Brain vulnerability and viability after ischaemia

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Abstract | The susceptibility of the brain to ischaemic injury dramatically limits its viability following interruptions in blood flow. However, data from studies of dissociated cells, tissue specimens, isolated organs and whole bodies have brought into question the temporal limits within which the brain is capable of tolerating prolonged circulatory arrest. This Review assesses cell type-specific mechanisms of global cerebral ischaemia, and examines the circumstances in which the brain exhibits heightened resilience to injury. We suggest strategies for expanding such discoveries to fuel translational research into novel cytoprotective therapies, and describe emerging technologies and experimental concepts. By doing so, we propose a new multimodal framework to investigate brain resuscitation following extended periods of circulatory arrest.

Delayed neuronal death
Morphological and histological features associated with neuronal cell damage and death that become apparent multiple days following an injury or insult.

Owing to its high metabolic demand and limited energy reserves[1], the brain is highly susceptible to ischaemic injury. The most common clinical manifestation of cerebral ischaemia is stroke, which results from interruptions in focal blood flow, and affects approximately 800,000 people per year in the United States[2]. A more extreme insult known as global cerebral ischaemia occurs when blood flow to the brain stops entirely, such as in the case of cardiac arrest (CA), affecting nearly 350,000 people per year in the United States[2]. The potential for positive outcomes with stroke treatments has steadily increased over the past decades, but brain resuscitation after CA remains a largely unsolved clinical problem.

To guide treatment options for global ischemia during CA, a three-phase, time-sensitive model was proposed in the early 2000s[3]. This model consists of electrical (0–4 min after CA), circulatory (4–10 min) and metabolic (more than 10 min) phases, which reflect the temporal progression of ischemic injury and resuscitation physiology. Most cases of out-of-hospital CA (OHCA) present in the metabolic phase, for which current treatment options are limited. Indeed, OHCA outcomes remain disappointingly poor, with approximately 10% of patients surviving to hospital discharge[2]. Furthermore, even if cardiac function is restored, more than half of surviving patients display persistent brain damage and reduced quality of life[2,4]. Therefore, the vulnerability of the brain to ischaemia is a major limiting factor for successful resuscitation with positive neurological outcomes.

Much of our current understanding of cerebral ischaemic injury comes from the use of experimental animal models. This Review centres primarily on knowledge derived from global ischaemia models (BOX 1); however, selected data from studies of focal injury are included given the overlap in several cellular mechanisms and the depth of the focal ischaemia literature. Complete cerebral ischaemia paradigms have established that brain function ceases within seconds of cerebrocirculatory arrest, with high-energy metabolites depleted within minutes[1,5,6]. Even 5 min of global cerebral ischaemia can induce delayed neuronal death in certain selectively vulnerable brain regions; by contrast, cardiac and renal cells can withstand 20–60 min of CA[1,7–9].

Nevertheless, earlier and more recent studies have questioned the temporal limits within which the mammalian brain is capable of tolerating ischaemia. Here we examine cell type-specific mechanisms of cerebral ischaemic injury and the circumstances in which neural tissues demonstrate resilience to cerebrocirculatory arrest. In doing so, we suggest ways in which these discoveries can fuel novel translational research. Unlike previous articles that have provided excellent overviews of stroke, here we synthesize our current basic and clinical knowledge of global ischaemia with the aim of developing new interventions to preserve whole-brain function following prolonged CA. Towards this goal, we highlight emerging concepts in ex vivo organ research and resuscitation, and their promise for advancing our understanding and treatment of global ischaemia. Overall, the data we present support the notion that the mammalian brain can withstand longer periods of ischaemia than widely appreciated under the appropriate

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Rational polytherapy
Combination therapy that is rationally designed to target multiple deleterious mechanisms simultaneously or in a deliberate sequence.

Extracorporeal perfusion
The use of a mechanical pump device with auxiliary components that circulates either a patient's blood or a specialized solution to provide circulatory support.

Electroencephalogram flattening
The absence of synchronous electrical activity in the brain, also known as a flat line or isoelectric reading.

Acidosis
The condition in which cellular or tissue pH decreases below the normal homeostatic range owing to the accumulation of protons.

Anoxic depolarization
An acute neuronal event involving the loss of cell membrane potentials caused by energy failure secondary to oxygen deprivation.

'No-reflow' phenomenon
The persistence of microvascular perfusion deficits despite the successful re-establishment of global circulation following ischaemia.

Haemodilution
The act of reducing the concentration of cells and components in the blood through the introduction of a fluid.

Erythrocyte
A red blood cell.

conditions. Ultimately, we provide an experimental framework for bridging basic mechanisms of ischaemic injury, ex vivo organ preservation, rational polytherapy and extracorporeal perfusion to push forward global ischaemia research.

Cerebral perfusion and metabolism

In adult humans, the average cerebral blood flow is nearly 50 ml per 100 g per minute (REF.[10]), with grey-matter and white-matter flows approximating 80 ml and 20 ml per 100 g per minute, respectively[11]. On average, the entire human brain consumes roughly 0.8–1.2 mmol ATP per 100 g per minute, whereas the rodent brain consumes nearly 2.1 mmol ATP per 100 g per minute[12–14]. Analysis of high-resolution physiological data reveals that the rodent neocortical grey matter expends roughly 3.3–5 mmol ATP per 100 g per minute[14], where approximately 1 mmol ATP per 100 g per minute is consumed for basic non-signalling needs, and 2.3–4.0 mmol ATP per 100 g per minute is consumed for signalling processes — 47% of which is used solely for action potential generation[14]. In the rodent white matter, about 55% of consumed energy is expended for housekeeping, 44% for maintaining resting potentials, 0.4% for generating action potentials and 0.1% for synaptic transmission[15].

In humans, electroencephalogram flattening occurs when cortical flow drops below approximately 20 ml per 100 g per minute[16]. In baboons, somatosensory evoked potentials are abolished when cortical flow rates fall below 15 ml per 100 g per minute[17], and exhaustive release of intracellular potassium occurs at 6 ml per 100 g per minute[18]. Entirely cut off from its nutrient supply, cerebral metabolism becomes wholly dependent upon its limited oxygen and energy reserves. The levels of energy substrates such as ATP, phosphocreatine and glucose fall precipitously within the first minute of global ischaemia[1], followed by derangements in other components of the cerebral metabolome[19]. Cerebrocirculatory arrest also leads to an accumulation of lactate, which results in a damaging acidosis that is proportional to pre-ischaemic glycaemic levels[20]. ATP converted to AMP during ischaemia is subsequently degraded to inosine and hypoxanthine[21], thereby reducing the concentration of adenine nucleotides available for metabolic resuscitation. As a result of ATP depletion, neuronal cells fail to maintain ionic balance and undergo anoxic depolarization with the influx of Na^+, Ca^{2+} and Cl^-, along with the efflux of K^+ (reviewed elsewhere[22]). These early metabolic and ionic disturbances precipitate various downstream injury mechanisms.

Cerebral hemodynamics in ischaemia

Even if cerebral blood supply is restored following global ischaemia, regional perfusion deficits, known as the 'no-reflow' phenomenon, may persist[23]. These reperfusion disturbances have been observed across multiple species, occurring in small, circumscribed vascular territories following brief ischaemia and encompassing greater vascular areas with increased ischaemic periods[23–25]. Notably, the vascular pattern of perfusion deficits occurs in a species-dependent manner[23–27], prompting caution when one is interpreting or extrapolating data across different animal models (BOX 1). However, studies have found that the no-reflow phenomenon can be mitigated by either driving reperfusion with elevated blood pressures or haemodilution[28], or alternatively by removing blood from the vasculature before recirculation using an arterial saline washout[29].

If the no-reflow phenomenon is prevented by such interventions, functional recovery may occur after normothermic cerebrocirculatory arrest lasting as long as 1 h (REF.[30]); that is, much longer ischaemic times than previously assumed to afford such recovery. In such instances, ischaemia is followed by post-ischaemic reactive hyperaemia, the duration of which roughly equals the length of the preceding circulatory arrest[6]. Subsequently, blood flow declines to or below control levels, a phenomenon termed 'secondary post-ischaemic hypoperfusion'[26,31,32]. These biphasic flow changes follow alterations in vascular tone — which is first reduced during ischaemia and early reperfusion (post-ischaemic vasoparalysis), and later returns to or reaches above normal when hyperaemia vanishes (post-ischaemic vasoconstriction)[33].

The mechanisms leading to post-ischaemic perfusion disturbances have yet to be fully elucidated. Analyses reveal that these reperfusion deficits occur primarily at the microcirculatory level[23,24]. In rats, in vivo multiphoton microscopy demonstrates that pial arteriolar constriction leads to erythrocyte stasis and increased transit time of capillary plasma after CA[32]. Other studies in rats have shown improved post-CA cerebral blood flow after inhibition of various vasoconstrictors, such as endothelin or 20-hydroxyeicosatetraenoic acid[31,34]. These molecules act on vasoactive mural cells, which contribute considerably to blood flow dynamics by regulating the luminal diameter of cerebral microvessels. However, whether the cells responsible for controlling vessel diameter following ischaemia are pericytes[35] or precapillary arteriole smooth muscle cells[36] remains controversial. Accurately identifying the cell type responsible will be critical for advancing therapies aimed at improving capillary flow during post-ischaemic reperfusion.

Other processes also have a role in microvascular disturbances following ischaemia. In both focal and global ischaemia, endothelial and perivascular glial

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Box 1 | **Models of global cerebral ischaemia**

Much of our understanding of the pathophysiology of global cerebral ischaemia has come from various animal models, each with intrinsic advantages and disadvantages.

Decapitation
With physical separation from the systemic circulation, this model offers an entirely closed system, providing unequivocal insights into cellular functions, metabolism and injury mechanisms as a function of ischaemic time[1,268,269]. Studies using this model have established that the brain has highly limited energy reserves, possibly underlying its susceptibility to ischaemic or anoxic conditions. Limitations include lack of the hypoxic hypoperfusion that precede various forms of cardiac arrest (CA), such as asphyxial, haemorrhagic or septic CA; exclusion of deleterious ischaemic metabolites derived from peripheral organs upon reperfusion; and difficulty achieving reperfusion without substantial intervention. However, recent advancements using a synthetic perfusate, an experimental perfusion platform and surgical procedures have converted this paradigm into an operational model of prolonged transient ischaemia in the isolated large mammalian brain[119].

Pneumatic neck cuff
The use of a pneumatic cuff around the neck leads to stagnant cerebral ischaemia. The initial validation of this model found that canines can withstand up to 6 min of cerebrocirculatory arrest with retained neurological function[270]. This apparatus was modified for humans to investigate the cause of fighter pilot unconsciousness[271], and showed that after 100 s of global ischaemia, participants exhibited reversible eye fixation, unconsciousness and anoxic convulsions. In non-human primates, the neck cuff (combined with a reduction in arterial blood pressure) established a 15-min threshold of tolerable global ischaemia[272]. This model does not capture hypotensive hypoperfusion preceding CA, and may induce increased intracranial and hydrostatic pressure.

Ventricular fibrillation
Induction of ventricular fibrillation recapitulates clinical CA, and can be used in larger mammals, including dogs[273] and pigs[95,212,274–276]. Large mammalian species are amenable to investigating the perfusion dynamics, cellular responses and surgical procedures that can be more readily translated to humans. Various methods are used to induce ventricular fibrillation[277], and this model is often coupled with resuscitative protocols. However, larger animals are more costly and labour-intensive in terms of surgery and post-operative care than smaller animals. Rodent models exist but are difficult to sustain given their high rates of spontaneous defibrillation[278].

Selective cerebrocirculatory arrest
Whole-body ischaemia makes the discernment of brain-specific biology and ischaemic thresholds difficult. Thus, a useful solution is selectively obstructing circulation to the brain in otherwise intact animals, either by inducing systemic hypotension and occluding the ascending arteries proximal to the aortic arch, or by increasing intraventricular pressure, which produces complete cerebral ischaemia. Several models of global cerebral ischaemia in large mammalian species without cardiac failure have been developed[30,207,279]. These models also lack the hypoxic hypoperfusion associated with distinct forms of CA, and do not capture widespread blood stagnation or the systemic metabolites produced by peripheral organ ischaemia.

