Cerebral preconditioning and ischaemic tolerance

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Abstract | Adaptation is one of physiology's fundamental tenets, operating not only at the level of species, as Darwin proposed, but also at the level of tissues, cells, molecules and, perhaps, genes. During recent years, stroke neurobiologists have advanced a considerable body of evidence supporting the hypothesis that, with experimental coaxing, the mammalian brain can adapt to injurious insults such as cerebral ischaemia to promote cell survival in the face of subsequent injury. Establishing this protective phenotype in response to stress depends on a coordinated response at the genomic, molecular, cellular and tissue levels. Here, I summarize our current understanding of how 'preconditioning' stimuli trigger a cerebroprotective state known as cerebral 'ischaemic tolerance'.

Ischaemic tolerance
A condition of transiently increased resistance to ischaemic injury as a result of the activation of endogenous adaptive mechanisms by preconditioning.

Preconditioning
Presenting a stressful but non-damaging stimulus to cells, tissues or organisms to promote a transient adaptive response so that injury resulting from subsequent exposure to a harmful stimulus is reduced.

Anoxia
Complete lack of oxygen; in contrast to hypoxia, or low oxygen.

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The brain's resistance to ischaemic injury, or ischaemic tolerance, can be transiently augmented by prior exposure to a non-injurious preconditioning stimulus. The first *in vivo* study of cerebral preconditioning documented an acute increase in the capacity of the rat brain for anaerobic glycolysis after brief anoxia, which increased the survival time of the animal following a subsequent exposure to prolonged anoxia[1]. That the amplitude of an electrically-evoked population spike from hippocampal CA1 neurons was maintained during anoxia if the slice was exposed earlier to brief anoxia led to the proposal in 1986 by Schurr *et al.*[2] that something akin to ischaemic tolerance might exist. The first landmark paper on cardiac preconditioning in dogs by brief coronary ischaemia was published in the same year[3]. The ability of brief hyperthermia to protect against subsequent focal stroke was documented shortly thereafter[4], but was not recognized at the time as a type of preconditioning-induced ischaemic tolerance. Rather, it was the dramatic finding that the delayed neuronal death of gerbil hippocampal CA1 pyramidal cells after global ischaemia could be completely prevented if carotid blood flow was briefly interrupted 2 days earlier[5] that launched the field of ischaemic tolerance research in the brain. Today, a number of robust and reproducible experimental models of cerebral ischaemic tolerance are recognized (see online Supplementary information S1 (table); for recent reviews, see REFS 6–10). Although rodent and neuronal cell culture models serve as the foundation for the field so far, emerging evidence indicates that ischaemic tolerance is an evolutionarily conserved form of cerebral plasticity that occurs in invertebrates and vertebrates, including humans.

Even without preconditioning, resident brain cells naturally respond to ischaemia by mobilizing a host of defences and counter responses to mitigate cell injury and death[11] (FIG. 1). However, preconditioning triggers a fundamentally different adaptive response and, in mammals, this response is characterized by at least two distinct time frames of induced tolerance relative to the preconditioning stimulus and the subsequent ischaemia. A short-lasting protective phenotype can be induced within minutes of exposure to preconditioning stimuli[2,12-14], as a result of changes in ion channel permeabilities, protein phosphorylation and other post-translational modifications; this is known as rapid preconditioning. However, the phenomenon of ischaemic tolerance is best appreciated as one that requires gene activation and *de novo* protein synthesis (FIG. 1); this 'classical preconditioning' requires many hours or even days to become fully manifest. Without another preconditioning stimulus to sustain it, the window for protection also wanes within days. The expression profile of gene activation and repression set in motion by preconditioning is not only temporally specific (see below), but also differs between neurons, glia and endothelial cells. The overall implication is that diverse families of pro-survival genes are activated and, in turn, encode proteins that serve to enhance the brain's resistance to ischaemia. Protection is achieved by the attenuation of broad categories of injury-inducing mechanisms, including excitotoxicity, ion/pH imbalance, oxidative and nitrosative stress, metabolic dysfunction, inflammation and, ultimately, necrotic and apoptotic cell death. Protection is also
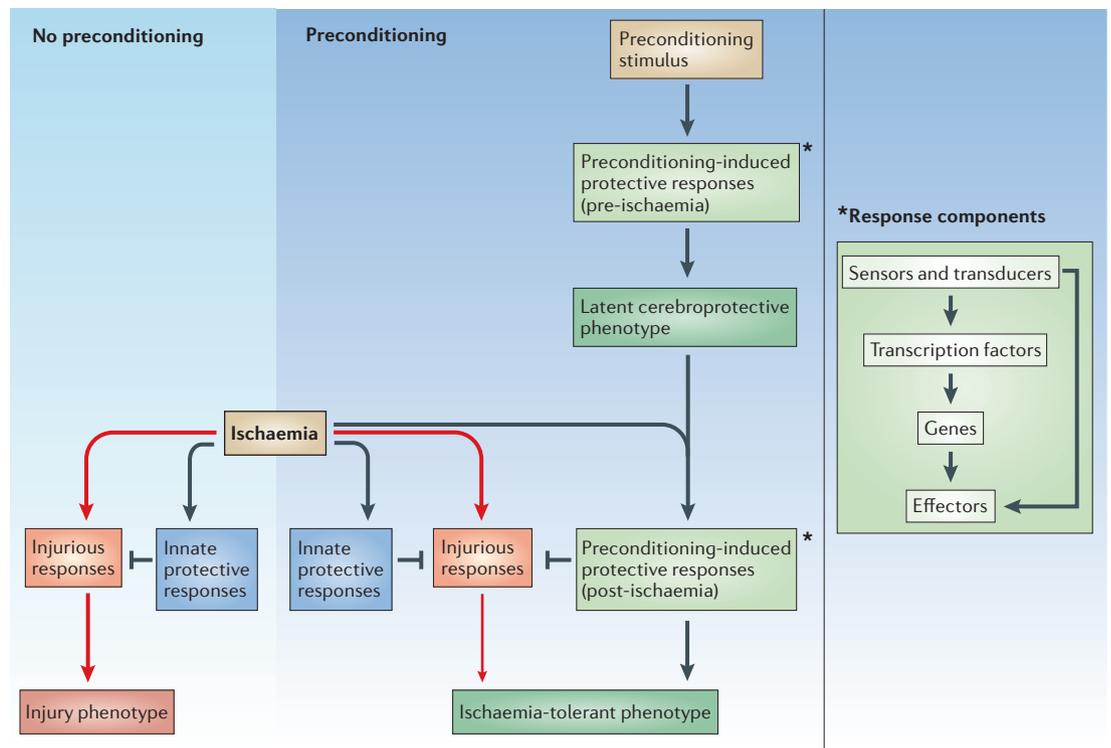
Figure 1 | Cerebroprotection by preconditioning. This protection requires several stimulus-specific and temporally-defined response cascades that, through a variety of effector molecules, are collectively responsible for establishing the ischaemia-tolerant phenotype. In the non-preconditioned brain, ischaemia-induced injurious responses (red arrows), tempered by innate protective responses that are also activated by ischaemia[11], lead to the well-known injury phenotype. However, the ischaemia-tolerant phenotype is the result of both pre- and post-ischaemic protective responses induced by preconditioning, each of which can be considered to be comprised of unique and shared effector cascade response components (see inset). The effectors in these cascades are either produced *de novo* as a result of changes in gene expression (triggered by distinct sensors, transducers and transcription factors) or are existing proteins that become activated by post-translational modifications. Therefore, even prior to ischaemia, preconditioning establishes a latent cerebroprotective phenotype. When ischaemia ensues, these effects contribute to the overall protection, joining together with other specific protective responses that ischaemia triggers in a 'reprogrammed', preconditioned brain. In addition to directly abrogating the injury-promoting cascades (thin red arrow), these responses render the brain more resistant to ischaemic injury and, collectively, serve as the mechanistic foundation of the ischaemia-tolerant phenotype.

