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Ras Family Small GTPase-mediated Neuroprotective Signaling in Stroke

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Abstract

Selective neuronal cell death is one of the major causes of neuronal damage following stroke, and cerebral cells naturally mobilize diverse survival signaling pathways to protect against ischemia. Importantly, therapeutic strategies designed to improve endogenous anti-apoptotic signaling appear to hold great promise in stroke treatment. While a variety of complex mechanisms have been implicated in the pathogenesis of stroke, the overall mechanisms governing the balance between cell survival and death are not well-defined. Ras family small GTPases are activated following ischemic insults, and in turn, serve as intrinsic switches to regulate neuronal survival and regeneration. Their ability to integrate diverse intracellular signal transduction pathways makes them critical regulators and potential therapeutic targets for neuronal recovery after stroke. This article highlights the contribution of Ras family GTPases to neuroprotective signaling cascades, including mitogen-activated protein kinase (MAPK) family protein kinase- and AKT/PKB-dependent signaling pathways as well as the regulation of cAMP response element binding (CREB), Forkhead box O (FoxO) and hypoxia-inducible factor 1(HIF1) transcription factors, in stroke.

Keywords

cell death; cell survival; ischemia; neuroprotection; Ras family GTPases; signal transduction; stroke; transcription factors

1. Introduction

Stroke results from blood vessel occlusion or hemorrhage, and is one of the leading causes of disability [1]. Although tremendous effort has been directed toward both basic and clinical studies of stroke, treatment for this disorder remains insufficient [2–4]. The pathophysiological mechanisms that contribute to ischemia-induced neuronal injury are complex [1]. Selective neuronal cell death, including programmed cell death (PCD, apoptosis and autophagy) and necrosis, plays a pivotal role in neuronal loss following ischemic injury [1, 3, 5–8]. Moreover, the vulnerability of neurons to ischemic stress differs in distinct neuronal populations [1]. Studies have identified multiple intrinsic and environmental agents that contribute to neural damage [1, 3, 5–6]. These include excitotoxicity and ion imbalance; oxidative and nitrosative stress; ER stress; tumor necrosis factor (TNF) family receptors and their ligands; and neurotrophic factor withdrawal. For example, excitotoxicity, arising from the engagement of glutamate family receptors (GluRs) and neurotransmitters such as glutamate, acts as one of the major factors responsible for

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neuronal death. Modulation of GluR activity has been shown to affect the susceptibility of neurons to ischemic injury [9–12]. Ischemic onset induces excessive neurotransmitter release and inhibits re-uptake. Subsequently, these neurotransmitters bind to ligand-gated ionotropic *N*-methyl-D-aspartate (NMDA) receptors, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, or G-protein coupled glutamate receptors to evoke excessive ion influx, which in turn triggers cell death. Also, following stroke, especially after reperfusion, the generation of excessive reactive oxygen species (ROS) leads to mitochondrial dysfunction, resulting in energy deprivation and release of pro-apoptotic proteins [1, 13–14]. Moreover, ischemia and reperfusion (I/R) result in inflammatory responses, which subsequently enhance injury. I/R-triggered signaling pathways ultimately activate both caspase and non-caspase proteases such as granzyme A, that result in cell death [6, 15].

Cerebral cells mount a robust physiological response to ischemia [4–6]. Neuronal cells naturally utilize host defenses to activate pro-survival and inhibit apoptotic signaling cascades to mitigate cell death and promote cellular recovery [4]. Growth factors like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), neurotrophic transmitters or small peptides released from the affected cells or the local environment are known to trigger intracellular survival signaling. In addition, the survival of developing neurons requires extracellular cues to actively inhibit apoptosis [16]. Such cues are provided in part by the engagement of neurotrophic factors and their receptors. For example, NGF and BDNF bind and activate the tropomyosin-related receptor tyrosine kinases (RTKs) TrkA and TrkB [17], while pituitary adenylate cyclase activating peptide-38 (PACAP38) engages the seven transmembrane spanning heterotrimeric G protein coupled receptor (GPCR) PACAP receptor I (PACR1) [18–19]. Receptor-ligand engagement activates diverse intracellular signaling cascades, including Ras family small GTP-binding proteins (Figures 1 and 2). Moreover, preconditioning facilitated by non-injurious ischemic stimuli can augment intrinsic neuronal resistance to ischemic injury (termed ischemic tolerance) [1, 6, 14, 20]. Beyond the prototypical preconditioning stimuli including brief cerebral ischemia and cerebral hypoxia [1, 20], diverse endogenous or exogenous stimuli (such as sub-lethal concentrations of NMDA [9], hyperoxia and oxidative stress, or hyperthermia and heat shock [20]) can also promote ischemic tolerance by activating common protective signaling pathways [1]. Similar intracellular signaling mechanisms are also required for neuronal regeneration and maintenance [16]. For example, NMDA receptor (NMDA-R)-mediated activity is critical for neuronal development, synaptic plasticity, learning and memory [9–12].

While neither comprehensive nor efficient treatments against ischemia/stroke currently exist, analysis of the cellular response to ischemic injury has provided mechanistic insight and the promise of novel therapies for victims of stroke, including several strategies currently in preclinical and clinical trials [1–4, 21–22]. In addition, the combination of thrombolytic agents and neuroprotective therapy has been found to be more beneficial than either approach in isolation [2–3]. Thus, the development of novel therapeutic strategies demands a more comprehensive understanding of the signaling networks involved in ischemic injury, neuronal regeneration and recovery after stroke. Ras family small GTPases serve to integrate diverse extracellular signals and control the activity of a variety of intracellular signaling pathways to regulate diverse cellular functions [20, 23–25]. This article will highlight Ras-family GTPase-mediated neuroprotective signaling pathways and the therapeutic advances made from targeting these neuroprotective signaling pathways in I/R animal models.

2. Ras family GTPases and their regulation in stroke

Ras family GTPases comprise a large group of small molecular weight GTP-binding proteins that share a high degree of amino acid conservation within the five Ras core regions (the G1-G5 domains) which are essential for GTP binding and hydrolysis [26]. Most members possess low intrinsic GTPase activity and do not have prominent functional motifs outside their effector loops [26]. Since the first member of the Ras G-protein superfamily was discovered three decades ago [26], over 150 Ras-related GTPases have been identified, and can be classified into several subfamilies based upon both sequence homology and the regulation of common cellular functions [23]. Within the Ras branch of the superfamily, these GTPases can be further subdivided into the RAS (H-, K-, and N-Ras), RAP (Rap 1 and 2), RIT [Rit1 and Rit2 (also called Rin)], RAL (Ral A and B), RHEB (Rheb and RhebL1), R-RAS (R-Ras1, R-Ras2 and M-Ras), and RGK (Rem1, Rem2, Gem, Rad, RERG, RasL11 and RasL12) subfamilies [23]. In response to extracellular signals, these GTPases are activated by the exchange of pre-bound-GDP for GTP, which results in a conformational change within the Ras core that exposes the effector domain. This domain in turn promotes the recruitment of proteins responsible for the activation of diverse signal transduction pathways, including those regulating cell proliferation, differentiation, motility, and/or cell survival [23, 27–29]. The cycling of Ras family GTPases between their GDP-bound inactive and GTP-bound active forms is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) [26]. GEFs facilitate the exchange of bound GDP for GTP, while GAPs accelerate the intrinsic GTPase activity, which catalyzes the hydrolysis of bound GTP, and returns Ras-related GTPases to their GDP-bound inactive conformation [26, 30].

Ras GTPases respond to a wide range of extracellular signals including those important for neuronal differentiation and survival. It is well-known that growth factors [such as neurotrophins, epidermal growth factor (EGF), IGF-1, vascular endothelial growth factor (VEGF) and FGF] [31–33] and neurotrophic polypeptides (such as PACAP38) [33–34] are capable of activating Ras family members including Ras [17, 26, 31–33, 35–37], Rap [32–34, 38], Rit [34, 37–46] and Rin [40, 44, 47–50] GTPases. For example, NGF binding results in the activation of the TrkA receptor, and the phosphorylation of tyrosine residues within the TrkA intracellular domain creates docking sites for the recruitment of both the Shc/Grb2/SOS complex which promotes Ras activation, and the FRS2/Crk/C3G complex which triggers Rap GTPase activation [17, 32]. PACAP peptides bind to the PACR1 receptor, triggering Epac (exchange protein directly activated by cAMP) GEF activity to induce Rap activation [34]. cAMP-dependent Epac activation also facilitates R-Ras [51] and H-Ras [52] activation. Although Epac [38, 41] and SOS1/2 [41, 50] are required for PACAP38- and NGF-mediated Rit and Rin activation, no individual GEF has been directly implicated in either Rit or Rin activation.