Four-vessel occlusion
The four-vessel occlusion model consists of a two-stage procedure in which the vertebral arteries are occluded and clasps are placed around the common carotid arteries in rodents (distinct from the techniques used in the selective cerebrocirculatory arrest models described above). This paradigm is successful in only approximately 75% of animals, with substantial interstrain variability in rats, potentially owing to variations in collateral circulation[280,281]. However, this model is relatively economical and accessible, and recapitulates selective neuronal vulnerability and post-ischaemic hemodynamic perturbations[282].

Other models and caveats
Other approaches have been introduced to produce global cerebral ischaemia in different species (for a review, see REF.[283]), including in adult[284], paediatric[285] and neonatal[286] contexts. Notably, the lack of successful translation of experimental treatments to humans may reflect differences in physiology and cellular composition between the brains of laboratory animals and the brains of humans, particularly in terms of grey matter to white matter ratios[287,288]. In addition, global ischaemia in humans is often co-morbid with underlying diseases such as atherosclerosis, which is not recapitulated in otherwise healthy laboratory animals. Therefore, it may be necessary to validate observations in multiple species, and in animals with co-morbidities, before drawing conclusions on the translatability of ischaemia-related findings and treatments.

swelling, haemoconcentration and the formation of microthrombi contribute to deficient microvascular reperfusion[23,24,28,37–39]. In baboons, microcirculatory thrombi following focal ischaemia contain erythrocytes, degranulated platelets[40] and polymorphonuclear leukocytes[41]. Following rapid endothelial cell expression of adhesion molecules[42,43], polymorphonuclear leukocytes can become trapped in the microvasculature, an obstructive process that can be exacerbated by concurrent luminal narrowing. Depletion of polymorphonuclear leukocytes mitigated reperfusion deficits following transient forebrain ischaemia in rats[44]; however, others have questioned the extent to which leukocyte adherence contributes to vascular plugging after global ischaemia[45].

The need for widespread patency at the microcirculatory level during recirculation is essential for nutrient exchange and cellular viability following ischaemia. In early experiments of prolonged cerebrocirculatory arrest in cats and monkeys, functional recovery was observed only in animals in which ischaemia was immediately followed by reactive hyperaemia, reflecting the need for instantaneous restoration of microcirculatory patency[6]. After CA lasting even short periods, such patency is not reliably achieved. Indeed, in a pig study of 3 min of CA followed by cardiopulmonary resuscitation (CPR), microcirculatory flow (as measured at the sublingual mucosa) did not strongly correlate with global recirculation[46]. However, this same discrepancy between microvascular flow and global recirculation was not observed in the cerebral microvasculature in another CA study of similar duration in pigs[47]. It is important to note, though, that this finding does not signify that deficits of cerebral microcirculatory patency may not occur following longer ischaemic intervals. More research is therefore necessary to investigate the relationship between cerebral microcirculation and global haemodynamics following prolonged CA, and how to ensure ubiquitous microvascular flow.

Haemoconcentration
The process of concentrating cells and components in the blood through the removal of fluid.

Mechanisms of cellular injury

The mechanisms of cellular injury can be separated into two broad groups: cellular responses during ischaemia (primary) and following reperfusion (secondary). Notably, reperfusion under untreated conditions exacerbates cellular injury by restoring flow in the highly toxic molecular milieu established during the ischaemic

period. Thus, correcting the physiological landscape immediately upon recirculation is crucial to limit injury and promote cellular recovery.

Here we highlight several deleterious mechanisms that affect major cell types within the brain (other mechanisms are reviewed elsewhere[48–50]). It is important to emphasize, however, that many of these mechanisms act synergistically to cause cerebral dysfunction by affecting multiple cell types simultaneously, instead of occuring in isolation.

Primary mechanisms

Endothelial cells and blood–brain barrier. Endothelial cells form the base layer of the neurovascular unit and blood–brain barrier (BBB) together with mural cells (that is, pericytes and smooth muscle cells) and astrocytic end-feet[51,52] (FIG. 1). Endothelial cells of the BBB are closely linked via tight junctions (FIG. 1b), which restrict free water and ion flow by increasing transendothelial electrical resistance[53]. However, during ischaemia, the endothelium undergoes rapid activation and becomes increasingly permeable to blood constituents (FIG. 1b). Reductions in oxygen levels impair endothelial barrier and BBB function by causing aberrant cAMP signalling, which increases cell monolayer permeability[54]. BBB breakdown occurs shortly after CA in pigs[55], and the endothelial glycocalyx also loses integrity during ischaemia–reperfusion injury (IRI)[56], further contributing to vascular and barrier dysfunction (FIG. 1b).

In vitro, human umbilical vein endothelial cells are relatively resistant to hypoxia, with more than 90% of these cells exhibiting signs of viability after 6 h of reduced oxygen levels, and approximately 50% of cells remaining viable after 24 h of hypoxia[57]. Under hypoxic conditions, endothelial cells also upregulate proinflammatory and cell adhesion molecules, priming them for dysfunction following reoxygenation[58,59] (FIG. 1b). The speed at which the activated endothelium attracts circulating cells is mediated by the rapid shuttling of preformed P-selectin (a cell adhesion molecule) from Weibel–Palade bodies to the intraluminal cell surface[60] (FIG. 1b), even in the absence of reperfusion in both ischaemic cerebral[43] and ischaemic cardiac[61] vasculature. This occurs in response to various stimuli, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated either intracellularly or by surrounding cells[62] (FIG. 1b). Increased expression of adhesion molecules promotes endothelial cell–peripheral immune cell interactions, possibly contributing to injury in both focal[63] and global[64,65] ischaemia (FIG. 1b). Furthermore, intraluminal cellular adhesion compromises post-ischaemic reperfusion following focal ischaemia[41], and may play an analogous role in global insults[66], especially after extended no-flow periods[67] (FIG. 1b).

Ischaemia also disrupts normal endothelial production of nitric oxide (NO), a major physiological vasodilator, thereby exacerbating vascular dysfunction (FIG. 1b). Endothelial NO synthase (NOS3; also known as eNOS) is the primary source of endothelial NO, and its biochemical function depends on the presence of molecular oxygen[68]; although under anoxia NOS3 can produce NO by reducing nitrite[69], it can do this for only a limited time. As NO normally impedes leukocyte attachment to endothelial surfaces[70], its impaired production during ischaemia may further enable peripheral immune cell adhesion[71].

Neurons. A disproportionate amount of energy in the brain is required to maintain Na^+ homeostasis, which is largely governed by the Na^+/K^+-ATPase pump[72]. Thus, when energy failure occurs, neuronal ischaemic injury begins with cessation of Na^+/K^+-ATPase activity and consequent dysregulation of ionic balance. This leads to widespread anoxic depolarization with a dramatic neuronal influx of Na^+, Cl^- and Ca^{2+}, as well as an efflux of K^+, ultimately leading to cellular oedema, injury and aberrant neurotransmission[22,73,74] (FIG. 2). In a rat model of global ischaemia, blocking voltage-gated Na^+ channels with tetrodotoxin delayed the onset of anoxic depolarization and the shrinkage of the extracellular space, substantiating the importance of Na^+ channels in ionic and water dysregulation[75].

Importantly, ischaemia increases cytosolic Ca^{2+} concentration, which initiates a host of deleterious intracellular mechanisms. Many channels and pathways have been implicated in mediating the effects of supraphysiological increases in Ca^{2+} concentration in various models of anoxia and ischaemia, including ionotropic glutamate receptors and voltage-gated calcium channels, as well as the Na^+–Ca^{2+} exchanger and its interplay with prostaglandin receptors[76–78]. Other studies have also implicated transient receptor potential channel 7 and acid-sensing ion channels[79,80].

Increased cytosolic Ca^{2+} concentration during ischaemia also drives the release of excessive glutamate, which is neurotoxic[81–83] (FIG. 2). Thus, much research has focused on glutamate-mediated excitotoxicity, and the roles of glutamate ionotropic NMDA receptor (NMDAR) subunits[84], along with other receptors[85,86], in facilitating its neurotoxic effects. NMDARs containing the subunit GRIN2A (also known as NR2A) or GRIN2B (also known as NR2B) are localized preferentially to synapses or extrasynaptic domains, respectively[87] (FIG. 2). Activation of synaptic GRIN2A-containing NMDARs has been associated with neuronal survival, whereas engagement of extrasynaptic GRIN2B-containing NMDARs drives pro-death mechanisms, such as antagonizing survival processes mediated by cAMP-responsive element-binding proteins (CREB proteins) and stimulating Ca^{2+}-activated chloride channels[88,89]. GRIN2B-containing NMDARs also interact with neuronal NO synthase (NOS1; also known as nNOS) via postsynaptic density protein 95 (PSD95), leading to dysregulated NOS1 activity and excessive NO production upon activation of GRIN2B-containing NMDARs[90] (FIG. 2). Notably, peptide blockage of interactions between GRIN2B-containing NMDARs and PSD95 is neuroprotective in a primate model of focal ischaemia[91], and phase II and phase III clinical trials suggest potential future efficacy after stroke[92,93]. During ischaemia, excessive glutamate release could diffuse beyond the synaptic cleft and activate extrasynaptic GRIN2B-containing NMDARs, thereby propagating neuronal damage (FIG. 2). In culture, prolonged

Neurovascular unit
A specialized functional and structural unit in the brain composed of endothelial, glial and neuronal cells that facilitate coupling between neuronal activity and blood flow.

Blood–brain barrier
(BBB). A specialized barrier within the vasculature of the brain that limits the non-selective movement of peripheral blood components into the brain.

Glycocalyx
A network of biomolecules that line the luminal surface of the cerebrovascular endothelium.

Weibel–Palade bodies
Storage granules in endothelial cells that contain multiple biomolecules, such as P-selectin.

Excitotoxicity
A pathological process by which neurons are damaged and killed through the overactivation of cellular receptors by the excitatory neurotransmitter glutamate.

