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SYMPOSIUM REVIEW

Targeting the Nrf2–Keap1 antioxidant defence pathway for neurovascular protection in stroke

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Abstract Endogenous defence mechanisms by which the brain protects itself against noxious stimuli and recovers from ischaemic damage are a key target of stroke research. The loss of viable brain tissue in the ischaemic core region after stroke is associated with damage to the surrounding area known as the penumbra. Activation of the redox-sensitive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) plays a pivotal role in the cellular defence against oxidative stress via transcriptional upregulation of phase II defence enzymes and antioxidant stress proteins. Although recent evidence implicates Nrf2 in neuroprotection, it is not known whether activation of this pathway within the neurovascular unit protects the brain against blood–brain barrier breakdown and cerebrovascular inflammation. Targeting the neurovascular unit should provide novel insights for effective treatment strategies and facilitate translation of experimental findings into clinical therapy. This review focuses on the cytoprotective role of Nrf2 in stroke and examines the evidence that the Nrf2–Keap1 defence pathway may serve as a therapeutic target for neurovascular protection.

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Abbreviations ARE, antioxidant response element; DHA, dehydroascorbate; GSH, glutathione; HO-1, haem oxygenase-1; Keap1, Kelch-like ECH-associated protein 1; MCAO, middle cerebral artery occlusion; Nrf2, nuclear factor erythroid 2-related factor 2; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; ROS, reactive oxygen species; tBHQ, *tert*-butylhydroquinone; Trx, thioredoxin.

Giovanni Mann (left) obtained his BSc in Zoology from George Washington University, USA and MSc and PhD in Physiology from University College London. As Professor of Vascular Physiology at King's College London, he has served as Chairman of the Executive Committee of The Physiological Society, President of the British Microcirculation Society and currently Secretary General of SFRRI, an Associate Editor for *Free Radical Biology & Medicine*, on Editorial Boards of *The Journal of Physiology*, *Microcirculation*, and *Free Radical Research*, and an Editorial Advisor for the *Biochemical Journal*. He chairs Heart Research UK Translational Sciences panel and serves on the Royal Society International Networks panel, and as an External Referee for BBSRC and MRC. He heads an active research group investigating the role of Nrf2 as a regulator of vascular antioxidant defences and redox signalling in diseases associated with oxidative stress such as gestational diabetes, pre-eclampsia and stroke. **Alessio Alfieri** (right) obtained his Pharmacy degree (2004) and his PhD in Drug Science (2007) from the University of Naples. After working at the University of Sheffield (2008–2010), he joined the Cardiovascular Division at King's College London. His research interests include vascular physiology and pharmacology and the molecular mechanisms underlying endothelial dysfunction and inflammation.

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Introduction

Stroke is the second most common cause of death and the leading cause of adult disability (Donnan *et al.* 2008; Endres *et al.* 2008; Balami *et al.* 2011). Despite advances in the understanding of the pathophysiology of cerebral ischaemia, therapeutic options remain limited, with only recombinant tissue-plasminogen activator (rt-PA) currently approved for the treatment of stroke (Lakhan *et al.* 2009), but its use is limited by a brief therapeutic window (3–4.5 h) and potential side effects (intracranial haemorrhage). In stroke, cerebral ischaemia triggers the pathological mechanisms, collectively known as the ischaemic cascade, causing rapid and irreversible neuronal injury within the ischaemic core. However, the surrounding hypoperfused brain tissue, known as the penumbra, can be salvaged if flow is restored and/or efficacious therapies are applied. Notably, reperfusion from recanalised cerebral vessels can cause tissue injury due to cerebral oedema, brain haemorrhage and neuronal death (Jung *et al.* 2010). Acute responses of brain tissue to cerebral ischaemia and its chronic pathogenic progression involve many pathways, with accumulating evidence implicating reactive oxygen species (ROS) and inflammation as pivotal mediators (Lakhan *et al.* 2009; Jung *et al.* 2010). Early events following ischaemic damage, such as excitotoxicity induced by glutamate, calcium overload and ROS-mediated oxidative stress rapidly result in cell death within the infarct core, whereas later events precipitated by pro-inflammatory and pro-apoptotic mediators (interleukin-1 (IL-1), cyclooxygenase-2 (COX-2), matrix metalloproteinases (MMPs), caspases) escalate the progression of damage to the ischaemic penumbra (Dirnagl *et al.* 2003; Candelario-Jalil, 2009). Protective mediators are also released in the early (GABA, adenosine) and delayed (interlleukin-10 (IL-10), B-cell lymphoma 2 (Bcl_2), erythropoietin) phases of cerebral ischaemia, attenuating the damage to brain cells in the penumbra. Oxidative stress may function as a 'switch mechanism' tipping the balance between pro-death and pro-survival pathways in cerebral ischaemia (Crack & Taylor, 2005; Moskowitz *et al.* 2010).

Nrf2: a regulator of endogenous antioxidant defences

In addition to its high consumption of oxygen and glucose, the brain is enriched in peroxidisable fatty acids, iron and ascorbate (Zaleska & Floyd, 1985; Adibhatla & Hatcher, 2010). Moreover, several neurotransmitters are excitotoxic or auto-oxidizable (Halliwell, 2006). The brain has evolved endogenous defence mechanisms to counteract the damaging effects of ROS (Halliwell, 2001, 2011); however, antioxidant defences are much lower than other organs including liver and kidney (Marklund *et al.* 1982).