Ras GTPases are also activated in response to ischemic/excitotoxic insults, suggesting that these signaling molecules serve as mediators of the cellular signaling responsive to ischemia. For example, NMDA stimulation activates both Ras [53] and Rap [54] in hippocampal neurons. Oxygen-glucose deprivation (OGD)-hypoxia triggers Ras activation in cortical neurons [55] and PC12 cells [56], requiring the activity of both the NMDA-R and nitric oxide synthase (NOS) [55–56]. Ischemia-hypoxia induced Ras activation also requires NOS activity in rat cortex and hippocampus [57]. NMDA-R-dependent Ras activation is impaired in RasGEF GRF1/GRF2 double knock-out (dKO) mouse cortical brain slices, indicating that these calcium sensory RasGEFs play an important role in NMDA-mediated Ras activation [53]. Ischemic lesions are increased in GRF1/2-dKO mice after middle cerebral artery occlusion (MCAO) and reperfusion, further supporting the importance of GRF-Ras signaling in neuronal protection [53]. While GRF1 and GRF2 appear to dominate

Ras activation in post pubescent animals acting downstream of NMDA-R-induced Ca^{2+} influx, the SOS1/2 RasGEFs function to regulate Ras activity acting downstream of both the Shc/Grb2 complex and the calcium activated Pyk2 and Src kinase cascades in neonatal mice [53]. GRF1 and GRF2 [58] also play critical regulatory roles in long-term potentiation (LTP) and long-term depression (LTD) by coupling to distinct NMDA-R subunits NR2A or NR2B [59]. Finally, SOS1, but not SOS2, also contributes to NMDA-R-induced LTP [60].

The activation of Ras and Rap GTPases by the NMDA-R is terminated by several calcium sensory and neuronal specific GAPs including SynGAP for both Ras (via a GAP domain) [61] and Rap (via a C2 domain) [62], as well as Snk-SPAR [63–64] and SPAL [65] for the Rap GTPases. These GAPs are activated following NMDA stimulation [61, 63, 65] or transient ischemia [66–67], and are components of a macromolecular complex, which also contains postsynaptic density (PSD)-95 (PSD95)/synapse-associated protein-90 (SAP90) family scaffold proteins found within the postsynaptic density [65, 68–70]. Their GAP activity is regulated by phosphorylation [66–68, 70–71], with tyrosine phosphorylation of SynGAP following transient cerebral ischemia shown to increase its RasGAP activity [71]. However, whether NMDA-R activation induces SynGAP serine phosphorylation [66–67] or dephosphorylation [70] remains a matter of debate. SynGAP activation is also subjected to phosphorylation by Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) [66–68, 70, 72]. Again, whether CaMKII- or NMDA-R-mediated serine phosphorylation increases [72] or decreases [68, 70] the GAP activity of SynGAP remains controversial. What is clear is that SynGAP is critical for neuronal development and the induction of LTP [73]. Homozygous SynGAP knockout mice die within the first week after birth, and display both excessive neuronal apoptosis [73–74] and specific defects in hippocampal LTP [73]. Finally, heterozygous reduction of SynGAP results in both cognitive defects [75] and non-cognitive abnormalities [76–77]. Taken together, these studies indicate that the regulation of Ras-family GTPases is important for both neuronal development and ischemic responses.

3. Ras family GTPase-mediated neuroprotective signaling in stroke

3.1 Ras family GTPases and mitogen-activated protein kinase (MAPK)-mediated neuroprotective signaling in stroke

The MAPK family consists of several major groups of kinases – including extracellular-signal regulated kinase 1 and 2 (ERK1/2, also known as p44/p42 MAPKs), p38 MAPKs, c-Jun N-terminal protein kinase (JNK) and ERK5. These MAPKs are activated by numerous extracellular signals and serve to regulate diverse signal transduction pathways, which in turn control a variety of cellular functions from cell metabolism, motility, proliferation, differentiation, survival and death, to neuronal regeneration and plasticity [2, 17, 31–32, 78–80]. It is well-established that MAPK signaling plays a critical role in the cellular response to I/R-induced neuronal injury [2, 81–82]. However, whether the activation of these protein kinase cascades is harmful or beneficial remains controversial [12, 79, 82–86]. What is clear is that targeting MAPK signaling can have dramatic effects on neuronal survival, regeneration, rewiring and plasticity following I/R [12, 16, 79, 82–86]. (Figure 1)

3.1.1 Extracellular-signal regulated kinases 1/2 (ERK1/2)—ERK1/2 proteins belong to the threonine/tyrosine family of protein kinases, and are regulated specifically by the dual protein kinases MAPK kinases (MAP2Ks), MEK1/2, which in turn, are regulated by the upstream MAPK kinase kinases (MAP3Ks) Raf-1 (C-Raf), B-Raf and A-Raf [17, 23, 25–26, 31–32, 87]. The Raf-MEK1/2-ERK1/2 signaling cascade appears to be predominantly controlled by Ras family small GTPases [17, 23, 25–26, 31–32, 87]. It is well-known that Ras mediates transient ERK1/2 activation [17, 27, 29, 35], while Rit, Rin [37, 45, 47] and Rap [17, 27, 29, 35] GTPase signaling leads to sustained ERK1/2 phosphorylation. Ras GTPases are known to activate both C- and B-Raf kinases [17, 26, 31–

32], while Rap [88–89], Rit [37] and Rin [47] GTPases appear to couple exclusively to the neuronal specific B-Raf isoform. In agreement with the importance of this signaling cascade, *a-raf* knockout mice develop neurological and intestinal defects in later life, while *b-raf* and *c-raf* knockout mice are embryonic lethal [90].

Global and focal ischemia, as well as reperfusion post ischemia, strongly activates ERK1/2 [82, 91–93]. I/R-induced Ca^{2+} influx and ROS generation have also been shown to activate ERK1/2 [82, 92, 94–95]. Post-ischemic ERK signaling can be divided into three phases – an acute activation phase, followed by a period of reduced ERK activity, and finally a sustained (long-term) reactivation phase [96]. Multiple mechanisms that control ERK1/2 activity during the cellular response to ischemia have been identified [96–101]. For example, in the acute phase, Ca^{2+} overload-mediated activation of the calcium sensory RasGEFs (GRF1/2 [53, 58, 95, 102]) and non-receptor tyrosine kinase (Src or Lyn)- coupled activation of the Shc/Grb2/SOS complex [53, 96, 100], promote Ras-dependent ERK signaling. In the second phase, decreased ERK activity may result from the effects of ischemia-induced activation of calcium-sensitive RasGAPs [66–67], phosphatases such as PP2A [98], Raf inhibitory scaffold proteins such as protein 14-3-3 and Raf kinase inhibitor protein (RKIP) [96], or inhibitory scaffolding proteins such as Sprouty and Sped (Spry) [99]. Finally, the sustained phase of ERK1/2 activation involves stimulation arising from both original ischemic stimuli [4, 95] and extrinsic cues originating from the local environment [3–4, 6, 9, 17, 19, 82, 96].

Src- and CaMKII-mediated signaling underlies ischemic insult-induced Raf phosphorylation at Tyr340/341, and this modification of Raf contributes to both acute and sustained ERK activation [96–97, 100–101]. Activation of Src family non-receptor tyrosine kinases (SFKs) also modulates NMDA-R-dependent signaling via tyrosine phosphorylation [103] and contributes to NMDA-R-mediated synaptic plasticity, learning and memory [103–104]. SFKs serve as molecular sensors to integrate multiple signaling pathways such as GPCRs (for example, the $\text{Gq}\alpha$ -coupled M1 muscarinic receptor and the $\text{Gs}\alpha$ -coupled PACR1) and RTKs (such as the EphB receptor) to modulate NMDA-R function [103]. In addition to their ability to upregulate NMDA-R signaling, SFKs also mediate cross-talk between the IGF receptor and PACR1 (via Src) [105], the adenosine 2A (A2A) receptor and TrkA (via Src or Fyn) [106–108], the PACR1 and TrkA (via Src) [41, 109], and the IGF and EGF receptors (via Src) [110]. Transactivation modulates the activity of downstream signaling cascades including Ras family GTPase-dependent ERK1/2 activation, and functions to promote neuronal survival and differentiation [41, 105–109].

Whether the activation of ERK1/2 following I/R is protective or deleterious remains controversial [81–82]. The duration and magnitude of ERK1/2 activation appear to be critical for cell fate determination [81]. Strong ERK activation during the acute phase of I/R or the activation of ERK by ischemia itself appears pathophysiologically detrimental [82]. The negative effects of ERK activation seem to arise from its ability to promote the release of inflammatory mediators and the generation of oxidative stress [82, 111].

Treatment with the Raf inhibitor SB-386023-b at up to 6 h, but not more than 12 h after the induction of subarachnoid hemorrhage-mediated ischemia, impairs ERK activation and inhibits the increase in inflammatory mediators [112]. A similar time window for administration of the MEK1/2 inhibitor U0126 was found to inhibit inflammatory responses and ERK activation in the rat MCAO ischemic model [111]. As expected, the application of U0126 or PD98059 has been proven to reduce brain infarct volume, accompanied by a reduction in ERK activation within the injury site in rat [111, 113], mouse [114–115], and gerbil [116] MCAO models. SL327, another MEK1/2 inhibitor, blocks MCAO-induced ERK activation, and administration of SL327 at 15 min before or 25 min after I/R, dramatically reduces brain infarct volume and improves neurological deficits 1 to 3 days

after reperfusion [117]. The protective effects of SL327 are accompanied by a decrease in caspase-3 activation and DNA fragmentation, but not an improvement in mitochondrial function [117]. MEK inhibitor U0126 also protects *in vitro* mouse cortical neuron cultures against oxygen deprivation and nitric oxide toxicity [116].

