT controls [28]. Further, hypothermia was found to increase global SUMO-conjugation levels in SH-SY5Y cells and rat cortical neurons and protected the cells from oxygen-glucose-deprivation (OGD) induced cell death [28]. SUMO-2/3 silencing in cortical neurons decreased cell viability following OGD, which is consistent with prior observations [7]. Moreover, deep hypothermia induces translocation of SUMO conjugated proteins to the nuclei of neurons in rat brains, which resulted in decreased expression of several genes associated with pathological processes [69]. Importantly, SUMO conjugation is activated by a very rapidly cellular signaling pathway, as after 5 min of heat exposure to 43 °C there is already a decline in free SUMO-2/3 and an accumulation of conjugated SUMO-2/3 [54]. Regarding TTM in I/R injury, this rapid cellular signaling might explain the importance of early initiation of hypothermic management.

The key role of SUMOylation in TTM seems supported by findings in hibernation - the seasonal decrease of metabolism and core body temperature in hibernating species for a dedicated amount of time. A study in ground squirrels showed massive SUMOylation in the brain during hibernation [27]. In addition, free SUMO-2/3 disappeared and SUMO-2/3 conjugation of squirrels increased upon exposure to an ambient temperature of 4 °C. In contrast, free SUMO1 disappeared and conjugated SUMO1 increased only after onset of hibernation, and was rapidly normalized following rewarming [27]. While these observations are not conclusive, it is tempting to speculate that elevated global SUMOylation in the brain appears to be part of the neuroprotective effects against extreme circumstances seen during hibernation.

In summary, TTM inhibits the I/R injury induced endoplasmic reticulum stress and UPR. Part of this is due to TTM promoting SUMOylation, which likely represents an important mechanism to reduce overactivation of the UPR. Enhancing the cellular response

to ER stress and promoting SUMOylation constitute valuable targets for future protective strategies in I/R injury.

#### 4. Glutamate excitotoxicity as a mediator of ischemic neuronal injury

Another important factor induced by I/R injury is neurotoxicity, caused by the sudden release of high amounts of the excitotoxic neurotransmitter glutamate. Cellular energy reserves decrease due to reduced ATP production following I/R injury, which in neurons leads to increased release and impaired uptake of the excitatory neurotransmitter glutamate. Increased extracellular concentration of excitatory amino acids, particularly glutamate, act as mediators of neuronal damage during ischemia in the brain [63], the spinal cord [53], and the eye [55]. Glutamate is released from transmitter pools in glutamatergic neurons in the course of I/R injury, but also after traumatic insults and in neurodegenerative disorders [46]. By overstimulation of the *N*-methyl-D-aspartate (NMDA) receptor, glutamate leads to potentially lethal ionic derangements, such as excessive Na<sup>+</sup> and Ca<sup>2+</sup> influxes. Excitotoxicity is enhanced by depletion of cellular energy stores, thus leading to acceleration of neuronal damage when excessive glutamate release and I/R injury-induced mitochondrial dysfunction occur simultaneously. Neuronal excitotoxicity is difficult to investigate in human brains *in vivo*, because of ethical and technical difficulties. Ocular models for I/R injury allow *in vivo* investigation of neuronal excitotoxicity, due to similarities of retina to the brain in terms of anatomy, functionality, response to damage and immunology [31].

##### 4.1. Therapeutic hypothermia limits glutamate excitotoxicity

Experimental ocular injection of supraphysiological concentrations of glutamate provokes significant alterations in function and histology of the retina, which is prevented by hypothermic preconditioning of the eye to 33 °C [55]. In addition, mild ocular hypothermia applied 24 h before an ischemic event maintains normal retinal function and histology by preserving glutamate uptake and conversion of glutamate to the non-toxic glutamine by glutamine synthetase [55]. Glutamate levels not only increase following cerebral ischemia, but also after traumatic brain injury (TBI). In a rat model of TBI, mild hypothermia limited the increase in extracellular glutamate levels by precluding its release from astrocytes [29]. TTM mitigation of extracellular glutamate increase by astrocytes is accomplished by inhibition of its release through downregulation of connexin 43 (Cx43) and by promoting its uptake by upregulation of glutamate transporter 1 (GLT-1). Ultimately, TTM thus reduces brain edema and preserves neurocognitive dysfunction [29]. Further, microdialysis studies confirmed TTM to inhibit the release of glutamate, as selective cerebral deep hypothermia (18–20 °C) lowers glutamate levels following clamping of the common carotid arteries in rhesus monkeys [49] and reduces the release of glutamate in the spinal cord of a swine cardiopulmonary bypass model [53]. Moreover, TTM also protects cortical neurons and glia *in vitro* from excitotoxicity and apoptosis induced by glutamate and other excitatory amino acids, such as NMDA,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainite [63]. Thus, TTM can prevent excessive glutamate release and promote glutamate uptake *in vivo*.

Depth of TTM appears to play an important role in preventing glutamate induced neuronal injury. The impact of hypothermia on excitatory amino acid toxicity appears to be U shaped with an optimum between 20 °C and 35 °C, while temperatures of 12 °C are less effective [63]. Given the fact that mild hypothermia already inhibits release of excitotoxic neurotransmitters, there would be no additional benefit of lowering temperature beneath 30 °C. However, additional cooling from 30 °C to 20 °C conveys an additional protective effect, indicating that TTM at lower temperatures also inhibits excitotoxicity via postsynaptic mechanisms. Such postsynaptic protection by TTM is related to (temporary) maintenance of mitochondrial function and intracellular

Ca<sup>2+</sup> homeostasis (as described above) [63]. TTM precludes NMDA receptor mediated glutamate excitotoxicity and thereby maintains the activity of CaM kinase II  $\alpha$  (CaMK-II $\alpha$ ) and Ca<sup>2+</sup>-dependent PKC- $\alpha$ ,  $\beta$ ,  $\gamma$  isoforms, which all have specialized roles in Ca<sup>2+</sup> homeostasis and neuronal plasticity [5,42]. During I/R injury, both CaMK-II $\alpha$  and PKC isoforms translocate from the cytosol to the synaptosome [40]. Applying TTM (32 °C) inhibits this translocation of CaMK-II $\alpha$  and PKC isoforms after global forebrain I/R injury in rats [18]. Further, TTM prevents  $\alpha$ -fodrin proteolysis after I/R injury, likely by calpain inhibition, thus limiting neuronal damage during I/R injury and disruption of the cell membrane [18].