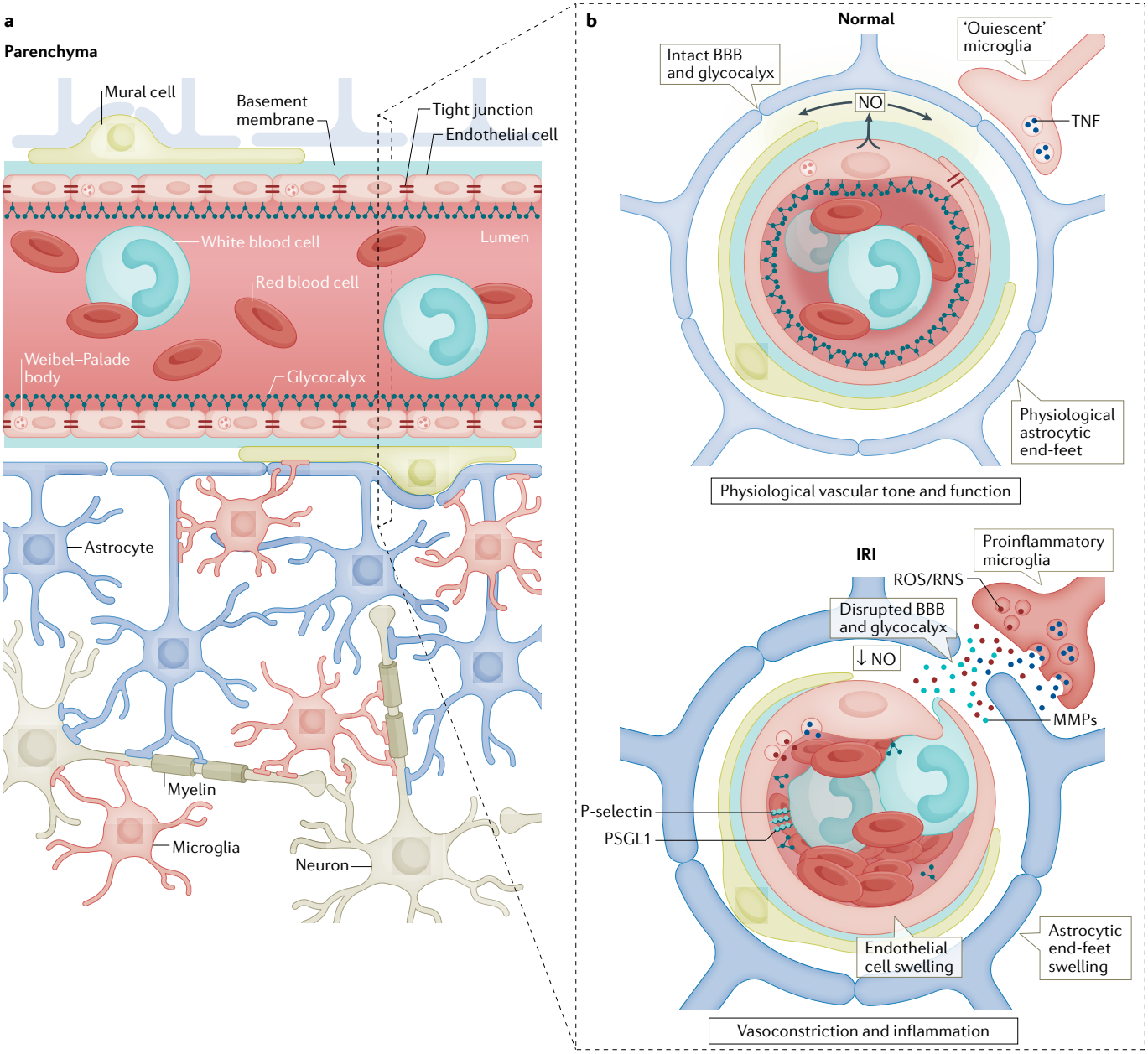
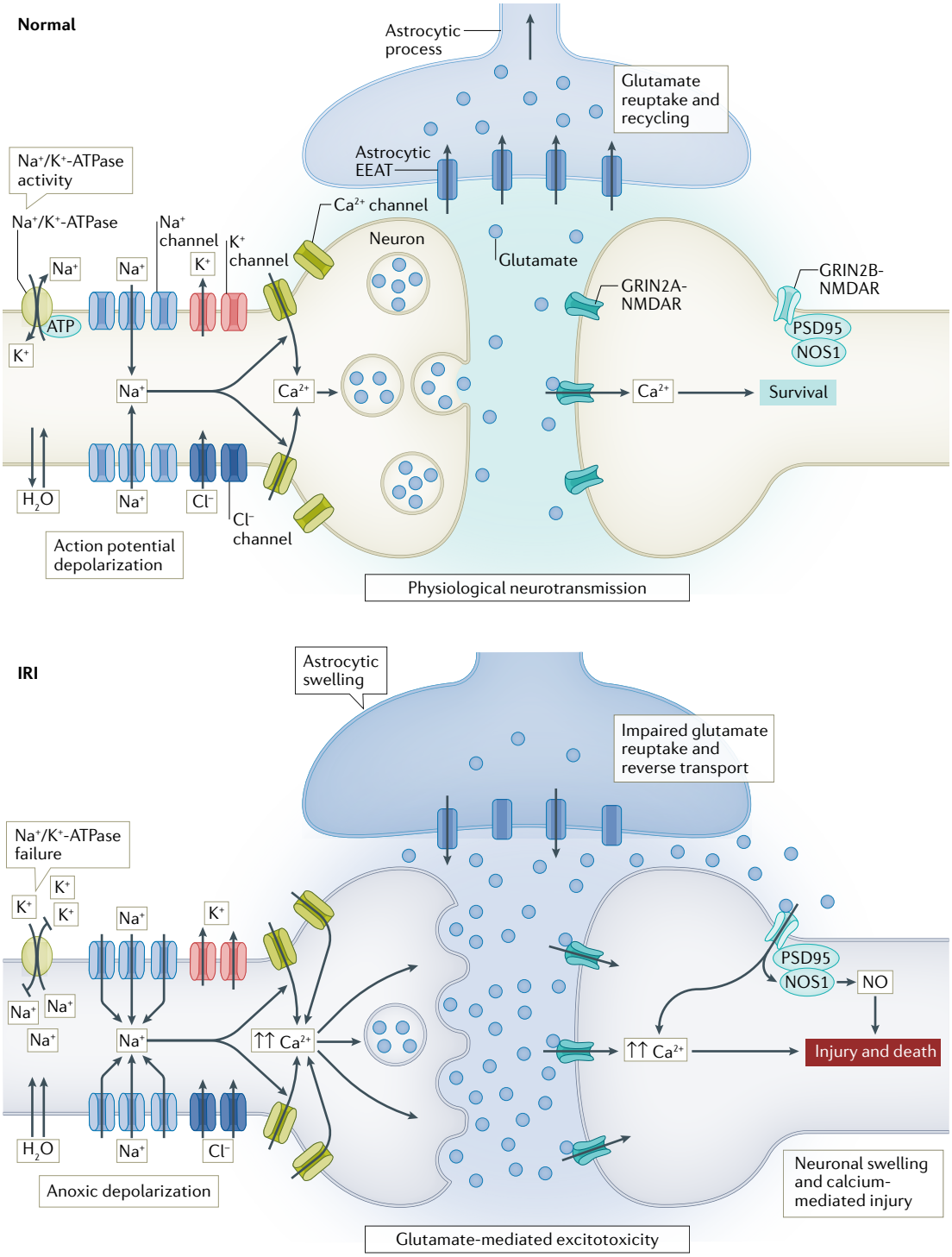


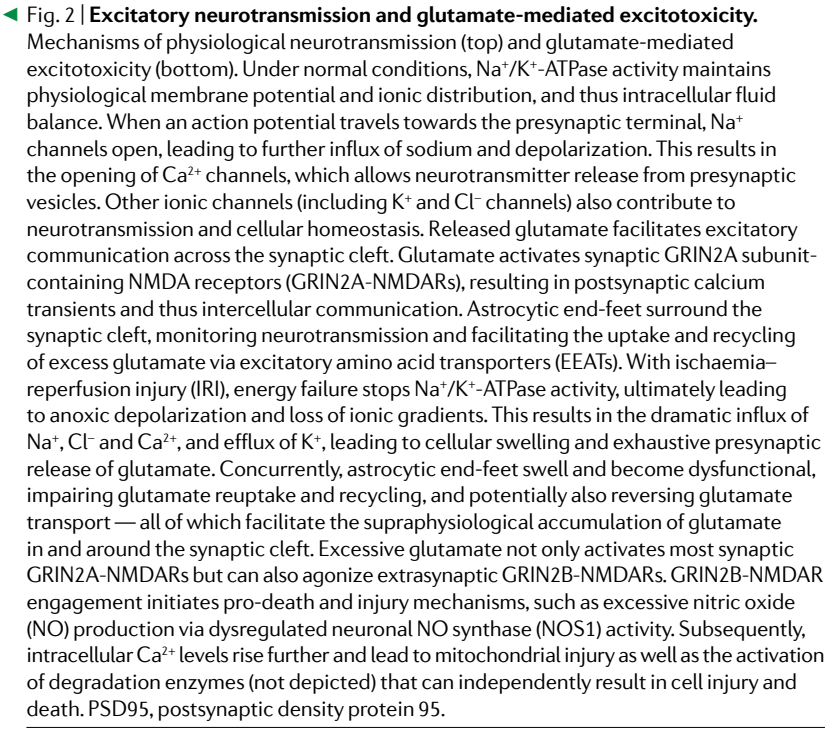
Fig. 1 | **Cerebral microvasculature structure, function and dysfunction.** **a** | A cerebral microvessel and neurovascular unit with associated cells in the brain. The cerebral microvasculature is composed of endothelial cells that contain Weibel–Palade bodies and are connected via tight junctions. The glycocalyx emerges from the luminal surface of the endothelium, contributing to vascular functionality. The basement membrane underlies the endothelial monolayer, which is abutted by mural cells (pericytes and smooth muscle cells) and astrocytic end-feet. In the parenchyma, astrocytes, microglia and neurons are intricately interconnected with multiple points of cellular communication. Oligodendrocytes are not depicted. **b** | Key cellular interactions under normal conditions (top) and ischaemia–reperfusion injury (IRI) conditions (bottom). Under physiological conditions, endothelial cells produce the vasodilator nitric oxide (NO), which maintains normal vascular tone and helps facilitate blood flow. The luminal surface of the endothelium normally maintains a non-thrombotic and non-inflammatory environment, preventing adherent interactions with white blood cells or other blood cells. The glycocalyx and blood–brain barrier (BBB) are structurally intact, protecting the brain from extravasation of blood components. Adjacent to the astrocytic end-feet are 'quiescent' microglia with processes that constantly survey the environment to assess and maintain homeostasis. In IRI, endothelial cells undergo rapid activation, release proinflammatory molecules and reactive oxygen species (ROS), and shuttle preformed adhesion molecules such as P-selectin to the luminal surface from Weibel–Palade bodies. Endothelial dysfunction results in the loss of NO production, leading to vasoconstriction and loss of vascular dilatory function. Furthermore, endothelial cell swelling and astrocytic end-feet swelling exacerbate intraluminal narrowing. This promotes intravascular coagulation of blood cell components and peripheral immune cell adhesion through P-selectin–P-selectin glycoprotein ligand 1 (PSGL1) interactions between endothelial cells and immune cells. Adhered immune cells become activated, and together with parenchymal microglial cells produce various proinflammatory molecules, ROS and matrix metalloproteases (MMPs) that facilitate the breakdown of the BBB and glycocalyx. Disruption of endothelial tight junctions enables the extravasation of peripheral immune cells to the brain parenchyma, which further contribute to the neurotoxic inflammatory response characteristic of IRI. RNS, reactive nitrogen species; TNF, tumour necrosis factor.



exposure to extracellular (that is, extrasynaptic) glutamate leads to acute Na^+-dependent swelling and terminal Ca^{2+}-dependent toxicity[94]. Following CA in pigs, depletion of Ca^{2+} with citrate during controlled reperfusion has shown promising neuroprotection[95], further implicating Ca^{2+} toxicity in the deleterious effects of ischaemia. Overall, the mechanisms underlying glutamate-mediated Ca^{2+} excitotoxicity comprise a wide range of cellular processes, including generation of ROS/RNS, activation of lipases and proteases, mitochondrial dysfunction and apoptosis[77,96–98].

Glia. Glia comprise oligodendrocytes, astrocytes and microglia. Here we discuss certain processes that affect astrocytes and microglia during ischaemia. Oligodendrocytes are not described, but their injury mechanisms have been covered previously[99].

Astrocytes can be considered the 'connectors' of the brain, bringing together endothelial, mural, perivascular glial and neuronal activity into the neurovascular unit[52] (FIG. 1a). Astrocytic dysfunction has been implicated in several of the sequelae of global ischaemia, such as impaired fluid regulation,



◀ Fig. 2 | **Excitatory neurotransmission and glutamate-mediated excitotoxicity.** Mechanisms of physiological neurotransmission (top) and glutamate-mediated excitotoxicity (bottom). Under normal conditions, Na^+/K^+-ATPase activity maintains physiological membrane potential and ionic distribution, and thus intracellular fluid balance. When an action potential travels towards the presynaptic terminal, Na^+ channels open, leading to further influx of sodium and depolarization. This results in the opening of Ca^{2+} channels, which allows neurotransmitter release from presynaptic vesicles. Other ionic channels (including K^+ and Cl^- channels) also contribute to neurotransmission and cellular homeostasis. Released glutamate facilitates excitatory communication across the synaptic cleft. Glutamate activates synaptic GRIN2A subunit-containing NMDA receptors (GRIN2A-NMDARs), resulting in postsynaptic calcium transients and thus intercellular communication. Astrocytic end-feet surround the synaptic cleft, monitoring neurotransmission and facilitating the uptake and recycling of excess glutamate via excitatory amino acid transporters (EEATs). With ischaemia–reperfusion injury (IRI), energy failure stops Na^+/K^+-ATPase activity, ultimately leading to anoxic depolarization and loss of ionic gradients. This results in the dramatic influx of Na^+, Cl^- and Ca^{2+}, and efflux of K^+, leading to cellular swelling and exhaustive presynaptic release of glutamate. Concurrently, astrocytic end-feet swell and become dysfunctional, impairing glutamate reuptake and recycling, and potentially also reversing glutamate transport — all of which facilitate the supraphysiological accumulation of glutamate in and around the synaptic cleft. Excessive glutamate not only activates most synaptic GRIN2A-NMDARs but can also agonize extrasynaptic GRIN2B-NMDARs. GRIN2B-NMDAR engagement initiates pro-death and injury mechanisms, such as excessive nitric oxide (NO) production via dysregulated neuronal NO synthase (NOS1) activity. Subsequently, intracellular Ca^{2+} levels rise further and lead to mitochondrial injury as well as the activation of degradation enzymes (not depicted) that can independently result in cell injury and death. PSD95, postsynaptic density protein 95.

glutamate-mediated excitotoxicity and disrupted astrocyte–neuron signalling.

