

# The Science of Stroke: Mechanisms in Search of Treatments

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This review focuses on mechanisms and emerging concepts that drive the science of stroke in a therapeutic direction. Once considered exclusively a disorder of blood vessels, growing evidence has led to the realization that the biological processes underlying stroke are driven by the interaction of neurons, glia, vascular cells, and matrix components, which actively participate in mechanisms of tissue injury and repair. As new targets are identified, new opportunities emerge that build on an appreciation of acute cellular events acting in a broader context of ongoing destructive, protective, and reparative processes. The burden of disease is great, and its magnitude widens as a role for blood vessels and stroke in vascular and nonvascular dementias becomes more clearly established. This review then poses a number of fundamental questions, the answers to which may generate new directions for research and possibly new treatments that could reduce the impact of this enormous economic and societal burden.

## Introduction

Few neurological conditions are as complex and devastating as stroke, the second leading cause of death worldwide. Also called a brain attack, victims may suddenly experience paralysis, impaired speech, or loss of vision due to interruption of blood flow (ischemia) caused by thrombosis or embolism. Less frequently (<15%), strokes are caused by hemorrhage or cardiac arrest. On average, strokes in the USA strike once every 40 s and cause death every 4 min, with an estimated 41.6% death rate in 2007 (Lloyd-Jones et al., 2009). With an aging population, the absolute numbers are likely to rise. Among survivors, work capacity is compromised in 70% of victims, and 30% need assistance with self-care. Hence, the disease burden is great. The estimated cost for stroke is 73.7 billion dollars in 2010 (USA) and projected to be 1.52 trillion dollars in 2050 (in 2005 dollars) (Lloyd-Jones et al., 2009). No racial or ethnic groups are spared, and the problem is global. For example, in the Russian Federation and China, the estimated death rates per 100,000 population are five to ten times higher than in the USA (Lloyd-Jones et al., 2009). Hence, stroke is an affliction of mankind.

For the above considerations and more, there is a compelling need to accelerate efforts to interrogate the stroke process and define the links that exist with other conditions such as vascular and neurodegenerative dementia. It is also crucial to expand the narrow repertoire of therapeutic opportunities for these devastating conditions. To accomplish this, novel approaches are required that expand upon our evolving mechanistic understanding of the fundamentals of cell survival and death processes as well as tissue repair. The future depends upon how successful we are in deciphering these mechanisms and bringing clarity to the complex interactions between the multiplicity of cell and tissue types within brain (Lo et al., 2003). Armed with this knowledge and its successful therapeutic application, the field of stroke could be transformed.

In this spirit then, this brief review addresses selected issues fundamental to the science of ischemic stroke and vascular dementia. It begins with posing questions about stroke risk factors followed by a discussion of key cell and tissue mechanisms that render brain susceptible as well as tolerant to ischemic injury, including those promoting tissue protection and repair. The review ends by highlighting promising treatment strategies, inspired by these endogenous mechanisms, which present the opportunity to open new avenues in stroke therapy.

## Stroke Risk Factors and Triggers

A stroke risk factor is a characteristic of an individual that increases the risk for stroke compared to someone without that characteristic (Hankey, 2006). Some risk factors cannot be modified, such as a family history of cerebrovascular diseases, older age, male sex, and Hispanic or Black race (Allen and Bayraktutan, 2008; Hankey, 2006). Other risk factors are modifiable, and their correction reduces the chance of having a stroke (Table 1). These factors, which often coexist, have been estimated to account for 60%–80% of stroke risk in the general population (Allen and Bayraktutan, 2008; Hankey, 2006). Genome-wide association studies are increasingly being employed to identify susceptibility genes for stroke (Hegele and Dichgans, 2010). Although several loci have been identified, the need for independent replication and the modest effect sizes have precluded the full assessment of the clinical relevance of these findings (Hegele and Dichgans, 2010).

### How Do Risk Factors Increase the Propensity to Stroke?

Risk factors have profound effects on the structure and function of blood vessels and on their interface with circulating blood. Many of the established risk factors alter vascular structure by promoting atherosclerosis and stiffening of arteries and by inducing narrowing, thickening, and tortuosity of arterioles and capillaries (Allen and Bayraktutan, 2008; Iadecola and Davisson, 2008). In brain, these morphological changes are often

**Table 1. Major Modifiable Risk Factors for Ischemic Stroke**

Classification	Risk Factor	References
Causal <sup>a</sup>	hypertension	Lawes et al., 2004a
	diabetes	Lawes et al., 2004b
	hypercholesterolemia	Amarenco et al., 2006
	cigarette smoking	Bonita et al., 1999
	atrial fibrillation	Hart et al., 1999
	valvular heart disease	Kizer et al., 2005
	ischemic cardiomyopathy	Loh et al., 1997
	carotid stenosis	Rothwell et al., 2003
	Probable <sup>b</sup>	hyperhomocysteinemia
increased inflammatory markers (WBC, CRP, infection)		Di Napoli et al., 2005
elevated plasma fibrinogen		Rothwell et al., 2004
dyslipidemia		Holme et al., 2009
patent foramen ovale		Nedeltchev et al., 2008
obstructive sleep apnea		Culebras, 2009
body mass index		Hu et al., 2007
lack of exercise		Hu et al., 2005
low fruit and vegetable diet		Dauchet et al., 2005
psychosocial stress		Simons et al., 1998

<sup>a</sup>Factors demonstrated in randomized clinical trials to reduce stroke risk if corrected, or for which a strong, consistent, and independent association with stroke has been established (Hankey, 2006).

<sup>b</sup>Factors for which the association with stroke is less firm and/or no causative role has been established. WBC, white blood cells; CRP, C-reactive protein.

associated with reductions in resting cerebral blood flow (CBF) and marked alterations in CBF regulation. Thus, aging, hypertension, diabetes, and hypercholesterolemia impair vital adaptive mechanisms that assure that the brain is adequately perfused (Arrick et al., 2007; Iadecola and Davisson, 2008; Iadecola et al., 2009; Kitayama et al., 2007). The ability of the endothelium to regulate microvascular flow is compromised, while the increase in blood flow evoked by neural activity is suppressed, resulting in a mismatch between the brain's energy supply and demand (Arrick et al., 2007; Iadecola and Davisson, 2008; Iadecola et al., 2009; Zou et al., 2004a).

Some risk factors, like hypertension and diabetes, impair protective vascular mechanisms that keep CBF stable during reductions in blood pressure (cerebrovascular autoregulation), facilitating the occurrence of ischemia if intravascular pressure drops (Imminck et al., 2004; Kim et al., 2008). These vascular alterations increase the brain's vulnerability to ischemia after arterial occlusion because they compromise the development of collateral flow arising from adjacent nonischemic vascular territories, which is vital to the survival of the ischemic perinfarct zone (see *Parenchymal Failure* section). In addition to their vascular effects, some risk factors, like aging and diabetes, may enhance the intrinsic susceptibility of brain cells to injury, amplifying the tissue damage produced by ischemia (Biessels et al., 2002), but the biological bases of this effect are not well understood. Little is known about the interaction among the

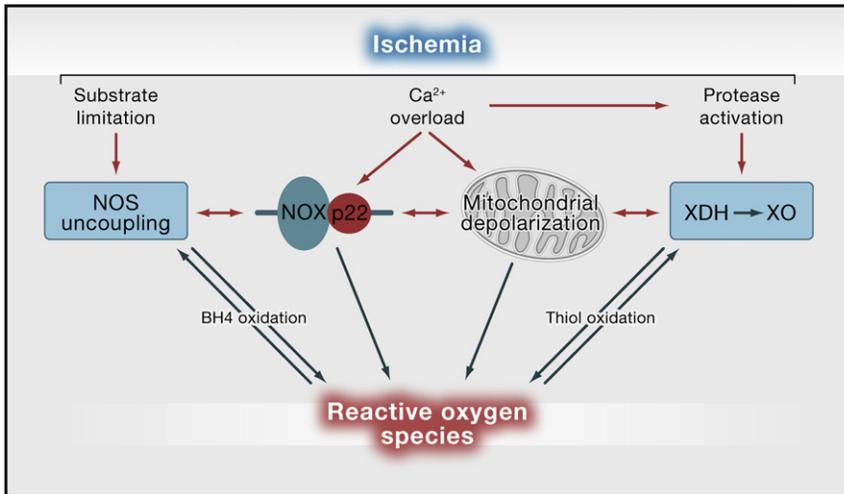
different stroke risk factors and whether their vascular effects are additive or synergistic. Furthermore, the relative contribution of parenchymal and vascular factors to stroke risk remains to be determined.