In addition, an antioxidant, S-Allyl-L-cysteine (SAC) has been found to inhibit ERK activation in the initial phase of ischemia, accompanied by a reduction in cerebral infarct volume following either transient MCAO or global ischemia [118]. SAC-mediated protection seems to result from a reduction in endogenous and exogenous ONOO⁻ production and ONOO⁻-mediated cytotoxicity, but not NMDA-R-mediated excitotoxicity [118]. Inhibition of ERK phosphorylation also contributes to Humanin (HN, a 24-amino acid peptide)-mediated reduction of cerebral infarct volume and neuronal death in a MCAO model [119]. Taken together, these studies suggest that inhibition of the Raf/MEK/ERK signaling cascade is protective against stroke. Small interference RNA (siRNA)-mediated ERK2 silencing reduces traumatic spinal cord injury, lending further support to the neuroprotective benefits of this therapeutic approach [120].

Despite the data suggesting that ERK inhibition is neuroprotective, numerous studies indicate that activation of ERK1/2 signaling in I/R is protective [82]. In agreement with this notion, persistent ERK phosphorylation is found in the relatively resistant CA2, CA3 and dentate gyrus (DG) regions of the hippocampus, but not in the vulnerable hippocampal CA1 region following cerebral ischemia [93, 121]. Moreover, ischemic tolerance induced by multiple agents or processes appears to rely in part upon ERK activation [20, 78]. Ischemic preconditioning prevents subsequent lethal cerebral ischemia-induced dephosphorylation of ERK and phosphorylation of JNK kinases, supporting a requirement for ERK activation in ischemic neuroprotection [122–123]. ERK also plays critical roles in regulating the signaling and physiological actions of growth factors, neurotransmitters, neuropeptides and estrogen [2–4, 6, 9, 12, 14–15, 17, 19, 25, 31–33].

Homozygous neurotrophin-4 (NT4) null mice (*nt4^{-/-}*) or heterozygous BDNF mutant mice (*bdnf^{+/−}*) exhibit higher susceptibility to ischemic injury induced by either MCAO/reperfusion, or permanent MCAO [124]. Moreover, expression of BDNF and its receptor TrkB is downregulated within the infarct core following MCAO, and grafts of BDNF expressing cells significantly enhance TrkB expression and reduce MCAO-induced cell death [125–126]. Serotonin 5-hydroxytryptamine receptor type 1A (5HT_{1A}R)-mediated protection against transient global ischemia also requires the NMDA-R subunit NR1-dependent induction of BDNF [127–128]. The neuroprotective effects of BDNF are in part mediated by ERK-phosphoinositide-dependent protein kinase 1 (PDK1)-dependent p90 ribosomal S6 kinase 1/2 (RSK1/2) signaling [129]. In addition, BDNF-mediated neuroprotection is facilitated by the scaffold protein, kinase suppressor of Ras-1 (KSR1) which increases the association of ERK1/2 with Raf and MEK1/2 kinases to promote ERK activation [130–131]. ERK signaling has also been implicated in the melatonin-mediated prevention of MCAO-induced brain injury and neuronal death [132], the protective effects of erythropoietin (Epo) following focal cerebral ischemia [133] and the glial-derived neurotrophic factor (GDNF)-mediated neuronal survival following NMDA exposure [134].

The Gα_i-coupled GPCR ligands, including PACAPs, have been shown to reduce brain infarct volume in multiple I/R animal models, while PACAP knockout mice display increased brain damage following ischemia [19]. In addition to its ability to transactivate RTKs [19, 105–106, 109], PACAP integrates multiple mechanisms to promote neuronal survival [19]. PACR1 stimulates Gα_i-PKA-ERK signaling but not p38 or JNK, which in turn modulates expression of Bcl-2 family members and decreases the release of cytochrome c and apoptosis-inducing factor (AIF), leading to a reduction in neuronal injury [19, 135–

136]. Additionally, PACAP38 triggers STAT3 and ERK activation via the upregulation of the neuroprotective cytokine IL-6 [137]. The essential role of IL-6 in PACAP38-mediated neuroprotection is supported by the fact that PACAP38 treatment fails to rescue brain damage in IL-6 null mice [137].

The activation of the Raf/MEK/ERK signaling cascade also contributes to the neuroprotection afforded by other neuropeptides, such as Apelin which couples to the GPCR APJ [138–139] and the cocaine and amphetamine-regulated transcript (CART) [140–141]. CART is also regulated by estradiol-mediated activation of estrogen receptor (ER), and contributes to estrogen-induced neuroprotection against MCAO ischemic injury [141].

Endogenous estrogen confers females with ischemic resistance via anti-inflammatory effects that reduce iNOS production [142]. Consistent with a protective role, exogenous estrogen has been shown to provide protection against ischemic injury to males and ovariectomized females [142]. MEK/ERK signaling is required for ER-dependent GPCR-kinase 2 (GRK2), c-Src and α -arrestin-1 activation [143], supporting a critical role for ERK signaling in estradiol-mediated neuroprotection [141, 143–144]. Transactivation of the IGF-1 receptor by estradiol also contributes to ER-stimulated ERK activation [144].

3.1.2 p38 mitogen-activated protein kinases (p38 MAPKs)—As with ERK signaling, the role of p38 MAPK in stroke is complex, with studies indicating that p38 contributes to ischemic damage but also promotes cerebral protection. p38 MAPK was initially identified as a kinase activated in response to bacterial lipopolysaccharide and osmotic stress, and implicated in promoting inflammatory mediator production and cellular apoptosis (thus, p38 MAPK is also termed a stress-activated protein kinase, SAPK) [145]. Four p38 MAPK isoforms have been identified in mammals, p38 α , β , γ and δ , which share a high degree of structural homology but differ in their tissue distribution and physiological functions [79]. p38 α knockout results in embryonic lethality, accompanied by deficits in the activation of MAPK-activated protein kinase 2 (MAPKAPK2 or MK2) and the response to IL-1 in embryonic stem cells [146]. Rap GTPase-dependent p38 signaling is reported to mediate NMDA-R-induced LTD [53, 58]. p38 signaling is also required for both Rit and Rin GTPase-induced neuronal differentiation [37–38, 47], and contributes to axonal and dendritic differentiation and neuronal survival [17, 24, 32, 147]. It is now believed that p38 MAPK signaling plays a critical role in cell fate decisions, and aberrant p38 signaling is linked with many human diseases including stroke [148–149].

The levels of active p38 are elevated in both neuronal and glial cells immediately following focal or global I/R, especially in vulnerable regions of the brain, including the cerebral cortex and hippocampal CA1 regions [2, 91–92, 150–155]. Similar to ERK, the duration and magnitude of p38 signaling appears to determine whether activation is detrimental or protective [81]. Sustained and exaggerated p38 activation in the ischemic brain is positively correlated with injury, accompanied by excessive inflammation, blood-brain barrier (BBB) dysfunction and cellular apoptosis [81, 151–152, 156]. p38 activation induces the production and release of various inflammatory factors including IL-1, TNF α , iNOS and cyclooxygenase-2 (COX-2) [145, 154]. Inhibition of p38 signaling using the p38 inhibitor SB203580 reduces global ischemia-induced cell death within the hippocampal CA1 region [150]. Interestingly, even when applied at 6 or 12 h after the onset of MCAO, *i.v.* administration of SB203580 dramatically reduces MCAO-induced infarct volume and improves neurological deficits [154]. A similar reduction in inflammatory signaling is seen in both moderate and severe focal cerebral ischemia following application of the more specific p38 inhibitor SB239063 [157–160]. Moreover, Apigenin, a flavonoid glycoside, reduces MCAO-induced infarct size via a similar anti-inflammatory mechanism [161].

These data suggest that p38-mediated inflammatory processes play a vital role in ischemic injury [154].

p38 activation also contributes to BBB disruption, leading to water influx, vasogenic edema, and secondary brain injury [156]. Intraventricular inhibition of p38 (using SB203580) significantly reduces transient cerebral ischemia-induced activation of phospholipase A₂ (cPLA₂), and attenuates BBB extravasation and edema [156]. cPLA₂ converts arachidonic acid to eicosanoids, leading to BBB dysfunction. In support of the importance of p38/cPLA₂ signaling to BBB leakage, cPLA₂^{-/-} mice display low levels of COX-2, PGE₂, and ROS in the ischemic cortex [162]. Atorvastatin, a member of the statin drug family, ameliorates BBB permeability through downregulation of p38/cPLA₂ signaling, and can prevent MCAO-mediated cerebral damage [163]. Finally, p38 is capable of directly activating caspase-3 [79, 164]. Together with p38-dependent mitochondrial dysfunction and generation of ROS, p38 MAPK signaling is believed to be a critical contributor to the regulation of cellular apoptosis and necrosis [79, 145, 148–149], suggesting that p38 inhibition may provide a reliable therapeutic strategy for stroke treatment.

Consistent with the neurotoxic consequences of p38 activation following ischemic injury, reduction of p38 activity in vulnerable neurons contributes to hyperbaric oxygen (HBO)-induced ischemic tolerance against subsequent lethal transient global ischemia [165–166]. Anisomycin, a p38 activator, abolishes HBO-mediated ischemic tolerance in a p38 inhibitor-sensitive fashion [166]. Moreover, fructose-1,6-bisphosphate prevents NMDA-mediated excitotoxicity by suppressing p38-mediated ROS production [167]. The improvement in cerebral lesion size and neurological deficits following I/R afforded by Activin A, the antioxidant thioredoxin (TRX), or the A2A receptor antagonist SCH58261 are also accompanied by p38 inhibition [168–171].