Compared with neurons, the effect of ischaemia on astrocytes and the roles these cells play during IRI are less well understood. However, ultrastructural evidence indicates that astrocytic swelling is an early response to focal[100] and global[66] ischaemia (for a review, see REF.[101]). Astrocytes may be less sensitive than neurons to hypoxic conditions in vitro[102], possibly owing to the former's ability to upregulate glycolytic capacity during hypoxia[103]. However, this resilience is diminished under hypoglycaemic and acidotic culture conditions[104,105], suggesting that, in circumstances simulating global ischaemia, astrocytes may be as vulnerable as neurons. Furthermore, astrocytes may be particularly sensitive to the exacerbated acidosis associated with hyperglycaemic global ischaemia[106,107] and, during permanent focal ischaemia in rats, astrocytic decline may precede neuronal demise within the lesion core[108].

The temporal relationship between astrocytic death and neuronal death has far-reaching implications, given their cellular interplay. Astrocytes may shuttle lactate to neurons for metabolism in vivo[109], and astrocyte-derived growth factors protect neurons following oxygen–glucose deprivation in culture[110]. Importantly, astrocytic processes intricately wrap around synapses[111], facilitating the removal of approximately 90% of synaptic glutamate[112] (FIG. 2) — a process that is essential for neuronal homeostasis. Indeed, loss of astrocytic glutamate uptake in the hippocampal CA1 field may contribute to selective neuronal vulnerability following global ischaemia[113], and may exacerbate injury during stroke[114]. Ischaemia may also induce reversal of glutamate uptake, causing glutamate to be transported from astrocytes back into the synaptic cleft[115] (FIG. 2). Overall, astrocytic dysfunction allows excessive glutamate to remain intrasynaptically and extrasynaptically, increasing the likelihood of activating GRIN2B-containing NMDARs and subsequent neuronal injury (FIG. 2). Thus, astrocytic responses during ischaemia are very important and require further investigation.

The other major glial cell type we discuss here is microglia, the resident immune cell of the brain. Under physiological conditions, microglia typically display a 'resting' profile during which they are highly dynamic — continuously sampling and surveying the brain parenchyma with their fine processes[116]. Our understanding of the primary microglial mechanisms during ischaemia is relatively limited, as most studies have examined microglia as effector cells during reperfusion-mediated inflammation.

In vivo two-photon imaging of microglia before and after induction of ischaemia in mice reveals a dramatic 'stalling' of their dynamic processes that does not reverse until a capillary near the cell soma is reperfused[117]. Thus, microglia may sense local microcirculatory dynamics, and potentially halt rearrangements of their cytoskeleton to conserve ATP for subsequent activation upon reperfusion. Under permanent focal ischaemia, microglia die in the lesion core within 24 h (REF.[118]), although this time course may be shorter in global ischaemia[119] and simulated in vitro ischaemia[120]. As explained later, microglia have various effects on tissue following ischaemia. Therefore, more research is needed to uncover how to promote their survival and beneficial functions upon reperfusion.

Secondary mechanisms

If reperfusion and reoxygenation resume after the critical period of ischaemic tolerance, secondary mechanisms exacerbate tissue damage through reperfusion injury. Considering that oxygen is necessary to sustain cellular viability, yet simultaneously drives subsequent damage, the readmission of molecular oxygen following ischaemia is known as the oxygen paradox[121].

The extent of reperfusion injury is proportional to the duration of the ischaemic interval, which establishes an increasingly deranged cellular milieu. Therefore, by correcting the physiological landscape immediately upon reperfusion, it may be possible to counteract damaging molecular programmes that were initiated during the ischaemic period. In the following sections we highlight several particularly salient and therapeutically tractable mechanisms. Other secondary mechanisms, including cerebral oedema formation, have been discussed elsewhere[122].

Inflammation. Following reperfusion, a new phase of neuroinflammation, involving microglia and peripheral immune cells, begins. Under normal conditions, microglia maintain a quiescent immunophenotype through the interaction of the inhibitory member of the cluster of differentiation 200 receptors (CD200R1; also known as OX2 receptor 1) on their cell surface with neuronal CD200 (REF.[123]). However, during IRI-induced neuronal injury, this interaction becomes disrupted, leading to microglial activation (FIG. 3). Neuronal damage also induces the release of damage-associated molecular

Simulated in vitro ischaemia
An experimental method through which cultured cells and tissues are subjected to conditions similar to in vivo ischaemia by the combination of hypoxia and hypoglycaemia.

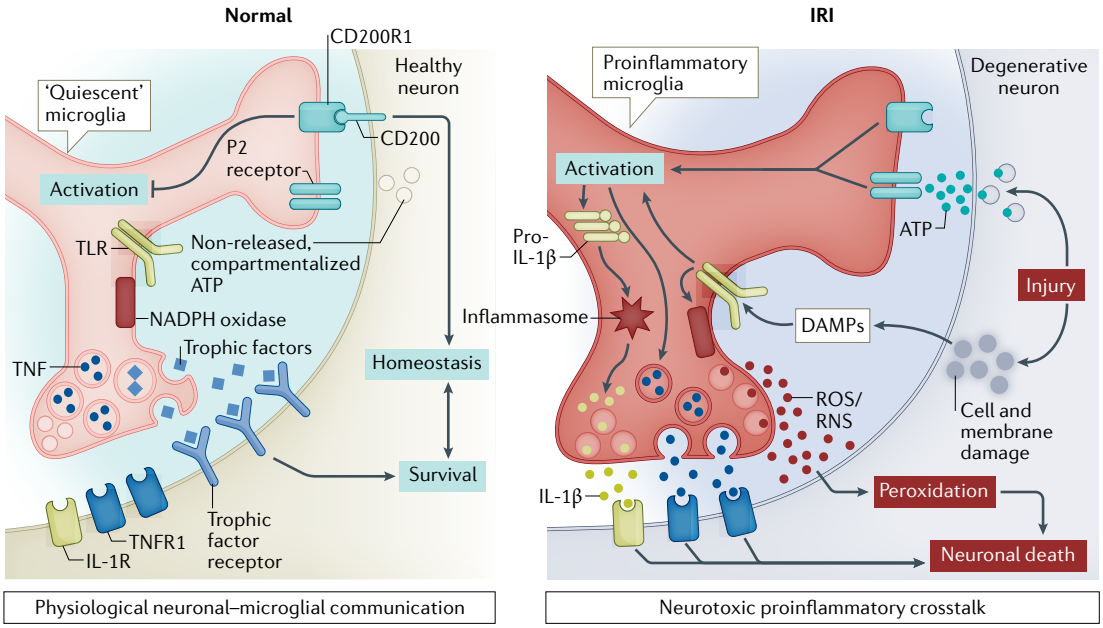


Fig. 3 | **Neuron–microglial cell interplay and pro-inflammatory signalling.** Neuron–microglial cell communication under normal conditions (left) and ischaemia–reperfusion injury (IRI) conditions (right). Physiological signalling between neurons and microglia is essential for maintaining neuronal health and function. Microglia maintain a quiescent immunophenotype through the interaction of the inhibitory member of the cluster of differentiation 200 receptors (CD200R1) on their cell surface with neuronal CD200, an interaction that also facilitates neuronal homeostasis and survival. Furthermore, microglia release trophic factors that engage neuronal surface receptors and thus promote neuronal survival. During IRI, early neuronal release of nucleotides such as ATP signals to microglia through microglial purinergic 2 (P2) receptors to initiate classical activation. Concurrently, the inhibitory effects of the CD200–CD200R1 interaction are lifted through the diminished expression of neuronal CD200, further contributing to microglial activation. The release of various damage-associated molecular patterns (DAMPs) from neighbouring neurons activates microglial Toll-like receptors (TLRs), further driving a proinflammatory immunophenotype. In turn, microglia upregulate NADPH oxidase (NOX), which produces reactive oxygen species (ROS) that lead to neuronal membrane peroxidation and contribute to cellular death. Microglia also release proinflammatory molecules such as soluble tumour necrosis factor (TNF). By binding TNF receptor 1 (TNFR1) on the surface of neurons, soluble TNF can initiate an intracellular cascade that results in mitochondrial dysfunction and apoptosis via complex II formation, caspase 8 activation, mitochondrial pore formation and cytoplasmic release of cytochrome *c* (not depicted). Upon activation, microglia also upregulate the transcription of pro-interleukin-1β (pro-IL-1β), an integral component of post-ischaemic inflammation. Subsequently, the inflammasome complex is assembled; this multisubunit complex mediates not only the cleavage of pro-IL-1β to IL-1β but also IL-1β release. Once released, IL-1β can bind to neuronal IL-1 receptor (IL-1R), ultimately contributing to neuronal damage and death. RNS, reactive nitrogen species.

patterns such as ATP and high mobility group protein B1 (HMGB1), which are detected via microglial purinergic and Toll-like receptors (TLRs), respectively, to elicit microglial activation[124] (FIG. 3). ATP is quickly released from cells during focal ischaemia[125], and thus can stimulate the secretion of proinflammatory molecules from microglia[126] (FIG. 3), contributing to neuronal death[127]. Moreover, HMGB1 release further drives proinflammatory microglial activation by engaging TLR2 and TLR4 (along with receptor for advanced glycosylation end products (RAGE))[128] (FIG. 3). Knockout of the gene encoding TLR4 in mice subjected to global ischaemia reduces HMGB1 release and microglial activation and increases neuronal survival, underscoring the potential deleterious interplay between neurons and glia in IRI[129].

TLR activation leads to a proinflammatory microglial immunophenotype, characterized by the upregulation of tumour necrosis factor (TNF), interleukin-1β (IL-1β), IL-6, IL-8 and matrix metalloproteases (MMPs)[124,130,131]. In mice subjected to global ischemia, MMPs have an important role in delayed hippocampal damage[132], and their pharmacological inhibition preserves BBB and neuronal integrity[133]. TLRs also interact closely with NADPH oxidase (NOX) to facilitate the release of ROS through nuclear factor-κB (NF-κB)-dependent mechanisms[134] (FIG. 3), a finding corroborated in a mouse model of focal ischaemia[135]. Other receptors, such as CD36, similarly converge on NF-κB to potentiate post-ischaemic inflammation and the activation of inducible NO synthase (NOS2; also known as iNOS) following focal injury[136]. After focal ischaemia, NOS2 is expressed throughout the cerebral vasculature in both rats[137] and humans[138]. In global ischaemia, inhibiting NF-κB with minocycline blocks NOS2 and microglial activation, while also protecting hippocampal neurons[139]. Concurrent upregulation of NOS2 and NOX facilitates the production of NO and the superoxide anion (O_2^-), which react to generate peroxynitrite (NO_3^-) — a highly damaging RNS that can cause either apoptosis or necrosis in a dose-dependent manner[140].

Necrosis
A form of cell death that results from unregulated digestion or autolysis of the cell.

Pharmacological inhibition[141] or loss[142] of NOS2 in rodent models of focal ischaemia results in marked cerebroprotection, implicating NOS2 as a major driver of post-ischaemic injury.

Proinflammatory cytokines also independently exert neurotoxic effects, as TNF and IL-1β signalling can promote cell death through engagement of TNF receptor 1 (TNFR1) and IL-1 receptor (IL-1R), respectively[143,144] (FIG. 3). Rather than remain local, these parenchymal proinflammatory mediators likely also propagate to the vascular compartment, exacerbating injury and activating both endothelial and peripheral immune cells. Notably, treatment with anti-TNF antibodies or IL-1R antagonists is neuroprotective in focal ischaemia in rodents[145] and humans[146], respectively.

