Novel Stroke Therapeutics: Unraveling Stroke Pathophysiology and Its Impact on Clinical Treatments

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Abstract

Stroke remains a leading cause of death and disability in the world. Over the past few decades our understanding of the pathophysiology of stroke has increased, but greater insight is required to advance the field of stroke recovery. Clinical treatments have improved in the acute time window, but long-term therapeutics remain limited. Complex neural circuits damaged by ischemia make restoration of function after stroke difficult. New therapeutic approaches, including cell transplantation or stimulation, focus on reestablishing these circuits through multiple mechanisms to improve circuit plasticity and remodeling. Other research targets intact networks to compensate for damaged regions. This review highlights several important mechanisms of stroke injury and describes emerging therapies aimed at improving clinical outcomes.

Pathophysiology of Stroke

The lack of blood flow during a stroke results in an intricate path-ophysiological response resulting in neural injury, as depicted in Figure 1 (Hossmann, 2006). Multiple mechanisms, including excitotoxicity, mitochondrial response, free radical release, protein misfolding, and inflammatory changes, lead to neural cell loss, but many of these pathways ultimately pave the way for recovery. Injury and death of astrocytes, as well as white matter injury, also contribute to cerebral damage. The delicate balance between detrimental or beneficial effect often relies on the timing and the magnitude of the factors involved. The inflammatory response is a prime example of a system that both propagates ischemic injury and helps promote recovery. Inflammation initially contributes to cellular injury through the release of cytokines and harmful radicals but eventually helps to remove damaged tissue, enabling synaptic remodeling. Glial cells also serve dual roles, helping to regulate the blood-brain barrier, promoting angiogenesis and synaptogenesis, but conversely forming the glial scar that may prevent further plasticity (Gleichman and Carmichael, 2014). The goal for this review is to provide a brief overview of the pathophysiology of stroke followed by a discussion of the current state of stroke recovery research with an emphasis on those approaches that target multiple mechanistic pathways. Many of these therapies are aimed at

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up-regulating pathways that enhance recovery while reducing the deleterious pathways triggered by the initial ischemic insult. Further understanding and optimizing this delicate balance may facilitate development of effective stroke therapeutics.

Excitotoxicity

CNS ischemia results in a deficiency of glucose and oxygen leading to the inability of neuronal cells to maintain normal ionic gradients. Depolarization of these neurons leads to excessive glutamate release resulting in the intracellular influx of calcium, triggering cell death pathways such as apoptosis, autophagocytosis, and necrotic pathways (Lipton, 1999). This process has been termed excitotoxicity and is mediated largely through the glutamatergic pathways involving N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPARs), and kainate receptors (Dirnagl et al., 1999; Moskowitz et al., 2010). The role of calcium in excitotoxicity also remains complex and has numerous effects in the ischemic environment. The intracellular increase in calcium triggers mitochondrial dysfunction and activation of free radicals, phospholipases, and proteases, which lead to cell death or injury (Szydlowska and Tymianski, 2010). Interestingly, the interplay between the cells is also critical to the spread of injury after ischemic insults. Blockage of the gap junctions between cells in the adult brain reduces neuronal death (Wang et al., 2010), potentially indicating the important interactions that occur between cells during neuronal damage. These processes also promote cerebral edema, which has clinical import in the first few days after a stroke. Numerous therapeutic approaches have centered on interrupting pathways triggered by excitotoxicity to improve stroke recovery, and while often successful in animal models (Yenari et al., 2001; Namura et al., 2013), translation of these findings into the clinic remains challenging.

Mitochondrial Alterations

The mitochondria play a critical role in cell energy homeostasis and are thus prominently involved during ischemia when the energy balance is disrupted and ATP synthesis is altered. The rapid influx of calcium experienced with excitotoxicity leads to excess accumulation in the mitochondria, causing dysfunction, which leads to mitochondrial permeability transition pore (mtPTP) opening and cytochrome c release (Liu et al., 1996; Murphy et al., 1999). These events create mitochondrial swelling and membrane collapse, initiating cell death cascades such as apoptosis (Liu et al., 1996). The reactive oxygen species (ROS) created by the mitochondria also play a prominent role in reper-fusion injury and cell death in the ischemic environment (Kalogeris et al., 2014). Maintaining mitochondrial integrity and limiting their induction of apoptotic and oxidative stress pathways in the cell are important avenues to preventing widespread cell toxicity from an ischemic insult.