Additional evidence has accumulated from preconditioning studies suggesting that p38 activation may also be neuroprotective in I/R. Nishimura *et al* [172] found that 2 min global ischemia results in long-lasting p38 activation in CA1 and CA3 neurons, while pre-treatment with SB203580 reduces p38 signaling, resulting in attenuated ischemic tolerance and a reduction in hippocampal CA1 neuronal survival following subsequent lethal ischemia. Limb ischemic preconditioning (LIP) induces brain ischemic tolerance [173]. LIP increases p38 activity within the CA1 and later in the hippocampal CA3 and DG regions, and reduces neuronal death following global ischemia in a SB203580-sensitive fashion [173]. Preconditioning using isoflurane reduces brain infarct volume and improves neurological deficit after permanent MCAO, which is also reversed by SB203580 administration [174]. In contrast to the role of p38 signaling in promoting BBB leakage discussed above, inhibition of p38 significantly aggravates cerebral vascular leakage and increases infarct volume following transient MCAO in mice [175].

In part, the controversy surrounding the role of p38 signaling in I/R may arise from the differential activation of the many upstream regulators of p38 and the activation of distinct p38-dependent downstream signaling pathways, which together allow p38-signaling to control a wide range of distinct cellular functions [79, 84, 149]. Like other MAPKs, p38 MAPKs are activated by dual protein kinase MAP2Ks including MKK3, MKK4 and MKK6, which in turn are activated by diverse upstream MAP3Ks, including MEKK1-4, apoptosis signal-regulating kinase 1 (ASK1), TGFβ-activated kinase 1 (TAK1)/TAB1, TAO1/2, mixed lineage kinase 3 (MLK3), and dual leucine zipper-bearing kinase 1 (DLK1) [79]. Most of these MAP3Ks are also capable of activating JNK1/2 signaling [79], which has greatly complicated the *in vivo* analysis of the cellular effects of these regulatory cascades.

Caspase-3 activation results in MEKK1 cleavage and activation [164]. Activated MEKK1 serves as part of a positive feedback loop to amplify p38-caspase-3-dependent cell death [164]. Application of kainite (KA) receptor GluR6 inhibitor NS102 or GluR6 receptor silencing inhibits I/R-induced p38, MLK3, MKK3/4/6 and MK2 phosphorylation, suggesting that a GluR6-PSD95-MLK3 signaling complex contributes to the activation of a MKK3/4/6-p38-MK2 cascade and ischemia-induced neuronal injury [176]. Indeed, administration of either GluR6 inhibitor NS102 or p38 inhibitor SB239063, or GluR6 silencing, significantly decreases cell death at 3 days after reperfusion and increases the survival of CA1 pyramidal cells at 5 days after reperfusion following transient ischemia [176]. Another MAP3K, ASK1, mediates ROS- and TNF α -dependent activation of both p38 and JNK kinases, and promotes apoptosis [177–181]. This apoptotic activity can be inhibited by treatment with the antioxidant agent, TRX [180]. Conversely, the ability of ROS to induce activation of p38 and JNK, and apoptosis, is impaired in ASK1^{−/−} mice [181]. Moreover, I/R trigger ASK1 activation and hippocampal apoptosis, which is suppressed by exposure to the antioxidant *N*-acetylcysteine [182] or by ischemic preconditioning [183]. Taken together, these data support a role for ASK1-p38/JNK signaling in cellular apoptosis following cerebral ischemia.

p38 MAPKs act by either directly phosphorylating cellular substrates or inducing their phosphorylation by activating a second tier of cellular kinases, including MK2, MSK1/2, MNK1 and GSK3 β [79]. The MK2 kinase is a direct substrate of p38, and has been shown to contribute to p38-mediated apoptotic signaling [79]. MK2 deficient mice are resistant to MCAO-induced ischemic injury, displaying reduced infarct size and an improvement in neurological deficits [184]. This resistance is accompanied by a reduction in IL-1 β production, but no alteration is seen in hemodynamic or systemic variables, TNF α production, caspase-3 activation or apoptosis [184]. MK2^{−/−} mice display a similar resistance to myocardial I/R-induced injury [185]. However, interpretation of these studies is complicated by the finding that ischemic tolerance in myocardial cells depends upon p38 and MK2 activation [186–188], suggesting that p38-MK2 signaling is also capable of promoting cell survival.

Indeed, MK2 phosphorylates heat shock protein 27 (HSP27), a molecular chaperone known to promote neuronal survival [189]. HSP27 expression and phosphorylation are increased upon either global cerebral ischemia or preconditioning in hippocampal CA1 and DG regions or in cultured hippocampal slices [190–191]. Moreover, preconditioning reduces subsequent lethal I/R-induced neuronal death in the hippocampal CA1 region, which is accompanied by a reduction in phosphorylated HSP27 but an increase in cellular HSP27 protein levels [190], suggesting that HSP27 plays a critical role in neuroprotection against I/R. Indeed, transgenic mice over-expressing HSP27 display a reduction in kainite-induced neuronal death and caspase-3 activation within the hippocampal CA3 region [192]. It is believed that HSP27-mediated protection relies upon its chaperone activity, which is generated by a high molecular mass homo-oligomer of the unphosphorylated protein [190]. HSP27 also suppresses the release of cytochrome c and blocks the association of cytochrome c with Apaf-1, resulting in disruption of the mitochondrial apoptotic pathway [193].

MSK1, another p38 MAPK substrate, is also required for p38- but not AKT-dependent ischemic preconditioning in MK2^{−/−} mice, suggesting that MSK1/2 may act as an alternative downstream target of p38 MAPK-dependent survival signaling [194].

3.1.3 c-Jun N-terminal protein kinases (JNKs)—The JNK family protein kinases, another branch of the SAPKs, consist of three genes, *jnk1*, *jnk2* and *jnk3*, which encode 10 kinase isoforms in mammalian cells [195–196]. JNKs have well-established roles in the

regulation of diverse cellular functions, but play particularly critical roles in the regulation of cell death and survival. While JNK1 and JNK2 are ubiquitously expressed, JNK3 is exclusively expressed in the brain and testes [195–196]. Like all other MAPK members, JNKs are activated by dual protein kinases (MKK4 and more specifically, MKK7), which in turn are regulated by an array of upstream MAP3Ks (see description in section 3.1.2) [79, 195–196]. In agreement with the importance of JNK-dependent signaling in normal development, deletion of the JNKs, MKK4 or MKK7 results in embryonic lethality [195–196]. However, activation of JNKs following I/R has been associated with induction of brain damage [2, 81, 85, 122, 195–196]. I/R trigger a persistent JNK1/2 activation in the vulnerable hippocampal CA1 region, as well as within the striatum and cortex bordering the infarct core following either global or transient ischemic insult [91, 93, 197–198]. Neuronal specific JNK3 phosphorylation is found to be upregulated in the hippocampus, neocortex and striatum [198]. Importantly, activated JNKs co-localize with apoptotic cerebral cells [91, 93, 197–198]. Furthermore, JNK3 deficient mice display tolerance to cerebral ischemia/hypoxia-induced brain damage [199–200], further supporting the detrimental role of JNK signaling in stroke.

Both nuclear and non-nuclear apoptotic pathways contribute to JNK-mediated cell death following I/R [81, 85, 195–196, 199–200]. Activated JNK kinases translocate to the nucleus and activate pro-apoptotic transcription factors including c-Jun and FoxO (see discussion in section 4.2). The activation of these transcription factors controls the expression of a variety of pro-apoptotic genes including the Bcl-2 family member Bim, Fas-ligand (Fas-L), TNF α and TNFR1 [81, 85, 197–198, 201]. JNK signaling also triggers NF κ B-dependent inflammatory responses [202]. In the cytoplasm, JNKs activate apoptotic molecules including Bcl-2 family members. Once activated, pro-apoptotic Bcl-2 family members such as Bad, Bid, Bim and Bax translocate to the mitochondria and trigger cytochrome c release, which in turn induces caspase-9- and caspase-3-mediated apoptosis [81, 85, 197–198, 201]. As expected, JNK inhibitors (both SP600125 and AS601245) attenuate JNK-dependent nuclear and non-nuclear apoptotic signaling pathways and prevent cerebral cell death and brain injury in a variety of ischemic models [196, 203–209].

Multiple signaling mechanisms have been reported to regulate the activation of JNKs, and subsequently to modify JNK-mediated neurotoxicity. For example, JNK activation is negatively regulated by AKT signaling (see discussion in section 3.2). JNK interacting protein 1 (JIP1) inhibits JNK phosphorylation and nuclear translocation via its scaffolding activity [196, 210]. Following I/R, JIP1 expression within the brain is increased, but at a delayed rate when compared to that of JNK activation and increase in JNK expression [211]. However, the delayed pattern of JIP1 expression is strongly correlated with a reduction in neuronal death, suggesting that targeting JIP1 may provide an innovative therapy for stroke [196, 210]. Dickens *et al* [210] identified a 20 amino acid (aa) conserved sequence in JIP1 critical for JNK binding, and demonstrated that this peptide alone was sufficient to inhibit JNK activity. Coupling the *D*-retro-inverso form of this inhibitory peptide to the HIV-TAT peptide generates a competitive and cell permeable JNK inhibitory peptide, D-JNKI1 [212–213]. D-JNKI1 inhibits JNK activation, reduces excitotoxicity- and OGD-induced neuronal death and improves neural recovery *in vitro* [212–213]. Importantly, administration of D-JNKI1 after I/R significantly reduces infarct volume and improves functional recovery in multiple ischemic models [212–215]. The combination of HBO preconditioning and D-JNKI1 treatment synergistically reduces ischemic cerebral damage, resulting in an up to 78% decrease in infarct volume [214].