In rodent models of CA, peripheral leukocytes and lymphocytes have been found to rapidly infiltrate the brain following reperfusion[64,65] (FIG. 1b). However, more research is needed to fully investigate the role peripheral immune cells play in IRI following global ischaemia. Much of our current understanding is extrapolated from ischaemic stroke, after which peripheral immune cells adhere to the cerebral endothelium and release proinflammatory cytokines, ROS/RNS and MMPs, thereby contributing to microvessel occlusion, BBB breakdown and tissue damage[147–149] (FIG. 1b). Moreover, following focal injury, brain-specific expression of CD36 drives the endothelial release of colony-stimulating factor 3, which promotes proinflammatory activation of infiltrated peripheral neutrophils[150]. Infiltrating neutrophils express high levels of NOS2 following focal ischaemia in rats[151], and loss of NOS2 expression in neutrophils reduces cerebral damage in chimeric mice after such ischaemia[152].

In addition to contributing to neuronal damage, microglia can also have a beneficial role following ischaemia. In vitro, exogenous BV2 (microglial-like) cells exert neuroprotective effects in hippocampal slice cultures deprived of oxygen and glucose[153]. Likewise, following global ischaemia in mice, exogenously administrated microglia (that is, those not subjected to ischaemia) migrate to hippocampal lesions, causing an overall reduction in cell damage[154]. Furthermore, microglial depletion in ischaemic slice cultures[155] and in a mouse model of focal injury exacerbates cellular damage[156]. Microglia exert their protective effects by promoting tissue repair through an 'alternative' activation profile characterized by the induction of transforming growth factor-β (TGFβ) and various interleukins, such as IL-10 and IL-4 (REF.[157]). As macrophages, microglia also assist in clearing apoptotic cells, which reinforces their anti-inflammatory immunophenotype[158,159]. Moreover, microglia produce trophic factors that promote cellular homeostasis and survival, such as brain-derived neurotrophic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF)[154,156,160] (FIG. 3). Future research aimed at selectively stimulating alternative microglial activation may promote brain tissue repair and limit cellular damage during IRI.

Oxidative stress and mitochondrial dysfunction. ROS/RNS are members of a class of toxic free radicals, and are formed by various pathways under normal[161] and IRI[162] states. The mechanisms leading to the production of ROS/RNS upon reperfusion have generally centred on mitochondrial respiration, NOX activity and xanthine oxidase activity[163].

The mitochondrial electron transport chain accounts for approximately 95% of ROS production under normal conditions[164] (FIG. 4), and the primary species produced is O_2^- (REF.[165]) (FIG. 4). This ROS normally undergoes neutralization by superoxide dismutase to H_2O_2, which is further degraded to O_2 and H_2O by catalase or glutathione peroxidase[166]. However, during IRI, endogenous antioxidant defences become depleted[167] (FIG. 4), allowing O_2^- to form other reactive species, such as H_2O_2, HO_2^- and OH^-, or combine with NO to create NO_3^- (REF.[168]). The importance of oxidative stress following global ischaemia has been substantiated by the exacerbation of cellular injury in rodents genetically lacking endogenous antioxidants[169] and, conversely, the protective effects of antioxidant overexpression[170].

The pathological production of ROS during IRI is a multistep process that displays a biphasic temporal profile, with peaks occurring at 3 h and 72 h following reperfusion in mice[171]. Complex I is a major source of mitochondrial O_2^- (reviewed in REF.[172]), producing free radicals during reperfusion through reverse electron transfer[173] via the oxidation of accumulated succinate[174] (FIG. 4). During reverse electron transfer, the complex I cofactor flavin mononucleotide becomes overly reduced, leading to extensive ROS and H_2O_2 formation, and secondary mitochondrial energy failure through feedback oxidative damage of complex I[173,175,176] (FIG. 4). Further evidence suggests that complex III activity may also become compromised in the brain following reperfusion[177]. In the presence of divalent transition metal ions (such as Fe^{2+}), H_2O_2 forms the most damaging oxidant, the hydroxyl radical ($OH•$)[178], which drives lipid peroxidation.

ROS/RNS also exacerbate IRI-mediated increases in cytoplasmic Ca^{2+} concentration, promoting mitochondrial dysfunction and activation of pro-death pathways[179–181]. Central to these injury mechanisms is the formation of the mitochondrial permeability transition pore (mPTP), which increases mitochondrial permeability to various low molecular weight solutes[182,183] (FIG. 4). The mitochondrial pro-apoptotic factor cytochrome *c* is released into the cytosol through the mPTP, initiating apoptosis[184] (FIG. 4). Following focal ischaemia in mice, mPTP formation may also lead to necrotic cell death by facilitating the interaction of p53 with cyclophilin D (CYPD)[185].

Overall, ROS/RNS and mPTP formation are viable targets to mitigate IRI. In piglet[186] and gerbil[187] models of global ischaemia, delivery of superoxide dismutase results in neuroprotection, a finding also observed with other antioxidants[188]. Likewise, inhibitors of iron-dependent lipid peroxidation have also prevented neuronal loss following focal ischaemia in gerbils[189] and helped prevent post-ischaemic hypoperfusion after global ischaemia in cats[190]. Moreover, a combination of superoxide dismutase and the iron chelator deferoxamine enhanced cerebral recovery following CA in dogs[191]. Additionally, inhibition of mPTP opening with cyclosporine A treatment diminished IRI in the rat

Reverse electron transfer
The process by which electrons are transferred in the reverse order in the electron transport chain, leading to the reduction of NAD^+.

Non-shockable
Describing cardiac rhythms that are incompatible with electrical defibrillation, such as pulseless electrical activity and asystole.

brain after selective global cerebral ischaemia[192,193], and in the rat brain[194] and rabbit brain[195] after CA. Clinically, although cyclosporine A did not improve neurological outcomes following non-shockable OHCA when administered at resuscitation[196], a subsequent trial found that it reduced infarct volume in a subset of stroke patients with proximal occlusions and successful recanalization[197]. Considering the comparable dosages between the two trials (2.0–2.5 mg per kilogram), a higher dose may be necessary to observe cerebroprotective effects in the context of CA, given the increased pathological burden.

Viability after prolonged ischaemia

Although the brain is highly susceptible to interrupted blood flow, several lines of investigation have questioned the inevitability of neural cell death after prolonged

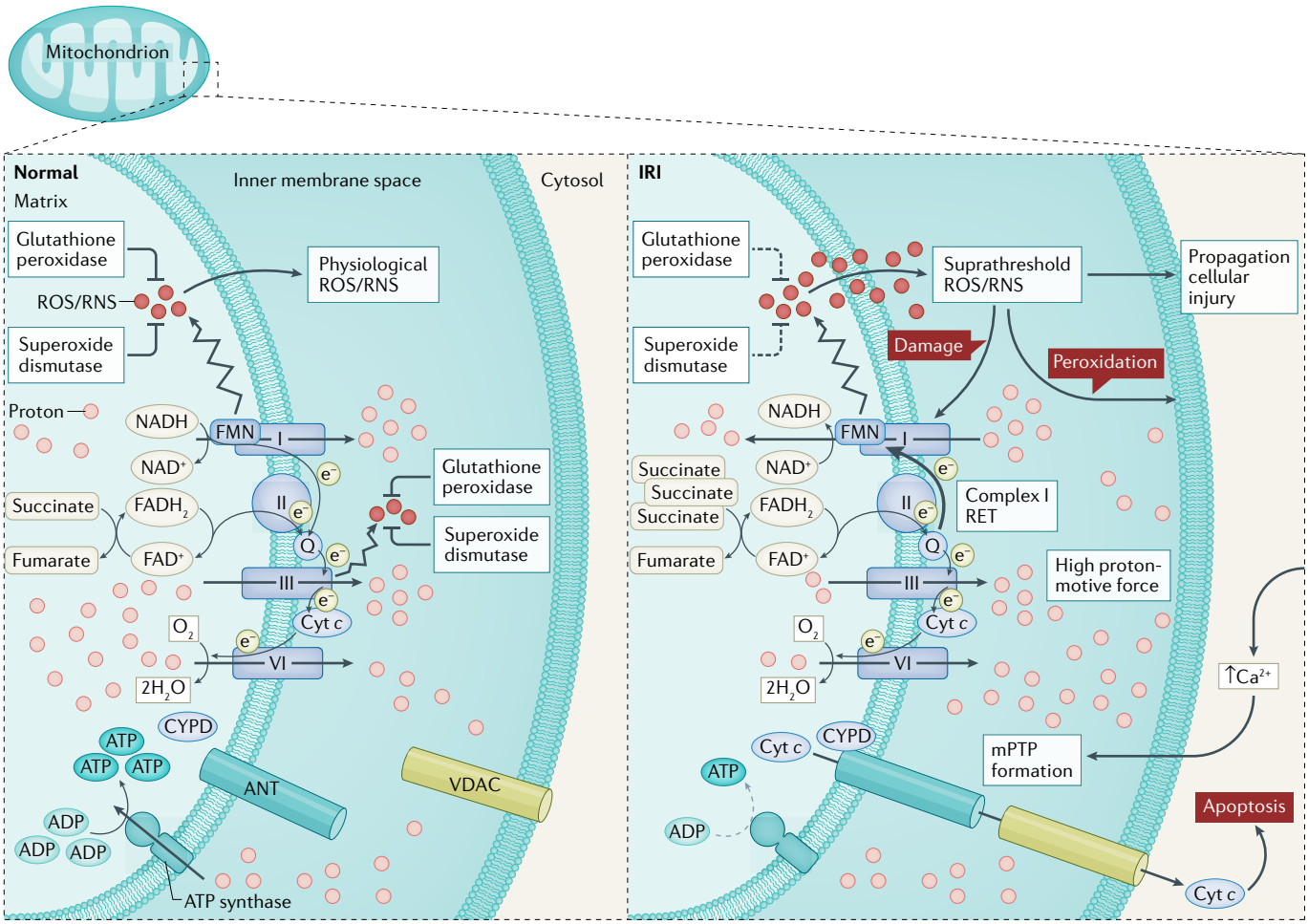


Fig. 4 | **Mitochondrial function and dysfunction during ischaemia–reperfusion injury.** Mechanisms of mitochondrial function and dysfunction under normal conditions (left) and ischaemia–reperfusion injury (IRI) conditions (right). The mitochondrial electron transport chain (ETC) is composed of complexes that transfer electrons through redox reactions with high-energy carriers such as NADH and $FADH_2$. Electrons from NADH and $FADH_2$ are sequentially passed through each complex, ultimately leading to the reduction of O_2 to H_2O. The passage of electrons down the chain drives the generation of the electrochemical gradient across the inner mitochondrial membrane, which is utilized by ATP synthase to produce ATP. Under normal conditions, the ETC produces physiological levels of reactive oxygen species (ROS), formed via univalent electron leakage by flavin mononucleotide (FMN) in complex I, as well as by complex III (red arrows). However, these reactive species are quenched by endogenous antioxidant defences. One of the major ROS is superoxide, which is quenched via dismutation to H_2O_2 by superoxide dismutase and further degraded to harmless O_2 and H_2O by catalase (not depicted) or glutathione peroxidase. Superoxide can also react with nitrogenous molecules to form reactive nitrogen species (RNS). During IRI, the ETC becomes highly reduced, mitochondrial damage accumulates and endogenous antioxidant defences become depleted. During the ischaemic period, succinate accumulates, and levels of ADP (necessary for ATP synthase activity) also become depleted. Following reperfusion, the oxidation of suprathreshold succinate maintains a pool of reduced coenzyme Q, contributing to the establishment of a proton-motive force through the proton-pump function of complexes III and IV. This proton-motive force grows in magnitude in the setting of limited ATP synthase activity, owing to reduced ADP pools. The high proton-motive force subsequently drives reverse electron transfer (RET) via complex I, during which FMN becomes overly reduced by coenzyme Q, leading to extensive generation of ROS and RNS (red arrows). This results in damage to complex I, with subsequent secondary mitochondrial energy failure. Furthermore, the suprathreshold levels of ROS and RNS lead to peroxidation of mitochondrial membranes, increasing their permeability and propagating cytosolic injury. Last, increases in intracellular Ca^{2+} concentration following ischaemia initiate the formation of the mitochondrial permeability transition pore (mPTP) through the assembly of cyclophilin D (CYPD) in the mitochondrial matrix, adenine nucleotide translocator (ANT) in the inner membrane and voltage-dependent anion channel (VDAC) in the outer membrane. This leads to a loss of tight compartmentalization in the mitochondrion, resulting in the cytosolic release of the pro-apoptotic factor cytochrome *c* (cyt c), which activates the intrinsic apoptotic cascade.