In summary, TTM limits glutamate induced excitotoxicity thus reducing related neuronal cell death by the mitigation of the increase in extracellular glutamate - by inhibiting its release and promoting its re-uptake - and by affecting cellular processes downstream of the NMDA receptor. However, depth of TTM appears to critically influence its therapeutic efficacy to limit ischemic injury, as found in cell cultures of cortical neurons. This observation indicates that there is a need for randomized controlled trials addressing the issue of temperature levels in order to optimize TTM.

#### 5. Cerebral ischemia induces a neuroinflammatory response

Ischemia induces the production of pro-inflammatory (chemotactic) cytokines, such as interleukin 1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) by glial cells and endothelial cells, as well as by circulating macrophages and neutrophils. Although the neuroinflammatory response is primarily neuroprotective, neuroinflammation can further progress and exacerbate neuronal injury. The release of cytokines in the extracellular space modulates the progression of neuronal I/R injury as neuroinflammatory cells are attracted to the site of injury through chemotaxis. Migrating neuroinflammatory cells are glial cells (astroglia and microglia), which can induce regional apoptotic cell death via mitochondria [39]. Moreover, TNF- $\alpha$  causes mitochondrial damage via the activation of tumor necrosis factor receptor 1 (TNF-R1) mediated release of the pro-apoptotic cytochrome c, decrease of mitochondrial  $\Delta\Psi$  and as a consequence, reduction of ATP production, which ultimately results in apoptosis of the cell [11]. On the other hand, anti-inflammatory cytokines, such as IL-4, IL-9, and IL-10, are considered to counterweigh the inflammatory cascade, and serve as a negative feedback system when propagated.

In addition, systemic inflammation may also provoke neuroinflammation and neuronal cell death through release of damage-associated molecular patterns (DAMPs). DAMPs can increase permeability of the blood-brain barrier (BBB) and initiate its breakdown [59]. Further, I/R injury upregulates brain Toll-like receptors (TLRs), a group of pathogen recognition receptors of the innate immune system. Circulating DAMPs activate TLRs after I/R injury, specifically TLR2 and TLR4 [24]. Consequently, intracellular pathways become activated, such as IKK-NF $\kappa$ B, and p38 mitogen-activated protein kinase (MAPK) and JNK1/2, which induce the transcription of inflammatory cytokines and chemokines, and apoptosis. Neuroinflammation is therefore an important target for neuroprotective strategies after I/R injury.

##### 5.1. The effect of hypothermia on neuroinflammation in neurons and glial cells

*In vitro*, low temperature reduces the TLR2-induced activation of NF- $\kappa$ B. Hypothermia induces downregulation of NF- $\kappa$ B by preventing the degradation of its inhibitor I $\kappa$ B- $\alpha$  in microglia [10]. Hypothermia induced reduction or delayed degradation of I $\kappa$ B- $\alpha$  may explain the decreased secretion of the pro-inflammatory cytokines TNF- $\alpha$  and monocyte chemoattractant protein-1 (MCP-1) of stimulated microglial cells even during and after rewarming [10]. Further neuroprotective effects of TTM are exemplified by attenuation of TLR2 mediated TNF- $\alpha$  and nitric oxide (NO) production at 33 °C in cultured rat microglia

[39]. Conversely, hyperthermia (39 °C) produces the opposite response and increases activation of NF- $\kappa$ B and subsequent production of TNF- $\alpha$  and NO in cultured microglia [39]. In addition to limiting production of pro-inflammatory cytokines including TNF- $\alpha$  and MCP-1, deeper cooling towards 20 °C was found to also increase anti-inflammatory components, especially cytokines IL-1 and IL-10, as found in activated primary mouse microglial cells at 72 h after LPS challenge [10].

In vivo, cooling to 30 °C in a rat stroke model inhibited cerebral mRNA expression of anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines INF- $\gamma$ , TNF- $\alpha$ , IL-2, IL-1 $\beta$  and MIP-2, and limited the infiltration of systemic inflammatory cells [17].

In addition to the production of inflammatory cytokines, microglia can also induce ROS associated damage. Moderate hypothermia decreases the production of ROS and reduces the proliferation of microglia in cell cultures [60]. Interestingly, moderate hypothermia affects the proliferation of microglia more than the proliferation of astrocytes and fibroblasts [60], thus suggesting that TTM primarily mitigates microglia-mediated neuroinflammation and apoptosis.