Ischaemia
The condition of reduced or blocked blood flow to a tissue, which, as a result of reduced oxygen and nutrient delivery, can lead to tissue injury.

Stroke
A cerebrovascular injury in which blood supply to part of the brain is suddenly interrupted by vessel occlusion (ischaemic stroke) or by vessel wall rupture (haemorrhagic stroke); brain damage and death can rapidly ensue.

Cerebral plasticity
The ability of the brain to reorganize or change both structurally and functionally in response to a challenge, stressor or new experience; this change can be short- or long-lasting, or permanent.

Oxidative and nitrosative stress
A potentially helpful or harmful condition that is due to the actions of molecular compounds with reactive oxygen or reactive nitrogen groups, respectively.

manifested by engaging innate survival mechanisms and enhancing endogenous repair processes (for example, the proliferation and mobilization of bone marrow and other stem cells) that lessen the overall extent of injury and concomitantly facilitate the recovery of brain function.

Broadly speaking, classical preconditioning and the acquisition of the ischaemia-tolerant phenotype involves particular adaptation response cascades to specific stimuli occurring over distinct time frames[15,16]. Whether the triggering stimulus is preconditioning alone, ischaemia alone, or ischaemia with prior preconditioning, each of the cascades can be characterized by gene-dependent responses involving a unique family of sensors, transducers and transcription factors that activate them and the effector proteins that are produced; gene-independent, post-translational modifications of existing proteins also contribute concomitantly to the response (FIG. 1). In brief, preconditioning itself initiates many adaptive reactions in cells and tissues that lead, after some time, to the establishment of what might be

known as a 'latent' cerebroprotective phenotype, priming the tissue for the actual injurious ischaemic event. Ischaemia in a preconditioned brain then activates a unique and separate set of adaptive responses. Although changes in the expression of some genes and the modification of some proteins may be shared among these two protective responses, the results of studies carried out so far suggest that their genetic and molecular basis is distinct. In addition, with respect to the response to ischaemic injury, preconditioning does not simply activate competing mechanisms to counteract these injury cascades, but actually serves to genetically 'reprogramme' the response to ischaemia[16–18].

In this review I describe the molecular and physiological regulation of these survival-promoting mechanisms and examine their integration, from the level of subcellular organelles to that of the whole brain. The investigation of endogenous pathways by which the brain protects itself from ischaemia represents a new paradigm for research into stroke — one that holds promise for identifying unique stroke therapeutics.

Preconditioning stimuli

Differences in the intensity, duration, and/or frequency of a particular stress stimulus determine whether that stimulus is too weak to elicit any response, of sufficient magnitude to serve as a preconditioning trigger, or too robust and therefore harmful (for a review, see REF. 8). Therefore, molecules known to cause ischaemic brain injury — such as glutamate, reactive oxygen species, inflammatory cytokines and caspases — might, at the lower levels achieved in response to a particular preconditioning stimulus, trigger adaptive rather than deleterious responses in resident brain cells[19–30].

Although brief cerebral ischaemia, or cerebral hypoxia (for a review, see REF. 10), serve as prototypical preconditioning stimuli, ischaemic tolerance can be induced by exposing animals or cells to diverse types of endogenous and exogenous stimuli that are not necessarily hypoxic or ischaemic in nature (see online Supplementary information S1 (table)). These include spreading depression, hyperoxia and oxidative stress, prolonged hypoperfusion, and, as alluded to above, hyperthermia or heat shock. Therefore, one stressor can promote 'cross-tolerance' to another. The sheer variety of stimuli capable of inducing an ischaemia-resistant phenotype in the brain indicates that the signalling pathways activated by these different triggers converge downstream on some common, fundamental mechanisms that ultimately account for the protection (see below). Many exogenously delivered chemical preconditioning agents (for example, inflammatory cytokines, anaesthetics and metabolic inhibitors) can also induce ischaemic tolerance, raising the hope that in the future it will be possible to pharmacologically activate these distal pathways in the human brain. Moreover, although they have yet to be identified, the molecular triggers capable of establishing ischaemic tolerance can be blood-borne: brief ischaemia of skeletal muscle promotes 'remote' tolerance against cerebral ischaemia[30], as does physical exercise[31,32].

CNS-specific antigens such as myelin basic protein[33], myelin oligodendrocyte glycoprotein[34] and even E-selectin[35] can also serve as preconditioning stimuli when administered systemically. This results from a type of immunological tolerance, known as bystander suppression[33], whereby the subsequent release of the same antigens in response to ischaemia-induced blood–brain barrier damage triggers the recruitment of regulatory T cells that produce anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming factor-β (TGFβ). Therefore, if a particular preconditioning challenge exposes CNS antigens to the blood, secondary to a transient opening of the blood–brain barrier, it is possible that bystander suppression could contribute to the ischaemia-tolerant phenotype induced by that stimulus.