As summarised in Fig. 1, the redox-sensitive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) plays a key role in the cellular defence against oxidative stress (Ishii *et al.* 2000, 2004; Kensler *et al.* 2007; Kaspar *et al.* 2009). Under quiescent conditions, Nrf2 is sequestered by its cytosolic repressor Keap1 (Kelch-like ECH-associated protein 1), a cytoskeletal protein that anchors and represses its transcriptional activity (Itoh *et al.* 1999; McMahon *et al.* 2003; Tong *et al.* 2007). Keap1 promotes rapid proteasomal degradation of Nrf2 via ubiquitination and also acts as a sensor to oxidative and electrophilic stress (Itoh *et al.* 1999). It has been suggested that alterations in the structure of Keap1 leads to dissociation of the Nrf2–Keap1 complex (Motohashi & Yamamoto, 2004), but site-specific modification of Keap1 may also cause an altered E3 ubiquitin ligase function and subsequent reduction in Nrf2 degradation (Tong *et al.* 2007).

Phosphorylation of serine/threonine residues in Nrf2 may be an alternative mechanism by which Nrf2 dissociates from Keap1 (Surh *et al.* 2008), and kinases, including protein kinase C (PKC), c-jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), casein kinase 2 (CK2), p38 mitogen-activated protein kinase (p38 MAPK) and phosphoinositide 3-kinase (PI3K), appear to modulate nuclear import and export of Nrf2 (Jain & Jaiswal, 2007; Surh *et al.* 2008). In summary, oxidation of redox-sensitive cysteines within Keap1 constitute the molecular basis for Nrf2 activation (Tong *et al.* 2007), but ROS-induced Nrf2 phosphorylation may provide an alternative mechanism (Burdette *et al.* 2010). Moreover, there are reports of crosstalk between Nrf2, cFOS, peroxisome proliferator-activated receptor γ (PPARγ), p53 and nuclear factor κB (NFκB) signalling pathways (Wakabayashi *et al.* 2010).

The induction of phase II defence enzymes and antioxidant stress proteins by Nrf2 is regulated via the antioxidant response element (ARE, or electrophile response element EpRE) in the promoter region of target genes (Fig. 1). Notably, many Nrf2 inducers exhibit hormetic properties with beneficial effects reported at nanomolar concentrations but toxic effects at higher concentrations (Mann *et al.* 2009; Siow & Mann, 2010).

Nrf2 is ubiquitously expressed (Moi *et al.* 1994) and, in the brain, may act as one of the most important defences against oxidative stress by modulating microglial dynamics (Rojo *et al.* 2010), protecting astrocytes (Vargas & Johnson, 2009) and neurons (Lee *et al.* 2003) from toxic insults, regulating the expression of inflammatory markers (Innamorato *et al.* 2008) and antioxidant enzymes (Shah *et al.* 2007; Yan *et al.* 2008). It has also been proposed that Nrf2 plays a protective role in neurodegenerative disorders, including Parkinson's (Cuadrado *et al.* 2009), Alzheimer's (Kanninen *et al.* 2008), and Huntington's

(Stack *et al.* 2010) disease as well as traumatic brain injury (Yan *et al.* 2008).

This review focuses on the cytoprotective role of Nrf2 in stroke and examines the evidence that the Nrf2–Keap1 defence pathway may serve as a therapeutic target for neurovascular protection in cerebral ischaemia. In addition to reviewing the findings of previous studies in experimental stroke, we discuss possible therapeutic strategies aimed to protect the penumbra from cell death following cerebral ischaemia.

Stroke, the neurovascular unit and antioxidant defences

With its special anatomical characteristics, the brain circulation plays a critical role in the pathogenesis of cerebrovascular disorders (Abbott *et al.* 2010) (see Fig. 2). Microvascular alterations after ischaemia–reperfusion, including disruption of the blood–brain barrier, oedema and haemorrhage, can induce swelling of astrocytic foot processes and neurodegeneration. Despite its high energetic demands, the brain does not possess reserves of oxygen or nutrients such as ATP, glucose, glycogen, phosphocreatine, and thus relies on cerebral blood flow for normal function. Notably, the interaction of astrocytes and neurons with the vasculature is essential for local regulation of cerebral blood flow (Abbott *et al.* 2006; Attwell *et al.* 2010). The close relationship between neural activity and cerebral blood flow is described as neurovascular coupling, with the neurovascular unit comprised of neurons, glia (astrocytes, microglia, oligodendrocytes) and vascular cells (endothelial, smooth muscle, adventitial cells and pericytes) (Iadecola, 2004).

Despite knowledge of the cellular function of neurons, astrocytes and vascular cells, information on intercommunication between these cell types in response to ischaemic injury and the molecular mechanisms underlying damage and repair processes in the neurovascular unit following an ischaemic episode is limited. The fine match between neural energetic demands and vascular flow is disrupted in stroke. Oxidative stress is a major contributor to cerebrovascular dysfunction (Faraci, 2005). Interestingly, oxidative stress seems to be a common pathway within the neurovascular unit, affecting neurons (Niizuma *et al.* 2009), astrocytes (Simpson *et al.* 2010) and the endothelium (Rizzo & Leaver, 2010). Cellular defences in the brain involve a number of endogenous protective enzymes, including superoxide dismutase (SOD), glutathione peroxidase, catalase, thioredoxin, peroxiredoxins and haem oxygenases. In