### 5.2. Effects of hypothermia on endothelial cell-mediated neuroinflammation

The blood-brain barrier – consisting of endothelial cells – plays a pivotal role in the balance of inflammatory processes. Endothelial cells are part of the inflammatory pathway because of their ability to produce and regulate inflammatory cytokines. In a recent study, in vitro endothelial cells were activated with TNF- $\alpha$  and incubated under mild (32 °C) and deep (17 °C) hypothermia [9]. Downregulation of NF- $\kappa$ B dependent pro-inflammatory gene expression by hypothermia caused a decrease in chemotactic cytokines that recruit leukocytes in vivo [9]. Mild hypothermia (33 °C) inhibited TNF- $\alpha$ -induced phosphorylation of p38 MAPK and JNK1/2 in endothelial cells treated with TNF- $\alpha$ , leading to a decrease in apoptotic cell death. TTM also induces MAPK phosphatase 1 (MKP-1), which serves as a protective mechanism of mild hypothermia against TNF- $\alpha$ -induced hyperpermeability, actin reorganization, and apoptosis [68]. Downstream effects of hypothermia comprise decreased protein expression of pro-inflammatory chemokines and cytokines, such as IL-8, MCP-1 and COX-2 as a consequence of downregulation of NF- $\kappa$ B. Further, mild hypothermia up-regulated Bcl-2 and IL-6 in endothelium cells compared to the normothermic cells. Bcl-2 protects mitochondria by increasing the ability to resist high calcium levels. IL-6 has potent pro- and anti-inflammatory and cytoprotective properties and inhibits apoptosis via different mechanisms including the upregulation of Bcl-2 family proteins [9].

Thus, protective effects of TTM on neuroinflammation rely not only on the regulation of Toll-like receptors, but also on intracellular pathways, such as IKK-NF $\kappa$ B, p38 MAPK and JNK1/2, and regulation of both pro- and anti-inflammatory cytokines and chemokines, both in neuronal and endothelial cells in the brain.

## 6. Neuroprotection by upregulation of cold shock proteins

During hypothermia, a small subset of proteins, called cold shock proteins, escapes the downregulation of general metabolism and protein synthesis. The cold shock proteins RNA binding protein 3 (RBM3) and cold-inducible RNA binding protein (CIRP) are expressed at high levels in the brain during cooling and hibernation [47]. The neuroprotective properties of RBM3 have been well documented, e.g. by RBM3 protecting against neuronal cell death induced by prion disease [47]. In addition, elevated levels of RBM3, induced either by hypothermia or through lentiviral delivery, protect synapses, prevent behavioral deficits and neuronal loss, and prolong survival in an Alzheimer mice model [47]. Further, RBM3 is also involved in the protection of neurons in hypoxic ischemia by blocking the UPR. This mechanism underlying RBM3 neuroprotection was identified in organotypic slice cultures from RBM3 knockout mice, in which both hypothermia and RBM3 were found to suppress the PERK-eIF2 $\alpha$ -CHOP pathway in vitro and

in vivo. RBM3 accomplished hypoxic survival via inhibition of PERK phosphorylation, ultimately resulting in reduced apoptosis [73]. The effect of temperature on induction of RBM3 expression has been investigated in brain slices and several neuronal cell cultures were exposed to mild and deep hypothermia. Mild hypothermia increased mRNA and protein levels of RBM3 and CIRP in brain slices and neuronal cell lines, without any additional effect upon deeper cooling [62]. In conclusion, hypothermia might increase RBM3 levels and thereby decrease ER stress and prevent apoptosis in neurons after I/R injury. Upregulation of RBM3 during cooling and hibernation suggests that the elevation of cold shock protein is a protective mechanism of hypothermia. Other protective proteins and signaling pathways remain subject of investigation.

## 7. Mimicking the effects of TTM pharmacologically

Despite the protective effects of TTM, its clinical use may be hampered by side-effects, such as the production of ROS upon rewarming, but also electrolyte shifts, hemodynamic changes, cardiac arrhythmias, and seizures. Pharmacologically mimicking the cellular effects of TTM may constitute an alternative approach to specifically target the molecular pathophysiology of I/R injury. The section below addresses only experimental studies, as the substances have not yet been tested for their neuroprotective effect after I/R injury in patients.

### 7.1. Targeting mitochondrial dysfunction

Cerebral I/R injury can improve by pretreatment with the H<sub>2</sub>S releasing agent NaHS via reducing ROS production and inhibiting OGD-induced mitochondrial dysfunction in rats [70]. Additionally, NaHS was proven to sustain  $\Delta\Psi$  and inhibit caspase 3-mediated apoptosis in cortical neurons [34]. Thus, NaHS mimicks the effect of TTM on mitochondrial function after I/R injury. Activation of  $\Delta$ -opioid receptors (DOR) was found to be neuroprotective by regulating ion homeostasis, which is crucial for mitochondrial function. Beneficial effects are found when using the synthetic  $\Delta$ -opioid peptide [D-Ala<sup>2</sup>,D-Leu<sup>5</sup>] enkephalin (DADLE), which prevents secondary neuronal injury after I/R injury [2]. Release of opioid-like proteins has been hypothesized to play a protective role during hibernation states in hibernating mammals, as it appears that activation of opioid receptors prolongs metabolic suppression [21].

### 7.2. Targeting the UPR

Sodium phenylbutyrate (4-PBA), an aromatic fatty acid known to improve protein folding, is a new strategy targeting ER stress as found in a rat stroke model with induced type 2 diabetes featuring severe and prolonged activation of the UPR [61]. 4-PBA was shown to reduce infarct size and improve neurobehavioral function outcome [61]. It was then theorized to improve ER folding capacity and inhibit the UPR by diminishing the induction of CHOP and caspase 12. Manipulating levels of ER stress by targeting the UPR might be a novel strategy in mimicking TTM. ER stress targeted compounds are fairly novel, but show promising results as protective agents in the experimental setting. For example, binding immunoglobulin protein inducer X (BIX) was identified in a screen for compounds that induce the expression of binding immunoglobulin protein (BiP), a molecular chaperone present in the ER. BIX treatment reduced the infarct volume in mouse models of middle cerebral artery occlusion and protected photoreceptors against light-induced cell death [20]. Salubrinal is a specific inhibitor of eIF2 $\alpha$  phosphatase enzymes, which is capable of inhibiting I/R injury-induced ER stress. Inhibiting eIF2 $\alpha$  phosphatase increases the levels of eIF2 $\alpha$ -phosphorylation, reducing translation rates and activating downstream ATF4 signaling. ATF4 is a major component in regulated cell death, and sustained ATF4 expression can result in apoptosis.