JNK activation following I/R is also regulated by multiple upstream MAP3Ks including ASK1 and MLK3 [79, 85–86, 195–196]. ASK1-mediated JNK signaling promotes apoptosis, which can be blocked by HSP27 overexpression [216]. HSP27 associates with

ASK1, disrupting ASK1-dependent JNK activation and leading to long-lasting tissue preservation and neurobehavioral recovery following focal cerebral ischemia [216].

MLK3 is activated in cerebral vulnerable regions following I/R [217]. The Zhang laboratory has identified several mechanisms that contribute to MLK3-dependent JNK signaling following ischemic insult, and have demonstrated that inhibition of the MLK3-JNK signaling cascades provides neuroprotection. For example, the antibiotic geldanamycin destabilizes the I/R-induced MLK3-HSP90 interaction and promotes MLK3 degradation, which reduces MLK3-dependent JNK activation, resulting in reduced ischemic injury [217]. In addition, I/R promote long-lasting Rac1 GTPase activation [218]. Rac1 activation enhances the assembly of the POSH-Rac1-MLK3 signaling complex, resulting in MLK3 and JNK activation in the hippocampal CA1 region [218]. Administration of the Rac1 inhibitor NSC23766 or Rac1-silencing attenuates this signaling cascade and reduces hippocampal CA1 injury following cerebral ischemia [218]. Furthermore, I/R also increase GluR6-PSD95-MLK3 signaling module-dependent JNK activation, neuronal death and cerebral damage [219]. Conversely, disruption of GluR6-PSD95-MLK3-dependent JNK signaling leads to cerebral protection [220]. Disruption of this cascade also contributes to the neuroprotection afforded by multiple agents including hypothermic and ischemic preconditioning [221–222], exposure to the antioxidant NAC [223], or expression of the isolated PDZ1 domain from PSD95 [220]. Mehta *et al* [224] identified a conserved motif RLPGKERMA (GluR6-9C) within the C-terminus of GluR6, and demonstrated that this peptide is capable of disrupting the GluR6/PSD95/SAP90 complex. Administration of TAT-GluR6-9c, a fusion peptide generated by coupling GluR6-9C to the HIV-TAT peptide, perturbs the GluR6-PSD95-MLK3 interaction and facilitates the association of MLK3 with JIP1, reducing both JNK activation and OGD- or MCAO-induced injury [225–226]. Together, these studies suggest that JNK signaling plays a critical role in promoting ischemic cerebral injury and that strategies designed to inhibit this cascade may result in the development of novel stroke therapies.

3.2 Ras family GTPases and AKT-mediated neuroprotective signaling in stroke

AKT/protein kinase B (PKB), a serine/threonine protein kinase, plays a central role in the control of diverse cellular processes including cell proliferation, differentiation, migration, nutrient metabolism, survival and death [227]. AKT activation is mediated by phosphorylation at two distinct regulatory sites. Phosphorylation at Thr308 in the activation loop is mediated by the PDK1 kinase pathway, and is essential for AKT activity [228]. However, maximum AKT activation requires a second phosphorylation event at Ser473 within the C-terminal hydrophobic motif mediated by the PDK2-like kinase pathway [229–230]. Ras family small GTPases are known to serve as important regulators of AKT signaling [228, 231–233]. Ras-dependent phosphoinositide 3-kinase (PI3K)/PDK1/AKT signaling has been reported to be critical for neurite extension/retraction [234]. As a physical interacting partner for class 1A PI3Ks [228, 232], Ras plays a critical role in RTK-mediated AKT phosphorylation at Thr308 [232–233]. Neurotrophic factors (such as NGF [235–236], BDNF [237] and GDNF [238]), as well as growth factors (such as VEGF [239–240] and IGF-1 [241]), have been shown to activate AKT through this Ras-dependent PI3K/PDK1 signaling cascade.

The Ras-AKT signaling axis is also important for NMDA-R signaling [242–243]. Activation of the NMDA-R has been shown to increase the levels of GTP-bound Ras [244], which in turn associates with the p110 catalytic subunit of PI3K to enhance PI3K activity [228, 232]. These data support the existence of a NMDA-R-Ras-PI3K signaling complex and implicate this cascade in ischemia-dependent AKT activation [245]. NMDA exposure leads to PI3K-dependent AKT activation in cortical neuron cultures [243]. AKT expression and phosphorylation are elevated several hours after I/R, and tend to return to basal levels after

24 hours [246–248]. Activated AKT is found predominantly within the ischemic resistant penumbral region but not in the ischemic core [247], suggesting that AKT signaling is crucial for neuronal protection after I/R. Indeed, PI3K inhibition treatment exacerbates neuronal loss after ischemia [247]. Moreover, carboxyl-terminal modulator protein (CTMP), an endogenous inhibitor of AKT, is upregulated in hippocampal neurons following global ischemia [249]. CTMP abolishes AKT kinase activity and promotes neuronal death [249]. Conversely, overexpression of constitutively active AKT protects primary cultured hippocampal neurons from NMDA exposure [250]. Transgenic mice overexpressing AKT display a significantly smaller infarct volume after MCAO surgery [251], further supporting a pro-survival role for AKT signaling in stroke.

As a critical pro-survival molecule, AKT controls multiple anti-apoptotic pathways in response to growth factors and a variety of cellular stresses [227]. AKT-mediated protective signaling involves both the activation of pro-survival mediators and the inactivation of pro-apoptotic proteins (Figure 2). For example, AKT phosphorylates Bad, pro-caspase-9 and pro-apoptotic kinases such as the mammalian Ste20-like kinases 1/2 (MST1/2, see discussion in section 3.3), inhibiting their pro-apoptotic functions [228, 233, 252–254]. AKT also promotes survival through the regulation of gene transcription. For example, AKT phosphorylates MDM2, a p53-directed E3 ubiquitin ligase, which acts to downregulate p53-dependent pro-apoptotic pathways at the transcriptional level [233, 255]. AKT is also known to activate CREB (see discussion in section 4.1) and inactivate FoxO family (see discussion in section 4.2) transcription factors [228, 255]. Finally, AKT activates I κ B kinase (IKK), which in turn promotes the phosphorylation and subsequent degradation of I κ B, resulting in the release of NF κ B. Once free of I κ B, NF κ B translocates to the nucleus and stimulates the transcription of anti-apoptotic molecules such as the cellular inhibitor of apoptosis protein (cIAP) [228].

AKT also negatively regulates JNK-dependent death signaling to prevent ischemic injury [256–258]. The lipid phosphatase PTEN (phosphatase and tensin homolog) can inhibit AKT-mediated JNK blockade and thus promotes neuronal death following ischemic injury [257]. The toxicity of PTEN is attenuated by calpain-mediated PTEN degradation, and application of the calpain inhibitor A6185 has been shown to reverse calpain-mediated PTEN inhibition and neuroprotection [257]. Active AKT also inhibits JNK-mediated phosphorylation of Bad at Ser128 and increases Bad phosphorylation at Ser136, resulting in a blockade of Bad-induced mitochondrial permeability [252]. Ca²⁺ influx triggers the dissociation of Src and HSP90, resulting in Src-dependent AKT activation [217].

This AKT signaling pathway inhibits ASK1-dependent JNK activation, serving to mitigate ischemic injury [217]. Moreover, geldanamycin administration triggers AKT-dependent ASK1 inactivation, and thus prevents ischemic injury [217]. AKT signaling also elevates the antioxidant capacity of neurons. Papadia *et al* [259] reported that synaptic NMDA-R activity triggers PI3K/AKT-dependent FoxO3a inactivation, which results in the downregulation of Txnip, a FoxO targeted gene. Txnip is a thioredoxin (Trx) inhibitor, and thus the downregulation of Txnip increases Trx enzymatic activity, which in turn reduces oxidized peroxiredoxin (Prx) enzymes and increases the cellular capacity to detoxify peroxides and ROS [259–260]. In addition, NMDA-R-mediated synaptic activity enhances C/EBP β - and AP-1-mediated gene transcription, resulting in the expression of Sestrin2 and sulfiredoxin enzymes, which also serve to reduce peroxiredoxin [259–260]. These orchestrated transcriptional events ultimately promote oxidative stress resistance and neuroprotection against I/R [259].