#### **How Do Risk Factors Alter Cerebral Blood Vessels?**

Many cardiovascular risk factors increase production of reactive oxygen species (ROS) and promote inflammation in systemic and cerebral blood vessels. The predominant vascular sources of ROS are the superoxide-producing enzyme NADPH oxidase, xanthine oxidase, mitochondrial enzymes, and uncoupling of nitric oxide synthase (NOS), a state in which this enzyme generates superoxide instead of NO (Faraci, 2006) (Figure 1). Many of the damaging effects of oxidative stress on blood vessels are related to the biological inactivation of NO by the free radical superoxide, which reduces NO bioavailability and prevents its beneficial effects (Pacher et al., 2007; Schulz et al., 2008). Thus, loss of the vasoregulatory effects of endothelial NO leads to vasoconstriction and reduced NO-dependent vascular responses, with a negative impact on the regulation of microvascular flow (Schulz et al., 2008). Loss of the antiaggregant, anti-proliferative, and anti-cell adhesion effects of NO promotes platelet aggregation, leukocyte adhesion to endothelial cells, and smooth muscle proliferation, key steps in vascular inflammation (Pepine, 2009; Zou et al., 2004b). In addition, ROS can directly promote inflammation by increasing blood-brain barrier (BBB) permeability through upregulation of vascular endothelial growth factor (VEGF) and by inducing the expression of cytokines, matrix remodeling enzymes, including metalloproteases, and proinflammatory genes through NF $\kappa$ b activation (Marchesi et al., 2008). One such NF $\kappa$ b-dependent gene product, inducible nitric oxide synthase, produces large amounts of NO, which alter vascular structure and function through nitration and nitrosylation of critical proteins (Gunnnett et al., 2005; Guzik et al., 2003; Lima et al., 2010). Peroxynitrite, a potent nitrating agent produced by the reaction of NO with superoxide, alters vasomotor reactivity by inactivating critical endothelial and smooth muscle enzymes and by activating the DNA repair enzyme poly-ADP-ribose polymerase (PARP), which leads to ATP depletion and vascular ion channel dysfunction (Pacher et al., 2007). Whereas ROS may set the stage for inflammation, vascular inflammation, in turn, leads to ROS production, creating a vicious circle that enhances vascular damage. Activation of the plasminogen system by oxidative stress and inflammation promotes matrix remodeling, smooth muscle cell migration, and intimal hyperplasia (Nicholl et al., 2006), factors that promote atherosclerosis and alterations in vascular structure. Thus, oxidative stress and vascular inflammation are major pathways through which risk factors exert their deleterious effects on blood vessels. However, it remains to be determined how individual risk factors trigger the activation of one or both of these processes. This is a critical question for targeting preventive strategies in patients with specific risk factors.

#### **Why Now? Role of Stroke Triggers**

While vascular risk factors increase the likelihood of stroke in affected individuals, precipitating factors that act as "stroke triggers" must also be postulated. In some patients, the specific event responsible for the arterial occlusion can be identified, e.g., neck trauma leading to dissection and occlusion of the



**Figure 1. Major Sources of ROS in Brain and Blood Vessels**

Stroke risk factors and ischemia lead to the activation of several ROS-generating enzymatic systems. In ischemia, the increase in cytosolic  $Ca^{2+}$  activates the superoxide-producing enzyme NADPH oxidase (NOX), through PKC and NO derived from neuronal NOS (Brennan et al., 2009; Girouard et al., 2009). Mitochondria become depolarized by the  $Ca^{2+}$  overload (Nicholls, 2008) and, possibly, by ROS derived from NADPH located near mitochondria (Girouard et al., 2009) producing large amounts of superoxide. The  $Ca^{2+}$ -induced activation of proteases and/or the pro-oxidant environment convert xanthine dehydrogenase (XDH) to xanthine oxidase (XO) (Abramov et al., 2007), a superoxide-generating enzyme involved in purine degradation. Finally, the substrate deprivation induced by ischemia and the oxidative inactivation of essential cofactors like tetrahydrobiopterin (BH4) uncouple L-arginine oxidation from NO formation by NOS, resulting in superoxide

production (NOS uncoupling) (Förstermann, 2010). Other potential sources of ROS include enzymes for arachidonic acid or catecholamine metabolism, but their participation in stroke-induced oxidative stress remains in question (Adibhatla and Hatcher, 2010; Kunz et al., 2007a).

internal carotid artery (Kim and Schulman, 2009). But in most instances the factors precipitating the ischemic event cannot be established. Although systemic infections, pregnancy and puerperium, use of illicit drugs, and mental stress often trigger a stroke (Elkind, 2007), precisely how these factors exert their effect remains unclear. Exacerbation of vascular inflammation and activation of the coagulation cascade are likely to play a role (Welsh et al., 2009). For example, the added vascular dysfunction and blood clotting abnormalities, superimposed on those induced by stroke risk factors, could precipitate vascular occlusion or hemodynamic insufficiency. This view is supported by the fact that acute stroke often occurs in the setting of increased circulating leukocytes and elevated plasma markers of systemic inflammation and vascular activation, which also predict a poor outcome (Elkind, 2007; Welsh et al., 2009). Thus, our understanding of stroke triggers is limited, and mechanistic studies addressing these issues would be valuable in the identification of high-risk cases.

### Parenchymal Failure: Why and How Does the Brain Die during Ischemia?

Because of its high intrinsic metabolic activity and large concentrations of the neurotransmitter-excitotoxin glutamate (Choi, 1992), the brain is especially vulnerable to ischemic insult. This can develop either as a consequence of thrombosis in situ or following embolic occlusion of a cerebral blood vessel, the latter usually arising from the heart or atherosclerotic plaques in the carotid artery and aortic arch. Although neurological dysfunction occurs within seconds to minutes of vessel occlusion, the evolution of ischemic injury and cell death continues in stages for minutes, hours, and even days, depending, in part, upon the vulnerability of the particular brain region, its cellular constituents, and the extent of residual perfusion.

The responses of blood vessels and their perfusion are important to the outcome as well. For example, a deficiency of vasodilating molecules such as endothelial NO enlarges stroke size (Huang et al., 1996). Early restoration of blood flow by clot

lysis and reperfusion decreases ischemic injury, and this may be achievable by giving recombinant tissue plasminogen activator (tPA), the only FDA-approved treatment for reestablishing flow and salvaging brain tissue. However, tPA is used in <10% of patients and even less frequently after 3 hr because of the risk of hemorrhage into ischemic tissue (NINDS tPA Study Group, 1995). Moreover, tPA may have other unintended risks (Kaur et al., 2004), such as injury to the blood-brain barrier by activating matrix metalloproteinases (Wang et al., 2003) or excitotoxicity in experimental models (Nicole et al., 2001). Combination therapies that ameliorate these effects may extend tPA's therapeutic window while mitigating the untoward effects of reperfusion and plasminogen activation (Liu et al., 2004; Cheng et al., 2006; Murata et al., 2008). Theoretically, that could prolong tissue viability for hours or even longer, depending upon the rapid delivery of drug to compromised brain (Ginsberg, 2008).

Whether or not vulnerable brain tissue can be rescued or protected after a protracted time period in the absence of partial or complete restoration of blood flow is an unanswered question. In all likelihood, areas of vulnerable brain do remain viable for hours after vessel occlusion, at least in some instances. For example, the hemiparesis that follows middle cerebral artery occlusion in the baboon was reversible in 73% of animals with reperfusion at 2–4 hr, compared to 33% and 17% in animals with reperfusion at 8 and 16–24 hr, respectively (Marcoux et al., 1982; Crowell et al., 1981; Jones et al., 1981). The challenge then is to restore adequate blood flow quickly after vessel occlusion and to protect viable tissue from unleashed mechanisms that lead to cell and tissue demise.