### 7.3. Targeting excitotoxicity

Salubrinal can also reduce neuronal death after excitotoxicity in the hippocampus, and also alleviate neurodegeneration in models of Parkinson's disease and ALS [20]. Attempts to limit glutamate excitotoxicity by inducing overall downregulation of ion channels, e.g. with the  $\text{Na}^+/\text{K}^+$  ATPase inhibitor ouabain, have failed [8]. Other approaches might be to actively reduce glutamate via tempol [12] or to antagonize NMDA, e.g. with Memantine [4] or PSD-95 inhibitor [6], or AMPA, e.g. with Perapamel [51].

### 7.4. Targeting neuroinflammation

Antibodies against TNF- $\alpha$  and TNF binding proteins demonstrated protection against I/R injury in a stroke model [38]. In addition, non-steroid anti-inflammatory drugs (NSAIDs) are considered to potentially inhibit neuroinflammation. However, regular NSAIDs have limited penetration in the brain. Newly synthesized NSAID conjugates, some of them with  $\text{H}_2\text{S}$  releasing properties, suppress the production of inflammatory cytokines and NO after LPS stimulation in various neuronal and glial cell lines [67]. Nevertheless, such compounds are still far away from clinical usage, as in vivo data on brain penetration and subsequent effects are lacking.

Adenosine monophosphate-activated protein kinase (AMPK) might be a target, as it is known that AMPK inhibition after cerebral ischemic injury inhibits excessive activation of astrocytes and microglia and release of microglial pro-inflammatory factors [36]. 5'-AMP is implied in stimulation of a hypometabolic state after its activation by the adenosine A2B receptor. The signaling pathways activated by 5'-AMP might be an important pharmacological target to safely suppress both metabolism and the immune system [1], leading to neuroprotection following I/R injury. In addition, central A1 adenosine receptor (A1AR) activation or administration of 5'-AMP can chemically induce hypothermia in mice [44]. Also, the cannabinoid receptor agonist, WIN55, 212-2 has anti-inflammatory properties and chemically induces hypothermia. It has shown higher survival rates after cardiac arrest in a rat model [35]. Chemically induced hypothermia approximates induction of hibernation. This might also prove beneficial on other molecular pathways, either known to be influenced by TTM or to usher protective properties in hibernating species.

Potential disadvantages of mimicking hypothermia pharmacologically in permanent stroke are the limited penetration of drugs in the ischemic area, as blood flow can be halted here. Also, changes in BBB function in the course of stroke might alter brain distribution of drugs. To address this problem, gene therapy has been proposed as an alternative strategy in stroke, in which injection of adeno-associated viruses (AAVs) in the brain I/R injury area delivers therapeutic genes enhancing e.g. the UPR [20], for instance with RBM3 [47]. Nevertheless, efficacy may be seriously jeopardized since gene therapy relies on new protein synthesis, which is markedly impaired after I/R injury in the ischemic area.

## 8. Conclusion

I/R injury results in neurological damage because of mitochondrial dysfunction and oxidative injury, ER stress, glutamate excitotoxicity, and neuroinflammation. TTM has been proven to encompass a beneficial action in brain I/R injury, which depends on the targeted temperature and may in turn dictate the specific defense pathways recruited. TTM mitigates I/R injury induced apoptosis pathways by addressing all before mentioned pathways. TTM may also act as a neuroprotective agent by promoting global SUMOylation and inhibiting excessive UPR. Druggable targets in the latter pathways are relatively new, yet promising. Problems lie in the limited penetration of drugs in brain areas affected by I/R injury and stroke. Gene therapy has potential benefits, yet is far from suitable to apply in humans at this day and age.

Another way to mimic TTM is by pharmacological induction of hypometabolism, for instance by administration of 5'-AMP. Thus far, TTM forms the best therapeutic strategy to clinically deploy hypothermia-induced neuroprotective pathways against I/R injury. Therefore, we suggest combining TTM with specific pharmacological interventions to further boost protective temperature-related molecular pathways without inducing the negative effects associated with deep cooling, may improve neurological outcome in different I/R injury related neurological conditions.

## Transparency document

The [Transparency document](#) associated with this article can be found, in online version.

## Acknowledgements

None of the authors has any external funding or competing interest to declare.

