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SYMPOSIUM REVIEW

Protective conditioning of the brain: expressway or roadblock?

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Abstract The brain responds to noxious stimulation with protective signalling. Over the last decades, a number of experimental strategies have been established to study endogenous brain protection. Pre-, per-, post- and remote 'conditioning' are now widely used to unravel the underlying mechanisms of endogenous neuroprotection. Some of these strategies are currently being tested in clinical trials to protect the human brain against anticipated damage or to boost protective responses during or after injury. Here we summarize the principles of 'conditioning' research and current efforts to translate this knowledge into effective treatment of patients. Conditioning to induce protected brain states provides an experimental window into endogenous brain protection and can lead to the discovery of drugs mimicking the effects of conditioning. Mechanisms of endogenous brain tolerance can be activated through a wide variety of stimuli that signal 'danger' to the brain. These danger signals lead to the induction of regulator and effector mechanisms, which suppress death and induce survival pathways, decrease metabolism, as well as increase substrate delivery. We conclude that preclinical research on endogenous brain protection has greatly benefited from conditioning strategies, but that clinical applications are challenging, and that we should not prematurely rush into ill-designed and underpowered clinical trials.

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Abbreviations Akt, Akt-kinase; CIDS, CNS injury-induced immunosupression; DAMP, damage-associated molecular patterns; EPO, erythropoeitin; ERK, extracellular signal-regulated kinase; G-CSF, granulocyte colony stimulating factor; HIF-1, hypoxia-inducible factor-1; HK, hexokinase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; miRNA, micro RNA; mTOR, mammalian target of rapamycin; PAMP, pathogen-associated molecular patterns; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; PHD, prolyl-hydroxylase; PI3K, phosphatidylinositol 3-kinases; RCT, randomized controlled trial; ROS, reactive oxygen species; TLR, toll-like receptor; VEGF, vascular endothelial growth factor.

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'That which does not kill us makes us stronger' Friedrich Nietzsche, Ecce Homo – Warum ich so weise bin 2 (1908)

The multiple flavours of 'conditioning'

Cells, tissues, organs, as well as whole organisms respond to sublethal stress by activating protective signalling cascades (Dirnagl *et al.* 2003). In aerobic organisms, strong evolutionary pressure for the development of endogenous mechanisms of protection is generated by hypoxia, as well as by infection and inflammation. Protective responses may be generated either in anticipation of a stressor, during stress against its immediate harmful consequences, or as a response to delayed secondary mechanisms after stress. Various medical disciplines, in particular cardiology, neurology/neurosurgery, anaesthesiology, as well as transplantation medicine, are studying the pathways underlying this signalling of endogenous protective responses. It is the ultimate goal of this research to develop therapeutic organ protection based upon nature's own strategies (Dirnagl *et al.* 2009; Keep *et al.* 2010; Gidday, 2010). Early milestones in this quest were the discoveries that pre-exposure to hypoxia can prolong anoxic survival by preserving brain metabolism (Dahl *et al.* 1964); that brain can adapt to anoxia by hypoxic pre-exposure (Schurr *et al.* 1986); and the description of ischaemic preconditioning in ischaemic myocardium (Murry *et al.* 1986) and brain (Kitagawa *et al.* 1990). Since then, various types of organ 'conditioning' have been described (Fig. 1): *preconditioning*, in which the conditioning stimulus (e.g. ischaemia, hypoxia, metabolic inhibition or inflammation below the threshold of damage) is given several days ('delayed preconditioning') or minutes ('classic preconditioning') before a noxious stimulus presents (e.g. ischaemia); *perconditioning*, in which the conditioning stimulus is given while the noxious stimulus is still present; *postconditioning*, in which the conditioning stimulus is given shortly after the noxious stimulus (e.g. after reperfusion), and *remote conditioning*, in which not the organ which is affected by the noxious stimulus is conditioned, but another, remote organ or bodily system (e.g. limb ischaemia to induce protection of heart or brain). Robust experimental protocols have been developed for each of these types of conditioning, and a number of the underpinning signalling pathways have been established (Kirino, 2002; Gidday, 2006; Dirnagl & Meisel, 2008; Obrenovitch, 2008; Zhao, 2009; and see below). Some of these conditioning strategies are either directly applicable to patients (e.g. remote conditioning by limb ischaemia), or can be pharmacologically mimicked, such as prolyl-hydroxylase (PHD) inhibitors activating hypoxia inducible factor (HIF)-related pathways, or growth and survival factors such as erythropoeitin (EPO) or granulocyte colony stimulating factor (G-CSF). A number of clinical trials have been concluded in cardiology, and several are underway in patients with brain disease (see below and Table 1). At least in cardiology, where clinical development of conditioning-related strategies is most advanced, their translation into effective therapies has so far been hugely disappointing (Ludman *et al.* 2010). It is therefore timely to ask what we know about the mechanisms underlying brain conditioning, and what the chances are that brain conditioning will become a clinical reality in the near future.