Free Radicals

Brain ischemia also triggers free radicals, which contribute to the oxidative stresses on neural tissue. The influx of calcium triggers nitric oxide (NO) production by nitric oxide synthase (NOS) that leads to injury through the formation of oxygen free radicals and the production of peroxynitrite (ONOO–) (Iadecola, 1997). The mitochondria undergo dysfunction during ischemia, leading to further oxidative stress (Kalogeris et al., 2014). NADPH oxidase also plays a critical role in ROS production in the setting of excitotoxicty

and ischemia (Moskowitz et al., 2010). Furthermore, chimeric bone marrow studies have shown that inflammation contributes with neutrophils releasing inducible NOS (iNOS), which leads to toxic levels of NO (Garcia-Bonilla et al., 2014; Moro et al., 2004). Free radicals trigger the PI3-kinase/Akt pathway as well as upregulate the transcription factor NF-κB. Interestingly, the timing and environment of activation of this pathway likely determine whether stroke recovery is improved or impeded by this signaling cascade (Crack and Taylor, 2005). Other pathways of interest are the transient receptor potential (TRP) channels. TRP channels, TRPM7 specifically, are linked to free radicals in ischemia and likely contribute to increasing the influx of calcium and cellular toxicity experienced during decreased oxygenation (Sun et al., 2009). Not only do free radicals contribute to initial toxicity, they also prevent recovery, which makes them an important post-stroke therapeutic target (Miyamoto et al., 2013). Numerous methods have reduced the oxidative stress from free radicals in ischemic injury and shown neurologic improvement in preclinical models. Combining the regulation of these pathways with other ischemic injury mechanisms may lead to novel therapeutics.

Protein Misfolding

The largest stores of intracellular calcium reside in the endoplasmic reticulum (ER), an organelle that regulates protein synthesis and responds to protein misfolding (Zhang et al., 2014). These processes are largely affected by ER stress induced by ischemic injury (Roussel et al., 2013). As excitotoxic changes occur in neural cells, the sarcoplasmic/ER calcium ATPase (SERCA) pump fails due to energy depletion and adds to the occurrence of cell death (Szydlowska and Tymianski, 2010). The increased accumulation of misfolded proteins also trigger the protein kinase-like ER kinase (PERK) pathway regulating eIF2α kinase activation, which halts new protein synthesis (Althausen et al., 2001). The phosphorylation of eIF2α has been explored as a means to alter damage in cerebral ischemia. Inositol requiring enzyme 1 (IRE1) is another protein involved in the misfolding of proteins that has been shown to induce apoptotic pathways during periods of ER stress (Morimoto et al., 2007). Chaperones (such as oxygen-regulated protein 150 kDa and binding immunoglobulin protein), which normally guide protein synthesis, are also altered in ischemia, and upregulation of these chaperones may reduce apoptosis and limit damage from ischemia (Roussel et al., 2013). The cumulative effect of SERCA pump failure and chaperone misfunctioning make ER stress and its role in protein misfolding important targets for acute stroke therapies.

Astrocytic Changes and White Matter Injury

The glial cells (astrocytes and oligodendrocytes) surrounding neurons and their connections play an integral role in the brain's response to ischemia and recovery. Axons and glial cells are intimately interwoven, forming the connections and signals that compose neural activity and are poised as key therapeutic targets to enhance recovery mechanisms and reduce injurious ones. At baseline, white matter receives less blood supply than gray matter, and this may predispose white matter to ischemic damage with milder variations in blood flow. During ischemic injury, glial cells are damaged by similar injury pathways to neurons including glutamate toxicity (Sánchez-Gómez et al., 2011). Ischemia also triggers P2X7 receptors on oligodendrocytes, which contribute to calcium overload and mitochondrial

depolarization (Wang et al., 2009). One of the key differences between the effects of ischemia on white matter compared with gray matter is the reliance on oligodendrocytes for functional deficits as well as the reduced influence of NMDA-type glutamate receptors on white matter injury (Matute et al., 2013).

After the acute response to hypoxic conditions, the glia also help to modulate inflammation and recovery. Although the glial scar has been shown to prevent new growth, it also exhibits positive effects of helping to restore the integrity of the blood-brain barrier. Additionally, reactive astrocytes, associated with formation of the glial scar, also modulate trophic factors, which enhance recovery (Rolls et al., 2009). Thus, glia play a prominent role in modulating the injury cascade and eventual recovery after stroke.