### Salvageable versus Nonsalvageable Tissue: Salvageable Tissue Is the Target for Therapy

Tissue distal to an occluded blood vessel is often heterogeneous, meta-stable, and differentially susceptible to ischemic injury. This meta-stable zone, termed the ischemic penumbra or perinfarct zone (Astrup et al., 1981), is characterized by significantly depressed tissue perfusion that is barely sufficient to

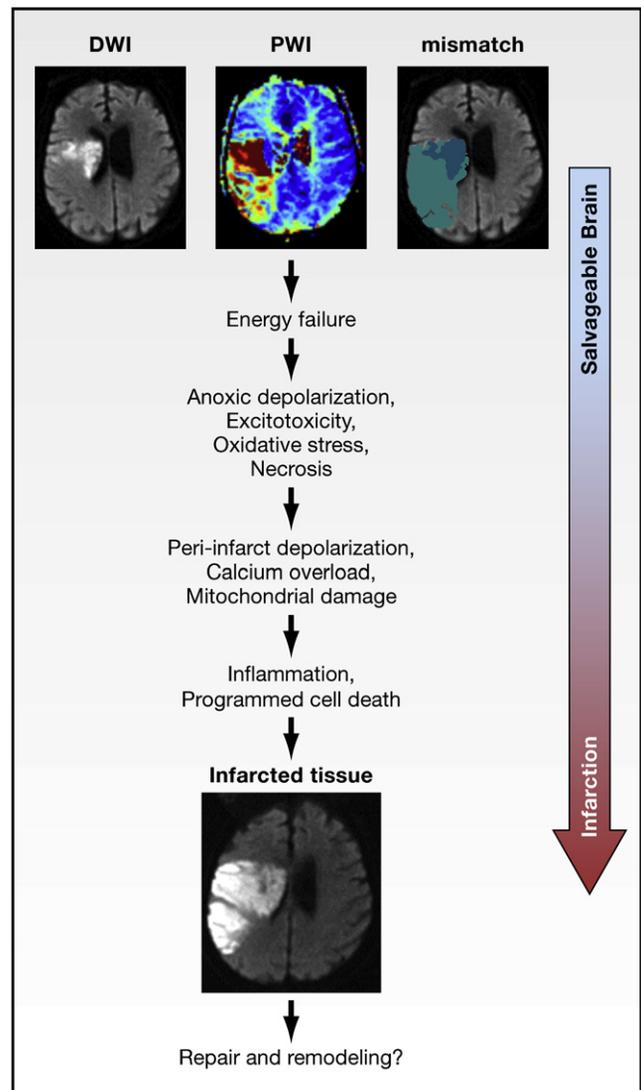
support basal ATP levels and oxygen metabolism as well as normal ionic gradients in the presence of electrical silence and suppressed protein synthesis. The ischemic penumbra then denotes an “at risk” region that is functionally impaired but potentially salvageable. However, unless perfusion is improved or cells made relatively more resistant to injury, the tissue at risk dies within a few hours (Lo, 2008a, 2008b). The ischemic core by contrast refers to the irreversibly damaged tissue distal to an occluded blood vessel, characterized by <20% of baseline blood flow levels, depleted ATP stores, and irreversible failure of energy metabolism (Lo, 2008b). With time, the infarct core expands into the ischemic penumbra, and therapeutic opportunity is lost. Therefore, detecting a penumbra in patients can help to identify those who might benefit most from acute treatments that restore blood flow (thrombolysis or endovascular clot retrieval) or treatments for the future that render viable brain more resistant to ischemic injury. For this purpose, magnetic resonance imaging methods such as perfusion-weighted and diffusion-weighted imaging are most often used. Perfusion-weighted imaging measures the spatial extent of blood flow compromise, whereas diffusion-weighted imaging is thought to detect the region of attenuated diffusion of water, a putative surrogate marker for severely damaged brain tissue. The difference is thought to reflect the ischemic penumbra (Figure 2) (Ebinger et al., 2009). Although the predictive value of this method is still debated, perfusion-diffusion mismatch provides a useful first attempt to define the tissue at risk.

**What Are the Prominent Mechanisms Leading to Cell and Tissue Demise?**

Within the ischemic penumbra, multiple mechanisms have been identified over the past few decades that irreversibly damage brain tissue (Figure 2). Understanding the contribution of each informs us about potential therapeutic opportunities and treatment targets. In the area of severely limited blood supply, ATP utilization continues in the presence of minimal synthesis and levels drop, leading to acidosis and loss of ionic homeostasis. As a consequence, cells swell and membranes rupture. However, ischemic cell death cannot be equated with limited ATP availability; rather, tissue death develops as a consequence of numerous ionic, biochemical, and cellular events that impose overwhelming stresses upon already compromised tissue (see for review Dirnagl et al., 1999; Lo et al., 2003).

**Excitotoxicity**

Excitotoxicity and calcium overload are major factors contributing to the early stages of ischemic cell death. The canonical pathway asserts that glutamate, the most abundant neurotransmitter, accumulates into the extracellular space as a consequence of energy and ion pump failure, as well as failure of reuptake mechanisms (Choi and Rothman, 1990). The glutamate overload leads to prolonged stimulation of AMPA and NMDA ionotropic receptor subtypes to dramatically enhance the influx of calcium, sodium, and water into neurons. Massive calcium influx activates catabolic processes mediated by proteases, lipases, and nucleases (Ankarcrona et al., 1995). In addition, activation of nNOS, PLA2, and other Ca<sup>2+</sup>-dependent enzymes leads to production of NO, arachidonic acid metabolites, and superoxide, which act as additional triggers of cell death (Dirnagl et al., 1999; Lo et al., 2003). For these and other reasons,



**Figure 2. The Ischemic Penumbra**

The ischemic penumbra represents compromised but viable brain tissue lying distal to an occluded blood vessel. The top three MR images in this figure were taken in the same patient early after occlusion of the middle cerebral artery. The images are shown in the horizontal plane passing through the lateral ventricles. “Stunned but not dead tissue” is detected by magnetic resonance imaging (MRI) techniques that assess (1) the spatial extent of the perfusion or blood flow deficit that presents as red and yellow areas on perfusion-weighted imaging (PWI, top middle image) and (2) severely damaged tissue in the ischemic core that presents as high-intensity areas on diffusion-weighted imaging (DWI, top left image) (Ebinger et al., 2009). In this patient, the perfusion deficit is larger than diffusion lesion. The top right image depicts the mismatch between core areas with a diffusion lesion (dark blue) and the larger areas with low perfusion (light blue-green). Over hours to days (see text), the core territory expands due to the complex cascades involving excitotoxicity, oxidative stress, programmed cell death mechanisms, and inflammation, as the initial trigger of energy failure ultimately progresses to infarcted tissue. The resulting infarct is shown in the image at the bottom of the figure and is about the size of the initial perfusion deficit in this untreated patient. In later stages, some patients may also partially recover, in part due to endogenous mechanisms of repair and remodeling (Chopp et al., 2007). MRI scans courtesy of G. Albers, Stanford.

oxidative phosphorylation becomes uncoupled, leading to further ATP depletion, ROS production, and release of stored  $\text{Ca}^{2+}$  from mitochondria, further accelerating a series of catastrophic events that lead to acute cell death.

Despite the validation of glutamate toxicity in the laboratory setting and its enormously positive impact on the quest for new therapeutics in neuroscience, numerous clinical trials targeting NMDA and AMPA receptors have failed to improve outcome in stroke patients (Ginsberg, 2009). Although replete with shortcomings in trial design and implementation, the failure to translate has reinforced the notion that the glutamate hypothesis may have oversimplified the complexity of the cell death process and underestimated the diversity of expressing cell types as well as the heterogeneity of glutamate responses following receptor overstimulation in stroke. Moreover, it is likely that NMDA-AMPA pathways comprise only a subset of routines disrupting ionic imbalance following glutamate receptor activation. As originally conceived, the NMDA-AMPA model did not consider the delayed and invariably fatal glutamate-independent calcium influx following oxygen-glucose deprivation or the failure of glutamate receptor blockade to halt cell death after very intense oxygen-glucose deprivation (Aarts et al., 2003). Equally importantly, it could not account in an obvious way for the death of nonneuronal cell types such as astrocytes, vascular cells, or microglia in ischemic tissue. An alternative viewpoint is that the drugs tested in clinical trials were poorly chosen, because many of them block all receptor functions (Lipton, 2007a). As a result, the dosing may have been suboptimal, due to the development of untoward side effects that prevented dose escalation to neuroprotective levels. A more suitable approach would be to use therapeutic agents that selectively target excessive channel opening (e.g., uncompetitive inhibitors with a relatively fast off-rate, such as memantine [Orgogozo et al., 2002; Möbius and Stöffler, 2002; Lipton, 2006; Kavirajan and Schneider, 2007]), thereby retaining normal receptor functions (Lipton, 2007a).

To add to the complexity, it is now known that NMDA receptor location and subunit composition differentially regulate neuronal survival or death, although the strength of the calcium signal may specify the fate of neurons best following NMDA overactivation (Stanika et al., 2009). With notable exceptions, selective enhancement of synaptic receptors (in which NR2A peptide subunits predominate over NR2B) promotes neuronal survival, whereas activating extrasynaptic receptors (in which NR2B peptide subunits predominate over NR2A) promote cell death (Chen et al., 2007), in part, by activating death-associated protein kinase 1 (Tu et al., 2010). Turning CREB-dependent signaling and BDNF expression up or down is critical for cell fate determination during NMDA receptor activation. The MAP kinase family members P38 and ERK1/2 participate, most likely by phosphorylation-dependent regulation of submembrane scaffolding proteins and NR2A and NR2B receptor subunits (Hardingham et al., 2002). Under ischemic conditions, the vulnerability to cell death is reduced when postsynaptic scaffolding proteins are modified, and PSD-95 binding is prevented (Cui et al., 2007). Moreover, successfully targeting the glutamate receptor complex early on would also diminish the opening of downstream channels, e.g., transient receptor potential channels (TRP) and acid-sensing ion channels, as well as

suppress the frequency of cortical spreading depolarizations (see below), ongoing within the ischemic penumbra. Hence, understanding and leveraging the repertoire of diverse responses and mediators at the receptor and second messenger level can only enhance possibilities for successfully targeting excitotoxicity for the treatment of ischemic injury.