Sensors and transducers

To induce tolerance, the preconditioning stimulus must be recognized by molecular sensors as a sign of something potentially much more severe to come. So far, numerous types of sensor have been identified, including neurotransmitter, neuromodulator, cytokine and toll-like receptors[36], as well as ion channels and redox-sensitive enzymes. In turn, these sensors activate transduction pathways that initiate the adaptive response. Although dependent in part on the nature of the preconditioning stimulus, members of these transduction pathways for which there is strong general support include mitogen-activated protein kinases (MAPKs) and their phosphorylated Ras, Raf, MEK and ERK subfamilies[6,37,38], mitochondrial ATP-sensitive K^+ (K_{ATP}) channels[39,40], Akt (also known as protein kinase B)[41–43] and the protein kinase C-ε isoform[44].

The possibility that the nitric oxide-based adaptive response to hypoxia in *Drosophila*[45] is evolutionarily conserved suggests that this multifunctional modulator might be a logical choice as an autocrine and paracrine mediator of preconditioning stress. Indeed, pharmacological and genetic evidence supporting the involvement of nitric oxide (derived from the endothelial[30,43,46–49], neuronal[37,48] and inducible isoforms of nitric oxide synthase (NOS)[50,51]) in the transduction process is continuing to mount. Given the redox sensitivity of many kinases and transcription factors, reactive oxygen species might also serve as transducers[28,29,52]. Adenosine, another prototypical paracrine mediator and 'retaliatory metabolite', the production of which is linked to ATP degradation, seems integral to tolerance induction in some models[39,40,53,54]. Finally, caspases might be essential induction catalysts, given that cyclic AMP responsive element-binding protein (CREB), the p50 and p65 subunits of nuclear factor-κB (NF-κB), and protein kinase C and other kinases are caspase substrates[26,55]. Notably, some of the aforementioned molecular transducers and signalling intermediates also serve as post-ischaemic effectors of the ischaemia-tolerant phenotype[8] (see below).

Transcription factors. Preconditioning-activated signalling pathways converge to induce post-translational modifications of existing proteins and/or to activate transcription factors that drive the genomic response. Several transcription factors are known to be sensitive to regulation by hypoxia/ischaemia and probably participate in this response, including activating protein 1 (AP1)[56], CREB[57–59], NF-κB[52,60], early growth response 1 and the redox-regulated transcriptional activator SP1(REF.61). However, the hypoxia-inducible factor (HIF) isoforms have garnered the most experimental support so far with respect to mediating the transactivation of adaptive, pro-survival genes, particularly those involved in glucose metabolism and angiogenesis[10,62–64]. Transcriptional regulation of the genome by HIF is similar in *Drosophila*, *C. elegans* and mammals. In mammals, the HIF1α isoform is hydroxylated during normoxia by an oxygen- and iron-dependent prolyl hydroxylase, allowing interaction between HIF1α and an ubiquitin ligase that targets HIF1α for degradation by the proteasome pathway. However, hypoxia renders the hydroxylase enzyme nonfunctional, and HIF1α, no longer able to associate with the ligase in its non-hydroxylated form, then enters the nucleus, dimerizes with HIF1β, and promotes the transcription of genes

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Reactive oxygen species
Highly reactive compounds containing oxygen with an unpaired electron; at low concentrations they subserve signalling functions, but at higher concentrations they can damage cellular macromolecules.

Inflammatory cytokines
Members of a group of intercellular signalling molecules, produced by stimulated immune cells and other cells, that trigger and/or amplify inflammatory responses.

Caspases
A family of aspartate-specific cysteine proteases most well known for their involvement in promoting apoptotic cell death, although they may also exhibit apoptosis-independent signalling functions.

Spreading depression
A decrease in neuronal activity evoked by local stimulation of brain tissue leading to a wave of depolarization that spreads slowly across the entire tissue.

Autocrine and paracrine
A form of localized signalling in which a cell secretes a given substance that then acts on the same cell, or neighbouring target cells to achieve a biological effect.

Normoxia
Normal (sea level) oxygen levels.

Proteasome pathway
A mechanism whereby proteins are degraded by other proteins; these other proteins often exist as a complex of various proteases.

that enhance hypoxic resistance. Prolyl hydroxylase inhibitors such as deferroxamine, cobalt chloride and other 'HIF-mimetics' are therefore attracting attention as potential preconditioning therapeutics (for reviews, see REFS 10,65). The HIF2α isoform is regulated in a similar way. It exists primarily in endothelial cells and, although it seems to be important in embryonic vasculogenesis, it does not seem to be induced in response to hypoxia in the neonate brain[15] as it is in the adult brain[66]. Details regarding transcriptional regulation by HIF2α are still unclear; although it may co-transactivate some genes with HIF1α, through the influence of kinases and other regulators, HIF2α might be more active on the promoters of endothelial cell-specific survival genes[67] associated with angiogenesis, vascular remodelling, and endothelial cell homeostasis.

Effectors

Primarily through recent oligonucleotide-based DNA microarray investigations[15–18,68] and novel gene identification methods[69], the transcriptome of the ischaemia-tolerant brain is gaining definition. Several themes are emerging from this work. First, genes representing many larger 'families' participate in the response — given the robustness of the protection in most tolerance models, this was not unexpected. Many share common promoter sequences that are responsive to preconditioning-activated transcription factors. On balance, genes functionally related to the cell cycle, metabolism, inflammation, excitotoxicity, ion homeostasis, signal transduction and so on are differentially expressed in response to preconditioning[15–18,68].

Second, genes activated by preconditioning stimuli are often quite distinct from those associated with ischaemia alone; similarly, the genomic expression pattern in response to ischaemia is unique in a preconditioned animal, and differs considerably from the pattern activated by either preconditioning or ischaemia in a non-preconditioned animal[16].