## References

- [1] H.R. Bouma, J.N. Mandl, A.M. Strijkstra, A.S. Boerema, J.W. Kok, A. van Dam, A. Ijzerman, F.G. Kroese, R.H. Henning, 5'-AMP impacts lymphocyte recirculation through activation of A2B receptors, *J. Leukoc. Biol.* 94 (1) (2013) 89–98, <http://dx.doi.org/10.1189/jlb.1212613>.
- [2] D. Chao, Y. Xia, Ionic storm in hypoxic/ischemic stress: can opioid receptors subside it? *Prog. Neurobiol.* 90 (4) (2010) 439–470, <http://dx.doi.org/10.1016/j.pneurobio.2009.12.007>.
- [3] H. Chen, L. Qi, SUMO modification regulates the transcriptional activity of XBP1, *Biochem. J.* 429 (1) (2010) 95–102, <http://dx.doi.org/10.1042/BJ20100193>.
- [4] Z.Z. Chen, D.D. Yang, Z. Zhao, H. Yan, J. Ji, X.L. Sun, Memantine mediates neuroprotection via regulating neurovascular unit in a mouse model of focal cerebral ischemia, *Life Sci.* 150 (2016) 8–14, <http://dx.doi.org/10.1016/j.lfs.2016.02.081>.
- [5] Y. Chu, D. Fioravante, M. Leitges, W.G. Regehr, Calcium-dependent PKC isoforms have specialized roles in short-term synaptic plasticity, *Neuron* 82 (4) (2014) 859–871, <http://dx.doi.org/10.1016/j.neuron.2014.04.003>.
- [6] D.J. Cook, L. Teves, M. Tymianski, Treatment of stroke with a PSD-95 inhibitor in the gyrencephalic primate brain, *Nature* 483 (7388) (2012) 213–217, <http://dx.doi.org/10.1038/nature10841>.
- [7] A.L. Datwyler, G. Lättig-Tünnemann, W. Yang, W. Paschen, S.L. Lee, U. Dirnagl, M. Endres, C. Harms, SUMO2/3 conjugation is an endogenous neuroprotective mechanism, *J. Cereb. Blood Flow Metab.* 31 (11) (2011) 2152–2159, <http://dx.doi.org/10.1038/jcbfm.2011.112>.
- [8] K.R. Dave, S.L. Christian, M.A. Perez-Pinzon, K.L. Drew, Neuroprotection: lessons from hibernators, *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 162 (1–3) (May 2012) 1–9, <http://dx.doi.org/10.1016/j.cbpb.2012.01.008>.
- [9] A. Diestel, J. Roessler, F. Berger, K.R. Schmitt, Hypothermia downregulates inflammation but enhances IL-6 secretion by stimulated endothelial cells, *Cryobiology* 57 (3) (2008) 216–222, <http://dx.doi.org/10.1016/j.cryobiol.2008.08.005>.
- [10] A. Diestel, S. Troeller, N. Billecke, I.M. Sauer, F. Berger, K.R. Schmitt, Mechanisms of hypothermia-induced cell protection mediated by microglial cells in vitro, *Eur. J. Neurosci.* 31 (5) (2010) 779–787, <http://dx.doi.org/10.1111/j.1460-9568.2010.07128.x>.
- [11] D.N. Doll, S.L. Rellick, T.L. Barr, X. Ren, J.W. Simpkins, Rapid mitochondrial dysfunction mediates TNF-alpha-induced neurotoxicity, *J. Neurochem.* 132 (4) (2015) 443–451, <http://dx.doi.org/10.1111/jnc.13008>.
- [12] P. Dohare, M.C. Hyzinski-García, A. Vipani, N.H. Bowens, J.W. Nalwalk, P.J. Feustel, R.W. Keller Jr., D. Jourdain, A.A. Mongin, The neuroprotective properties of the superoxide dismutase mimetic tempol correlate with its ability to reduce pathological glutamate release in a rodent model of stroke, *Free Radic. Biol. Med.* 77 (2014) 168–182, <http://dx.doi.org/10.1016/j.freeradbiomed.2014.08.029>.
- [13] M. Forkmann, S. Kolschmann, L. Holzhauser, K. Ibrahim, M. Guenther, M. Christoph, J.T. Fuhrmann, A. Boscheri, A. Schmeißer, R.H. Strasser, C. Wunderlich, Target temperature management of 33 degrees C exerts beneficial haemodynamic effects after out-of-hospital cardiac arrest, *Acta Cardiol.* 70 (4) (2015) 451–459.
- [14] M.Y. Globus, R. Busto, B. Lin, H. Schnipper, M.D. Ginsberg, Detection of free radical activity during transient global ischemia and recirculation: effects of intrasystemic brain temperature modulation, *J. Neurochem.* 65 (3) (1995) 1250–1256.
- [15] P. Gong, R. Hua, Y. Zhang, H. Zhao, Z. Tang, X. Mei, ... C. Li, Hypothermia-induced neuroprotection is associated with reduced mitochondrial membrane permeability in a swine model of cardiac arrest, *J. Cereb. Blood Flow Metab.* 33 (6) (2013) 928–934, <http://dx.doi.org/10.1038/jcbfm.2013.33>.
- [16] P. Gong, C.S. Li, R. Hua, H. Zhao, Z.R. Tang, X. Mei, ... J. Cui, Mild hypothermia attenuates mitochondrial oxidative stress by protecting respiratory enzymes and upregulating MnSOD in a pig model of cardiac arrest, *PLoS One* 7 (4) (2012), e35313, <http://dx.doi.org/10.1371/journal.pone.0035313>.
- [17] L.J. Gu, X.X. Xiong, T. Ito, J. Lee, B.H. Xu, S. Krams, ... H. Zhao, Moderate hypothermia inhibits brain inflammation and attenuates stroke-induced immunodepression in rats, *CNS Neurosci. Ther.* 20 (1) (2014) 67–75, <http://dx.doi.org/10.1111/cns.12160>.