A window into endogenous brain protection

Pre-, per-, post-, remote as well as pharmacological ('mimics') conditioning serve as highly valuable tools to understand the mechanisms of endogenous brain protection. It appears that these mechanisms are independent of the conditioning strategy, or have at least a vast overlap. This reflects the fact that these mechanisms have evolved as unspecific responses to a number of challenges to the organism (hypoxia, injury, infection). In the following we discuss them briefly and without reference to specific conditioning strategies (Fig. 2).

Sensors of danger. Mechanisms of endogenous brain tolerance can be activated through a wide variety of stimuli that signal 'danger' to the brain. Both hypoxia and infection endanger the entire organism and are fundamental challenges for most organisms and hence for organs and their cells. Therefore, multiple redundant cascades to adapt to these conditions have evolved. Many aspects of (anti-)inflammatory and hypoxic signalling overlap, and cascades mediating endogenous tolerance are very similar in different organs. For comprehensive overviews the reader is referred to Dirnagl *et al.* (2003); Gidday (2006); Dirnagl & Meisel (2008); Obrenovitch (2008), and the references therein.

Hypoxia-inducible factor-1 (HIF-1) is the key regulator of cellular oxygen homeostasis. Under hypoxic conditions HIF-1 activates highly conserved transcriptional profiles ultimately geared to adapt cellular homeostasis to reduced oxygen availability. Among others, these adaptations include changes in cellular energy metabolism, regulation of Bcl2-family proteins, cell proliferation, cell cycle control and vasomotor control or angiogenesis (Sharp & Bernaudin, 2004; Semenza, 2009). Likewise, toll-like receptors (TLRs), which are present on many if not all mammalian cells, are responsible for inducing cellular responses to counteract infection.

HIF-1 induces adaptation to decreased oxygen on two levels. First, cellular homeostasis, including mitochondrial respiration, is adapted to hypoxic conditions rather quickly (within hours, Semenza, 2010). Second, chronic hypoxia induces angiogenesis to increase blood supply to hypoxic tissue through a variety of HIF-1-dependent factors such as vascular endothelial growth factor (VEGF;

Table 1. Examples of currently recruiting clinical trials with neurological endpoints as listed at ClinicalTrials.gov (as of 3/2011): preconditioning, remote conditioning and agents that mimic endogenous neuroprotection

Trial name	Condition	Intervention	NCT registration
Preconditioning for aneurismal subarachnoid haemorrhage	Subarachnoid haemorrhage	Remote limb preconditioning	NCT01110239
Remote ischaemic preconditioning in subarachnoid haemorrhage (RIPC-SAH)	Subarachnoid haemorrhage Aneurysmal subarachnoid haemorrhage Cerebral vasospasm Intracranial aneurysm	Remote ischaemic preconditioning	NCT01158508
The neuroprotection of sevoflurane preconditioning on intracranial aneurysm surgery	Brain ischaemia	Sevoflurane continuous inhalation	NCT01204268
Effect of remote ischaemic preconditioning on clinical outcomes in CABG surgery (ERICCA)	Coronary heart disease	Remote ischaemic preconditioning	NCT01247545
Effect of remote ischaemic preconditioning on cognitive function after cardiac surgery	Cardiac surgery	Remote ischaemic preconditioning	NCT00877305
New acute treatment for stroke – the effect of remote PERconditioning	Acute stroke	Remote preconditioning	NCT00975962
Neuroprotective study of electroacupuncture pretreatment in patients undergoing cardiac surgery	Stroke Brain injuries	Electroacupuncture pretreatment	NCT01020266
Thrombolysis and deferoxamine in middle cerebral artery occlusion (TANDEM-1)	Acute ischaemic stroke	Deferoxamine	NCT00777140
AX200 for the treatment of ischaemic stroke (AXIS 2)	Acute ischaemic stroke	Filgrastim (G-CSF)	NCT00927836