Proximal occlusions
Clots or blockages in the proximal parts of large vessels (for example, of the neck or base of the brain).

ischaemia. Post-mortem cell culture is a long-standing technique that provides fundamental evidence that brain cells (including those harvested from dead humans[198–200]) retain sufficient viability to withstand isolation procedures multiple hours after death. Furthermore, tissue specimens with sufficient viability can also be harvested from the post-mortem brain for both acute and organotypic slice cultures. In rodents, post-mortem slice cultures have been paired with electrophysiological interrogations to reveal that neuronal activity is recoverable even up to 6 h after death[201]. In humans, cortical mitochondria remain functional for multiple hours after death[202], and adult tissue specimens harvested up to 8 h post-mortem can be maintained in culture for more than 30 days with robust markers of cellular viability and integrity[203]. Moreover, viable tissue specimens of the developing human brain can be harvested even 18–49 h after death following cold storage for use in neural cell proliferation and differentiation studies[204,205].

It is important to emphasize that the highly controlled and artificial conditions in these experiments undoubtedly contributed to the observed retention of cellular viability many hours after death. Nonetheless, these studies indicate that brain cells and tissue can tolerate prolonged periods of ischaemia, retaining their capacity for cellular recovery under the appropriate circumstances. Importantly, these observations suggest that the fully intact brain may exhibit a similar resilience to extended periods of interrupted blood flow.

Resilience of intact brains

Over the past century, multiple observations have brought into question the conventional 4–5-min window of cerebral ischaemic tolerance in the fully intact brain. In the subsequent sections, we detail evidence demonstrating the resilience of the non-human large mammalian brain to global ischaemia, and outline several key principles that may afford the brain greater resistance to prolonged periods of circulatory arrest. We also describe the clinical circumstances in which the human brain exhibits a heightened tolerance to global ischaemia.

Resilience of non-human brains

One of the earlier studies extending the timeframe in which large mammals can survive CA found that canines can withstand 10 min of global normothermic ischemia with full neurological recovery[206]. However, to test brain-specific tolerance, selective cerebrocirculatory arrest was produced in a later study by increasing intraventricular pressure above systemic circulatory pressure, creating a 'bloodless' ischaemia[207]. Under these circumstances, animals withstood 25 min of cerebrocirculatory arrest if artificial ventilation was maintained[207], indicating that the brain parenchyma may be more resilient to global ischaemia in the absence of intravascular blood. This time window was later extended in cat and non-human primate brains, with the restoration of cerebral metabolism, intact pyramidal cell response and even integrative neurological function following 1 h of complete bloodless ischaemia with high reperfusion pressure[30,208,209]. These findings not only corroborate that the brain can withstand prolonged global ischaemia with the elimination of intravascular blood[207], but also demonstrate that ischaemic tolerance could be broadened to 1 h in the presence of controlled reperfusion at increased blood pressures.

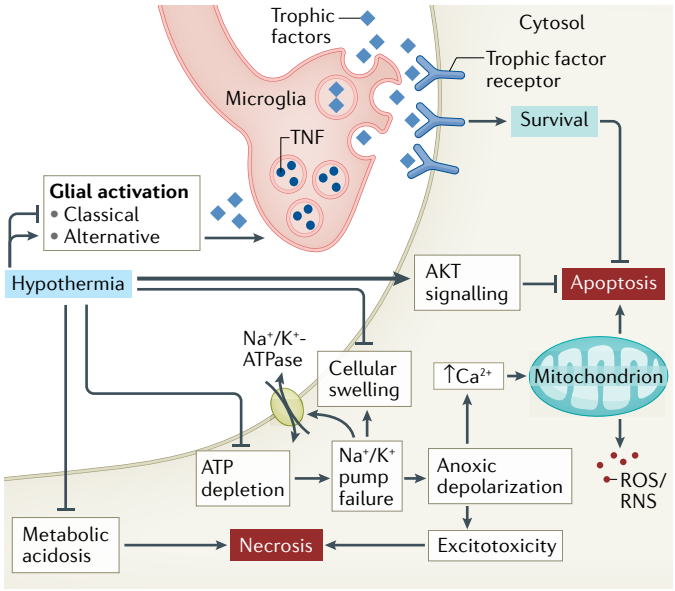
More recent studies have shown that cell functions in the fully intact, isolated porcine brain can be restored ex vivo 4 h after death using a combination of bloodless ischaemia, hypothermia, controlled reperfusion and a cytoprotective acellular perfusate[119]. Although no global cerebral function (that is, in the form of synchronized cortical network activity) was observed after 6 h of reperfusion, parenchymal and vascular cells demonstrated intact structural and functional viability multiple hours after death[119], indicating that the brain may retain even greater resilience to ischaemia with the appropriate set of multimodal interventions.

However, these extended timepoints have yet to be translated to a whole-body model of CA. Indeed, it is possible that the aforementioned resilience of the brain to selective cerebrocirculatory arrest may be due to the lack of global corporeal ischaemia, and that, following prolonged periods of CA, successful neurological resuscitation may be limited by the aggregate release of toxic by-products from each ischaemic organ. Nevertheless, advancements in CA research corroborate the necessity of controlling both recirculation with high mean arterial pressure[210,211] and pulsatility[212,213] to achieve full brain reperfusion, as well as controlling the molecular composition of the reperfusate to limit IRI[95]. With such parameters in place, adult pigs demonstrated survival with intact neurological function after 20 min of CA[95].

From these studies, several principles can be distilled about the factors that can augment the ability of the brain to tolerate prolonged periods of global ischaemia. These include intravascular blood removal, controlled reperfusion, hypothermia and molecular control of the cellular and tissue environment. As observed in ex vivo cell/tissue culture[201–204,214,215] and brain[119] studies, along with the selective cerebrocirculatory arrest models[30,207–209], neural cells and tissue can withstand prolonged periods of ischaemia in the absence of intravascular blood. The benefits of blood removal are probably multifactorial, but likely centre on diminishing the peripheral inflammatory response and mitigating the no-reflow phenomenon during recirculation. Furthermore, controlled reperfusion with high mean arterial pressure and pulsatility is necessary to preserve brain functionality following 1 h of selective cerebrocirculatory arrest in cats and non-human primates[30,208,209], and to maintain neurological function following 20 min of CA in pigs[95]. Similar to intravascular blood removal, high mean arterial pressure and pulsatility facilitate widespread microvascular perfusion to overcome post-ischaemic circulatory deficits. In addition, a form of hypothermia along with biochemical control of the cellular and tissue environment was used in post-mortem ex vivo cell/tissue culture[201–204,214,215] and brain[119] studies, as well as in full-body CA models[95]. The protective effects of hypothermia involve multiple mechanisms simultaneously (FIG. 5a), chief among which is the delay of fulminant energy failure and metabolic

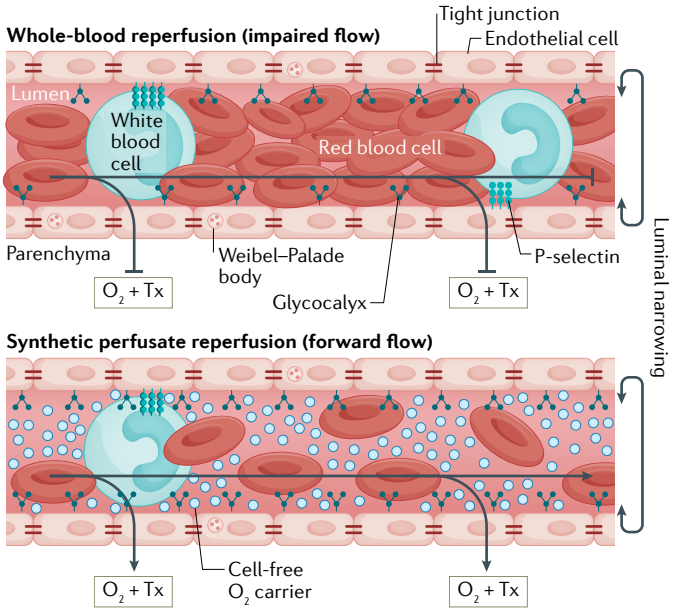
a

Therapeutic hypothermia
- Controls metabolism
- Delays energy failure
- Decreases metabolic acidosis
- Promotes prosurvival pathways
- Quells inflammation



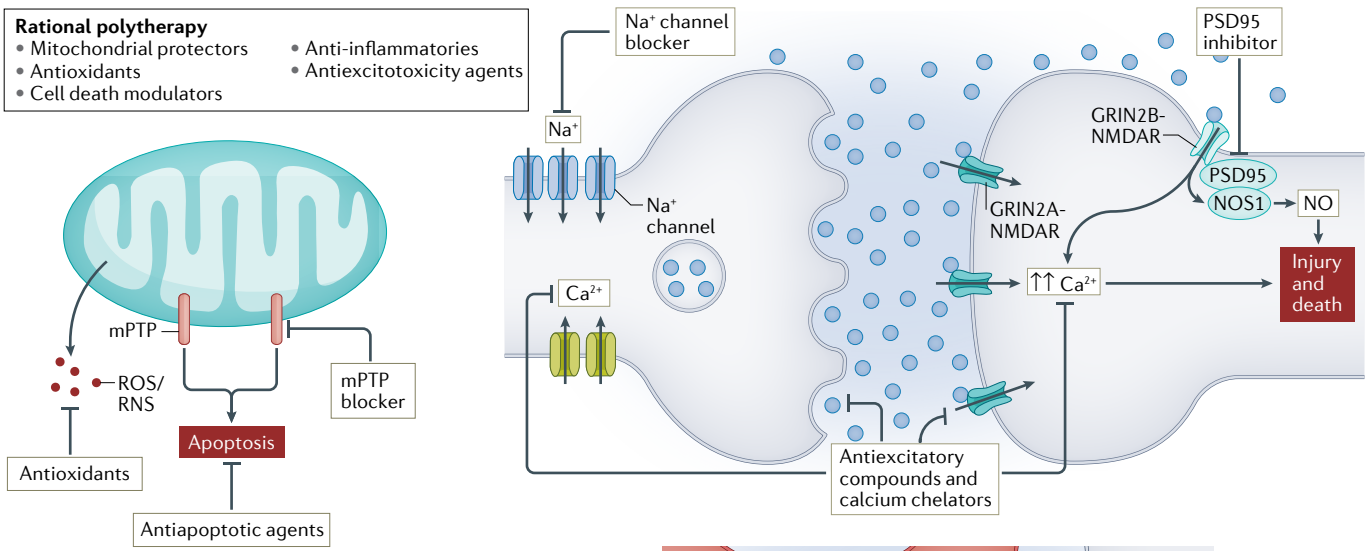
b

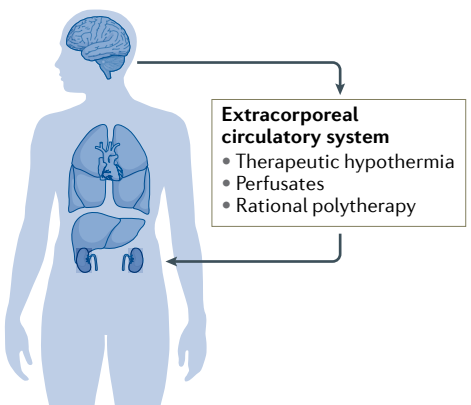
Perfusates
- Acellularity (circumnavigate occlusions)
- Robust oxygen delivery
- Delivery vehicle for rational polytherapies
- Non-coagulative
- Osmolarity (limit oedema)