Inflammatory Response and the Role of the Blood-Brain Barrier

The immune system plays a vital role in the CNS's response to ischemia and to eventual recovery of function. An intricate cascade of immune cells and inflammatory factors cause blood-brain barrier breakdown, remodeling of the post-stroke tissue, and also offer a margin of neuroprotection from the harsh excitotoxic post-stroke environment of increased free radicals and enzymes (Iadecola and Anrather, 2011). Initially, microglia respond to the ischemic insult followed by an increase of dendritic cells, macrophages, and lymphocytes, and as astroglia are reduced and blood-brain barrier breakdown occurs, an influx of neutrophilic cells permeates the infarct and peri-infarct region (Gelderblom et al., 2009). Proinflammatory cytokines (i.e., tumor necrosis factor-α and interleukin-1β) are also released as well as free radicals by the immune cells in the post-stroke tissue, which increase the inflammatory response and upregulate cell adhesion molecule expression, further propagating the immune response (Huang et al., 2006). Immune cells also release inducible NO synthetase, which contributes to the detrimental effect of NO in brain ischemia, as noted above (Moro et al., 2004). Additionally, matrix metalloproteins (MMPs) and myeloperoxidase (MPO) production are elevated by the immune response, both of which are major factors leading to blood-brain barrier breakdown (Bao Dang et al., 2013). Inhibiting the acute inflammatory response after stroke has been shown to decrease injury and improve neurologic outcome in rodent stroke models (Arac et al., 2011), but has not yet been translated into the clinic.

Components of the complement cascade play a role in ischemic injury and recovery. The amount of complement proteins increases after ischemia (Pedersen et al., 2004). Evidence suggests that complement proteins tag synapses for removal by microglia to enable synaptic pruning and remodeling (Stephan et al., 2012). Another role of complement proteins (C3a and C5a, in particular) is protecting neurons from the NMDA excitotoxicity that occurs post-stroke (van Beek et al., 2001; Mukherjee et al., 2008). Immune cells such as eosinophils also produce trophic factors such as nerve growth factor (NGF) and neurotrophin-3 that promote neuronal outgrowth and may have a significant impact on post-infarct plasticity (Foster et al., 2011). Microglia also play a prominent role producing glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), which promote neural growth and healing (Wang et al., 2013; Yang et al., 2012). Insulin-like growth factor (IGF-1), another molecule modulated by microglia, enhances axonal growth as well as neurogenesis

in the subventricular zone (SVZ) to improve stroke recovery (Butovsky et al., 2006; Lalancette-Hébert et al., 2007). Cytokines, such as transforming growth factor-β and interleukin-10, often serve dual roles of driving the inflammatory response but also promoting tissue repair and resolution of inflammation depending on timing and the environment (Iadecola and Anrather, 2011).

The multifaceted immune response has both a beneficial and deleterious effect on the surviving tissue. The timing and levels of inflammatory factors and cells contribute to the balance of post-stroke injury and the restorative process (Peruzzotti-Jametti et al., 2014). The immune response has a positive role on recovery by pruning unwanted synapses and allowing for the formation of new growth and connections. However, there is also a negative effect of the inflammatory response with rodent models showing decreased stroke volume and infarct size in immunodeficient animals (Hurn et al., 2007). While neutrophils release cytokines and radicals that worsen the inflammatory response, inflammatory cells also help remove debris and damaged tissue to facilitate recovery. The balance of the inflammatory response after stroke is critical for recovery, and investigation into the components that lead to improved recovery and plasticity versus those that worsen ischemic damage is an exciting area for further research and translational investigations.

Stroke Therapies

The complex injury pathways described above often disrupt the cortical maps that form the neural representation of our body. Increased spine formation and axonal sprouting weeks after ischemia demonstrate enhanced neural plasticity in the peri-infarct area and contralesional hemisphere as brain regions reorganize, likely to restore function (Brown et al., 2007). Alterations in synaptic function and vasculature have been shown to correlate with behavioral improvement after stroke as the brain remaps to compensate for damaged networks (Winship and Murphy, 2009). Because of the complexity of the restorative processes that occur after the initial ischemic damage, a single mechanistic pathway will likely not be sufficient to greatly improve functional outcomes. Strategies such as cell therapies, stimulation, or mild hypothermia that affect several of these pathways, or a combination of therapeutic approaches, may prove to be the most promising for clinical translation.