#### **Calcium Dysregulation**

It is also known that increased calcium influx from glutamate receptor overactivation combined with release of  $\text{Ca}^{2+}$  from mitochondria and other stores (e.g., endoplasmic reticulum) may not fully account for the irreversible buildup of intracellular  $\text{Ca}^{2+}$  after excitotoxic stimulation. Other channels and ion pumps activated during ischemia in addition to glutamate receptors have been implicated in the  $\text{Ca}^{2+}$  accumulation. These include the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (Bano et al., 2007) hemichannels (Contreras et al., 2004), acid-sensing ion channels (Xiong et al., 2004), volume-regulated anion channels (Kimelberg et al., 2006; Simard et al., 2007), and TRP channels (Aarts and Tymianski, 2005). In particular, ASIC1a, activated by ischemia-induced acidosis, is involved in the  $\text{Ca}^{2+}$  influx and its inhibition is neuroprotective (Simon, 2006). ASICs are stimulated within the pH range commonly found in ischemic brain tissue, thus explaining the well-established link between acidosis and worsening of ischemic outcome in animals and humans. Failure of  $\text{Ca}^{2+}$  efflux mechanisms, especially the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger also contributes to the  $\text{Ca}^{2+}$  accumulation. Prostaglandin E2 EP1 receptors have a role in the failure of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger during ischemia, and their inhibition is markedly neuroprotective (Abe et al., 2009; Kawano et al., 2006). Therefore, targeting  $\text{Ca}^{2+}$  influx-efflux mechanisms may be a valuable approach to counteract  $\text{Ca}^{2+}$  dysregulation and associated injury in conjunction with novel drugs targeting glutamate receptors.

#### **Oxidative and Nitrosative Stress**

Oxidative and nitrosative stress are also powerful mediators of ischemic injury. In one scenario, the redox environment of cells modulates signal transduction cascades that tip the balance between prodeath and prosurvival pathways (Crack and Taylor, 2005). In the second scenario, ROS and perhaps reactive nitrogen species, including peroxynitrite, act directly as executors of cell death (Chan, 2001). The brain appears particularly vulnerable to radical-mediated attack because of its limited antioxidant defenses (Adibhatla and Hatcher, 2010). Until recently, it was accepted that during ischemia ROS were generated principally by mitochondria, a well-known ROS source during electron transport and oxidative phosphorylation. However, it was recently shown that NADPH oxidase generates the majority of superoxide anion produced in vivo and in vitro during NMDA receptor activation and ischemia (Brennan et al., 2009; Girouard et al., 2009). Thus, the mitochondrion does not appear to be the major source of radicals following NMDA receptor activation. However, the close proximity of NADPH oxidase to neuronal mitochondria (Girouard et al., 2009) raises the possibility that this enzyme generates “kindling” ROS that promote mitochondrial uncoupling, triggering a secondary ROS surge from mitochondria (Figure 1). These observations point to NADPH oxidase as a potentially important therapeutic target in stroke but also highlight the need for a better understanding of the interaction among the different ROS sources

and the relative contribution of vascular versus neuronal sources. In this regard, a surprising finding has been that activation of NMDA receptors increases ROS both in neurons and vascular cells (Girouard et al., 2006), suggesting a role of vascular ROS in excitotoxic brain damage. Although this observation emphasizes the close relationships between vessels and neurons (see *Protecting the Neurovascular Unit*), it has not been established how vascular oxidative stress may contribute to tissue outcome in excitotoxic brain injury.

NO and related oxidation products are key players in excitotoxicity. Reactive nitrogen species have important cellular effects, such as inhibition of key mitochondrial enzymes, facilitating mitochondrial transition pore formation, DNA damage, PARP activation, and activation of  $\text{Ca}^{2+}$ -permeable TRPM7 channels (Aarts and Tymianski, 2005; Pacher et al., 2007). As a potential second mechanism, NO modifies protein groups by covalently attaching to cysteine residues forming S-nitrosothiol derivatives. This posttranslational modification significantly impacts cell survival by altering, for example, the function of critical regulatory proteins such as caspases, metalloproteases (Gu et al., 2002), and the glycolytic enzyme GAPDH (Nakamura and Lipton, 2009). As NO has the potential to target a number of specific cysteine residues to alter protein function, the impact of S-nitrosylation is (largely) underexplored in the ischemic process.

Taken together, the experimental evidence implicating oxidative and nitrosative stress in stroke is strong. But the translation of these fundamental concepts into clinical applications has proven to be challenging. Most recently, a nitron-based radical spin trap failed in the final phase 3 clinical trial for acute ischemic stroke (Shuaib et al., 2007). And more broadly, the history of antioxidant therapies in clinical trials for neurodegeneration has been littered with many disappointments (Kamat et al., 2008). Overproduction of reactive nitrogen and oxygen species are surely damaging to brain cells. But at lower, homeostatic levels, radicals are critical signaling molecules participating in normal neuronal and vascular function (Faraci, 2006). Finding novel ways to scavenge or suppress deleterious radicals without interfering with endogenous signaling will be important to design effective therapies. As discussed above for excitotoxicity, a desirable strategy would be to develop drugs that only become activated by the pathological state intended for inhibition, i.e., during oxidative stress (Lipton, 2007b). Another approach would be to test antioxidant molecules with pleiotropic properties, such as activated protein-C (APC). APC blocks ROS generation, suppresses postischemic inflammation, and blocks apoptosis in neurons and endothelium, thereby providing both parenchymal and vascular protection (Yamaji et al., 2005; Cheng et al., 2006; Liu et al., 2004). APC as well as other pleiotropic agents with a broad spectrum of activity in experimental stroke are undergoing clinical trial testing (Ginsberg, 2009).

### **Cortical Spreading Depolarizations**

In addition to excitotoxic events at the molecular and cellular level, massive release of glutamate and ionic imbalance negatively impact the evolution of ischemic injury at the tissue level. Cortical spreading depolarization (CSD), a high energy consuming phenomenon akin to the cortical spreading depression of Leao, is triggered by high levels of extracellular glutamate

and  $\text{K}^+$  and is characterized by slowly propagating massive depolarization of neurons and astrocytes along with drastic disruption of ionic gradients (Somjen, 2001). Electrophysiological and imaging data convincingly demonstrate CSD in patients with ischemic stroke (Dohmen et al., 2008). In experimental animals, a single cortical spreading depolarization expands the area of severe hypoperfusion by more than 20% (Shin et al., 2006). In fact, it appears that a greater frequency but more importantly, duration of DC shifts correspond to accelerated infarct maturation and core expansion (Mies et al., 1993; Back et al., 1996). The infarct expansion is probably due to mismatch between high metabolic demand to support membrane repolarization and marginal tissue perfusion due to constrained penumbral blood flow (Back et al., 1996). Hence, suppressing CSD may prove worthwhile for reducing infarct maturation. Notably, the noncompetitive NMDA receptor antagonist ketamine markedly suppressed CSD frequency in head injury patients (Sakowitz et al., 2009), but it is unclear whether it improved clinical outcome. By contrast, irreversible depolarizations resistant to NMDA receptor antagonists (anoxic depolarization) develop in the ischemic core territory (Nellgård and Wieloch, 1992) and are probably terminal events contributing to severe ionic imbalance and tissue failure.

### **Inflammation**

Focal ischemia evokes a robust inflammatory response that begins within a few hours of onset and typifies the secondary or delayed response to ischemia. It involves activation of microglia and astrocytes as well as influx of hematogenous cells recruited by cytokines, adhesion molecules, and chemokines across the activated blood vessel wall. Stimulated brain cells and blood vessels then communicate with one another through a complex network of paracrine and autocrine signaling. The highly choreographed response evolves over many days, with multiple signals and targets triggering expression of novel proteins and secondary infarct expansion (Kleingin and Vink, 2009). Innate and adaptive immune systems participate as well. At least early on, inflammation amplifies the ischemic lesion, but, as discussed later, it may set the stage for tissue repair in the late postischemic period. A number of therapeutic trials designed to target the inflammatory response have failed to show clinical benefit (Sughrue et al., 2004), underscoring the need to clarify further the complexities of acute and delayed postischemic inflammatory signaling within brain.