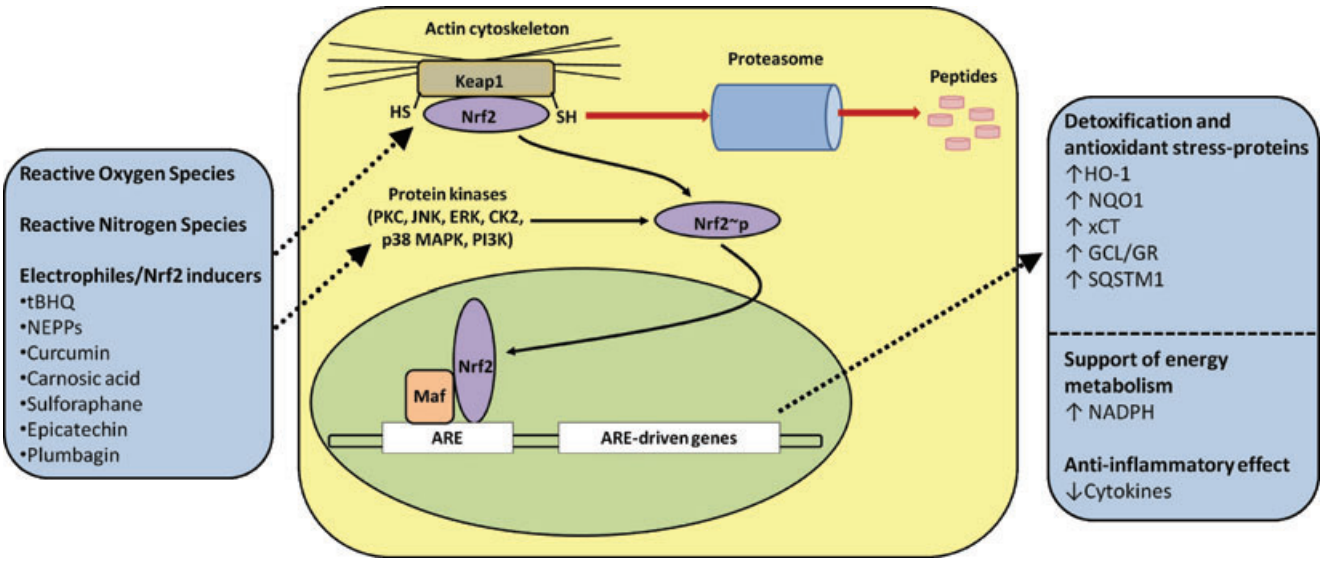

Figure 1. Activation of the Nrf2–Keap1 defence pathway in oxidative stress
Nrf2 is normally retained in the cytosol bound to the actin-binding protein Keap1 and targeted for proteasomal degradation. Reactive oxygen species, reactive nitrogen species and endogenous and exogenous electrophiles/activators (see text for more details) can alter the Nrf2–Keap1 complex by modifying cysteine (-SH) residues on Keap1. Subsequent phosphorylation of Nrf2 by cytoplasmic kinases may increase its nuclear translocation, where it binds with small Maf proteins to the antioxidant response element (ARE). Induction of ARE-driven genes results in upregulation of haem oxygenase-1 (HO-1), NAD(P)H-quinone oxidoreductase-1 (NQO1), glutamate-cysteine ligase (GCL), glutathione reductase, sequestosome-1 (SQSTM1) and the cystine/glutamate anionic amino acid transporter (xCT). Nrf2/ARE-linked detoxification and antioxidant stress proteins restore the basal redox status in cells exposed to oxidative stress and inflammatory mediators. tBHQ, *tert*-butylhydroquinone; NEPPs, neurite outgrowth-promoting prostaglandins. Adapted from Itoh *et al.* (1999); Ishii *et al.* (2000); Kensler *et al.* (2007); Innamorato *et al.* (2008); Siow & Mann (2010).

addition to these antioxidant enzymes, small non-protein compounds (glutathione, dietary vitamins C and E) essentially contribute to defend the brain against oxidative stress (Halliwell, 2001).

Ischaemia–reperfusion in the brain triggers oxidative and nitrative injury in the neurovascular unit (Gursoy-Ozdemir *et al.* 2004). Using fluorescent probes in mouse brains subjected to 2 h of ischaemia and 3 h of reperfusion, high levels of superoxide and peroxynitrate production have been observed in neurons, astrocytes and the endothelium. Oxidative and nitrative stress is also associated with markers of vascular injury, e.g. metalloproteinase-9, and blood–brain barrier breakdown, e.g. leakage of Evans blue, suggesting that oedema and haemorrhage may result from reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated in the neurovascular unit during stroke. However, after longer time-points of reperfusion an infiltration of leukocytes may further exacerbate oxidative stress and inflammation.

Due to their more efficient synthesis of glutathione and ARE-linked gene expression, astrocytes are more protected than neurons against basal levels of oxidative stress (Vargas & Johnson, 2009). As astrocytes closely interact with neurons to provide protection from noxious stimuli, Nrf2 activation in astrocytes has been proposed as a therapeutic target for neuroprotection. As illustrated in Fig. 2, glutathione (GSH) released from astrocytes may protect neurons against oxidative stress. As neurons are not able to take up glutathione, additional mechanisms may underlie the neuroprotection afforded by astrocytes: (i) radical-scavenging action by GSH in the extracellular space; (ii) extracellular hydrolysis of GSH by γ-glutamyltranspeptidase present on the external surface of astrocytes, yielding cysteine and glycine, which in turn can be taken up by neurons and used for intracellular GSH synthesis; (iii) GSH may act as a neuromodulator/neurotransmitter by binding membrane receptors on neurons (Halliwell, 2001; Vargas & Johnson, 2009). Moreover, astrocytes also release glutamine, thus providing all the precursors for GSH synthesis in neurons (Vargas & Johnson, 2009). Another example of cross-talk in the antioxidant defences between neurons and astrocytes is ascorbate transport and recycling: neurons are able to take up ascorbate, whereas astrocytes take up the precursor dehydroascorbate (DHA) and then convert it to ascorbate intracellularly (Rice, 2000). This is considered as an important mechanism in brain