Third, although important and interesting from a cell survival standpoint, the genomic response is not simply one of activation of normally quiescent survival genes. Rather, gene repression also occurs, and, in fact, dominates the overall response to ischaemia in a preconditioned brain[16,18]. Finally, changes in gene transcription after preconditioning, or after ischaemia in a preconditioned brain, have distinct temporal profiles. For example, following preconditioning, some genes are expressed or repressed within minutes or hours (adenosine A$_{2a}$ receptor and vascular endothelial growth factor (VEGF)[15,18]), whereas others are affected days later (β-actin, serine/threonine protein kinase, arachidonate 12-lipoxygenase, calretinin, the S100A5 calcium-binding protein, dihydropyrimidine dehydrogenase and the zinc transporter ZnT1 (REFS 15–18). Some change transiently (CCAAT/enhancer-binding protein-related transcription factor, adenosine A$_{2a}$ receptor[18] and metallothionein II (REFS 15,17,18)), whereas others change for a protracted time (heat shock proteins, BCL2, p38-MAPK, TGFβ1, Iκ-Bα, glial fibrillary acidic protein and β-tubulin[15,17,18]).

The generation of these rapidly expanding genomic datasets must ultimately be coupled to the identification of translational expression patterns that define the ischaemia-tolerant proteome, as not all of the changes in mRNA levels identified by the previously mentioned microarray studies will be realized as proportional changes in protein levels. Findings so far indicate that cellular-level effectors of ischaemic tolerance are ubiquitous, and include structural and functional proteins of the cell membrane, cytoskeleton, mitochondria, and other organelles that have widespread actions on cellular metabolism. At least three unique time windows of phenotypic, effector mechanism expression — that is, before, during and after ischaemia — are worthy of closer examination.

The first is the period of time that follows preconditioning but is prior to the lethal ischaemic insult. At the cellular level, significant alterations in protein composition (such as elevations in the concentrations of various stress proteins, kinases and phosphatases, transcription factors, metabolic enzymes, transport and structural proteins, trophic factors and plasticity-related molecules, and cell cycle/apoptosis-related proteins) are underway or established prior to ischaemia. These new protein signatures are representative of a tissue that is prepared to resist the threat of an impending ischaemic event (FIG. 1).

The second window of effector mechanism expression relates to the period of ischaemia itself, such that an identical insult is experienced as less severe in a preconditioned brain compared with a naive brain. In part, this occurs through increased inhibitory neurotransmitter levels, lower intra-ischaemic levels of extracellular glutamate, reductions in calcium influx secondary to altered AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor subunit compositions, adenylation states, and desensitization status[70–73]. Whether levels of high-energy phosphate reserves and glycolytic and oxidative intermediates are altered during the ischaemic interval is controversial and may be age- and model-dependent[74–76].

Pathophysiological events occurring during post-ischaemic reperfusion are also positively modulated in ischaemic tolerance. Effector mechanisms acting during this time window broadly serve to stabilize the cell's energy and protein metabolism, ameliorate the actions of glutamate, reactive oxygen and nitrogen species and other injurious mediators, and reduce post-ischaemic inflammation. Tolerance mechanisms working at the level of endoplasmic reticulum function result in improved rates of recovery of neuronal protein synthesis[75,77]; specifically, better preservation of the reinitiation and elongation steps of transcription, and increased levels of the chaperone glucose regulated protein 78 kDa (GRP78) are realized[78,79]. In addition, post-ischaemic protein aggregation, redistribution and ubiquitin-conjugation[80] are reduced. The rate of repair of oxidative DNA damage is also enhanced[81,82]. Overall, many facets of ischaemic mitochondrial dysfunction, including changes in the redox activity of the respiratory chain components, oxidative phosphorylation

Table 1 | **Molecular mediators of programmed cell death and programmed cell survival**

Cellular localization or activity	Programmed cell death	Programmed cell survival
Membrane receptors	TNFα; FasL; DR3/4/5; adaptor proteins (TRADD, FADD)	Adenosine A1; metabotropic glutamate; P2Y2; dopamine D2; GCSF; PPARα/β/δ; neurotrophin; TNFα
Signal transduction	Caspases; ASK1; GSK3β; neuronal NOS	Raf, MEK and ERK kinases; PI3K/pAkt; JAK2; STAT5; PKCε; ceramide; endothelial NOS; MLK3
Transcription factors	NF-κB; JNK; HIF; FOXO	CREB; SP1; HIF; NF-κB; AP1; SRF; FOXO; MEF2
Mitochondrial effectors	BAX, BAD, BID, BIM; loss of mitochondrial membrane potential; release of cytochrome c, SMAC/DIABLO and AIF; production of ROS	BCL2; BCL-X$_L$; BCL-W; stabilization of mitochondrial permeability pore; inhibition of cytochrome c and SMAC/DIABLO release; inhibition of AIF translocation; elaboration of thioredoxin; uncoupling protein 2
Nuclear effectors	PARP; p53; c-jun; endonucleases; pro-apoptotic genes	IAPs (XIAPs, survivin); DNA repair enzymes; survival genes
Miscellaneous		Reduction in caspase activation; endoplasmic reticulum stabilization (ORP150); increases in trophic factors (NGF, BDNF, IGF1, bFGF); synthesis of heat shock proteins and other molecular chaperones; post-translational modifications; production of VEGF, erythropoietin and adenosine

Inhibition of programmed cell death and augmentation of programmed cell survival contribute to preconditioning-induced ischaemic tolerance. AIF, apoptosis-inducing factor; AP1, activator protein 1; ASK1, apoptosis signal-regulating kinase 1; BAD, BCL-associated death promoter; BAX, BCL2-associated protein X; BCL2, B-cell leukaemia/lymphoma 2; BCL-W, BCL2-like protein 2; BCL-X$_L$, BCL2-like protein 1; BID, BH3-interacting domain death agonist; BIM, BCL2-interacting mediator of cell death; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CREB, cyclic AMP responsive element-binding protein; DR, death receptor; ERK, extracellular signal-regulated kinase; FADD, Fas-associated protein with death domain; FasL, Fas ligand; FOXO, forkhead family of transcription factors; GCSF, granulocyte colony-stimulating factor; GSK3β, glycogen synthase kinase 3β; HIF, hypoxia-inducible factor; IAP, inhibitor of apoptosis protein; IGF1, insulin-like growth factor 1; JAK2, Janus tyrosine kinase 2; JNK, c-jun amino terminal kinase; MEF2, myocyte-enhancing factor 2; MEK, mitogen-activated protein kinase kinase; MLK3, mixed lineage kinase 3; NF-κB, nuclear factor-κB; NGF, nerve growth factor; NOS, nitric oxide synthase; ORP150, oxygen-regulated protein 150; P$_2$Y$_2$, purinergic receptor P$_2$Y; p53, tumour suppressor p53; pAkt, phosphorylated Akt; PI3K, phosphotidyl inositol-3-phosphate; PARP, poly(ADP-ribose)polymerase; PKCε, ε isoform of protein kinase C; PPAR, peroxisome proliferator activator; Raf, small G-protein; ROS, reactive oxygen species; SMAC/DIABLO, second mitochondria-derived activator of caspase/direct IAP binding protein with low pI; SP1, specificity protein 1; SRF, serum response factor; STAT5, signal transducer and activator of transduction 5; TNFα, tumour necrosis factor-α; TRADD, TNF receptor-1-associated death domain protein; VEGF, vascular endothelial growth factor; XIAP, X-linked IAP.