Several other molecular mechanisms that regulate AKT signaling reduce ischemic injury. Insulin/IGF-1 is known to mediate PI3K/PDK1-dependent neuroprotective effects [261–

262], protecting both hippocampal DG and CA2 neurons against OGD [262]. VEGF triggers both PI3K/AKT and ERK1/2 signaling to mediate cerebral protection [263], and administration of VEGF significantly improves recovery following ischemia [240, 264]. In addition, sphingosine-1-phosphate/PI3K/AKT signaling is essential for cerebral protection mediated by geranylgeranylacetone (GGA) [265] and isoflurane [266] following hypoxia/ischemia. Gly14-Humanin (HNG), a potent Humanin variant, dramatically increases PI3K-dependent AKT phosphorylation, leading to a reduction in cerebral infarct volume following MCAO [267]. Moreover, ischemic preconditioning is known to promote AKT phosphorylation, as one mechanism to induce ischemic tolerance [1, 20].

Besides the PI3K/PDK1 pathway, AKT is also regulated by PDK2-like kinase signaling pathways including mammalian target of rapamycin (mTOR) complex 2 (mTORC2) [268], which is an evolutionarily conserved complex consisting of the mTOR, mLST8, Rictor and SIN1 proteins [261]. mTORC2 was initially identified as the kinase required for AKT-Ser473 phosphorylation in response to insulin/IGF-1, without affecting PDK1-mediated AKT phosphorylation at Thr308 [261]. However, recent studies indicate that mTORC2 may also be essential for AKT-Ser473 phosphorylation in response to TGF- β [269] and nutrient stress [270]. Importantly, mTORC2-dependent AKT signaling is required for insulin and IGF-dependent neuronal protection against OGD [262]. Ras family members are believed to be involved in the regulation of PDK2/AKT signaling pathways. For example, RasC, a homolog of H-Ras in *Dictyostelium*, activates the mTORC2-AKT (Ser473) pathway [271–272], indicating that the Ras family GTPase-mediated PDK2/AKT signaling cascade may also contribute to neuroprotection in stroke.

3.3 Ras family GTPase-mediated apoptotic signaling

With the discovery of a new class of Ras effectors, the Ras association domain family (RASSF) proteins, a role for Ras in the induction of apoptosis is now emerging [273]. RASSF proteins were first identified as tumor suppressors [274]. Recent mechanistic studies indicate that RASSF family proteins relay apoptotic signals by forming a complex with both activated Ras proteins (preferentially K-Ras) and MST1/2 proteins [274–277]. In response to stress, MST1 is autophosphorylated at Thr183 (Thr180 for MST2) [278–280], which promotes caspase-3-mediated apoptosis. MST1 is also a caspase-3 substrate [281], and the MST1 fragment generated by caspase-3 cleavage translocates to nucleus and phosphorylates histone H2B at Ser14, resulting in chromatin condensation [282–283]. MST1/2 activation also promotes JNK-mediated apoptosis (see discussion in section 3.1.3) and FoxO3a-controlled pro-apoptotic gene expression [284–285] (see discussion in section 4.2). MST1/2 has also been shown to bind and activate WW45/LATS, which inhibits the mammalian transcriptional activator yes-associated protein (Yap), leading to the downregulation of IAPs, to promote apoptosis [286–289]. Taken together, these data suggest that Ras-mediated apoptotic signaling may contribute to the pathogenesis of stroke (Figure 2).

4. Regulatory roles of Ras family GTPases on transcription factors in stroke

I/R trigger the activation of a complex array of transcription factors that control both pro-survival and pro-apoptotic gene expression, and the balance of protein expression following injury appears to play a crucial role in cell fate determination [1–4, 20]. The regeneration of new neurons, the rewiring of neural networks, neural plasticity, learning and memory also require new protein synthesis [1–4, 16, 20]. A variety of transcription factors, including NF κ B, MEF2C, CREB, AP1, HIF1, p53, Sp1, c-Jun and FoxO proteins, have been implicated in the regulation of gene expression during and after stroke [1–4, 20]. Ras family GTPases are known to function as upstream regulators of these transcriptional events [1–4,

20]. Here, we will focus on the role of Ras family GTPase-mediated regulation of CREB, FoxO and HIF1 transcription factors in the pathophysiological regulation of stroke (Figures 1 and 2).

4.1 cAMP response element binding (CREB) transcription factor

In the central nervous system, CREB plays a pivotal role in the regulation of gene expression underlying neuronal development, survival, regeneration, memory, synaptic plasticity and cell repair [290]. CREB is activated primarily by elevated cellular Ca^{2+} and cAMP levels. Phosphorylation at Ser133 is crucial for the transcriptional activity of CREB [290]. Phosphorylated CREB recruits co-activators such as CREB-binding protein (CBP) and RNA polymerase, which further enhance CRE-mediated gene expression [290]. In addition, growth factor-mediated receptor tyrosine kinase activation is also capable of activating CREB. Ras-family GTPases play critical roles in coupling these diverse cellular stimuli to CREB activation [290]. For example, NMDA-R mediated Ras-ERK signaling activates CREB, which is important for both neuronal survival and LTP [12, 53, 291]. Activation of Ca^{2+} -dependent CaMKs (I, II and particularly IV), cAMP-PKA signaling cascades, as well as AKT, PKC, RSK1, MK2, MSK1/2 and MNK1 kinases have each been shown to phosphorylate CREB [12, 25, 290–292]. In addition, both ERK and p38 signaling cascades regulate RSK1, MK2, MSK1 and/or MNK1 kinase activity, allowing them to serve as upstream regulators of CREB-dependent transcription [79, 290] (Figures 1 and 2).

CREB phosphorylation but not CREB expression is dynamically regulated following I/R [290]. Despite the fact that CREB phosphorylation is downregulated during global ischemia, reperfusion induces a profound increase in phosphorylated CREB within the resistant hippocampal DG region, but only a slight increase is observed within the vulnerable hippocampal CA1 region [293–300]. Focal brain ischemia also induces significant and persistent phosphorylation of CREB in the peri-ischemic area of the cerebral cortex where neurons remain viable [296–298]. Only a transient increase in CREB phosphorylation is observed within the ischemic core where neurons undergo death [296–298]. Although the dynamics of CREB phosphorylation is variable among different ischemic models, the pattern of CREB activation is strongly correlated with that of CRE-mediated gene transcription, neuronal survival and ischemic infarct size following ischemia. Despite the fact that hypoxic and ischemic preconditioning only induce modest CREB phosphorylation, the levels of phosphorylated CREB in primed neurons are dramatically enhanced during subsequent severe hypoxia/ischemia [301–305]. This appears to rely upon the NR2A but not the NR2B subunit of the NMDA-R, since NR2A selective inhibition, but not NR2B blockade, attenuates CREB activation and ischemic tolerance [301, 303]. Moreover, ischemic tolerance and CREB activation are blocked by NMDA-R inhibition, Ca^{2+} removal, or inhibition of PKA, CaMKs or MEK1/2 signaling [295, 301], suggesting that cells mobilize diverse signaling pathways to mediate ischemia-induced CREB activation. By using CRE-*LacZ* reporter transgenic mice, Sugiura *et al* [306] found that CRE-mediated transcription also occurs in astrocytes, oligodendrocytes, endothelial and microglia cells, suggesting that CREB-dependent signaling is a common protective mechanism for a variety of cerebral cells within the ischemic brain [306]. Indeed, phosphorylated CREB is detected in oligodendrocytes following focal ischemia [307]. Together, these studies indicate that CREB signaling is critical for neuroprotection [301–304].

Increasing CREB activation appears to be a viable therapeutic strategy for stroke [290]. For example, exogenous 17- β -estradiol administration reduces focal ischemic injury in a CREB-dependent manner [308–309]. VEGF-A/VEGF receptor 2 (VEGFR2) signaling increases CREB activation and prevents OGD-mediated death of both neurons and cerebral vascular endothelial cells [310–311]. These protective effects are attenuated by the inhibition of either CREB or ERK signaling [310–311]. Activation of CREB is also required for adult

neurogenesis in the DG region. Zhu *et al* [312] found that the number of newborn neurons in the DG region is increased following focal cerebral ischemia in adult rats, accompanied by an increase in CREB phosphorylation. Expression of dominant-negative (DN) CREB mutants, CREB-S133A or -R287L, abolishes CREB activation and the survival of precursor cells that ultimately differentiate into new neurons in adult rats [312]. Conversely, the expression of active CREB, VP16-CREB, greatly increases the number of newborn neurons within the DG region [312].

CREB controls the expression of numerous genes including the immediate-early genes *c-fos* and *zif/268*, as well as genes encoding the K^+ channel, CaMKII α , Bcl-2, BDNF, antioxidant enzymes and HSP70 [290]. *c-fos* induces the synthesis of neurotrophic factors by increasing AP-1 binding activity, which promotes neuronal survival and neurogenesis to mitigate ischemic injury [290] (see discussion in section 3.1.1). The expression of the anti-apoptotic Bcl-2 proteins is increased following both I/R and ischemic preconditioning in a CREB-dependent manner [293, 295, 302, 307]. Accordingly, inhibition of CaMK II–IV or attenuation of CRE transcription suppresses Bcl-2 expression and exacerbates neuronal damage [295, 302].