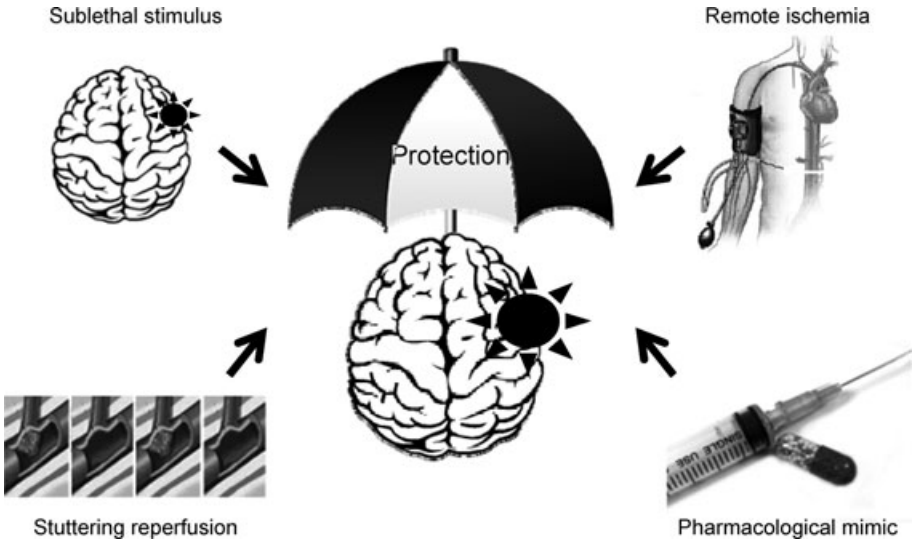

Figure 1. 'Conditioning' paradigms to protect the brain
Typically, preconditioning uses a sublethal stimulus given minutes or days before the insult against which it aims to protect. Stuttering reperfusion is the prototypical per- or postconditioning strategy, by which one aims to prevent 'reperfusion damage' by repetitively opening and blocking brain perfusion before permanent reperfusion is allowed. Remote ischaemia is another per- or postconditioning strategy which typically produces repetitive, short phases of ischaemia of a peripheral limb to induce humoral and neural mechanisms of protection of a remote organ, such as the brain. Pharmacological mimics are drugs that either boost endogenous protective signalling cascades (such as the HIF pathway), or exogenously provide the effectors of endogenous protection, such as EPO.

Semenza, 2009). In general, under normoxic conditions the HIF-1α subunit is targeted to rapid proteasomal degradation through post-translation modification by prolyl-hydroxylation, whereas under hypoxic conditions HIF-1α is stabilized and HIF-1-dependent transcription is initialized (Sharp & Bernaudin, 2004; Semenza, 2009). Furthermore, transcription of HIF-1α is increased upon growth-factor signalling, in particular upon activation of the PI3K–Akt–mTOR (mTOR, mammalian target of rapamycin) pathway (DeBerardinis *et al.* 2008). While the brain has developed some unique sensors of systemic hypoxia, such as central and arterial chemoreceptors (Sharp & Bernaudin, 2004), the molecular cascades involved in oxygen sensing are highly conserved in all cell types (Sharp & Bernaudin, 2004; Semenza, 2009, 2010).