deficits[83], calcium overload and the initiation of apoptosis (see below), are abrogated in ischaemic tolerance[84,85]. Compared with the naive brain, the preconditioned brain also shows post-ischaemic increases in the Mn and CuZn isoforms of superoxide dismutase[86,87], glutathione peroxidase and glutathione reductase[88], uric acid[89], and haeme oxygenase-1 (REF. 87), which enhance the tissue's free radical scavenging capabilities. Reductions in pro-inflammatory cytokine synthesis[90], and an upregulation of other feedback inhibitors of inflammation, including decoy receptors and intracellular signalling inhibitors[36], contribute to the promotion of an anti-inflammatory phenotype following ischaemia.

Programmed cell survival

Counterbalancing the well-established programmed cell death pathways activated by ischaemia are the less well-understood 'programmed cell survival' pathways. These schemes might represent two sides of the same ischaemic tolerance coin (TABLE 1). However, we know relatively little about the organelle- and cell-specific molecular underpinnings of these survival pathways. Although apoptotic cell death, as an endpoint, is reduced in ischaemic tolerance[91–93], so far only changes in recognized apoptotic mediators have been measured to explain this protective effect. For example, we know that preconditioning-induced, CREB-mediated increases in the synthesis of the anti-apoptotic proteins BCL2 (REFS 58,59,91,94,95) and BCL-X$_L$ (REF. 95) occur, as well as stabilization of the mitochondrial membrane potential[95], decreases in mitochondrial cytochrome c[96] and

SMAC/DIABLO release[97], and reductions in caspase 3 synthesis[98] and p53 activation[99]. We do not yet know if the production of members of the caspase-neutralizing inhibitors of apoptosis (IAP) family, the interference with the mitochondrial-to-nuclear translocation of apoptosis-inducing factor (AIF), or the stabilization of intramitochondrial redox and membrane potential by uncoupling proteins[100] take place. In addition, the ability of preconditioning to reduce apoptotic cell death is likely to involve groups of as yet unidentified, evolutionarily conserved proteins that have direct and unique cell survival functions, and is not dependent on the inhibition of existing pro-apoptotic pathways alone.

The survival-promoting effects of some endogenous molecules, such as the endoplasmic reticulum-associated chaperone ORP150 (REF. 101) and serum response factor[102], as well as survival genes such as alivin 1 (REF. 103), are now being identified, but their role in ischaemic tolerance remains unknown. Other survival-promoting effector proteins, such as those of the neurotrophin family, have been implicated directly in ischaemic tolerance. For example, preconditioning-induced increases in neurotrophin 'tone' are one strategy that the tolerant brain uses to derive the various trophic benefits from nerve growth factor[104], brain-derived neurotrophic factor (BDNF)[104,105], basic fibroblast growth factor[106], insulin-like growth factor[107] and the neuregulins[108]. Several studies have now identified Akt, which is phosphorylated by phosphotidylinositol-3-kinase, as crucial to establishing the tolerant phenotype, secondary to an Akt-mediated phosphorylation of mixed lineage kinase 3 (REF. 109),

Decoy receptors
Soluble or cell-surface-binding proteins that bind the ligand with high affinity and specificity, but do not induce a biological response; used in immunological regulation.

Box 1 | **Erythropoietin: translational research success in the making?**

Originally identified as a kidney-derived glycoprotein hormone responsible for erythroid progenitor cell proliferation, erythropoietin is now recognized as a pleiotropic cytoprotective cytokine and a prominent hypoxia-inducible factor (HIF) gene target[147]. Although exogenous erythropoietin is neuroprotective in stroke[148–150], preconditioning with hypoxia[63,151] or deferroxamine[152] increases endogenous cerebral erythropoietin mRNA and protein levels. Even erythropoietin itself, administered 24 h before cerebral ischaemia, promotes tolerance[153,154]. Blockade of the protective effects of preconditioning by soluble erythropoietin receptors or erythropoietin antisense[112,151,155] provides causal evidence for erythropoietin's participation in ischaemic tolerance.

The beneficial cytoprotective effects of erythropoietin are mediated by erythropoietin receptors (EPORs, yet to be subclassified) located on neurons, astrocytes, microglia and endothelial cells[153]. Lumenally-oriented EPORs on endothelial cells[149] may transcytose exogenously delivered erythropoietin from blood to brain; in particular, to the EPORs on the astrocytic end-feet that ensheath cerebral capillaries[153], to initiate astrocyte-mediated protective mechanisms[129].

Mechanistically, erythropoietin-mediated protection involves a multidimensional array of actions, triggered by the phosphorylation of Janus kinase 2 (JAK2), signal transducer and activator of transcription 5 (STAT5), Akt (also known as protein kinase B) and other signalling complexes that ultimately reduce apoptosis, reactive oxygen species production and inflammation[156]. Erythropoietin also has pro-angiogenic effects[157] and regulates stem cell trafficking and neurogenesis[158].

As millions of patients with anaemia tolerate erythropoietin therapy, its safety profile supports the use of short-term erythropoietin application for acute stroke. In the first Phase I/II clinical trial, high-dose recombinant erythropoietin administered within 8 h of symptom onset significantly improved early clinical outcome measures[159]; a Phase III, multicentre trial is now underway in Germany. Efforts to create a designer cytokine with fewer erythropoietic and prothrombotic effects relative to its non-haematopoietic effects that crosses the blood–brain barrier have proved successful in several different animal models of neurological disease[160,161], and may be the preferred construct for long-term preconditioning of patients at high risk for stroke.