As noted above, cerebral blood vessels are the first to be exposed to the ischemic insult, and their reaction to injury sets the stage for the inflammatory response. Early on, production of cytokines, such as  $\text{TNF}\alpha$  and  $\text{IL}1\beta$ , in vascular cells and perivascular microglia-macrophages upregulates adhesion molecule expression (e.g. ICAM-1, P and E-selectin) and, along with integrins, promote leukocyte rolling and sticking to the vessel surfaces (Zhang et al., 1998). Neutrophils are the first blood-borne cells to be recruited into the brain, followed by monocytes and, starting on days 1–2, lymphocytes. As the brain's primary immune cell, activated microglia transform into macrophages and accumulate at the border zone along with blood-borne macrophages to clear debris and dead cells and produce proinflammatory mediators and toxic molecules (Schilling et al., 2003).

Molecules released from injured or dying brain cells also contribute to the inflammatory response. In particular, products from cells undergoing oxidative stress and necrosis, for example lipopeptides, advanced glycation end-products (AGE), modified lipids, heat shock proteins, hyaluronic acid, and the nuclear protein HMGB1, lead to activation of the innate immune system by engaging toll-like receptors (TLRs). TLRs are expressed in multiple cells and, in conjunction with CD36, receptors for AGE (RAGE), and other scavenger receptors, may serve to detect “danger signals” triggering NF $\kappa$ b-dependent inflammatory signaling (Oppenheim and Yang, 2005; Wang et al., 2007). PARP activity appears to function as an NF $\kappa$ b coactivator (Chiariugi and Moskowitz, 2003), with therapeutic potential for delayed administration of PARP-1 inhibitors (Kauppinen et al., 2009). Beside NF $\kappa$ b, other transcriptional activators, including activator protein-1, STAT-3, interferon regulatory factor 1, and C/EBP beta, exert a proinflammatory effect (Kapadia et al., 2006; Iadecola et al., 1999), while PPAR $\gamma$  acts as a negative modulator of postischemic inflammation (Kapadia et al., 2008). In addition to neutrophils and monocytes, T cell subsets have recently been implicated as modulators of secondary infarct progression. T<sub>reg</sub> cells express the antiinflammatory cytokines IL10 and TGF $\beta$  and normally dampen the immune response by suppressing T cell effector activity (Liesz et al., 2009). Secondary infarct progression is impacted by a second subset of invading T lymphocytes, gamma-delta T cells, a source of the proinflammatory cytokine IL-17. After deletion of IL-17, infarct size decreases (Shichita et al., 2009).

These findings support the notion that taming postischemic inflammation may block secondary events that extend brain injury. However, as noted below, during the later stages in the injury process, inflammation promotes critical events necessary for tissue repair. Therefore, therapeutic interventions targeting postischemic inflammation should be mindful of temporal considerations by minimizing the destructive potential of inflammation in the acute phase while enhancing its beneficial contributions to tissue repair in the late stages of cerebral ischemia.

#### **Necrosis, Necroptosis, and Autophagy: The Execution**

Necrosis and apoptosis are the principal mechanisms of cell death after ischemic injury, and the details of both have been reviewed (Yuan, 2006, 2009). Depending in part upon the magnitude, type of stimulus, and cell type, a number of cell death triggers promote necrosis or apoptosis, although cellular features of apoptosis and necrosis are sometimes found together in dying ischemic cells. Probably due to its ATP dependence, apoptotic cell death is prominent after milder injury and is more delayed (Bonfoco et al., 1995), although severe ischemia is associated with early-onset caspase cleavage, enzyme activation, and acute cleavage of substrate proteins (Namura et al., 1998). Apoptosis and necrosis can be induced by multiple triggers, such as Ca<sup>2+</sup> overload or oxidative stress. Mitochondria play an essential role in both (Schinzel et al., 2005), serving as a reservoir for proapoptotic and antiapoptotic proteins and cytochrome C. One such protein apoptosis-inducing factor (AIF) is released from the outer membrane of mitochondria in response to activation of the nuclear protein PARP and generation of a PAR polymer, its toxic product (Yu et al., 2006). AIF then translocates to the nucleus to promote a caspase-independent form

of cell death, in part by promoting DNA fragmentation and chromatin condensation. Release of mitochondrial proteins sets in motion (or suppresses) multiple caspase-dependent and -independent processes to dismantle critical homeostatic and repair mechanisms. Despite the expression of executioner procaspases, activated caspases, and caspase cleavage products, classic apoptotic morphology is rarely found in humans within adult ischemic brain. However, it does appear more prominently in the ischemic brains of neonates (Ferriero, 2004).

Until now, necrotic cell death was defined as unregulated, unprogrammed, and lacking cell signaling events. It is the predominant form of ischemic cell death. It was recently shown that certain cells, including those within brain, possess a molecular switch that determines whether cells die by necrosis or apoptosis in a TNF-dependent way (Holler et al., 2000). In this cell death variation, necrosis is triggered by two kinases, RIP 1 and RIP 3, reciprocally interacting, often in the presence of an apoptosis inhibitor (Cho et al., 2009). Activated RIP3 is capable of stimulating enzymes such as the glycogen-degrading enzyme, glycogen phosphorylase, to steer TNF-induced apoptosis toward necrosis, in part through bursting energy metabolism and ROS generation (Zhang et al., 2009). Nec-1, a small-molecule inhibitor of RIP-1, decreases ischemic cell injury in models of ischemia-reperfusion and improves neurological outcome, suggesting RIP-1 as a potential therapeutic target (Degterev et al., 2005).

Autophagy, an energy-dependent process implicated as a cell death mechanism, is used by eukaryotes to degrade and recycle subcellular organelles. Its precise role in the death process is controversial because autophagic activity may be cytoprotective, at least in some disease models, e.g., polyglutamine expansions or in aging models (Levine and Kroemer, 2009). In other disease models, such as in neonatal hypoxia-ischemia, autophagy reportedly promotes cell death (Puyal et al., 2009). Precisely how this is accomplished remains for further study.

#### **Stroke and Dementia**

The major mechanisms of tissue dysfunction and death reviewed above develop as a consequence of acute vessel occlusion and the attendant activation of endogenous mechanisms of cell injury and tissue demise. More subtle and less well understood mechanisms are beginning to define a new research frontier linking cerebrovascular dysfunction and small strokes to the development of vascular cognitive impairment (VCI) and Alzheimer's disease (AD), the most common causes of dementia (Fotuhi et al., 2009). AD and “vascular” dementia have traditionally been considered distinct entities (Iadecola, 2004). Thus, AD, the most prevalent of the two, is characterized pathologically by deposition of the amyloid  $\beta$  peptide (A $\beta$ ) in the brain parenchyma (amyloid plaques) and blood vessels (amyloid angiopathy) and by neurofibrillary tangles (Querfurth and LaFerla, 2010). On the other hand, cerebrovascular diseases can lead to cognitive impairment in different ways. For example, a single stroke can cause dementia by damaging brain regions critical for cognition (strategic infarct dementia), while multiple strokes can cause stepwise cognitive deterioration through cumulative brain damage (multi-infarct dementia) (Leys et al., 2005; Pinkston et al., 2009). Most often, VCI is due to small white matter lesions (leukoaraiosis) that are thought to interrupt neural pathways

involved in cognition (Pinkston et al., 2009). A recent realization has been that AD and cerebrovascular diseases coexist in up to 60% of cases (Leys et al., 2005; Pinkston et al., 2009). Thus, although cases of “pure” AD and vascular dementia can exist, in most cases the cognitive decline can be attributed to overlapping cerebrovascular and AD pathologies (Querfurth and LaFerla, 2010). The causes of leukoaraiosis and the overlap between AD and cerebrovascular diseases are discussed in the next sections.

#### **What Causes the White Matter Damage Underlying VCI?**

In the cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL syndrome), a monogenic disease associated with dementia, extensive white matter damage is caused by notch-3 mutations in vascular smooth muscle leading to vascular insufficiency (Chabriat et al., 2009). In common forms of leukoaraiosis, however, the mechanism of the white matter damage remains unclear, although family history, hypertension, diabetes, and chronic smoking are well-established risk factors (Selnes and Vinters, 2006). The white matter lesions consist of demyelination, axonal loss, enlarged perivascular spaces, astrogliosis, and microglial activation (Fernando et al., 2006; Pantoni and Garcia, 1995). The wall of arterioles is thickened by accumulation of hyaline material (lipoinosclerosis) or completely disrupted (fibrinoid necrosis), leading to microhemorrhages (Fisher, 1968; Pantoni and Garcia, 1995). Capillary density, resting CBF, and cerebrovascular reactivity are reduced both in normal and affected white matter (Brown et al., 2007; Mandell et al., 2008; O’Sullivan et al., 2002).