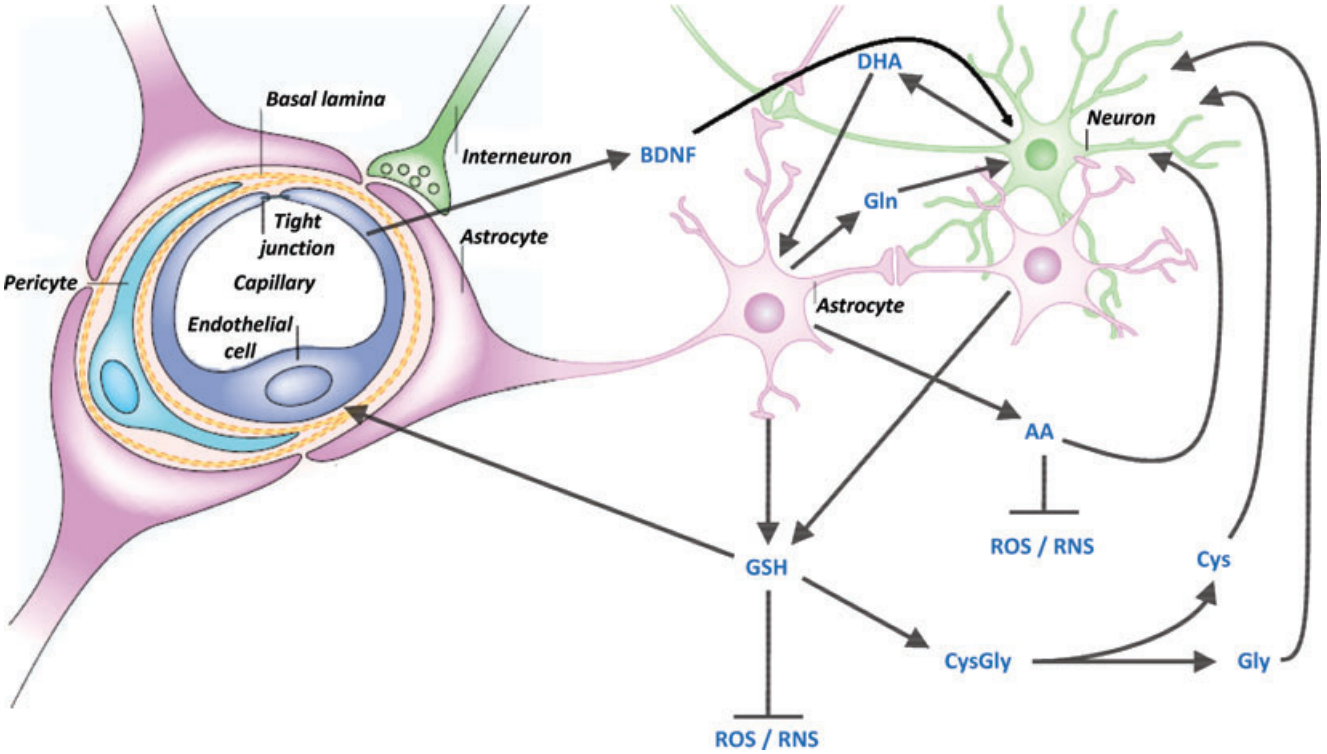

Figure 2. Communication between astrocytes, neurons and brain endothelial cells in the defence against oxidative stress
Glutathione (GSH) and its precursors, ascorbic acid (AA) and dehydroascorbate (DHA), and brain-derived neurotrophic factor (BDNF) provide protection of the neurovascular unit against free radical-mediated injury (see text for more details). Cys, cysteine; Gln, glutamine; Gly, glycine; RNS, reactive nitrogen species. Modified from Fig. 2 in Abbott *et al.* (2006) with permission from the Nature Publishing Group.

homeostasis, as neurotransmission requires high ascorbate and low DHA concentrations in the extracellular fluid (Wilson, 2002). It has been suggested that neurons release DHA which is subsequently taken up and converted to ascorbate in astrocytes (Swanson *et al.* 2004). Moreover, astrocytes release ascorbate into the extracellular fluid, which can be taken up by neurons and/or elicit antioxidant actions extracellularly (Wilson, 2002).

Although brain endothelial cells are more resistant to oxidative stress and ischaemia compared to astrocytes and neurons (Lee *et al.* 2010), sub-lethal levels of free radicals affect brain endothelial cell function (Guo *et al.* 2008; Arai & Lo, 2009). Notably, the brain-derived neurotrophic factor released from brain endothelial cells protects neurons from oxidative stress and oxygen-glucose deprivation *in vitro* (a condition which resembles cerebral ischaemia) (Guo *et al.* 2008). Recent evidence shows that GSH released from astrocytes protects brain endothelial cells against hemin-induced apoptosis (Sukumari-Ramesh *et al.* 2010). However, it remains to be resolved whether GSH released from astrocytes acts as an extracellular free radical scavenger and/or exerts cyto-protective effects intracellularly after uptake by endothelial cells (Kannan *et al.* 2000). Notably, co-cultures of brain capillary endothelial cells and astrocytes show a decreased content of GSH compared to cells in monoculture, but this is accompanied by an increased antioxidant activity and reduced free radical-induced lipid peroxidation (Schroeter *et al.* 1999).

However, approaches exclusively targeted to protect neurons have proven to be of limited value in clinical trials (del Zoppo, 2010). Thus, future research and therapeutic interventions in stroke should target all components of the neurovascular unit, with an aim of trying to further resolve the time course of repair and injury processes, and whether neurodegeneration promotes vascular dysfunction or vice versa.