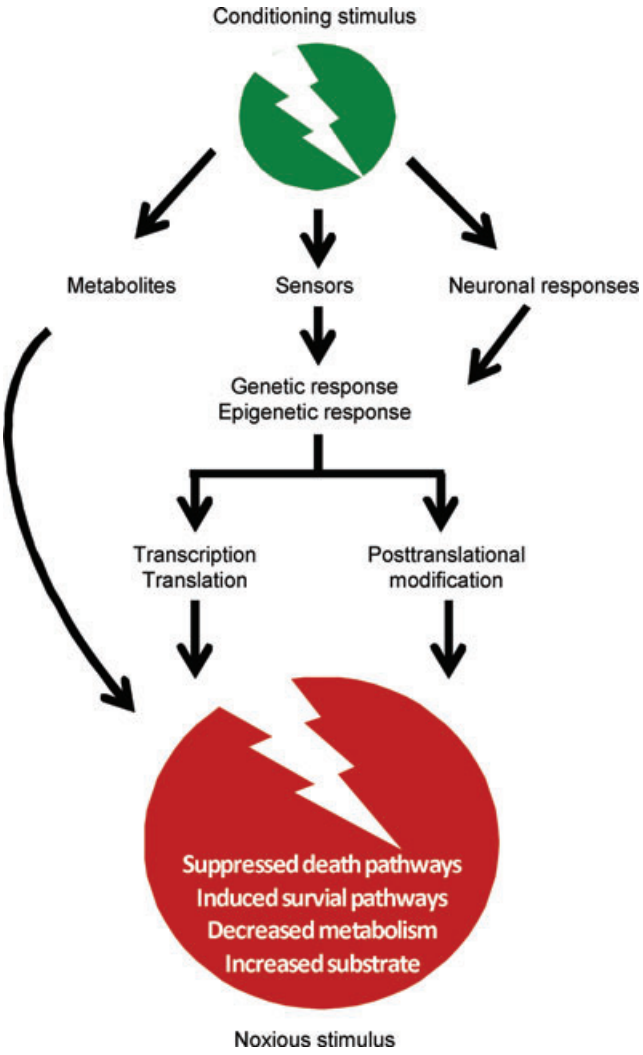

Figure 2. General principles of action of 'conditioning strategies' to protect the brain
A pre-, per-, post-, remote-conditioning stimulus may either: directly protect the brain via release of locally or remotely acting metabolites (e.g. adenosine); after activation of sensors (e.g. HIF-1) lead to a complex signalling cascade which may include genetic as well as epigenetic responses; or activate genetic and epigenetic responses via neuronal pathways (e.g. activating the sympathetic nervous system or the hypothalamic–pituitary axis). The signalling pathways of the various conditioning strategies may converge in similar or even identical effector mechanisms, such as suppressed death pathways, induced survival pathways, decreased metabolism ('hibernation'), and increased substrate delivery.

TLRs are an integral part of the innate immune system, providing the first line of defence against pathogens at the cellular level. TLR signalling is an important mediator of ischaemic damage in the brain, but it can also mediate inflammation-induced cross tolerance such as through stimulation with lipopolysaccharide (LPS) or tumour necrosis factor-α (TNF-α) (Marsh *et al.* 2009). In general, TLRs activate transcription factors through common intracellular pathways, with distinct effects in different cell types or tissues (Marsh *et al.* 2009). TLRs are a major discriminator between 'self' and 'foreign' (Akira & Takeda, 2004). A signalling cascade resulting in activation of nuclear factor NF-κB transcription and an inflammatory response are initiated following ligation of pathogen-associated molecular patterns (PAMPs) with TLRs (Liew *et al.* 2005). Host-derived damage-associated molecular patterns (DAMPs), which are released upon ischaemic injury (Vartanian & Stenzel-Poore, 2010), can also induce inflammatory signalling through the TLR pathway (Seong & Matzinger, 2004) and contribute to ischaemic damage in the brain (Kariko *et al.* 2004; Lehnardt *et al.* 2007; Ziegler *et al.* 2007; Dirnagl *et al.* 2009). In turn, inflammatory signalling can boost HIF-1 transcription, which controls many genes involved in regulation of inflammation and host defence (Nizet & Johnson, 2009) and which has been found to be essential for the cellular innate immune response in inflammation (Cramer *et al.* 2003). Furthermore, mitochondria are very sensitive to changes in homeostasis, and are important sensors of cellular stress (see below). TLRs in the brain are constitutively expressed in astrocytes, microglia and endothelial cells. TLRs can be upregulated upon inflammatory stimulation in these cells, but also in neurons and oligodendrocytes (Marsh *et al.* 2009). Little is known about the differential role of the various TLR types and cell types which express those TLRs in mediating endogenous neuroprotection.

Regulators and effectors. Hypoxia induces substantial changes in gene expression patterns in the brain (Bernaudin *et al.* 2002). Preconditioning is thought to reprogram the brain's genomic response to a noxious stimulus (Stenzel-Poore *et al.* 2007). For example, many of the HIF-1 target genes are involved in regulating cellular metabolism, survival, proliferation and angiogenesis (Semenza, 2009). Furthermore, increasing evidence suggests that different epigenetic regulatory mechanisms are activated in the context of conditioning

paradigms, and regulate the endogenous protective response. Inhibition of DNA methylation and histone deacetylation reduce ischaemic damage by altering the transcriptional profile (Endres *et al.* 2000; Meisel *et al.* 2006; Yildirim *et al.* 2008). Micro RNAs (miRNAs), small RNA molecules that function as post-transcriptional regulators of gene expression (Lagos-Quintana *et al.* 2001), are important regulators of diverse aspects of brain function, including development and maintenance of brain plasticity (Saugstad, 2010). Furthermore, miRNAs have emerged as important mediators of endogenous tolerance in the brain (Dharap & Vemuganti, 2010; Lusardi *et al.* 2010), and both HIF-1 and TLR signalling can be modulated by miRNAs (Crosby *et al.* 2009; O'Neill *et al.* 2011).