Disruption of the arteriolar wall by amyloid, as observed in genetic or sporadic forms of cerebral amyloid angiopathy and in AD, is also associated with white matter lesions (Weller et al., 2009). Hypoxia-inducible genes and markers of endothelial activation are upregulated, suggesting hypoxia-ischemia (Fernando et al., 2006). A causative role for hypoxia-ischemia is also suggested by the fact that the blood supply to the subcortical and basal ganglia white matter is provided by terminal arterioles located at the watershed between two vascular territories: perforating arteries from the neocortex and penetrating branches arising from larger arteries at the base of the brain (Iadecola et al., 2009). Therefore, these areas may be particularly susceptible to vascular insufficiency, especially if the structure of the arterioles and their vasomotor function are altered. On the other hand, energy and blood flow requirements of white matter are much lower than those of gray matter, rendering the tissue, in theory, less vulnerable to anoxia-ischemia (Hertz, 2008). Therefore, alternative mechanisms leading to an increased intrinsic vulnerability to injury may also play a role. For example, risk factors for dementia could enhance white matter susceptibility to anoxia, as aging has been described to do (Baltan et al., 2008). Disruption of the BBB, an early event in leukoaraiosis, could lead to injury by producing perivascular edema, microhemorrhages, and inflammation, contributing to demyelination (Farrall and Wardlaw, 2009). Demyelinated axons may be more susceptible to ischemic injury due to their heightened metabolic requirements caused by loss of energy-efficient saltatory conduction and leaky sodium channels (Trapp and Stys, 2009). Furthermore, dysfunction within the endothelium may compromise the trophic effect that these cells exert on neurons and

glia, contributing to brain dysfunction and damage (see section on repair mechanisms). Aging, inflammation, hypoxia and, possibly, other risk factors can also reduce the repair potential of the damaged white matter by impairing oligodendrocyte progenitor recruitment and differentiation (Back et al., 2002; Pang et al., 2010; Sim et al., 2002). Therefore, the mechanisms underlying leukoaraiosis might be distinct from those of ischemic cell death and are a fertile ground for new investigations.

#### **Why Does Stroke Increase the Risk of Dementia?**

Stroke doubles the risk of developing dementia (Leys et al., 2005). Although, as discussed above, location, size, and number of ischemic lesions, as well as leukoaraiosis can account for the cognitive impairment in some cases, epidemiological and pathological evidence indicates that stroke also increases the risk for AD. Alzheimer pathology can facilitate the development of dementia in patients with ischemic injury, and, vice versa, ischemic injury can aggravate the cognitive deficits induced by AD (Fotuhi et al., 2009). These findings have suggested a previously unrecognized interaction between amyloid pathology and ischemic injury. This is not surprising, considering that A $\beta$  has powerful cerebrovascular effects. A $\beta$  constricts cerebral vessels, suppresses vascular reactivity, and impairs autoregulation, leading to vascular insufficiency and increased susceptibility to ischemic injury (Iadecola, 2004; Zhang et al., 1997). Conversely, ischemia and hypoxia can induce A $\beta$  accumulation by promoting its cleavage from APP (Tesco et al., 2007) and by downregulating the lipoprotein-related receptor protein-1, a critical receptor for the vascular clearance of A $\beta$  (Bell et al., 2009; Tesco et al., 2007; Wu et al., 2005). Therefore, in a vicious cycle, the vascular effects of A $\beta$  aggravate ischemia, and, in turn, brain ischemia enhances A $\beta$  accumulation (Iadecola, 2004). Recent studies have also linked plasma A $\beta$  to the white matter damage underlying leukoaraiosis (Gomis et al., 2009; Gurol et al., 2006). The significance of this finding and its underlying mechanisms remain to be defined and placed in context with other findings showing that A $\beta$  could promote axonal injury directly through its cytotoxic effects (Querfurth and LaFerla, 2010) or indirectly by inducing vasoconstriction and ischemia (Iadecola, 2004). Therefore, the vascular dysfunction induced by circulating and parenchymal A $\beta$  may act in concert with the structural and functional vascular changes induced by risk factors to amplify the vascular insufficiency in small white matter arterioles leading to white matter injury. However, factors other than A $\beta$  could also contribute to the interplay between AD pathology and cerebrovascular diseases. For example, cerebral arterioles of AD patients exhibit a tendency to constrict due to increased activity of transcription factors (serum response factor/myocardin) that control the expression of contractile proteins in smooth muscle cells (Chow et al., 2007). This finding may play a role in the increased vasoconstrictor tone and reduced CBF observed in AD (Iadecola, 2004). It remains to be established whether these changes in gene expression are related to A $\beta$  or to upstream molecular events independent of it.

#### **How Does the Brain Repair Itself after Stroke?**

A well-known fact in clinical stroke is that most patients show some degree of recovery over time. As the brain recovers, considerable remapping of cortical areas takes place. Functional

MRI studies demonstrate that peri-infarct areas are highly dynamic (Dijkhuizen et al., 2003; Chopp et al., 2007). Representational areas shift as latent networks are unmasked and parallel circuits are recruited adjacent to damaged regions (Murphy and Corbett, 2009). More recently, advanced optical imaging techniques combined with electrophysiology demonstrated that this remodeling at a circuit level can be correlated with specific processes of dendritic and synaptic plasticity at a cellular level (Li and Murphy, 2008). Imaging of mitochondrial function (Liu and Murphy, 2009) and vascular dynamics (Schaffer et al., 2006) have provided new insight into how the brain responds to microvascular perturbations following ischemia. These powerful tools may eventually allow one to interrogate mechanisms of stroke recovery at a molecular and cellular level while testing prorecovery therapies and strategies (Murphy and Corbett, 2009; Zhang and Chopp, 2009).

#### **Repair and Remodeling Processes after Stroke**

A major advance within this past decade has been the realization that adult mammalian brains also exhibit focal areas of ongoing neurogenesis in the subventricular zone and subgranular zone. Under normal conditions, these newborn neurons migrate to olfactory regions and hippocampus. But after cerebral ischemia or hemorrhage, the birth of new cells seems to increase and neuroblasts reroute toward damaged brain tissue (Arvidsson et al., 2002; Parent et al., 2002), perhaps as part of an endogenous attempt by the brain to repair itself. However, whether these new neurons significantly contribute to functional recovery remains unclear. Neurogenic responses may persist for surprisingly long periods of time, but the majority of these newborn cells survive only for a few days (Arvidsson et al., 2002). Whether these cells develop normally, express neurotransmitters, or even integrate into existing neural networks remains to be fully elucidated. Future attempts to promote endogenous neurogenesis for therapeutic purposes will have to address ways to modify parenchymal microenvironments to enhance neuronal survival and integrate newly born cells into viable circuitry.

In concert with neurogenesis and neural plasticity, the brain recovering from stroke exhibits complex patterns of vascular remodeling. Angiogenesis and vasculogenesis in peri-infarct regions have been detected in rodent models of cerebral ischemia (Ding et al., 2008) as well as in human stroke (Krupinski et al., 1996). Indeed, it is now recognized that angiogenic and neurogenic responses are tightly coregulated after stroke and brain injury (Arai et al., 2009). This might not be surprising, because molecular mechanisms of neurogenesis and angiogenesis have been evolutionarily conserved so that similar mediators and pathways are involved in both phenomena (Carmeliet and Tessier-Lavigne, 2005). In the normal brain, the neurovascular niche defines these complex mechanisms of cell-cell signaling between cerebral endothelium and neural precursors in the subventricular and subgranular zones of ongoing neurogenesis. In the context of poststroke recovery, these close relationships between neurogenesis and angiogenesis are maintained. Neuroblasts migrate along perivascular routes (Thored et al., 2007). Promotion of neurogenesis enhances vascular regrowth and, conversely, angiogenic stimulation enhances neurogenesis (Ohab et al., 2006; Taguchi et al., 2004). Hence, it seems likely that brain recovery after stroke comprises interdependent