Nrf2-mediated neurovascular protection in stroke

Two research approaches have been employed *in vivo* to investigate the role of the Nrf2–Keap1 defence pathway in stroke: genetic modification of mice ($Nrf2^{-/-}$) and use of activators of Nrf2-linked gene transcription. Several natural and synthetic compounds, including isothiocyanates, flavonols, heavy metals and hydroperoxides, are potent Nrf2-inducers (Rushmore & Kong, 2002; Shah *et al.* 2010) (Fig. 1). Liverman *et al.* (2004) first reported an upregulation of Nrf2 in the brain following oligaemia, a pathological condition characterized by blood flow reduction and oxidative stress in the absence of cell death, mimicking events in the ischaemic penumbra (Baron, 2001). In this study, Nrf2-positive neurons, while absent in brains from control mice, were detected following oligaemia in the Purkinje cells of the cerebellar cortex and pyramidal neurons of the cingulate cortex. Conflicting findings have been reported for Nrf2-deficient mice (see Table 1), with one study reporting that loss of Nrf2 exacerbates cortical infarction after 7 days, but not 24 h, after permanent middle cerebral artery occlusion (MCAO) (Shih *et al.* 2005). Notably, NAD(P)H-quinone oxidoreductase-1 (NQO1) and glutathione S-transferase activity is reduced in the brain of Nrf2-deficient mice. Another study, however, reports an increase in infarct volume (∼10%) 24 h after transient MCAO (Shah *et al.* 2007) (see Fig. 3). The discrepancy between these two studies may be explained by the difference in the mouse genetic background (C57B/SV129 *vs.* CD1) and/or by the type of occlusion used (permanent *vs.* transient). As a consequence of reperfusion, oxygen and other free radical species are rapidly generated (Allen & Bayraktutan, 2009) with oxidative stress providing a key stimulus for Nrf2-mediated neuroprotection. A delayed reperfusion and inflammatory response, particularly within the peri-infarct region and cortex, with secondary generation of oxidative stress and subsequent cell death, may explain the difference between the wild-type and $Nrf2^{-/-}$ genotypes after cerebral ischaemia–reperfusion injury (Nagayama *et al.* 2000; Carmichael, 2005).

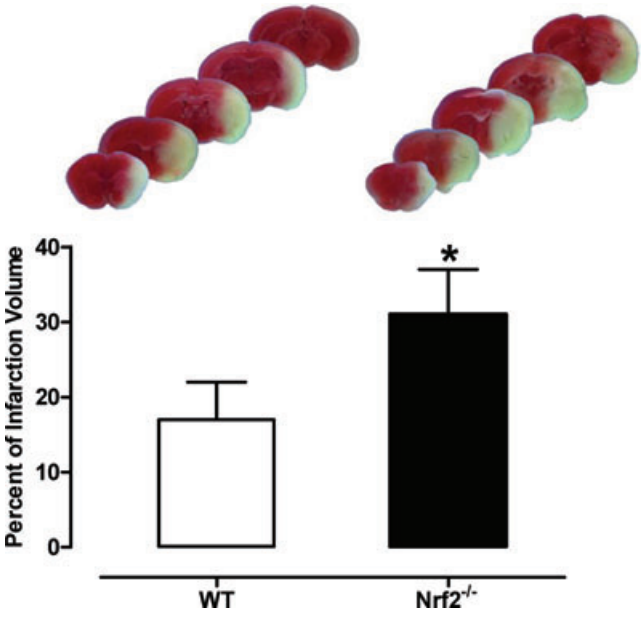

Figure 3. Cerebral infarct volumes in wild-type (WT) and Nrf2-deficient mice subjected to 90 min MCAO and 24 h reperfusion
Upper panel: representative images of serial brain sections stained with TTC (2,3,5-triphenyltetrazolium chloride). The light areas denote the infarct region. Lower panel: quantification of infarct areas in WT and $Nrf2^{-/-}$ (mean ± SEM, $P < 0.01$; $n = 8$ per group). Adapted from Fig. 1 in Shah *et al.* (2007) with permission from Elsevier.

Table 1. Activation of the Nrf2–Keap1 defence pathway in experimental stroke

Strain and species	Experimental model	Findings	Reference
Sprague–Dawley rats	Permanent MCAO–intraluminal filament method	Nrf2 and HO-1 expression was up-regulated between 3 and 72 h after cerebral ischaemia. Curcumin, 100 mg kg^{-1} I.P., 15 min after the onset of stroke reduced cerebral infarct, neurological deficit and brain oedema at 24 h of ischaemia.	Yang *et al.* (2009)
Sprague–Dawley rats	MCAO–intraluminal filament method for 90 min	tBHQ 16.7 mg kg^{-1} I.P. three times (−24, −16 and −8 h) before stroke reduced infarct size and sensorimotor deficit at 24 h and 1 month after ischaemia–reperfusion.	Shih *et al.* (2005)
Wistar rats	MCAO–intraluminal filament method for 90 min	ICV infusion of tBHQ 1 mM for 72 h reduced cerebral infarct size and sensorimotor deficit at 24 h after ischaemia–reperfusion.	Shih *et al.* (2005)
Long–Evans rats	MCAO and CCAO: vessel clip for 3 h	Sulforaphane 5 mg kg^{-1} I.P. 15 min after the onset of ischaemia decreased the infarct volume at 3 days of reperfusion.	Zhao *et al.* (2006)
ICR mice	MCAO: intraluminal filament method for 60 min	Stroke induced Nrf2 expression in neurons of the peri-infarct region between 2 and 72 h, peaking at 8 h. Keap1 expression declined after stroke in neurons of both the peri-infarct and infarct region between 2 and 72 h.	Tanaka *et al.* (2011)
CD1 mice	MCAO: intraluminal filament method for 90 min	Loss of Nrf2 function in KO animals increased cerebral infarct and neurological deficit at 24 h of reperfusion.	Shah *et al.* (2007)
C57B/SV129 mice	MCAO: permanent vessel cauterization	Loss of Nrf2 function in KO animals increased cerebral infarct at 7 days but not 24 h after ischaemia.	Shih *et al.* (2005)
C57B/SV129 mice	Intracortical injection of endothelin-1	Neuroprotection by dietary 1% tBHQ observed in wild-type was lost in $Nrf2^{-/-}$ animals.	Shih *et al.* (2005)
C57BL/6 mice	MCAO: intraluminal filament method for 2 h	NEPP11 1 mg kg^{-1} I.P. 1 h before and 4 h after the onset of stroke reduced brain infarct at 24 h of reperfusion.	Satoh *et al.* (2006)
C57BL/6 mice	MCAO: intraluminal filament method for 2 h	Carnosic acid 1 mg kg^{-1} I.P. 1 h before the onset of stroke reduced cerebral infarct at 24 h of reperfusion.	Satoh *et al.* (2008)
C57BL/6 mice	MCAO: intraluminal filament method for 1 h	Plumbagin 3 mg kg^{-1} I.V. 6 and 24 h before (but not 1 h after) the onset of stroke reduced cerebral infarct and neurological deficit at 72 h of reperfusion.	Son *et al.* (2010)
C57BL/6 mice	MCAO: intraluminal filament method for 90 min	Epicatechin given orally by gavage 30 mg kg^{-1} 90 min before the onset of stroke reduced cerebral infarct and neurological deficit at 24 h of reperfusion, and this effect was lost in Nrf2-deficient animals. Post-treatment with the same dose and by the same route 3.5 h but not 6 h after the onset of stroke reduced cerebral infarct and neurological deficit at 72 h of reperfusion.	Shah *et al.* (2010)
C57BL/6 mice	Intra-striatal injection of NMDA	Epicatechin given orally by gavage 30 mg kg^{-1} 90 min before the onset of stroke reduced NMDA-induced excitotoxicity at 48 h.	Shah *et al.* (2010)