Ischemia also induces the expression of diverse stress proteins including HSP27 (see discussion in sections 3.1.2 and 3.1.3), HSP70, HSP72, HSP90 and HSP94 [313–316]. HSP proteins possess chaperone activities that prevent protein aggregation and promote protein re-folding and play a critical role in cells adapting to stress [314]. In particular, HSP70 has been shown to play a neuroprotective role [316]. HSP70 has been found to inhibit NF κ B-mediated inflammatory gene expression by preventing IKK-mediated I κ B phosphorylation [317]. Moreover, HSP70 may directly block AIF translocation [318–319] and reduce cytochrome c release [318, 320]. These studies have led to a notion that increased HSP protein expression will be neuroprotective. Indeed, transgenic mice overexpressing HSP70 display both a reduction in cerebral infarct size and an improvement in neurological deficits following either hypoxia/ischemia or MCAO-induced brain injury [317–318]. In addition, p38-dependent limb ischemic tolerance requires HSP70 up-regulation [321]. In agreement with the neuroprotective role of HSP70, HSP70^{−/−} mice display significantly increased cerebral infarct volume following focal cerebral ischemia [322].

4.2 Forkhead box O (FoxO) transcription factor

The FoxO family of transcription factors consists of four members in mammals, including FoxO1, FoxO3a, FoxO4 and FoxO6 [323]. The transcriptional activity of FoxO proteins is controlled by nuclear-cytoplasmic trafficking. The shuttling of FoxO proteins is regulated by an array of post-translational control mechanisms, including phosphorylation, acetylation, deacetylation and proteolytic degradation [323–325]. Upon growth factor deprivation, oxidative stress, or NMDA-R activation, FoxO1 is dephosphorylated at Ser256, resulting in its translocation to the nucleus. Ras-Ral-JNK signaling can also trigger FoxO translocation to the nucleus through C-terminal phosphorylation of FoxO [323–328]. Additionally, in response to oxidative stress, MST1 kinase-mediated phosphorylation of FoxO1 at Ser212 (Ser207 in FoxO3a) facilitates nuclear translocation of FoxO [285, 329]. Once located in the nucleus, FoxO proteins become active and regulate gene expression. The transcriptional activity of nuclear FoxO is enhanced by p300/CBP-mediated acetylation and is inactivated by SIRT1 (a mammal homolog of the yeast Sir2 deacetylase)-mediated deacetylation [323–325, 330].

The export of FoxO proteins from the nucleus is driven by sequential phosphorylation events [323–325, 331]. Several signal transduction cascades regulate the cytoplasmic translocation and inactivation of FoxO, including PI3K/AKT, serum and glucocorticoid-regulated kinase (SGK), casein kinase 1 (CK1), and dual-specificity tyrosine

phosphorylation regulated kinase (DYRK) [324]. In contrast with its ability to activate FoxO via Ral GTPase-JNK signaling [323–325, 327–328], Ras has been reported to inhibit FoxO by activating the PI3K/AKT signaling [332]. FoxO proteins that relocate to the cytosol following insulin or growth factor stimulation are often subjected to ubiquitination-dependent proteasomal degradation [323]. Both PI3K/AKT and ERK signaling have been reported to regulate FoxO protein turnover through phosphorylation [323, 333–334]. For example, AKT-phosphorylated cytosolic FoxO1 (Ser256) creates a binding site for the Skp2 E3 ubiquitin ligase [334]. ERK phosphorylates FoxO3a at Ser 294, 344 and 425, resulting in FoxO3a degradation through MDM2-dependent ubiquitination [333].

FoxO-mediated transcription plays critical roles in cell cycle regulation, defense against oxidative stress, DNA repair, life span control and apoptosis [325]. FoxO-mediated cell cycle arrest involves upregulation of p27^{kip1}, cyclin D and the tumor suppressor protein, pRb [325]. FoxO also regulates the expression of antioxidant enzymes to confer cellular oxidative stress resistance [325] (see discussion in section 3.2). In addition, FoxO signaling regulates the expression of a variety of pro-apoptotic proteins, including FasL, Trail, Bim, and Bcl-6 (Bcl_{XL} promoter repressor) [325]. Following I/R, FoxO activation within the vulnerable hippocampal CA1 region leads to neuronal death, accompanied by enhanced FoxO DNA-binding activity and the upregulation of FoxO-target genes such as FasL and Bim [335–336]. Increases in FoxO signaling are normally accompanied by the inactivation of AKT [335–336], suggesting that AKT plays a critical role in regulating FoxO function. Indeed, application of the tyrosine phosphatase inhibitor, vanadate, elicits neuroprotection against I/R-induced cerebral injury in part by activation of PI3K/AKT signaling and subsequent FoxO inactivation, leading to reduced FasL and Bim expression as well as reduced caspase-3 activation [335, 337]. In addition, treatment with the PTEN inhibitor, bisperoxovanadium, prevents neuronal apoptosis via downregulation of the FoxO3a-Bim axis in response to hypoxic/ischemic brain injury [338]. Furthermore, I/R promote calpain-mediated activation of the calcineurin (CaN) phosphatase in vulnerable neurons. CaN dephosphorylates both AKT and FoxO proteins, and enhances FoxO nuclear translocation [336]. As expected, treatment with a CaN inhibitor attenuates I/R-mediated FasL expression and neuronal death [336]. Moreover, Miawaki *et al* [249] found that ischemic insult increases expression and activation of the AKT inhibitor, CTMP, in vulnerable hippocampal neurons. CTMP eliminates AKT kinase activity, resulting in FoxO (Ser256) dephosphorylation and neuronal apoptosis [249]. Accordingly, CTMP silencing attenuates CTMP-mediated neuronal death following ischemia [249]. Preconditioning has also been shown to disrupt the CTMP-AKT complex, increasing hippocampal neuron survival following global ischemia [249]. Taken together, these studies suggest that targeting FoxO proteins is a viable strategy for promoting ischemic neuroprotection.

4.3 Hypoxia inducible factor 1 (HIF1) transcription factor

HIF1 (comprised of a heterodimer of HIF1 α and HIF1 β subunits) is a transcription factor that is strongly induced by hypoxia [339]. In normoxic cells, HIF1 α and HIF1 β are bound to HSP90, subjected to hydroxylation and subsequent ubiquitination-mediated degradation. Hypoxia leads to the phosphorylation of the HIF1 α and HIF1 β subunits, which increases their stabilization and promotes their dimerization and nuclear translocation [339]. Once in the nucleus, HIF1 binds hypoxia-response elements (HRE) to regulate a diverse array of hypoxia-responsive genes [339–340]. Growth factors such as IGF-1 and neurotrophins facilitate HIF1 activation, and both ERK1/2 and PI3K/AKT signaling pathways have been implicated in the regulation of HIF1 activation [341–345]. Ras family GTPases are established HIF1 regulators through their control of both ERK1/2 and PI3K/AKT signaling cascades [346–350]. For example, H-Ras/ERK signaling is required for HIF1-dependent VEGF expression, while both H- and K-Ras promote PI3K/AKT signaling that results in

HIF1 activation [346–350]. PI3K/AKT signaling also activates the Rheb GTPase, which in turn triggers mTORC1 and results in HIF1 activation [351–353]. To date, over 100 target genes have been identified for HIF1, including both protective and detrimental effectors [339–340]. Thus, the effect of HIF1 activation during hypoxia/ischemia is complex and is capable of promoting both cell survival and death [339–340].

In addition to hypoxia, Bergeron *et al* [354] found that permanent MCAO enhances HIF1 α expression within the peri-infarct area, which is correlated with increased HIF1 transcriptional activity and HIF1-dependent gene expression. However, whether HIF1 activation is protective or deleterious appears to depend upon the duration and magnitude of HIF1 activation. Acute or excessive HIF1 activation promotes cerebral injury [339]. The harmful effects of HIF1 activation result from its ability to induce apoptotic signaling pathways, inflammatory reactions, and BBB dysfunction [340].

Global ischemia-hypertension markedly increases HIF1 α expression within the hippocampus and cortex, accompanied by elevated levels of p53, caspases-3 and -9, and a massive loss of hippocampal neurons via apoptosis [355]. Moreover, HIF1 regulates caspase-3 gene expression, which is augmented by cerebral ischemia [356]. Global ischemia has also been found to increase expression of the pro-apoptotic Bcl-2 family protein, Bcl-2/adenovirus E1B interacting protein 3 (BNIP3), especially within the vulnerable hippocampal CA1 region in a HIF1-dependent manner [357]. In addition, Ras/MEK/ERK/HIF1 signaling leads to BNIP3 upregulation, contributing to NO-induced cell death [346]. Following MCAO, HIF1 increases iNOS in the ischemic core and hippocampus [358] and enhances the expression of inflammatory mediators MMP2 and MMP9 [359]. HIF1 is also responsible for hypoxia-induced expression of monocyte chemoattractant protein-1 (MCP-1) and MCP-5 [360]. Furthermore, severe hypoxia and/or cerebral ischemia trigger HIF1-mediated VEGF expression. Excessive VEGF production induces BBB leakage, resulting in water influx and vasogenic edema [361]. HIF1 inhibition attenuates the increases in VEGF, MMP-2 and MMP-9 levels following hypoxia/ischemia, leading to a reduction in infarct volume and brain edema [361–362]. Conversely, application of the HIF1 activator, dimethylxylglycine (DMOG), increases BBB permeability and brain edema [361]. Deletion of HIF1 α in the hippocampus and cortex reduces global I/R-induced neuronal death, which in part appears to be mediated by the down-regulation of apoptotic genes [363]. These results are consistent with a role for HIF1 α as a regulator of pro-apoptotic signaling.