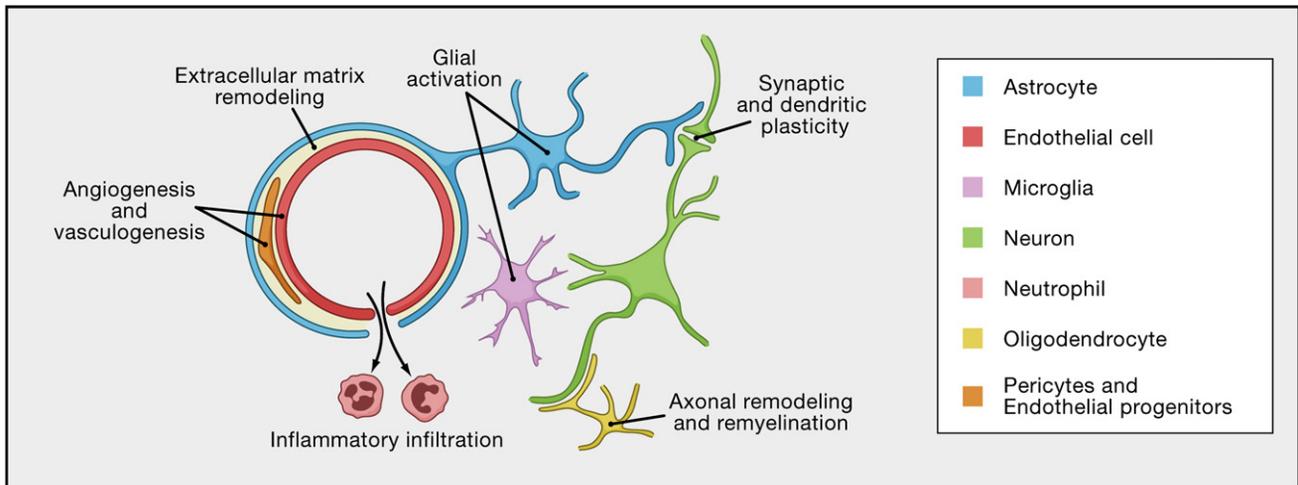
neurovascular plasticity and remodeling processes that recruit multiple common mediators and signals (Snappy et al., 2009). The new frontier of neurovascular repair may require a detailed exploration of how these multicell and multisignal phenomena are regulated in the context of stroke. Recent experimental studies have suggested that pharmacologic interventions such as EPO, statins, activated protein C, and phosphodiesterase inhibitors may promote functional recovery after cerebral ischemia (Wang et al., 2004; Zhang et al., 2006; Thiyagarajan et al., 2008; Chen et al., 2009). Ultimately, therapies that can augment these endogenous signals and substrates of neurovascular remodeling may have long therapeutic time windows in stroke (Zhang and Chopp, 2009).

#### **Moving Forward: Charting a Course toward New Stroke Therapies**

Tremendous progress has been made in the understanding of fundamental mechanisms of neuronal cell death. However, the translation of these powerful molecular and cellular principles into clinically effective neuroprotective therapies in stroke has been challenging. More recently, emerging data from both experimental and clinical studies now suggest that all cell types in the brain participate in the complex pathophysiology of brain injury following stroke. Consequently, therapeutic approaches should target multiple cell types in an attempt to protect their structural and functional integrity and their reciprocal interactions.

#### **Protecting the Neurovascular Unit**

The so-called “neurovascular unit” provides a conceptual framework that integrates responses in all cell types, including neuronal, glial, and vascular elements (del Zoppo, 2009; Iadecola, 2004; Zacchigna et al., 2008) (Figure 3). Cell-cell interactions in the neurovascular unit form the basis for brain function. Dysfunctional signaling in the neurovascular unit underlies the basis for disease. Whereas the intricate molecular pathways of cell death in the neuron have been dissected in detail, the mechanisms of how the entire neurovascular unit responds to stroke are not completely understood. As discussed in the previous sections, cerebral ischemia can rapidly induce perturbations in cell-cell signaling and function within the neurovascular unit. How each cell type succumbs or adapts to injury is dependent on responses in other cells. For example, genomic and proteomic responses in neurons subjected to metabolic stress have been fairly well described. But how each cell type might alter its response to vascular risk factors and ischemia in the context of other elements in the neurovascular unit remains to be elucidated. As the nexus of cell-cell interactions grows, a systems biology approach may eventually be required to rigorously define mechanisms as well as targets for complete neurovascular protection. The importance of cell-cell signaling in the neurovascular unit has been underscored by many studies (Arai and Lo, 2009a, 2009b; Dugas et al., 2008; Eroglu et al., 2009; Guo et al., 2008). Hence, under normal conditions, trophic coupling within the neurovascular unit helps maintain homeostasis. In contrast, under diseased conditions, it is likely that loss of coupling would lead to pathophysiology (Grammas et al., 1999; Nagai et al., 2007). Loss of trophic coupling is also likely to play a role in the way vascular risk factors render neurons



**Figure 3. Remodeling the Neurovascular Unit**

This schematic depicts a cerebral blood vessel and surrounding brain parenchyma that comprise the neurovascular unit. The neurovascular unit encompasses cell-cell and cell-matrix interactions between component vascular, glial, and neuronal cells. Homeostatic signaling in the neurovascular unit sustains normal brain function. Dysfunctional neurovascular signaling mediates injury after stroke. During the recovery phase after stroke, neurovascular signaling may also be critically important with repair mechanisms that may involve angiogenesis, vasculogenesis, and endothelial precursor mechanisms. As the blood-brain barrier heals, inflammation may convert from a deleterious to beneficial role. Reactive glia (astrocytes, microglia, pericytes) may secrete trophic factors. Synaptic and dendritic plasticity as well as axonal remyelination may provide parenchymal substrates for functional recovery. Emerging data now suggest that these phenomenon do not occur in isolation. All components of the neurovascular unit are likely to remodel coordinately (Arai et al., 2009; Murphy and Corbett, 2009; Zhang and Chopp, 2009).

and glia more vulnerable, such as in the link between leukoaraiosis and brain dysfunction underlying cognitive impairment. Recently, BDNF has been implicated as a candidate factor that mediates trophic coupling between vascular and neuronal compartments (Arai and Lo, 2009b; Guo et al., 2008). Insofar as BDNF polymorphisms have been implicated in modulating synaptic plasticity (Kleim et al., 2006), these neurovascular trophic signals may also influence mechanisms of recovery after stroke.

The concept of the neurovascular unit may provide an integrated framework for investigating mechanisms and therapies. Salvaging neurons alone may not be sufficient. One has to protect glial and vascular elements as well. Future investigations might be better served by pursuing targets that are expressed on multiple cell types in brain. Furthermore, prevention of cell death alone may also not be sufficient. In order for therapies to be clinically meaningful, cellular function must be preserved and/or restored. Neurons that are alive but lack proper connectivity or do not express the correct array of transmitter release-reuptake and synaptic activity may not be functional. Astrocytes that survive but cannot provide hemodynamic coupling would be unable to link neuronal activation with the required vascular responses. Oligodendrocytes that are respiring but metabolically impaired might not provide the necessary myelination for axonal conduction. Endothelial cells that survive but do not maintain blood-brain barrier properties would facilitate damage to adjacent parenchyma. Ultimately, any stroke therapy must include both prevention of cell death as well as rescue of integrated neurovascular function. Ischemic tolerance and postischemic hypothermia are some of the most potent protective strategies for ischemic brain injury and represent examples of multimodal therapies targeting the

neurovascular unit as a whole. These are described in the next sections.

#### **How Does the Brain Protect Itself? Lessons from Ischemic Tolerance**

Some of the very same mechanisms implicated in the tissue-damaging responses after stroke can also be engaged to protect the brain after threatened injury and to induce ischemic tolerance (IT). IT is a concerted organ response to protect itself against tissue injury and has been widely studied over the past three decades in an effort to identify endogenous mechanisms of protection and to exploit them for therapeutic purposes (Obrenovitch, 2008). In its delayed form, it is induced after challenge by a subthreshold noxious stimulus, developing within 1–3 days and lasting for several additional days. Experimentally it can be induced by exposure to preconditioning stimuli such as neurotoxins (e.g., glutamate), substrate deprivation (e.g., ischemia, hypoxia), or inflammation (e.g., cytokines and immune responses) and engages mechanisms that detect, transduce, and promote a complex tissue reaction that reflects a fundamental genomic reprogramming after threatened injury (Dirnagl et al., 2009; Gidday, 2006; Stenzel-Poore et al., 2004). In this altered physiological state, tissue damage is mitigated when challenged by exposure to a wide range of deleterious stimuli, even if unrelated to the initial challenge.