Abbreviations: CCAO, common carotid artery occlusion; HO-1, haem oxygenase-1; ICV, intracerebroventricular; I.P., intraperitoneal; I.V., intravenous; KO, knock-out; MCAO, middle cerebral artery occlusion; NEPP11, neurite outgrowth-promoting prostaglandin; NMDA, *N*-methyl-D-aspartate; Nrf2, nuclear factor erythroid 2-related factor 2; tBHQ, *tert*-butylhydroquinone.

tert-Butylhydroquinone (tBHQ) was the first Nrf2 inducer shown to be neuroprotective against experimental stroke (Shih *et al.* 2005) (Table 1). This molecule is a metabolite of the dietary antioxidant butylated hydroxyanisole which possesses an oxidisable 1,4-diphenolic structure that activates Nrf2, and it can increase Nrf2 phosphorylation via PKC (Huang *et al.* 2000). As shown in Fig. 4, tBHQ pre-treatment reduced cortical infarct size following transient MCAO in rats either after local intracerebroventricular delivery or multiple systemic

intraperitoneal injections (Shih *et al.* 2005). Furthermore, dietary administration of tBHQ also protects mice from endothelin-1-induced ischaemia/reperfusion injury, and this effect is lost in Nrf2-deficient animals. Thus, despite being a potent inducer of phase-2 defence enzymes via Nrf2 in astrocytes *in vitro*, protection afforded by tBHQ *in vivo* requires high doses and longer-term administration to reduce the damaging effects of stroke.

Several studies of stroke highlight the ability of drugs or natural plant-derived compounds to protect against stroke via activation of the Nrf2–Keap1 defence pathway (see Fig. 1). Neurite outgrowth-promoting prostaglandins (NEPPs) were synthesised based on the chemical structure of anti-cancer cyclopentenone prostaglandin derivatives and characterized by their neurotrophic effects. NEPP11 was shown to protect neurons *in vitro* against oxidative stress (Satoh *et al.* 2003) and to activate the Nrf2–Keap1 pathway in the HT22 neuronal cell line (Satoh *et al.* 2006). Moreover, NEPP11 reduces cerebral infarction by 30–50% after transient MCAO (Satoh *et al.* 2006). As summarised in Table 1, curcumin, a low molecular weight polyphenol found in turmeric, elicits antioxidant and anti-inflammatory actions; curcumin administration reduces infarct area, brain oedema and neurological deficits after 24 h of ischaemia (Yang *et al.* 2009). At this same time-point after ischaemia, Nrf2 protein levels are increased in neurons and astrocytes in the infarcted brain cortex. Carnosic acid, another polyphenolic compound with anti-inflammatory and free radical scavenging properties, reduced the infarct volume in mice subjected to transient MCAO and 24 h of reperfusion (Satoh *et al.* 2008). Notably, oral administration of the flavonol (−)-epicatechin prior to transient MCAO reduces infarct volume and neurological deficits after 24 h of reperfusion, but this protective effect is lost in Nrf2 and haem oxygenase-1 (HO-1)-deficient mice (Shah *et al.* 2010). It is interesting that the toxic plant repellent plumbagin protects mice from cerebral ischaemia and associated neurological deficits, but is not effective when administered 1 h after the onset of cerebral ischaemia (Son *et al.* 2010). Plumbagin-induced activation of Nrf2 was shown *in vitro* to involve PI3K and MAPK signalling pathways. Sulforaphane, abundantly present in cruciferous vegetables and readily bioavailable in rodents and humans, crosses the blood–brain barrier and is a well-known activator of Nrf2 (McWalter *et al.* 2004). Administration of sulforaphane after the onset of transient MCAO in mice reduces infarct size measured after 72 h of reperfusion (Zhao *et al.* 2006).

Therefore, a number of compounds known to activate the Nrf2–Keap1 defence pathway provide neuroprotection in experimental models of stroke. Notably, most of the Nrf2 inducers tested, including curcumin, NEPP11, plumbagin and sulforaphane, increased the levels of HO-1 in the brain. However, all of these compounds may also have additional biological activities and in most of the cases (e.g. NEPP11, carnosic acid, plumbagin and sulforaphane), it remains to be established whether protection in cerebral ischaemia is mediated entirely via Nrf2.