A variety of kinases involved in proliferation and survival are involved in eliciting an endogenous protective response in the brain. Among others, these include protein kinase C (PKC) (Speechly-Dick *et al.* 1994), mitogen-activated protein kinase (MAPK)/p38, extracellular signal-regulated kinase (ERK), Akt-kinase (Ruscher *et al.* 2002; Gao *et al.* 2008) and mTOR (Pagel, 2008; Swiech *et al.* 2008). The PI3K–Akt–mTOR pathway senses nutrient availability. Activation of this pathway leads to increased transcription of HIF-1α, further supporting adaptation of metabolism to substrate deprivation (DeBerardinis *et al.* 2008; Swiech *et al.* 2008).

Ischaemia is associated with profound metabolic imbalances and much of the cellular response initiated by conditioning events is geared to alter metabolic pathways to maintain basal metabolic integrity. Therefore, under hypoxia, glycolytic flux is diverted from oxidative phosphorylation to glycolysis. HIF-1 changes the expression of genes of the entire glycolytic cascade to adapt metabolism to hypoxic conditions (Iyer *et al.* 1998; Semenza, 2009). As a consequence, the glycolytic intermediate pyruvate is shunted away from the mitochondrial tricarboxylic acid (TCA) cycle by pyruvate dehydrogenase kinase 1 (PDK1). PDK1 inhibits pyruvate dehydrogenase (PDH), thereby reducing flux through the TCA cycle and ultimately reducing excess production of reactive oxygen species (ROS) (Kim *et al.* 2006; Papandreou *et al.* 2006). In addition, lactate dehydrogenase A (LDHA) converts pyruvate to lactate, which in the brain can be rapidly taken up and shuttled away by astrocytes (Gandhi *et al.* 2009). HIF-1 control over metabolism is not only limited to states of hypoxia, but appears to be of general relevance for survival and proliferation (DeBerardinis *et al.* 2008). Furthermore, glycolytic enzymes such as mitochondrial hexokinase (HK) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are emerging as important regulators of cell death (Majewski *et al.* 2004; Kim & Dang, 2005; Colell *et al.* 2007), with striking mechanistic similarities between neurons and cancer cells (Vaughn & Deshmukh, 2008).

Mitochondria, equipped to efficiently generate ATP through oxidative phosphorylation, also function as oxygen sensors by inhibiting PHD activity through ROS production, thereby stabilizing HIF-1α. Mitochondria are highly susceptible to changes in oxygen concentration and abruptly react by generating ROS (Kaelin, 2005; Kim *et al.* 2006; Klimova & Chandel, 2008; Semenza, 2010). Importantly, ROS signalling not only contributes to ischaemic damage, but is also involved in endogenous protection evoked by pre-, per-, post- and remote conditioning (Gidday, 2006; Tapuria *et al.* 2008; Hausenloy, 2009; Ovize *et al.* 2010; Saxena *et al.* 2010; Semenza, 2010; Xin *et al.* 2010).

As described above, inflammation mediated by the innate immune system as well as by the adaptive immune system (Yilmaz *et al.* 2006; Hurn *et al.* 2007; Liesz *et al.* 2011) contributes to brain injury following stroke. However, inflammatory stimulation using LPS can also induce endogenous tolerance (Bastide *et al.* 2003; Kunz *et al.* 2007; Orio *et al.* 2007). Additionally, cerebral ischaemia, as well as other insults to the central nervous system, lead to immunosuppression – a phenomenon termed CNS injury-induced immunosupression (CIDS) (Meisel *et al.* 2005). CIDS might therefore serve to contain an autoaggressive immune response following stroke (Gee *et al.* 2007). In humans, ischaemic preconditioning by transient forearm ischaemia changes gene expression patterns in circulating leukocytes, thereby suppressing leukocyte activation and potentially modulating innate and adaptive immune responses (Konstantinov *et al.* 2004; Saxena *et al.* 2010).

Clinical applications: expressway or roadblock?