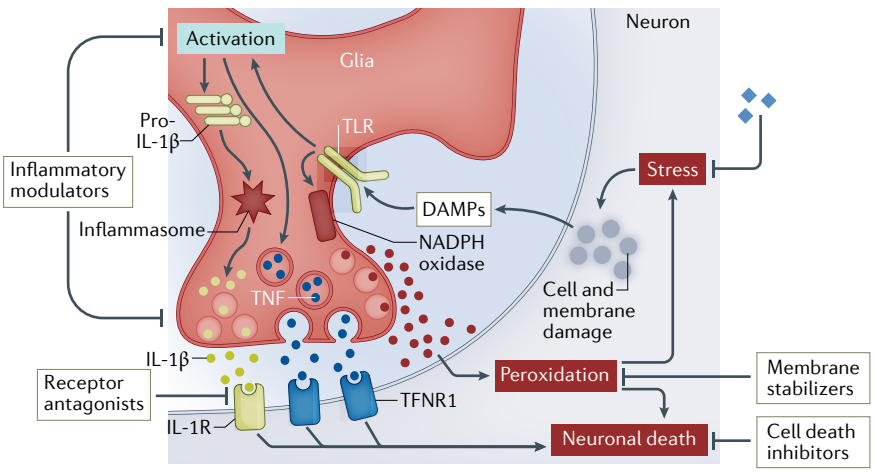
c

Rational polytherapy
- Mitochondrial protectors
- Antioxidants
- Cell death modulators
- Anti-inflammatories
- Antiexcitotoxicity agents





Extracorporeal circulatory system
- Therapeutic hypothermia
- Perfusates
- Rational polytherapy



◀ Fig. 5 | **A combined therapeutic strategy for reducing global ischaemic injury.** **a** | Therapeutic hypothermia exerts protective effects during global ischaemia through various mechanisms (upper left). Hypothermia inhibits the depletion of ATP, thus delaying Na^+/K^+ pump failure. In so doing, hypothermia preserves ionic balance, prevents cellular swelling and prevents downstream anoxic depolarization and excitotoxicity. By inhibiting anoxic depolarization, hypothermia mitigates supraphysiological accumulation of calcium, and therefore reduces mitochondrial dysfunction, apoptosis and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). In addition to blocking excitotoxicity, hypothermia slows the development of metabolic acidosis, thereby attenuating necrotic cell death during ischaemia. Lastly, cooling initiates pro-survival pathways that block apoptosis, such as AKT signalling, and prompts the release of trophic factors from supporting glial cells. **b** | The design and use of novel perfusates are an important avenue for the development of effective strategies in mitigating ischaemia–reperfusion injury and promoting cellular recovery. Acellular and non-coagulative perfusates could exert their beneficial effects by ensuring robust delivery of oxygen and treatments (Tx), while also being amenable to manipulations of osmolarity, for example, to limit oedema formation. During whole-blood reperfusion, various cellular components can form microthrombi, impeding vascular flow. Such microthrombi form in the context of increased blood stasis, cell adhesion molecules and vasoconstriction, resulting in impaired delivery of oxygen and therapeutic treatments (top vessel). Perfusion with an acellular perfusate containing cell-free oxygen carriers could flow around cellular obstacles, ensuring robust post-ischaemic reperfusion and maintenance of parenchymal oxygen and Tx levels (bottom vessel). Importantly, perfusates could act as a vehicle for the delivery of rational polytherapies. **c** | Rational polytherapy is characterized by the deliberate use of independent pharmacological agents that can simultaneously target multiple deleterious mechanisms known to cause cellular injury and death following ischaemia. For example, antioxidants, antiapoptotic agents and mitochondrial permeability transition pore (mPTP) blockers could act synergistically to promote mitochondrial health and function. These, together with antiexcitotoxicity agents, sodium channel blockers, inhibitors of postsynaptic density protein 95 (PSD95) and calcium chelators, could limit the extent of excitotoxicity during ischaemia–reperfusion injury. Furthermore, these agents can be coupled with inflammatory modulators as well as trophic factors and membrane stabilizers to limit injury caused by proinflammatory mediators. The correct timing and combination of each of these therapeutic approaches has not yet been fully explored, and further research is needed in this area. Currently, interventions are instituted at the beginning of the initial reperfusion. The strategies described in parts **a**–**c** could be administered via extracorporeal circulatory systems (bottom left) using targeted physiological parameters such as high mean arterial pressure and pulsatility. DAMP, damage-associated molecular pattern; GRIN2A-NMDAR, GRIN2A subunit-containing NMDA receptor; GRIN2B-NMDAR, GRIN2B subunit-containing NMDA receptor; IL-1β, interleukin-1β; IL-1R, interleukin-1 receptor; NO, nitric oxide; NOS1, neuronal nitric oxide synthase; TLR, Toll-like receptor; TNF, tumour necrosis factor; TNFR1, tumour necrosis factor receptor 1.

perturbations. Lastly, biochemical control of the cellular and tissue milieu is crucial to provide tissues with the corrective substrates necessary for survival, while also blocking the deleterious molecular programmes activated during the ischaemic period.

Human brain resilience

The human brain can also display remarkable resilience to interrupted blood flow, even several hours after CA, such as in the case of accidental hypothermia. Specifically, patients have been resuscitated from hypothermic arrest with intact neurological function using various means of extracorporeal perfusion[216–218]. Moreover, a 47% survival rate was reported in a cohort of patients who experienced accidental deep hypothermia followed by rewarming with extracorporeal circulation, with all survivors exhibiting long-term recovery of neurological function[219].

In addition to accidental circumstances, therapeutic hypothermia is commonly used in cardiovascular surgery to protect the brain during extended ischaemia[220,221], as well as during prolonged periods of CA[222,223]. Indeed, induced hypothermia has been associated with improved neurological outcomes in CA[224,225], leading to a formal recommendation for its use in resuscitation[226].

These clinical studies indicate that the human brain can withstand prolonged global ischaemia while under hypothermic conditions. Furthermore, extracorporeal perfusion, whether used to administer cooling procedures or to assist in rewarming, has also demonstrated efficacy in preserving neurological function after extended ischaemia. This is probably due to its ability to achieve targeted circulatory flow rates and overcome microcirculatory deficits. Indeed, if CPR does not achieve the return of spontaneous circulation (ROSC), extracorporeal perfusion can be administered in the form of extracorporeal CPR (eCPR) to maintain vital organ perfusion while the underlying cause of CA can be addressed[227]. Trials studying the effects of eCPR on neurological outcomes after CA are not yet definitive, but trend favourably[227–230]. Overall, it is clear that the use and development of advanced extracorporeal circulatory devices will be central for delivering novel therapies and maintaining adequate corporeal circulation for effective recovery.

Therapeutic implications

Taking the above studies into account, further research efforts should focus on merging and developing the aforementioned principles into an advanced, multimodal cerebroprotective strategy following prolonged global ischaemia. Specifically, a comprehensive approach would leverage the beneficial effects afforded by hypothermia, blood removal or haemodilution, pharmacological inhibition of injury mechanisms and controlled reperfusion with high pulsatile perfusion pressures. Such an approach could be achieved by combining therapeutic hypothermia with novel synthetic perfusates and rational polytherapies, all of which could be effectively administered via extracorporeal circulatory systems (FIG. 5). Some preliminary variations on these therapeutic principles have led to remarkable protection of brain structure and function after prolonged global ischaemia in experimental models[95,119,209].

Ex vivo whole-brain studies represent an opportune experimental paradigm for investigating the optimal combinations of such interventions, affording researchers the latitude of testing multiple variables in an isolated large mammalian brain (BOX 2). Furthermore, given the time-dependent pathophysiology of global ischaemia, future investigations will need to discern the ideal timing and duration of each component of a multimodal strategy after prolonged CA. Currently, interventions are deployed at the beginning of the initial reperfusion and maintained for varying lengths of time, which may influence their full therapeutic potential. For example, the administration of hyperosmolar therapy to mitigate oedema formation may be crucial early in reperfusion, but could cause damage with prolonged use. Importantly, such interventions could be first established and optimized in the isolated brain following varying lengths of ischaemia before being translated to the whole body, and used to guide clinical trial design (BOX 2).

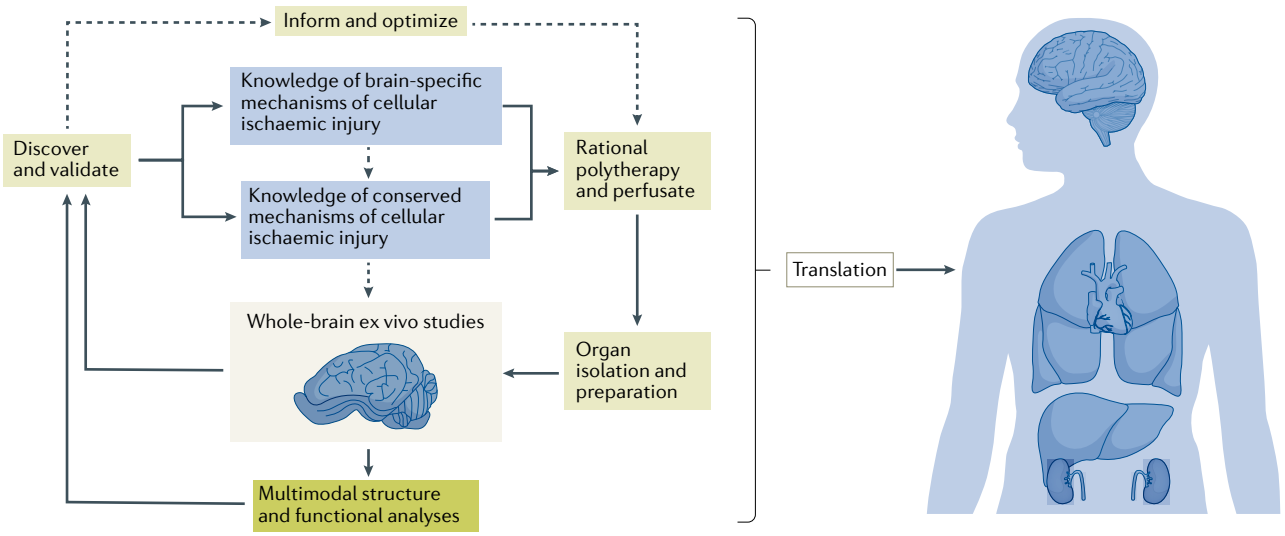
Box 2 | **Ex vivo brain studies as a tool for translation**

Isolated ex vivo brain studies are an ideal research platform for the development of new therapeutic strategies for ischaemia. Indeed, basic science knowledge of brain-specific and conserved mechanisms of ischaemic injury can guide the design of rational polytherapies and perfusates (see the figure; Supplementary Table 1). The efficacy of these interventions in promoting functional recovery following various lengths of ischaemia can be tested using multimodal structural and functional assays in isolated ex vivo brains (see the figure). The findings of these studies can then be used to both discover novel mechanisms of cellular injury and validate existing mechanisms of cellular injury in the brain for subsequent optimization of polytherapies and perfusates before being translated to the whole body (see the figure).

Ex vivo whole brains afford investigators with multiple scientific advantages. Chief among these is the latitude to experimentally manipulate physiological, mechanical and/or pharmacological parameters, such as the rate of cooling or rewarming, perfusion pressures and flows, dissolved gas levels and perfusate composition. Moreover, the ex vivo whole brain allows researchers to apply invasive experimental techniques to the tissue parenchyma, which is often not feasible in vivo. Specifically, this paradigm allows investigators to obtain real-time tissue and perfusate specimens during experimentation, helping to establish causal relationships between specific manipulations and cellular or global brain responses. Research efforts should leverage these experimental advantages to develop an optimized multimodal interventional strategy to address the complex pathophysiological consequences of reperfusion injury in the brain.

Electroencephalography is an important tool for determining the recovery of integrated brain functions in these experiments owing to its non-invasiveness and high temporal resolution. Quantitative electroencephalography can be combined with xenon-enhanced computed tomography and positron emission tomography to study the real-time effects of experimental interventions on global electrical activity, cerebral blood flow and the metabolic rate of oxygen consumption[289,290].