Analysis of Nrf2 mRNA and protein expression in rat brains after permanent MCAO reveals a time-dependent increase, starting at 3 h, peaking at 24 h and declining after 48 and 72 h (Yang *et al.* 2009). A recent study (Tanaka *et al.* 2011) investigated temporal changes in expression of Nrf2, Keap1 and the downstream antioxidant proteins thioredoxin (Trx) and HO-1 in murine brains after 60 min of transient MCAO and reported a difference in expression between the peri-infarct and the infarct region. Keap1 was found in the cytoplasm of cells and its expression declined

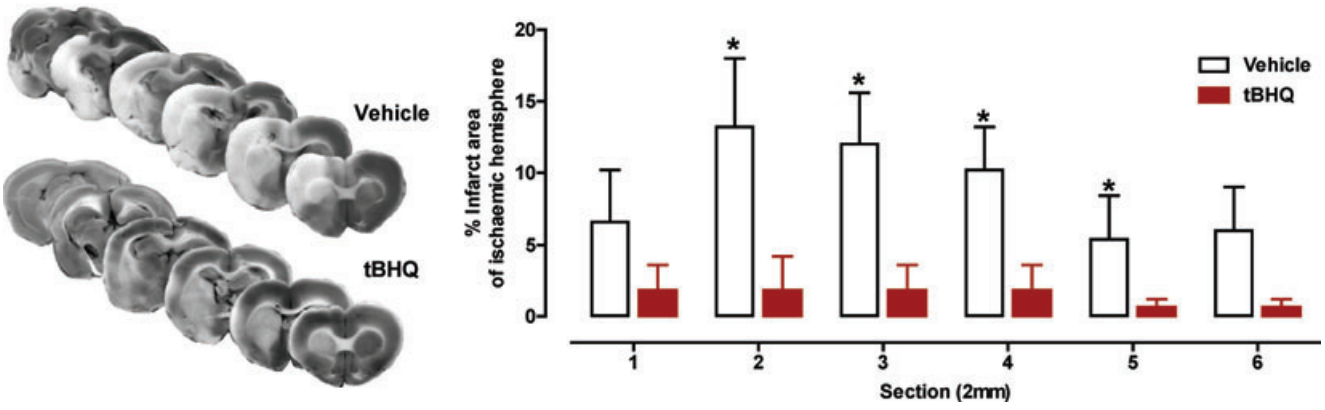

Figure 4. Reduction in cerebral infarct area in stroke following pre-treatment with the Nrf2-inducer *tert*-butylhydroquinone
Left panel: representative images of serial brain sections stained with TTC. Right panel: effect of intracerebral ventricular delivery of *tert*-butylhydroquinone (tBHQ, 1 mM for 72 h from pump implant) before 90 min MCAO and 24 h of reperfusion. Infarct areas are shown between section 1 (anterior) and section 2 (posterior). Data are expressed as mean ± SEM, $^*P < 0.05$; $n = 7$–9. Adapted from Fig. 2 in Shih *et al.* (2005) with permission from *The Journal of Neuroscience*.

in a time-dependent manner after transient MCAO. In both the peri-infarct and infarct regions, expression levels decreased from 2 to 24 h after reperfusion and remained at low levels after 72 h (see Fig. 5*A*). Although negligible levels of Nrf2 expression were detected in sham brain sections, Nrf2 was detected in both the cytoplasm and nucleus of the peri-infarct region 2 h after MCAO, peaking at 8 h and then declining at 24 and 72 h (Tanaka *et al.* 2011). Both Nrf2 and Keap1 seemed to be mainly expressed in neurons (Fig. 5*B* and *C*) and not in astroglial or microglial cells (data not shown). Moreover, GSH levels and Trx and HO-1 expression was low or absent in sham brain sections but increased in the peri-infarct region at 24 and 72 h after reperfusion. Notably, expression of Nrf2, Trx and HO-1 was very low in the infarct compared to peri-infarct region, possibly due to oxidative stress in these areas (Tanaka *et al.* 2011). As the infarct region is characterized by tissue necrosis and cell death (Yuan, 2009), loss of cellular activity may underlie the lack of free radical production and antioxidant enzyme expression. Moreover, as the area of lesion and relevant time-dependent progression of damage was not reported in this study, it remains unclear whether Nrf2 activation and subsequent anti-oxidant protein expression influence the recruitment of the penumbra into the infarct core following experimental stroke. Indeed, activation of the Nrf2–Keap1 pathway in the penumbra seems to be a major protective mechanism against oxidative stress-induced cell death.

Future research initiatives need to define the relative distribution of Nrf2 in different cell types of the neuro-vascular unit. Notably, traumatic brain injury induces Nrf2 activation in microvessels (Zhao *et al.* 2007) and larger arteries of the cerebral vasculature (Zhao *et al.* 2010), protecting the brain from blood–brain barrier breakdown. Thus, Nrf2 may improve cerebral vascular function in larger vessels as well as the blood–brain barrier. It has recently been reported that nerve blood flow is improved by Nrf2 activation (Negi *et al.* 2011). As anti-inflammatory and athero-protective roles for Nrf2 have already been described in the peripheral circulation (Siow & Mann,