The notion that HIF1 plays a predominantly neurotoxic role is challenged by numerous studies demonstrating that HIF1 plays critical roles in neural development and neuroprotection. Tomita *et al* [364] found that a conditional knockout of HIF1 α within the central nervous system leads to hydrocephalus, accompanied by loss of neurons (via apoptosis) and an impairment of spatial memory, which is restored by exogenous expression of HIF1 α in the embryo, supporting a critical role of HIF1 α in neuronal development and neuroprotection [364]. Conditional neuron-specific knockdown of HIF1 α has also been found to increase neural death and brain damage induced by either transient MCAO [365] or hypoxia/ischemia [366]. Administration of HIF1 specific activators reduces infarct volume in control mice; however, these compounds fail to protect HIF1 α mutant mice against transient MCAO [362]. The neuroprotective effects of HIF1 appear to require prolonged activation, which upregulates the expression of an array of protective genes including VEGF and VEGFR2; Epo and the Epo receptor (EpoR); and glucose transporter-1 (GLUT-1) among others [367–371]. The upregulation of these genes is seen within resistant brain regions where mild HIF1 activation is seen for up to 10 days following stroke [365] and is positively correlated with a reduction in tissue damage [367–371].

Similar mechanisms also contribute to the neuroprotection afforded by ischemic or hypoxic preconditioning [367–371], and antioxidants [such as vitamin E and deferoxamine (DFO)] [372–374]. Hypoxic preconditioning [342] or transient global ischemia [341] promotes IGF-1 expression within the cerebral cortex, which in turn, induces modest but sustained HIF1 activation, resulting in Epo and GULT-1 expression and cerebral cell survival. HIF1 activation appears to be mediated at least in part by ERK1/2 or PI3K/AKT pathway signaling. Treatment with either MEK1/2 or PI3K inhibitors significantly reduces HIF1 α activation, resulting in lower VEGF expression and reduced neuronal survival following hypoxia/ischemia [343–344]. Furthermore, hypoxic preconditioning results in a resistance of embryonic stem cell derived neural progenitor cells (ES-NPCs) to subsequent lethal hypoxia, in part through the upregulation of Epo, EpoR, Bcl-2, neurofilament (NF), and synaptophysin in a HIF1 α -dependent fashion [375]. Transplantation of these primed ES-NPCs induces neuronal differentiation in the ischemic brain [375]. In a similar fashion, transplantation of neural stem cells preconditioned by a HIF1-specific activator, DFO, significantly reduces the release of inflammatory mediators and the infarct volume following transient MCAO [376]. DFO increases HIF1 α activity and the HIF1-dependent expression of protective gene products including VEGF, BDNF and CXCR4 in neural stem cells [376].

5. Conclusions

A tremendous research effort has been directed toward understanding neuroprotective signaling. These studies have provided critical mechanistic insights into ischemic cerebral injury and identified a variety of molecular targets for the subsequent development of novel stroke therapies. Indeed, over 100 neuroprotective agents have resulted from these efforts and been subjected to clinical trials [22]. Unfortunately, many of these agents have failed to achieve the promising results first seen in animal models, often displaying modest tissue protection and/or undesired side effects [22]. In part, this may arise from the design of the initial animal studies, in which the anticipation of injury allowed either pretreatment or treatment rapidly following I/R. However, this narrow therapeutic window, which permitted efficient neuroprotection in animal models, is clinically difficult to achieve [22]. Therefore, a revision of the design of pre-clinical animal studies appears necessary to evaluate the efficacy of neuroprotective therapy within a wider therapeutic window. A second critical issue is that compounds designed to target a selective molecular pathway often have unintended cellular effects, including the off-target disruption of neuroprotective signaling cascades. For example, the JNK inhibitor SP600125 has recently been shown to inhibit a number of protein kinases in addition to JNK, resulting in cellular toxicity [196]. Thus, the development of more specific inhibitors is a high priority for the field.

Despite recent studies suggesting that neuroprotective therapy for victims of stroke may be practically achievable, many challenges remain before comprehensive therapy for stroke is realized [22]. In part, the challenge to developing comprehensive therapies arises from the realization that the same complex cellular signaling networks that play important neuroprotective roles also contribute to neurotoxicity following I/R. In addition, different cerebral cell types employ distinct survival mechanisms and display both spatially and temporally distinct responses to I/R. Therefore, carefully orchestrating the activation/inhibition of these complex signaling pathways is critical to the development of effective treatments. This demands a more sophisticated understanding of the dynamics of the signaling networks that are activated following I/R. The use of systems biology approaches may be required to provide this critical insight. The use of combination therapies, rather than targeting any single signaling pathway, may also be needed for more reliable and efficacious treatment. This might allow spatiotemporal control of both protective and injurious signaling pathways, and thus provide improved protection against I/R-injury. Indeed, the combination of tissue-type plasminogen activator (tPA), the only Food and Drug Administration (FDA)-

approved drug for thrombolytic therapy of acute ischemia, with neuroprotective therapy has been proven to deliver more effective stroke treatment, while also reducing the secondary neurotoxicity generated by tPA-treatment [2–3, 22]. In conclusion, it is clear that a greater understanding of cellular neuroprotective signaling mechanisms will continue to be critical to the development of innovative and effective therapeutic strategies for stroke.

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List of Abbreviation

A2A	adenosine 2A receptor
AIF	apoptosis-inducing factor
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASK1	apoptosis-signal regulating kinase 1
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
CaMK	calcium/calmodulin-dependent protein kinase
cAMP	cyclic AMP
CREB	cAMP response element binding protein
DG	dentate gyrus
EGF	epidermal growth factor
Epo	erythropoietin
ERK	extracellular-signal regulated kinase
ER	estrogen receptor
FGF	fibroblast growth factor
FoxO	forkhead box O transcription factor
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GluR	glutamate family receptor
GPCR	heterotrimeric G protein coupled receptor
HIF	hypoxia-inducible factor
HSP	heat shock protein
IAP	inhibitor of apoptosis protein
IGF	insulin-like growth factor
IκB	inhibitor of NF κ B kinase
IKK	I κ B kinase

IL	interleukin
iNOS	inducible nitric oxide synthase
I/R	ischemia and reperfusion
JNK	c-Jun N-terminal kinase
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
MEK1/2	MAPK kinase 1 and 2
MK2	MAPK-activated protein kinase 2 (MAPKAPK2)
MNK1	MAPK-interacting kinase 1
MSK1/2	mitogen- and stress-activated protein kinase 1 and 2
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
NFκB	nuclear factor kappa B
NGF	nerve growth factor
NMDA	<i>N</i> -methyl-D-aspartate
NTR	neurotrophin receptor
NTs	neurotrophins
PACAP	pituitary adenylate cyclase activating peptide
PCD	programmed cell death
PDK	phosphoinositide-dependent protein kinase
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B (AKT)
PSD	postsynaptic density
RSK	ribosomal S6 kinase
RTK	receptor tyrosine kinase
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
SFK	Src family non-receptor tyrosine kinase
Sos	son-of-sevenless homolog
TNFα	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

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Figure 1. Neuroprotective roles of Ras family GTPases in stroke: mitogen-activated protein kinase-mediated signaling pathways and regulation of transcription factors

Right Panel, during the acute phase, ischemia/reperfusion (I/R)-mediated induction of excessive Ca^{2+} influx and/or the generation of free radical species triggers Ras-dependent activation of mitogen-activated protein kinase (MAPK) cascades including ERK1/2, p38 and JNK. MAPK signaling induces mitochondrial dysfunction and apoptotic signaling, and enhances pro-apoptotic transcriptional events mediated by p53, c-Jun, FoxO and HIF1 among others. MAPKs also trigger the generation of free radical species, inflammatory reactions and blood brain barrier (BBB) dysfunction to amplify or maintain injury. *Left Panel*, sustained or long-term MAPK activation following I/R stimulates diverse neuroprotective pathways to moderate injury severity. Both intrinsic stimuli such as Ca^{2+} and free radical species, and extrinsic signals such as growth factors, neurotransmitters, neuropeptides, hormones and TNFR ligands derived from the affected cerebral cells or the local environment, are integrated to promote cerebral defenses. Ras-family GTPases serve as intrinsic molecular switches to control the integration of these varied stimuli and direct the activation of a diverse collection of signaling networks. The regulation of transcription factors are particularly critical, either promoting pro-survival or inhibiting pro-apoptotic gene expression to provide protection against stroke and to aid in subsequent cerebral recovery. CaMKs, calcium/calmodulin-dependent protein kinase; cyto c, cytochrome c; GAPs, GTPase-activating proteins; GEFs, guanine nucleotide exchange factors; NTs, neurotrophins; OGD, oxygen-glucose deprivation; SFKs, Src family non-receptor tyrosine kinases.

Figure 2. AKT-mediated neuroprotective signaling pathways

Right Panel, Ras-dependent RASSF/MST signaling promotes cellular apoptosis. *Left Panel*, Ischemia/reperfusion (I/R) induce Ras family GTPase-dependent activation of PI3K/AKT signaling, which promotes pro-survival signaling cascades to reduce cerebral injury. H2B, histone 2B; IAP, inhibitor of apoptosis protein; OGD, oxygen-glucose deprivation; SFKs, Src family non-receptor tyrosine kinases.