The discovery of ischaemic tolerance, the wealth of knowledge that has subsequently been gathered on mechanisms of endogenous organ protection, and the development of clinically applicable strategies of pre-, per- and postconditioning have precipitated a rush into clinical trials in cardiology, nephrology, anaesthesiology and neurology/neurosurgery, among other disciplines. Some of these rather small proof of concept trials, often using surrogate endpoints, have generated promising results (e.g. Chan *et al.* 2005; Bøtker *et al.* 2010; Lonborg *et al.* 2010; Schäbitz *et al.* 2010). For the brain, a putative beneficial effect of transient ischaemic attacks (as 'preconditioning equivalents') suggested the existence of endogenous neuroprotection in the human brain (Weih *et al.* 1999; Wegener *et al.* 2004), although this has been disputed (Johnston, 2004). Meanwhile, however, a number of negative or inconclusive randomized clinical trials (RCT, e.g. Hong *et al.* 2010; Rahman *et al.* 2010; Walsh *et al.* 2010) have been published on various forms of conditioning in several organ systems, including

heart and brain, and a less optimistic spirit prevails (Ludman *et al.* 2010). This is reminiscent of 'classical' neuroprotection trials, where promising preclinical and early clinical testing was not followed by evidence for efficacy in large RCTs (Green, 2008; Tymianski, 2010). Nevertheless, for conditioning strategies the jury is still out: the search term 'preconditioning OR perconditioning OR 'remote conditioning' AND brain' reveals 50 open clinical trials in the trial registry of the National Institute of Health (clinicaltrials.gov). This does not include RCTs testing pharmacological strategies of endogenous neuroprotection such as granulocyte-colony stimulating factor (G-CSF), AX200 for the treatment of ischaemic stroke (AXIS-2, NCT00927836) or erythropoietin (Safety Study of Carbamylated Erythropoietin (CEPO) to Treat Patients With Acute Ischemic Stroke, NCT00756249, publication of results pending). Table 1 lists a selection of currently recruiting RCTs with neurological endpoints in the field of 'conditioning' or endogenous neuroprotection.

It has been argued that in contrast to conventional neuroprotection trials, in which neuroprotective drugs are given after the ischaemic event, preconditioning strategies allow baseline assessment of neurological status before patients experience the index event: patients can be functionally tested before preconditioning them against an index event, such as focal neurological deficits after carotid or heart surgery, or delayed neurological deficits after subarachnoid haemorrhage. This may help cut down the variance in results and reduce the number of patients to recruit (Dirnagl *et al.* 2009), which for a Phase III neuroprotection trial in stroke may run into the thousands. However, the downside of this approach is that only a fraction of patients experience the index event (e.g. around 1% of strokes after coronary bypass surgery), potentially annihilating the advantage of an individual baseline and leading to the exposure of patients to possibly harmful treatments they do not actually need.

Outlook

Basic research on endogenous mechanisms has established a plethora of conditioning strategies and unravelled, among others, neurogenic, immunological, genetic and epigenetic mechanisms of brain protection. Nevertheless, many issues remain unsolved, including questions such as how remote preconditioning exerts its effects (humoral? neuronal?), or whether the dogma that the conditioning stimulus is subthreshold to damage is really true (it has been proposed that in many cases, damage was simply not assessed, or the tools were not sensitive enough; Dirnagl *et al.* 2003; Sommer, 2008). In the current clinical arena, many teams worldwide are testing the safety and efficacy of such diverse strategies as the prevention or amelioration of CNS damage when it can be anticipated (e.g. delayed vasospasm after subarachnoid haemorrhage), the prevention of CNS damage during potentially harmful interventions (e.g. neurosurgery), the induction of endogenous CNS protection by remote procedures (e.g. repeated limb ischaemia after acute stroke), or the pharmacological induction of endogenous CNS protection (e.g. HIF-1 induction via Desferoxamine) (see Table 1). Over the next few years some of those RCTs may provide evidence not only for the existence of endogenous neuroprotection, but also as to whether related mechanisms can be therapeutically exploited to benefit patients at risk for or with evolving CNS damage. Given the complexities and challenges of the underlying pathophysiology, as well as the design and implementation of clinical trials, and given the frustrating experiences regarding neuroprotection in the stroke field, we are well advised to learn from previous mistakes and to conduct preclinical research of the highest quality (Dirnagl, 2006) and not to prematurely rush into ill-designed and underpowered clinical trials (Weaver *et al.* 2004).

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