Neurovascular unit
A practical construct consisting of brain endothelium, astrocytes and microglia, neurons, and the extracellular matrix, and the dynamic interactions that occur between them in health and disease.

endothelial NOS[43], the IAP protein survivin[110] and, probably, many other cytoprotective effectors. *VEGF*, a HIF-driven gene, exhibits several distinct neurotrophic and neuroprotective effects relevant to post-ischaemic angiogenesis and neurogenesis[111]. Evidence is rapidly accumulating that erythropoietin, another HIF-regulated protein, participates as an endogenous pro-survival factor in ischaemic tolerance (BOX 1), and illustrates how the identification of endogenous survival-promoting molecules and their mechanisms of action may provide therapeutic targets for stroke.

Integrating the response
On the basis of studies in *in vitro* models, neurons, glia and endothelial cells can be rendered ischaemia-tolerant by preconditioning (see online Supplementary information S1 (table)), underscoring the capacity for intrinsic cellular-level tolerance without the need for communication between cell types, the involvement of the surrounding macro- and microvascular networks, or the participation of systemically-acting mediators. Nevertheless, achieving optimal levels of tolerance *in vivo* requires that the unique phenotypic response of each of these cells, as well as those invoked in resident immune cells, vascular smooth muscle cells and circulating leukocytes, are coordinated and assimilated to establish a tangible survival advantage across the whole tissue at risk. For example, co-culture studies show that the synthesis and release of erythropoietin[112] and other

molecules (see below) by astrocytes contribute importantly to the preconditioning-induced protection of neighbouring neurons. This section gives consideration to the adaptive responses that occur at the level of the neurovascular unit, which, when integrated temporally and spatially, provide robust protection in response to *in vivo* preconditioning.

Vascular ischaemic tolerance. Although neurons are inherently assumed to be the cellular target of cerebral preconditioning, ischaemic tolerance occurring at the level of endothelial and smooth muscle cells (and manifested across several levels of vascular organization) contributes importantly to neuronal protection. At the tissue level, absolute regional blood flow, either before[113] or during[113–115] ischaemia, does not seem to be altered by preconditioning; cerebral metabolism also remains unchanged[25,50,116]. However, reductions in absolute[114,117] and relative[118] blood flow after permanent focal stroke are lessened by prior preconditioning, concomitant with an increase in the number of histologically-identified patent microvessels[114]. Prior preconditioning also reverses the hypoperfusion that occurs early during reperfusion following global ischaemia[119]. With respect to vascular reactivity, post-ischaemic endothelium-dependent vasodilation is better preserved in the preconditioned brain[120]. Studies have yet to show that these improvements in post-ischaemic blood flow and reactivity contribute to the reduction in lesion magnitude that is associated with ischaemic tolerance.

Vasculoprotective tolerance mechanisms operating at the microvascular level account, in part, for the anti-inflammatory phenotype that characterizes ischaemic tolerance. For example, reductions in vasogenic oedema are evident in newborn[121] and adult[30,122,123] models of ischaemic tolerance, but how prior preconditioning enhances or maintains blood–brain barrier integrity in the face of ischaemia remains unclarified. Reductions in leukocyte–endothelial interactions and diapedesis of neutrophils and other leukocytes may be one explanation, as found in rats after exercise preconditioning[32] and in mice after lipopolysaccharide preconditioning[124], although investigations to establish a causal link between these changes and improved barrier function are required. Microarray studies[16,17], *in vivo* immunohistochemistry[32] and immunoblotting in a cerebral endothelial cell culture model of ischaemic tolerance[125] indicate that a reduction in the expression of members of the selectin and immunoglobulin adhesion molecule families following ischaemia may reduce leukocyte–endothelial rolling and adherence, and thereby attenuate infiltration. Moreover, even the proteome of circulating leukocytes is affected by preconditioning, as shown by transient suppression of the leukocyte genes encoding adhesion and migration, chemotaxis and cytokine synthesis after brief ischaemia in the human forearm[126]; indeed, blood monocytes show less activation after ischaemia in a preconditioned brain[124]. Such preservation of cell–cell homeostasis at the endothelial–blood interface might result from the anti-inflammatory effects of endothelially-derived

Epoxy eicosatrienoic acids
Epoxides of arachidonic acid generated by cytochrome P450 epoxygenases that have various regulatory actions.

nitric oxide, secondary to a preconditioning-induced, Akt-dependent phosphorylation of endothelial NOS[43,118] that enhances its activity[47,118]. Nothing is known about platelet dynamics at the endothelial–blood interface: although the blood of preconditioned mice has a prolonged coagulation time, this is not as a result of a reduction in platelet number, but might be secondary to preconditioning-induced increases in cyclooxygenase 1 and prostacyclin synthase[16]. If this were the case, then the resultant reduction in ischaemic microvascular thrombus potential would provide another advantage to the preconditioned brain.

A fully functioning cerebrovascular endothelium is also crucial to achieving optimal post-ischaemic angiogenesis and microvascular remodelling. The activation of endothelial NF-κB[127], the production of and responsiveness to VEGF[42], and the induction of distinct endothelial cell-specific anti-apoptotic response networks[128] may be just a few of the means by which prior preconditioning supports these neovascular responses that are so essential to long-term recovery. The selectively enhanced recruitment of endothelial progenitor cells to ischaemic brain regions would also be a beneficial feature of ischaemic tolerance, but this possibility has not yet been experimentally addressed.