- [18] K. Harada, T. Maekawa, R. Tsuruta, T. Kaneko, D. Sadamitsu, T. Yamashima, K. Yoshida Ki, Hypothermia inhibits translocation of CaM kinase II and PKC- $\alpha$ , beta, gamma isoforms and fodrin proteolysis in rat brain synaptosome during ischemia-reperfusion, *J. Neurosci. Res.* 67 (5) (2002) 664–669, <http://dx.doi.org/10.1002/jnr.10159>.
- [19] C. Hetz, The unfolded protein response: controlling cell fate decisions under ER stress and beyond, *Nat. Rev. Mol. Cell Biol.* 13 (2012) 89–102, <http://dx.doi.org/10.1038/nrm3270>.
- [20] C. Hetz, E. Chevet, H.P. Harding, Targeting the unfolded protein response in disease. *Nature reviews, Drug Discov.* 12 (9) (2013) 703–719, <http://dx.doi.org/10.1038/nrd3976>.
- [21] N.D. Horton, D.J. Kaftani, D.S. Bruce, E.C. Bailely, A.S. Krober, J.R. Jones, M. Turker, N. Khattar, T.P. Su, S.F. Bolling, P.R. Oeltgen, Isolation and partial characterization of an opioid-like 88 kDa hibernation-related protein, *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 119 (4) (1998) 787–805.
- [22] Y. Hua, K. Hisano, Y. Morimoto, Effect of mild and moderate hypothermia on hypoxic injury in nearly pure neuronal culture, *J. Anesth.* 24 (5) (2010) 726–732, <http://dx.doi.org/10.1007/s00540-010-0999-x>.
- [23] Hypothermia after Cardiac Arrest Study Group, Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest, *New. Engl. J. Med.* 346 (8) (2002) 549–556.
- [24] Y. Kong, Y. Le, Toll-like receptors in inflammation of the central nervous system, *Int. Immunopharmacol.* 11 (10) (2011) 1407–1414, <http://dx.doi.org/10.1016/j.intimp.2011.04.025>.
- [25] G. Kroemer, L. Galluzzi, C. Brenner, Mitochondrial membrane permeabilization in cell death, *Physiol. Rev.* 87 (1) (2007) 99–163 (doi:87/1/99).
- [26] M.C. Lai, S.N. Yang, Perinatal hypoxic-ischemic encephalopathy, *J. Biomed. Biotechnol.* (2011) 609813, <http://dx.doi.org/10.1155/2011/609813>.
- [27] Y.J. Lee, J.M. Hallenbeck, SUMO and ischemic tolerance, *NeuroMolecular Med.* 15 (4) (2013) 771–781, <http://dx.doi.org/10.1007/s12017-013-8239-9>.
- [28] Y.J. Lee, Y. Mou, D. Klimanis, J.D. Bernstock, J.M. Hallenbeck, Global SUMOylation is a molecular mechanism underlying hypothermia-induced ischemic tolerance, *Front. Cell. Neurosci.* 8 (2014) 416, <http://dx.doi.org/10.3389/fncel.2014.00416>.
- [29] Y.H. Li, C.L. Zhang, X.Y. Zhang, H.X. Zhou, L.L. Meng, Effects of mild induced hypothermia on hippocampal connexin 43 and glutamate transporter 1 expression following traumatic brain injury in rats, *Mol. Med. Rep.* 11 (3) (2015) 1991–1996, <http://dx.doi.org/10.3892/mmr.2014.2928>.
- [30] X. Liu, M. Wang, H. Chen, Y. Guo, F. Ma, F. Shi, Y. Bi, Y. Li, Hypothermia protects the brain from transient global ischemia/reperfusion by attenuating endoplasmic reticulum response-induced apoptosis through CHOP, *PLoS One* 8 (1) (2013), e53431 <http://dx.doi.org/10.1371/journal.pone.0053431>.
- [31] A. London, I. Benhar, M. Schwartz, The retina as a window to the brain—from eye research to CNS disorders, *Nat. Rev. Neurol.* 9 (1) (2013) 44–53, <http://dx.doi.org/10.1038/nrneuro.2012.227>.
- [32] G. Loor, J. Kondapalli, H. Iwase, N.S. Chandel, G.B. Waypa, R.D. Guzy, T.L. Vanden Hoek, P.T. Schumacker, Mitochondrial oxidant stress triggers cell death in simulated ischemia-reperfusion, *Biochim. Biophys. Acta* 1813 (7) (2011) 1382–1394, <http://dx.doi.org/10.1016/j.bbamcr.2010.12.008>.
- [33] J. Lu, H.Y. Qian, L.J. Liu, B.C. Zhou, Y. Xiao, J.N. Mao, ... C.L. Zhu, Mild hypothermia alleviates excessive autophagy and mitophagy in a rat model of asphyxial cardiac arrest, *Neurol. Sci.* 35 (11) (2014) 1691–1699, <http://dx.doi.org/10.1007/s10072-014-1813-6>.
- [34] Y. Luo, X. Yang, S. Zhao, C. Wei, Y. Yin, T. Liu, S. Jiang, J. Xie, X. Wan, M. Mao, J. Wu, Hydrogen sulfide prevents OGD/R-induced apoptosis via improving mitochondrial dysfunction and suppressing an ROS-mediated caspase-3 pathway in cortical neurons, *Neurochem. Int.* 63 (8) (2013) 826–831, <http://dx.doi.org/10.1016/j.neuint.2013.06.004>.
- [35] L. Ma, X. Lu, J. Xu, S. Sun, W. Tang, Improved cardiac and neurologic outcomes with postresuscitation infusion of cannabinoid receptor agonist WIN55, 212-2 depend on hypothermia in a rat model of cardiac arrest, *Crit. Care Med.* 42 (1) (2014) e42–e48, <http://dx.doi.org/10.1097/CCM.0b013e31829a8753>.
- [36] Y. Ma, J. Bu, H. Dang, J. Sha, Y. Jing, A.I. Shan-jiang, H. Li, Y. Zhu, Inhibition of adenosine monophosphate-activated protein kinase reduces glial cell-mediated inflammation and induces the expression of Cx43 in astroglia after cerebral ischemia, *Brain Res.* 1605 (2015) 1–11, <http://dx.doi.org/10.1016/j.brainres.2014.11.030>.
- [37] G.B. Mackensen, D.L. McDonagh, D.S. Warner, Perioperative hypothermia: use and therapeutic implications, *J. Neurotrauma* 26 (3) (2009) 342–358, <http://dx.doi.org/10.1089/neu.2008.0596>.
- [38] A. Martin-Villalba, M. Hahne, S. Kleber, J. Vogel, W. Falk, J. Schenkel, P.H. Kramer, Therapeutic neutralization of CD95-ligand and TNF attenuates brain damage in stroke, *Cell Death Differ.* 8 (7) (2001) 679–686.
- [39] T. Matsui, M. Tasaki, T. Yoshioka, Y. Motoki, H. Tsuneoka, J. Nojima, Temperature- and time-dependent changes in TLR2-activated microglial NF- $\kappa$ B activity and concentrations of inflammatory and anti-inflammatory factors, *Intensive Care Med.* 38 (8) (2012) 1392–1399, <http://dx.doi.org/10.1007/s00134-012-2591-3>.
- [40] S. Matsumoto, M. Shamloo, E. Matsumoto, A. Isshiki, T. Wieloch, Protein kinase C-gamma and calcium/calmodulin-dependent protein kinase II- $\alpha$  are persistently translocated to cell membranes of the rat brain during and after middle cerebral artery occlusion, *J. Cereb. Blood Flow Metab.* 24 (1) (Jan 2004) 54–61.
- [41] P. Mishra, D.C. Chan, Metabolic regulation of mitochondrial dynamics, *J. Cell Biol.* (Feb 8 2016) (pii: jcb.201511036).
- [42] M. Morioka, K. Fukunaga, S. Nagahiro, M. Kurino, Y. Ushio, E. Miyamoto, Glutamate-induced loss of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II activity in cultured rat hippocampal neurons, *J. Neurochem.* 64 (5) (1995) 2132–2139.
- [43] M.A. Moro, A. Almeida, J.P. Bolaños, I. Lizasoain, Mitochondrial respiratory chain and free radical generation in stroke, *Free Radic. Biol. Med.* 39 (10) (2005) 1291–1304.