Overall, this experimental workflow could help uncover optimal therapeutic strategies for brain protection and recovery following prolonged ischaemia. These optimized resuscitation parameters in the isolated brain can also be tested in peripheral organs (not depicted) before being translated to a whole-body model of cardiac arrest, or can be used to guide clinical trial design (see the figure). Overall, experimental workflows that juxtapose ex vivo isolated organs and whole-body research are a powerful way forward in the discovery of novel therapies for global ischaemia.



Hypothermia

The cerebroprotective effects of induced moderate hypothermia following CA have been confirmed in large clinical trials[224,225] and could even offer neuroprotection in patients with an initial nonshockable rhythm[231]. Although most effective when applied immediately after ROSC, moderate hypothermia (32–34 °C) may remain effective even when applied 4–16 h after the onset of CA[225]. Current guidelines recommend placing comatose adult patients with ROSC under moderate hypothermia, or strictly maintaining a temperature of 36 °C, for up to 24 h (REF.[232]). Rewarming is also an essential component of hypothermic therapy, with a currently recommended rewarming rate of 0.25–0.5 °C per hour[233]. However, no clear consensus exists, and some investigators indicate that a slower rewarming rate of 1 °C per day may improve neurological outcomes[234]. Overall, the ideal temperature management strategy remains under investigation.

In canines, resuscitation with mild hypothermia (34 °C) and circulatory support via cardiopulmonary bypass following 11 min of CA resulted in 100% survival and reduced histological brain damage compared with controls[235]. Furthermore, deep hypothermic CA (15 °C) administered via cardiopulmonary bypass for 60 min, 90 min and 120 min after 30 min of severe haemorrhagic shock resulted in 100% survival across all timepoints, with animals displaying good neurological function with hypothermic CA times of up to 90 min (REF.[236]). Profound hypothermic CA (10 °C) was found to exert greater cerebroprotection in the same canine model, resulting in intact neurological function even after 120 min of hypothermic CA[237]. Notably, the investigators in these studies also used a blood washout, further substantiating the beneficial effects of intravascular blood removal in the context of ischaemia. Similar results were observed in a separate exsanguination model in pigs, reporting improved outcomes with rapid cooling and slow rewarming[238,239]. Whole-brain ex vivo studies can serve as a fruitful adjunct to these models to determine the ideal temperature-management strategy for optimal cerebroprotection before translation to the whole body (BOX 2).

The protective effects of hypothermia are normally attributed to its ability to reduce metabolism and inhibit the breakdown of high-energy phosphates, such as ATP[240,241] (FIG. 5a). This not only maintains energy balance but also slows the cellular derangements known to occur following ischaemic injury. For example, cooling prevents the accumulation of lactate and succinate[242], potentially circumventing the deleterious effects of metabolic acidosis and mitochondrial ROS production (FIGS 4,5a). However, even if hypothermia slows ATP breakdown, it does not prevent its inevitable depletion[240]. Thus, its protective effects cannot be entirely explained by simple energetic budgeting, but may involve limiting other mechanisms associated with excitotoxicity, calcium-mediated cell damage, ROS production and apoptosis[243–245] (FIG. 5a). In addition, hypothermia upregulates many prosurvival programmes in the brain, such as AKT signalling, along with the expression of trophic factors such as BDNF and GDNF[246–248] (FIG. 5a). More molecular, cellular and organ-level research — potentially using an intact ex vivo brain model (BOX 2) — will be necessary to uncover the protective mechanisms of the effects of hypothermia.

Perfusates

Blood is a complex, fluidic tissue that responds independently to injury. Reperfusion with whole blood — whether in the ex vivo brain or in the whole body — carries its own disadvantages by promoting coagulation, microvascular plugging, inflammation and dysfunction of its cellular components. In multiple contexts, blood removal, or haemodilution, during ischaemia has been effective in mitigating the deleterious effects of post-ischaemic reperfusion deficits and IRI. However, inadequate circulatory volume and/or severe haemodilution in vivo may impair the delivery of various agents, especially oxygen, to the brain, thereby limiting the extent of achievable haemodilution. Thus, synthetic acellular perfusates with high oxygen-carrying capacities can be engineered to act as blood supplements, helping to prevent anaemia and reduce the no-reflow phenomenon and IRI through effective vascular distribution of molecular therapeutics (FIG. 5b).

To ensure appropriate oxygen delivery in the context of substantial haemodilution, considerable effort has been put into developing acellular solutions with sufficient oxygen-carrying capacities. These include haemoglobin-based and perfluorocarbon-based artificial oxygen-carrying systems[249,250] (Supplementary Table 1), which have been used for perfusion of various organs, including the brain[119], as well as in large mammalian models of resuscitation[251,252]. Importantly, acellular oxygen carriers can circumnavigate constricted or partially blocked capillaries during post-ischaemic recirculation, ensuring widespread oxygen delivery to the brain and body (FIG. 5b). Although acellular oxygen carriers should be considered integral components of future perfusates, their vasoactivity and oxidative potential should be assessed and potentially mitigated by incorporating antioxidants and/or free radical scavengers (Supplementary Table 1) — especially given the decrease in host antioxidant defences during IRI[167] (FIG. 4).

Perfluorocarbon
A class of organic molecules that form the basis of solvents with high oxygen-carrying capacities.

The composition of perfusates is of paramount importance in treating IRI and maintaining organ homeostasis following prolonged ischemia, or when one is translating these solutions to the whole body for resuscitation following CA. Given current understanding of the mechanisms of IRI, investigators may use novel acellular perfusates as a delivery vehicle to test the efficacy of various polytherapies (BOX 2). For example, the introduction of a perfusate or blood compounds devoid of immune cells and/or supplemented with anti-inflammatory agents may help distinguish the effects of peripheral immune cell activation versus microglial activation during IRI in a highly tractable system. Furthermore, other perfusates can be investigated in the intact large mammalian brain to study their effects on addressing IRI and post-ischaemic injury (BOX 2; FIG. 5b).

Importantly, comprehensive control of the initial reperfusate cannot be achieved during conventional resuscitation via intravenous administration alone due to unstable haemodynamics — especially in the absence of ROSC — as well as the inability to deliver large volumes of the reperfusion solution immediately. Rather, perfusates should be considered a vehicle for therapies and nutrients to be distributed throughout the body via advanced extracorporeal circulatory systems (FIG. 5c). Such perfusion devices allow real-time assessment and adjustment of physiological parameters, including flow rate and pH, which could be further manipulated to examine their effects on IRI. As controlling the mechanical and physiological aspects of reperfusion is crucial[210], it will be necessary to engineer perfusates that are compatible with various parameters and formulations.

Rational polytherapy

The notion that neuroprotective drugs work in animal models of ischaemia but fail to translate to humans is not new[253] (also reviewed elsewhere[254,255]). The various reasons that underlie the apparent failure of drug translation include the appropriateness of experimental models, treatment timing and clinical study design.

Another possible reason is that past neuroprotective strategies have largely targeted a single mechanism of cellular injury. As such, monotherapies are probably inadequate to treat ischaemia, given the various time-dependent and cell type-dependent mechanisms governing injury (FIGS 1–4). Thus, targeting multiple mechanisms could lead to synergistic effects and improved outcomes (FIG. 5c; Supplementary Table 1). Indeed, rational polytherapies have shown efficacy in several ischaemic models.

In a canine model of global ischaemia, the co-administration of mannitol (an anti-oedema agent), tocopherol (an antioxidant), dexamethasone (an anti-inflammatory) and a molecular oxygen carrier restored electrical activity more quickly than did administration of each compound alone[256]. In addition, basic fibroblast growth factor (a neurotrophin) and citicoline (a cell membrane stabilizer) reduced mortality and brain injury in a rat model of transient focal ischaemia, whereas either agent alone failed to show efficacy[257]. Notably, monotherapies may prove ineffective because they may

Histotoxic hypoxia
A chemically induced form of hypoxia in which cells are unable to utilize oxygen despite adequate delivery or concentration of oxygen.

Ventricular fibrillation
An abnormal cardiac rhythm in which the ventricles display erratic and uncoordinated contractions (fibrillation) owing to aberrant electrical conduction.

substitute one mechanism of cellular death for another, as demonstrated by the unexpected unmasking of apoptosis following the inhibition of excitotoxic necrosis in cultured cortical neurons[258,259]. Indeed, simultaneous inhibition of necrosis and apoptosis showed synergistic protection in a mouse model of histotoxic hypoxia[260].

Polytherapy has also been examined in the setting of CA. In a porcine model of 30-min ventricular fibrillation, streptokinase (a thrombolytic) plus eCPR and mild hypothermia (33 °C) reduced brain oedema and improved cardiac resuscitability compared with controls, although these interventions did not prevent neuronal injury[261]. Alternatively, a combination of adrenaline, atenolol (a β-adrenergic receptor antagonist) and levosimendan (a calcium sensitizer) during CPR following ventricular fibrillation in pigs reduced serum levels of astroglial and neuronal injury markers, and improved neurologic outcomes 48 h after resuscitation[262]. Similarly, a combination of noradrenaline and heparin (an anticoagulant), plus cerebral intravascular flushing and systemic haemodilution with dextran 40 (a colloid-like volume expander) following ventricular fibrillation in dogs resulted in 100% survival and positive neurological outcomes[263]. Furthermore, xenon, an anaesthetic that also displays NMDAR-antagonistic properties[264], conferred cerebroprotection during CA when combined with mild hypothermia[265], but not when administered separately[266]. This combination was also investigated in a clinical trial using diffusion tensor MRI, which revealed a decrease in white-matter microstructural damage in comatose patients who had experienced OHCA who received both therapies together compared with hypothermia alone[267].

Moving forward, brain-specific and conserved mechanisms of ischaemic injury should be at the centre of the rational design of novel polytherapies (BOX 2; Supplementary Table 1). Notably, the list of therapeutic categories in Supplementary Table 1 is not comprehensive, but instead includes examples of selected approaches and possible mechanisms to target. In designing polytherapies, investigators should include pharmacological compounds parsimoniously and deliberately to test specific hypotheses, and to establish the minimal components necessary to achieve maximum efficacy while avoiding possible adverse pharmacological interactions. Moreover, although polytherapy has been largely described as a pharmacological approach, its definition can be broadened to include additional interventions such as temperature management, perfusion dynamics and pH-controlling strategies. Overall, compelling evidence indicates that combination therapy holds promise for reducing IRI after CA.

Conclusions

Clinical observations maintain that the brain is intolerant of CA lasting more than several minutes. However, under specific circumstances, large mammals, including humans, can demonstrate resilience to prolonged ischaemia. Careful analysis of the conditions in which mammalian neural tissues can recover from extended global ischaemia has provided principles upon which to build future clinical solutions, as best exemplified in the case of therapeutic hypothermia.

However, given the many deleterious mechanisms that arise from prolonged global ischaemia, traditional monotherapeutic approaches have proved inadequate. A multifaceted strategy, combining advanced circulatory technologies compatible with perfusate delivery of rational polytherapies across a range of physiological parameters, may provide a more fruitful avenue for rigorous clinical and laboratory research on cerebral protection following extended ischaemia. Investigators now have various tools with which to test the efficacy of a multimodal approach for salvaging brain function after global ischaemia, such as the means of manipulating reperfusion conditions and the composition of reperfusion solutions to ameliorate IRI.

Evidence suggests that, under certain circumstances, the brain may be more resistant to circulatory arrest than widely appreciated. However, translating these findings from the laboratory to the clinical setting has proved difficult and often disjointed. New insights gained via bridging the knowledge of basic mechanisms of ischaemic injury, ex vivo organ preservation and extracorporeal perfusion have the potential to improve care for individuals with global cerebral ischaemia.

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S.G.D., G.T., K.A.H., Z.V., K.T.G., F.B. and N.S. researched data for the article. S.G.D., G.T., K.A.H., F.B. and N.S. wrote the article. All authors contributed substantially to discussion of the content and reviewed and/or edited the manuscript before submission.

Competing interests

S.G.D., Z.V. and N.S. are listed with J. Silbereis as inventors on a patent held by Yale University entitled "Methods, systems and compositions for normothermic ex vivo restoration and preservation of intact organs" (WO2019157277A1). F.B., C.B., and G.T. are shareholders in Resuscitec GmbH, a company originating from the University of Freiburg. K.A.H., K.T.G., D.A., D. Damjanovic, J-S.P. and D. Dellal declare no competing interests.

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