Glial ischaemic tolerance. Resident astrocytes and microglia, like vascular cells, also respond uniquely to preconditioning stimuli, and such responses are crucial to the foundation of the overall cerebroprotective phenotype (for a review, see REF. 129). Enhancements in astrocytic ion buffering, the transfer of energy substrates and neurotransmitter metabolites to neurons[130], func-

tional and structural blood–brain barrier support, free radical scavenging, glycogen storage and erythropoietin production[112] are some of the ways in which astrocytes are likely to enhance the ischaemic resistance of neighbouring neurons. The generation by preconditioned astrocytes of anti-inflammatory cytokines such as IL-10, heat shock proteins (for example, HSP27 and HSP32), and trophic factors such as TGFβ, BDNF, GDNF (glial-cell-derived neurotrophic factor) and VEGF might also contribute. Recently, evidence for the astrocytic production of epoxy eicosatrienoic acids secondary to a preconditioning-induced, HIF1α-dependent upregulation of the arachidonic acid epoxygenase P450 2C11 was advanced[64]. Morphologically, both astrocytes and microglia are transiently activated in response to preconditioning[131,132], which may be a reflection of the functional responses listed above. Conversely, the well-recognized, more robust activation of astrocytes and microglia/macrophages that is triggered by ischaemia is attenuated in previously preconditioned brains[124,131], which may be secondary to a repression of NF-κB-mediated transcription of inflammatory mediators[133].

Mechanistic parallels of note

Our understanding of the mechanisms by which preconditioning protects the brain will be facilitated by continued investigation of the mechanisms innate to the fetal brain (BOX 2) and the brain of the high-altitude native, both of which are able to function in low oxygen environments. Hypoxia- and anoxia-tolerant lower vertebrates and hibernating mammals also hold clues to ischaemic tolerance (see online Supplementary information S2 (table)); many survival responses in these animals are categorically similar, if not identical, to those used for protection in mammalian ischaemic tolerance. In vertebrates, adaptations to oxygen insufficiency are not found in every species in a genus, and therefore are not simply the phylogenetic consequence of some lower level of organization. This suggests the presence of distinct adaptive responses that allow tolerance to pure anoxia for days in some species and, under more hypothermic conditions, for weeks in others. The primary adaptive response invoked by anoxia-tolerant animals is not to switch to anaerobic metabolism and/or use alternative substrates (a distant second choice), but rather to induce a state of extremely low energy production and utilization (for a review, see REF. 134). In brief, we know that by reducing protein synthesis (metabolic arrest), and by stabilizing membrane function secondary to decreases in ion permeability and ion pumping (channel arrest), neurons can achieve a robust hypometabolic state. Other phenotypic features of anoxia-tolerant species include the avoidance of anoxic depolarization through the elevation of adenosine and inhibitory transmitter levels (and their receptors), more efficient clearance of extracellular glutamate, the storage of large amounts of glycogen, and the augmentation of glutathione peroxidase and ascorbate levels to handle oxidative stress — all complemented at the extracerebral level by circulatory redistribution and bradycardia (for a review, see REF. 135). The cerebral blood flow in the

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Box 2 | Endogenous tolerance in the neonate

Compared with adults of the same species, the intrinsic ability of the fetus and newborn to better tolerate hypoxia/ischaemia has long been recognized. Although this advantage is not surprising given the hypoxic intra-uterine environment, elucidating the mechanistic basis of this resistance at the level of the CNS might provide clues as to how preconditioning protects the adult brain. Reductions in oxygen demand are known to underlie the ability of the neonate brain to resist low oxygen tension. This hypometabolic state is achieved by many of the same fundamental alterations that characterize anoxia-tolerant species, including: lower rates of resting glucose metabolism; increases in glucose transport, glycolytic enzymes and glycogen stores; lower densities of NMDA (*N*-methyl-D-aspartate) channels and channel distribution patterns that reduce overall neuronal excitability and delay depolarization; and other adaptations that slow the rate of high-energy phosphate depletion and maximize ATP homeostasis during and after ischaemia[74,76,135,162].

Several models of neonatal ischaemic tolerance have been published, as well as a recent review[9]. The extent of protection afforded by hypoxic preconditioning in the neonatal rat is exceptionally robust[46,74], as is that resulting from hyperthermic[121,163] and xenon[58] preconditioning. Hypoxic preconditioning in the prenatal period is also effective for reducing hypoxic-ischaemic injury after birth[92]. It is not clear whether the neonatal brain is already 'primed' for preconditioning, and/or if preconditioning augments survival mechanisms already constitutively active in the hypoxia-adapted brain, but the genomic basis of ischaemic tolerance in neonates probably involves many distinct, age-dependent responses. For example, given the propensity for ischaemic neuronal death by apoptosis in the neonate, strong and potentially unique anti-apoptotic mechanisms must underlie ischaemic tolerance at this age[58,92,93,107]. Conversely, other molecular players and transcription factors involved in the induction phase of preconditioning in the neonatal brain[38,46,62,107,164] are not unique to this age group.

Box 3 | **Ischaemic tolerance in the human brain: therapeutic potential?**

So far, clinical trials of more than 50 compounds for acute ischaemic stroke have failed. Only thrombolysis-based therapy is currently approved for use in the United States, but treatment is limited to a small fraction of stroke patients meeting specific inclusion criteria. Most drugs that have advanced to clinical trials have been designed to block or limit the key molecular events identified in the laboratory as contributing to neuronal injury. Given the magnitude and reproducibility of cerebroprotection against ischaemia achieved in many diverse models of preconditioning, targeting mechanisms of innate cytoprotection present another strategy for drug development. That experimental studies have successfully used exogenously applied agents to activate dormant neuroprotective genes and cell survival pathways to achieve ischaemic tolerance further underscores the feasibility of identifying pharmacological approaches for increasing endogenous ischaemic resistance; for example, the use of prolyl hydroxlase inhibitors to stabilize HIF1α[65] and erythropoietin-based treatment. Patients at high risk for stroke might derive benefit from prophylactic treatment and, given the protracted therapeutic window over which ischaemic brain injury occurs, so might patients presenting shortly after stroke.

Ischaemic tolerance may already occur naturally in humans, in the form of short episodes of ischaemia without infarction, known as transient ischaemic attacks (TIAs). Three retrospective analyses of prospectively collected data[165–167] show, by several metrics, a reduced severity of stroke with antecedent ipsilateral TIA. But the number of investigations to date is limited, sample sizes are relatively small, and the clinical status of the patients is likely to be non-uniform. However, the results of large clinical studies support the parallel contention that, in myocardial ischaemia, prior angina is protective. Given the breadth and depth of research on myocardial ischaemic tolerance (with ~3500 papers published on this topic since 1986), preconditioning is much closer to becoming a routine clinical treatment for patients requiring ischaemia-inducing procedures such as coronary angioplasty and coronary artery bypass grafting[145].

anoxia-tolerant mammalian brain is maintained during anoxia onset, but thereafter is significantly reduced in parallel with metabolic rate.