- [44] M. Muzzi, F. Blasi, A. Masi, E. Coppi, C. Traini, R. Felici, M. Pittelli, L. Cavone, A.M. Pugliese, F. Moroni, A. Chiarugi, Neurological basis of AMP-dependent thermoregulation and its relevance to central and peripheral hyperthermia, *J. Cereb. Blood Flow Metab.* 33 (2013) 183–190, <http://dx.doi.org/10.1038/jcbfm.2012.157>.
- [45] N. Nielsen, et al., T.T.M. Trial Investigators, Targeted temperature management at 33 °C versus 36 °C after cardiac arrest, *N. Engl. J. Med.* 369 (22) (2013) 2197–2206, <http://dx.doi.org/10.1056/NEJMoa1310519>.
- [46] Y. Nishizawa, Glutamate release and neuronal damage in ischemia, *Life Sci.* 69 (4) (2001) 369–381 (doi:S0024320501011420).
- [47] D. Peretti, A. Bastide, H. Radford, N. Verity, C. Molloy, M.G. Martin, ... G.R. Mallucci, RBM3 mediates structural plasticity and protective effects of cooling in neurodegeneration, *Nature* 518 (7538) (2015) 236–239, <http://dx.doi.org/10.1038/nature14142>.
- [48] G.K. Poone, H. Hasseldam, N. Munkholm, R.S. Rasmussen, N.V. Grønberg, F.F. Johansen, The hyperthermic influence on CHOP and Ero1- $\alpha$  in an endoplasmic reticulum stress model of cerebral ischemia, *Brain Sci.* 15:5 (2) (2015) 178–187, <http://dx.doi.org/10.3390/brainsci5020178>.
- [49] J. Pu, X. Niu, J. Zhao, Excitatory amino acid changes in the brains of rhesus monkeys following selective cerebral deep hypothermia and blood flow occlusion, *Neural Regeneration Res.* 8 (2) (2013) 143–148, <http://dx.doi.org/10.3969/j.issn.1673-5374.2013.02.006>.
- [50] S. Pundik, K. Xu, S. Sundararajan, Reperfusion brain injury: focus on cellular bioenergetics, *Neurology* 79 (13 Suppl 1) (2012) S44–S51 (doi:79/13\_Supplement\_1/S44).
- [51] I. Rektor, Perampanel, a novel, non-competitive, selective AMPA receptor antagonist as adjunctive therapy for treatment-resistant partial-onset seizures, *Expert. Opin. Pharmacother.* 14 (2) (2013) 225–235, <http://dx.doi.org/10.1517/14656566.2013.754883>.
- [52] J.J. Reynolds, T.G. Hastings, Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation, *J. Neurosci.* 15 (5 Pt 1) (1995) 3318–3327.
- [53] C.K. Rokkas, C.S. Cronin, T. Nitta, L.R. Helfrich Jr., D.C. Lobner, D.W. Choi, N.T. Kouchoukos, Profound systemic hypothermia inhibits the release of neurotransmitter amino acids in spinal cord ischemia, *J. Thorac. Cardiovasc. Surg.* 110 (1) (1995) 27–35 (doi:S0022522395001395).
- [54] H. Saitoh, J. Hinchey, Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3, *J. Biol. Chem.* 275 (9) (Mar 3 2000) 6252–6258.
- [55] E.M. Salido, D. Dorfman, M. Bordone, M. Chianelli, M.F. Gonzalez Fleitas, R.E. Rosenstein, Global and ocular hypothermic preconditioning protect the rat retina from ischemic damage, *PLoS One* 8 (4) (2013), e61656, <http://dx.doi.org/10.1371/journal.pone.0061656>.
- [56] T.H. Sanderson, C.A. Reynolds, R. Kumar, K. Przyklenk, M. Huttemann, Molecular mechanisms of ischemia-reperfusion injury in brain: pivotal role of the mitochondrial membrane potential in reactive oxygen species generation, *Mol. Neurobiol.* 47 (1) (2013) 9–23, <http://dx.doi.org/10.1007/s12035-012-8344-z>.
- [57] A.F. Schinder, E.C. Olson, N.C. Spitzer, M. Montal, Mitochondrial dysfunction is a primary event in glutamate neurotoxicity, *J. Neurosci.* 16 (19) (1996) 6125–6133.
- [58] S. Shankaran, A.R. Lupton, R.A. Ehrenkranz, J.E. Tyson, S.A. McDonald, E.F. Donovan, ... A.H. Jobe, National Institute of Child Health and Human Development Neonatal Research Network, Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy, *New. Engl. J. Med.* 353 (15) (2005) 1574–1584.
- [59] T. Shichita, M. Ito, A. Yoshimura, Post-ischemic inflammation regulates neural damage and protection, *Front. Cell. Neurosci.* 14 (8) (2014) 319, <http://dx.doi.org/10.3389/fncel.2014.00319>.
- [60] Q.S. Si, Y. Nakamura, K. Kataoka, Hypothermic suppression of microglial activation in culture: inhibition of cell proliferation and production of nitric oxide and superoxide, *Neuroscience* 81 (1) (1997) 223–229 (doi:S0306-4522(97)00172-3).
- [61] K. Srinivasan, S.S. Sharma, Sodium phenylbutyrate ameliorates focal cerebral ischemic/reperfusion injury associated with comorbid type 2 diabetes by reducing endoplasmic reticulum stress and DNA fragmentation, *Behav. Brain Res.* 20:225 (1) (2011) 110–116, <http://dx.doi.org/10.1016/j.bbr.2011.07.004>.
- [62] G. Tong, S. Endersfelder, L.M. Rosenthal, S. Wollersheim, I.M. Sauer, C. Bührer, ... K.R. Schmitt, Effects of moderate and deep hypothermia on RNA-binding proteins RBM3 and CIRP expressions in murine hippocampal brain slices, *Brain Res.* 1504 (2013) 74–84, <http://dx.doi.org/10.1016/j.brainres.2013.01.041>.
- [63] M. Tymianski, R. Sattler, J.M. Zabramski, R.F. Spetzler, Characterization of neuroprotection from excitotoxicity by moderate and profound hypothermia in cultured cortical neurons unmasks a temperature-insensitive component of glutamate neurotoxicity, *J. Cereb. Blood Flow Metab.* 18 (8) (1998) 848–867, <http://dx.doi.org/10.1097/00004647-199808000-00005>.
- [64] W. Wang, F. Zhang, L. Li, F. Tang, S.L. Siedlak, H. Fujioka, ... X. Wang, MFN2 couples glutamate excitotoxicity and mitochondrial dysfunction in motor neurons, *J. Biol. Chem.* 290 (1) (2015) 168–182, <http://dx.doi.org/10.1074/jbc.M114.617167>.
- [65] H. Wei, H. Teng, W. Huan, S. Zhang, H. Fu, F. Chen, ... J. Zhao, An upregulation of SENP3 after spinal cord injury: implications for neuronal apoptosis, *Neurochem. Res.* 37 (12) (2012) 2758–2766, <http://dx.doi.org/10.1007/s11064-012-0869-z>.
- [66] V. Weinrauch, P. Safar, S. Tisherman, K. Kuboyama, A. Radovsky, Beneficial effect of mild hypothermia and detrimental effect of deep hypothermia after cardiac arrest in dogs, *Stroke* 23 (10) (1992) 1454–1462.
- [67] Z. Xu, J. Wu, J. Zheng, H. Ma, H. Zhang, X. Zhen, L.T. Zheng, X. Zhang, Design, synthesis and evaluation of a series of non-steroidal anti-inflammatory drug conjugates as novel neuroinflammatory inhibitors, *Int. Immunopharmacol.* 25 (2) (2015) 528–537, <http://dx.doi.org/10.1016/j.intimp.2015.02.033>.
- [68] D. Yang, P. Xie, S. Guo, H. Li, Induction of MAPK phosphatase-1 by hypothermia inhibits TNF- $\alpha$ -induced endothelial barrier dysfunction and apoptosis, *Cardiovasc. Res.* 85 (3) (2010) 520–529, <http://dx.doi.org/10.1093/cvr/cvp323>.