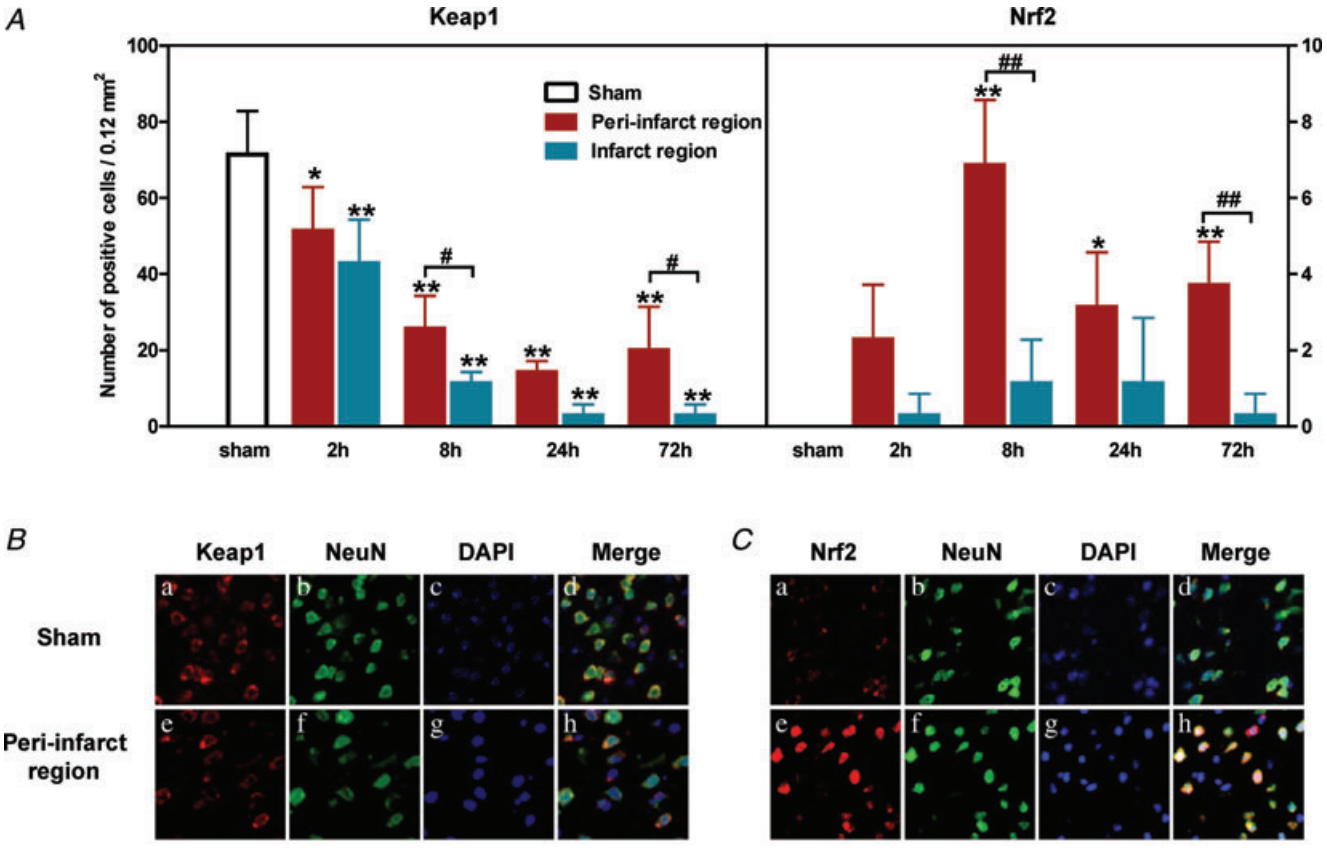

Figure 5. Keap1 and Nrf2-positive cells in the peri-infarct and infarct region of mouse brains after MCAO

A, brain sections were immunostained at different times of reperfusion after 60 min of MCAO (mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* sham; $\#P < 0.05$ and $\#\#P < 0.01$ *vs.* infarct region. $n = 5$ per group). Double immunofluorescent staining for the neural marker NeuN and Keap1 (*B*) and NeuN and Nrf2 (*C*) in brain sections from control (sham) mice and in the peri-infarct region of mice subjected to 60 min MCAO and 8 h of reperfusion. DAPI (4′,6-diamidino-2-phenylindole) was used for nuclear staining. Adapted from Figs 1 and 2 in Tanaka *et al.* (2011) with permission from Elsevier.

2010), it is possible that the Nrf2–Keap1 pathway elicits neurovascular protection via an improvement of cerebral blood flow.

Conclusions and future perspectives

The Nrf2–Keap1 defence pathway serves as a master regulator of endogenous antioxidant defences, and hence has been investigated as a potential therapeutic target for protection of the neurovascular unit in stroke. We have reviewed the evidence that Nrf2 is neuroprotective in experimental stroke, and that its activation may prevent the ischaemic penumbra from cell death. However, further research is required to establish the 'therapeutic window' during which activation of the redox-sensitive transcription factor Nrf2 is able to afford protection against cerebral ischaemia. Nrf2 inducers have been shown to be protective when administered before or after the onset of experimental stroke, but it seems likely that Nrf2-mediated increases in the activity of cytoprotective proteins require time. Based on the current understanding of the onset and progression of repair and injury processes in stroke and the need for alternative clinical treatment (Endres *et al.* 2008), studies are warranted to examine whether preconditioning of the Nrf2–Keap1 defence pathway *in vivo* offers significant protection against vascular dementia, where people experience small strokes over years. In our current studies, we have established that Nrf2 is expressed in different cell types of the neurovascular unit, and thus Nrf2 may have the potential not only to maintain cerebral blood flow, but also the survival of astrocytes and neurons following cerebral ischaemia–reperfusion injury in stroke.

As ageing-related changes in the brain are associated with an increased incidence of stroke (Chen *et al.* 2010), it is worth noting that both expression and activity of Nrf2 are diminished in ageing mice (Suh *et al.* 2004; Collins *et al.* 2009; Duan *et al.* 2009) and patients (Cheng *et al.* 2011; Demirovic & Rattan, 2011). Understanding the molecular mechanisms regulating Nrf2-mediated redox signalling in both young and aged rodent models of stroke should provide valuable insights for potential therapies targeted to protect the neurovascular unit.

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