It is mediated by highly redundant cellular, molecular, and physiological mechanisms involving neurons, astrocytes, and vascular cells (Gidday, 2006). Upstream sensors have been implicated, including adenosine and its A1 receptor, the NR2A NMDA receptor subunit, and the transcriptional regulator hypoxia inducible factor (HIF). HIF is an attractive candidate because it regulates a constellation of genes, including those whose protein products facilitate oxygen transport

(erythropoietin), regulate glycolytic metabolism (e.g., glucose transporters), and promote angiogenesis (e.g., VEGF), by way of example. Unexpectedly, genetic deletion of HIF1 $\alpha$  improves infarct volume after middle cerebral artery occlusion, implicating as yet unknown pathogenic mechanisms activated by ischemia (Helton et al., 2005). Erythropoietin, one of the gene products of HIF1 $\alpha$ , promotes PI3 kinase/AKT activity and the expression of NF $\kappa$ B-dependent protective genes (Digicaylioglu and Lipton, 2001). Erythropoietin also downregulates hypoxia-induced proapoptotic mechanisms (Noguchi et al., 2007; Prass et al., 2003; Ruscher et al., 2002), and erythropoietin-based therapies have been investigated in the clinical arena (Ehrenreich et al., 2009). Heat shock protein family members are upregulated in ischemic tolerance and, based on their multiple cytoprotective actions, may promote survival. Preconditioning stimuli preserve connexin 43 hemichannels in astrocytes facilitating ATP released from these cells and increasing the extracellular concentration of the beneficial nucleoside adenosine (Lin et al., 2008). At the same time, preconditioning safeguards the function of cerebral blood vessels from the deleterious effects of cerebral ischemia, resulting in improved penumbral flow and reduced brain damage (Kunz et al., 2007b). These astrocytic and vascular changes are coupled with mechanisms that reduce energy demands, lessen inflammatory responses and ion channel activity, and decrease cascades promoting blood clotting and apoptotic cell death (Dirnagl et al., 2009; Obrenovitch, 2008; Gidday, 2006). Factors involving mitochondria as well as its uncoupling proteins have also been suggested and include improved efficiency of ATP synthesis, preservation of the mitochondrial membrane potential, and delayed opening of mitochondrial transition pore formation, as well as opening of mitochondrial ATP-dependent K<sup>+</sup> channels (Dirnagl and Meisel, 2008).

One of the key questions defying explanation at the moment is how and why diverse preconditioning stimuli targeting single genes or key steps in seemingly unrelated cascades all impact infarct injury in genetically engineered and wild-type mice. It may be telling us that the mechanisms through which the brain protects itself involve a highly integrated network response that emphasizes multiple cells within the neurovascular unit. If true, comparable multicellular approaches are needed that capitalize on this integrated response in the design of successful treatments (see next section).

#### **Therapeutic Hypothermia: Engaging Pleiotropic Mechanisms in Multiple Cell Types**

The therapeutic hypothermia field has advanced significantly during the past decade, inspired by positive outcomes from clinical trials in cardiac arrest victims (Bernard et al., 2002). Satisfactory neurological recovery was achieved in about half of the treated patients when treatment was begun within 2 hr of cardiac arrest. Promising results were reported in small feasibility studies in neonatal ischemia (Shankaran et al., 2005). Current efforts are directed toward translating these successes in cardiac arrest and neonatal ischemia to patients with ischemic stroke, but there remain unresolved technical issues surrounding the timing and method for inducing hypothermia, and the deleterious effect of rewarming (Wagner and Zuccarello, 2005). It is now well accepted that hypothermia reduces ischemia in animal models of stroke, and so far, preclinical results have helped

guide the design of clinical trials in human subjects. Combining hypothermia with thrombolysis is one example. Experimental evidence suggests that hypothermia protects the brain through pleiotropic effects acting on multiple cell types within the neurovascular unit. Hypothermia reduces brain metabolism, thereby significantly decreasing oxygen consumption and the work load of the entire organ (Sakoh and Gjedde, 2003). Hypothermia also reduces glutamate release, ROS production, and peri-infarct depolarizations. Inflammatory markers decrease, as well as microglial activation, leucocyte infiltration, MMP activation, and BBB disruption. In addition, hypothermia suppresses apoptotic cell death, decreases mitochondrial release of cytochrome C and apoptosis inducing factor (see for review Tang and Yenari, 2010). Cell survival pathways are upregulated and appear to contribute to the multiplicity of neuroprotective mechanisms. To what extent each of these promotes tissue protection or interacts synergistically to enhance tissue survival is not known and remains for further investigation. Nevertheless, therapeutic hypothermia does illustrate the potential power of approaching stroke therapy using combinations of modalities that engage multiple cell types to enhance protection and recovery.

#### **Additional Strategies for Repair and Recovery: Trophic Factors and Cell-Based Therapies**

No matter how successful acute neuroprotective strategies become, many stroke patients may still fall outside of clinical time windows for effective treatment. Hence, approaches that promote repair and recovery will be essential for an integrated stroke armamentarium. Logically, these approaches can be divided into two categories—pharmacologic or trophic factor treatments that seek to amplify and augment endogenous processes of neurovascular plasticity and recovery and cell-based therapies that seek to replace damaged or lost brain cells. Many approaches that augment endogenous recovery have been explored. Growth factors such as NGF, FGF, GDNF, and BDNF have all met with variable degrees of success in animal models (Semkova and Kriegstein, 1999). But a limiting factor in many of these studies is their relatively large molecular weight, making transport into brain difficult. Some alternatives may yet exist, such as smaller peptides, transnasal delivery of growth factors to bypass the blood-brain barrier (Fletcher et al., 2009), peptidomimetics and compounds that can upregulate endogenous trophic factor production but do not require direct infusion into brain (Zhang and Chopp, 2009). Additionally, one may also target inhibitory molecules that accumulate in the area surrounding the injury and block neuronal repair, such as chondroitin sulfate proteoglycans, myelin associated proteins, Nogo, and semaphorins (Silver and Miller, 2004).

Although stroke-damaged brain usually exhibits varying degrees of spontaneous recovery, there will always be significant areas where endogenous repair is insufficient. In this regard, cell-based therapies may represent a new frontier. To date, a variety of cell sources have been tested, including neural stem cells, bone-marrow-derived mesenchymal cells, as well as endothelial progenitor cells (Koch et al., 2009). Treatments have typically involved direct transplantations into brain parenchyma, catheter infusions into cerebral ventricles, as well as systemic injections into circulating blood. The latter approach

is especially intriguing, as complex mechanisms of cell targeting seem to be operational in brain. Infarcted brain tissue upregulates and releases various chemokines, such as SDF-1, to establish gradients that broadly serve to attract stem and precursor cell populations (Madri, 2009). Induced pluripotent stem cells may also provide an alternative cell source to embryonic stem cells (Takahashi and Yamanaka, 2006). One advantage is the theoretical possibility that cell repair can be custom designed to suit specific diseases or individual needs, as described in a flurry of recent studies showing that neurons can be successfully derived from pluripotent cells taken from patients with Huntington's disease, ALS, and spinal muscular atrophy (Dimos et al., 2008; Ebert et al., 2009; Park et al., 2008). Whether similar methods can be applied to stroke victims remains to be tested.

Overall, cell-based therapies in models of experimental stroke suggest that delayed repair is a viable treatment approach. But more work is needed to translate these promising results into effective stroke therapies. Many questions remain, and the precise mechanisms of cell-induced repair are still unclear. For example, do the reported benefits occur because cells are being explicitly replaced or because transplanted cells serve as a source of trophic factor production? How well do transplanted cells survive? Will adjunctive therapies to decrease apoptotic dropout be necessary? If cells survive, how well do they integrate into existing neural or vascular networks? What is the optimum timing for such therapy? What types of cells should be used? And finally, what subtypes of stroke patients should be selected for robust clinical trial results?

As the basic mechanisms of trophic and cell-based repair continue to be defined, an interesting underlying theme has emerged that links the survival of newborn or transplanted cells to the host environment. As discussed in the previous sections, activated vascular cells and microglia may be detrimental to cell survival in the early stages of cerebral ischemia, due to production of cytokines, ROS, and toxic amount of NO. In the recovery phase, however, chronically activated microglia may now secrete beneficial factors such as BDNF and GDNF. Hence, prolonged inflammation can serve biphasic roles—dampening cellular recovery acutely but then enhancing tissue repair later on. In fact, this principle of biphasic mediators may be broadly applicable in stroke pathophysiology. There are many examples. Overactivation of NMDA receptors induces acute excitotoxicity (Besancon et al., 2008), but without NMDA signaling, chronic neuronal remodeling cannot take place (Young et al., 1999). Matrix metalloproteinases degrade and damage neurovascular substrates in the acute phase (Rosenberg, 2009), but the same proteases are critically important for neurovascular remodeling during the recovery phase (Zhao et al., 2006; Lee et al., 2006). The intracellular mediator HMGB1 promotes necrosis and expands the core infarct during acute cerebral ischemia (Qiu et al., 2008), but during the repair phase, HMGB1 release from reactive astrocytes promotes angiogenesis and synaptic plasticity (Hayakawa et al., 2010a, 2010b). Altogether, the boundaries between cell death and cell repair may be blurred (Lo, 2008b). Future studies that dissect these biphasic mechanisms should prove fruitful if we are to develop clinically meaningful strategies for both neurovascular protection as well as repair.

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