- [69] W. Yang, Q. Ma, G.B. Mackensen, W. Paschen, Deep hypothermia markedly activates the small ubiquitin-like modifier conjugation pathway; implications for the fate of cells exposed to transient deep hypothermic cardiopulmonary bypass, *J. Cereb. Blood Flow Metab.* 29 (5) (2009) 886–890, <http://dx.doi.org/10.1038/jcbfm.2009.16>.
- [70] Q. Yu, Z. Lu, L. Tao, L. Yang, Y. Guo, Y. Yang, X. Sun, Q. Ding, ROS-dependent neuroprotective effects of NaHS in ischemia brain injury involves the PARP/AIF pathway, *Cell. Physiol. Biochem.* 36 (4) (2015) 1539–1551.
- [71] Y. Yuan, X. Zhang, Y. Zheng, Z. Chen, Regulation of mitophagy in ischemic brain injury, *Neurosci. Bull.* 31 (4) (2015) 395–406, <http://dx.doi.org/10.1007/s12264-015-1544-6>.
- [72] D. Zhi, S. Zhang, X. Lin, Study on therapeutic mechanism and clinical effect of mild hypothermia in patients with severe head injury, *Surg. Neurol.* 59 (5) (2003) 381–385.
- [73] X. Zhu, A. Zelmer, J.P. Kapfhammer, S. Wellmann, Cold-inducible RBM3 inhibits PERK phosphorylation through cooperation with NF90 to protect cells from endoplasmic reticulum stress, *FASEB J.* 30 (2) (2015) 624–634, <http://dx.doi.org/10.1096/fj.15-274639>.