Active changes also accompany hibernation: metabolism and blood flow are lowered to levels less than 10% of baseline[136], body temperature and white cell counts are reduced, and stress kinases and heat shock proteins are activated. Unique lipid and protein sequestration patterns have been noted in neuronal endoplasmic reticulum[137]. The mechanisms by which hibernating animals handle the rapid increase in cerebral blood flow and severe oxidative stress during arousal are poorly understood, but it is possible that they could be applied in a translational way to meet the similar challenges faced by the human brain during post-ischaemic reperfusion.

Insights into ischaemic tolerance may also be forthcoming from studies of the high-altitude natives of the Andes and Himalayas. Positron emission tomography (PET) studies of the Andean Quechuas have revealed systematically lower region-by-region rates of cerebral glucose utilization compared with lowlanders[138]. This is consistent with the idea that the same hypometabolic strategy shown by the CNS of anoxia-tolerant animals is used by the human brain. In addition, it is worth noting that although the adaptations required by native lowlanders to withstand the hypoxia of high-altitude mountaineering may take weeks or months to establish, the resultant ability to maintain relatively unimpaired cognitive function at extreme altitude indicates that even those brain neurons most vulnerable to hypoxia have the endogenous capacity to adapt to, and successfully withstand, severe stress.

Arousal
The intentional act of periodic brief awakening characteristic of hibernating animals, characterized by distinct physiological changes and states of activity, depending on species.

Translational research
The process of taking results from the laboratory and translating them into therapies for clinical use.

Current questions and future studies

Despite coverage by ~450 publications since 1991, the field of cerebral preconditioning and ischaemic tolerance is still relatively immature. The same can be said, in general, of the so-called physiological 'stress response', which continues to challenge our understanding. Hundreds of unanswered questions result from the significant gaps that still exist in our knowledge base regarding ischaemic tolerance mechanisms. The few controversies surrounding particular results published so far are not unexpected in an emerging discipline such as this, not to mention one based on so many different experimental models. In addition to the need to develop well-characterized models of ischaemic tolerance in higher mammals, at least three basic issues regarding preconditioning efficacy in rodents still require more thorough documentation; these issues arise from the historical reliance on the use of either histological or morphological endpoints, or biochemical indices to document protection shortly after the ischaemic insult. First, it is imperative to thoroughly document whether the protection afforded by preconditioning is long-lasting and if injury is truly prevented, or whether preconditioning simply delays the development of infarction. Although a few studies have confirmed that sustained protection remains 2–4 weeks after cerebral ischaemia in neonatal and adult models[58,74,105,118], others have shown progressive cell loss at more protracted recovery times of 30 days post-stroke[139]. Although these outcome measures probably depend on the characteristics of the model under study, the intuitive qualitative prediction would be that tolerance is likely to be maximized in magnitude and duration (that is, permanent protection) when the preconditioning stimulus is optimized, and the severity of the subsequent ischaemic insult is not overwhelming. This was borne out experimentally in a study of global ischaemia in rats, in which sustained hippocampal CA1 protection was confirmed at 1 month following ischaemia under 'idealized' preconditioning and ischaemia regimens[140]. Regardless of this, additional work addressing this issue across various outcome measures is needed. A second issue is the functional documentation of ischaemic tolerance, both electrophysiologically and at the neurocognitive, sensorimotor and behavioural levels. In one study, preconditioned animals showed improved orientation and memory function relative to their naive counterparts[141]. However, another study measured functional deficits in tolerant animals even though, based on neuronal morphology, the tissue appeared ischaemia-tolerant[142]. It is also imperative to document that effective preconditioning stimuli are truly non-injurious; again, not only at histological levels but across more sensitive behavioural metrics as well[143]. Such studies have important therapeutic implications with respect to addressing the safety of different preconditioning regimens and their respective side effects. Studies designed to address the aforementioned issues will strengthen the experimental foundation for ischaemic tolerance and, in turn, promote translational research opportunities to further explore its clinical applicability (BOX 3).

Several time-dependent features of preconditioning and ischaemic tolerance deserve further exploration. For

example, almost every experimental model used so far has been based on a single preconditioning stimulus and the transient period of either rapid or classical ischaemic tolerance that it elicits. However, the more chronic adaptations shown by hibernating and diving mammals, and the acclimatization to hypoxia that occurs in humans with extended stays at high altitude, suggest that sustained augmentations in resistance to hypoxia-ischaemia can be realized in the brain. Is it possible that repetitive presentations of particular preconditioning stimuli could promote significantly longer-lasting changes in the neuroprotective phenotype? Can the brain exhibit a type of sustained adaptive plasticity akin to the idea of chronic ischaemic tolerance? The therapeutic implications of such a finding for patients at a high risk of stroke would be compelling.

Another example is the provocative finding from recent myocardial preconditioning studies that brief, transient interruptions in blood flow during early reperfusion, termed 'postconditioning', reduce ischaemic injury to levels similar to that achieved with preconditioning[144]. Moreover, as the beneficial effects of preconditioning and postconditioning do not seem to be additive, then perhaps some preconditioning mechanisms may be suitable for activating after ischaemia — a finding that has obvious clinical ramifications[145]. With the exception of a recent report showing that lipopolysaccharide, a well-established preconditioning agent[47,86,114,118,120,124,146], reduces leukocyte recruitment when given after ischaemia[146], whether this variation on reperfusion injury therapy would also trigger rapid ischaemic tolerance pathways in the brain that are uniquely different from those that underlie other efficacious post-ischaemic therapies in experimental stroke remains unexplored.

Conclusions

Studies of preconditioning and ischaemic tolerance are currently being published at what appears to be a near exponential rate, which should help to advance our understanding of its mechanistic basis and, in turn, its clinical potential. The endogenous survival mechanisms activated in response to preconditioning do not depend on differences in drug pharmacokinetics or administration protocols that can confound the translation of neuroprotective strategies from rodents to humans. Therefore, the identification of intrinsic cell survival pathways should provide more direct opportunities for translational neuroprotection trials. Although primarily focused on stroke at present, we might find that the innate regulatory schemes that underlie tolerance to ischaemia are also applicable to protecting the brain from other acute and chronic neurodegenerative disorders.

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