Currently, the mainstay of acute stroke therapy is intravenous administration of tissue plasminogen activator (tPA), which has been FDA approved within a narrow time window. Endovascular therapies utilizing intra-arterial mechanical or chemical thrombolysis also improve outcomes. After the acute time period, focused physical rehabilitation of the injured area is the primary current therapy that is proven to be effective (Veerbeek et al., 2014). Re-organization of the cortex has been observed with rehabilitation in pre-clinical models as well as in humans (Liepert et al., 2001). While rehabilitation can be effective, and encouraging results have been demonstrated with constraint induced movement therapy and other techniques (Hoare et al., 2007), the extent of neurologic recovery is still limited and novel approaches to augment or enhance the body's endogenous regenerative abilities are required (Table 1).

Restoring Circulation

Clinical treatments in use currently focus on restoration of blood flow to the penumbral tissue. Dendrites and their spine morphology are adversely affected by ischemia; however, recovery is possible even with severe ischemia if blood flow is restored quickly (Zhang et al., 2005). After many decades of pessimism surrounding stroke therapies, tPA given intravenously showed efficacy in a major clinical trial if given within 3 hr of symptom onset (National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). More recently, intravenous tPA was shown to be advantageous up to 4.5 hr after stroke in a large European trial (Hacke et al., 2008). Endovascular interventions of mechanical and chemical clot removal are often used clinically and have shown great promise with recent clinical trials demonstrating benefit within the acute timeframe (Berkhemer et al., 2015). The use of noninvasive transcranial Doppler ultrasound-assisted thrombolysis (in combination with intravenous tPA) is also being tested in early phase clinical trials (Barreto et al., 2013). Given the variety of strokes and patient differences in collaterals and vasculature, selecting the correct patients may be critical for the ultimate success of these therapies (Liebeskind et al., 2014). Unfortunately, a vast majority of stroke patients are not able to receive the acute treatments because of the narrow time windows. Further investigations studying the inflammatory and oxidative stresses as blood flow is restored will also help elucidate how the brain heals from ischemic injury. Therapies targeting later time windows are also needed to help recover from tissue that has been damaged before blood flow can be restored.

Disruption of Injury Pathways and Neuroprotection

The peri-infarct region appears to contain the highest potential for plasticity after stroke, with factors promoting growth and axonal sprouting expressed in this territory (Carmichael et al., 2005). Minimizing the damage to these areas and maximizing the potential for restoration are the goals of many of the neuro-protective strategies. Mechanisms that degrade and remodel the extracellular matrix (ECM), such as matrix metalloproteinases, are also upregulated in this region (Zhao et al., 2006). Additionally, angiogenesis and neurotrophic factors such as BDNF, which likely promote plasticity after stroke, are modulated in the area surrounding the infarct (Clarkson et al., 2011). Despite their success in animal models, clinical trials for multiple neuroprotective strategies have proven uninspiring. Multiple explanations exist for this (Dirnagl et al., 1999). One reason is the inadequacy of current animal models. The human brain and response to injury is far more complex than in the rodent. The variability in human anatomy is also difficult to represent in mouse models genetically engineered to be identical. Additionally, young rodents are most commonly used in the laboratory setting due to expenses, although differences in response to stroke are seen between young and older animals. Another difference is that outcome measures can be much more precisely designed and evaluated in the preclinical setting compared with clinical scales that may not truly assess the subtleties of stroke phenotypes. Timing of these therapies is another critical component and is much more controlled in the lab environment compared with clinical application, where administration of a drug can be delayed for hours or days depending on the patient's presentation. A recent trial studying the effect of magnesium given acutely after stroke developed a pathway for rapid delivery of medications. In this trial, magnesium was given in ambulances before the patients arrived at

the hospital (Saver et al., 2014). Although this trial was negative, the methodology will serve as a guide for future trials whose treatment effect is contingent on rapid drug administration.

While affecting a single pathway in an animal model is sufficient to prevent injury, multiple pathways may need to be disrupted in humans to yield similar results. Mild brain hypothermia (33°C), which has become the gold standard for acute neuroprotection in rodent stroke models (Zhao et al., 2007), improves neurologic outcomes for patients with a particular type of stroke (global cerebral ischemia) secondary to cardiac arrest and neonatal hypoxic-ischemic encephalopathy (Shankaran et al., 2012; Bernard et al., 2002). Therapeutic hypothermia after cardiac arrest has now become a recommended guideline for clinical care (Peberdy et al., 2010). Mild hypothermia is currently being investigated as an acute stroke therapy, with trials to date proving the feasibility of this approach (Piironen et al., 2014). Another promising acute neuroprotective strategy targets the post-synaptic density-95 protein (PSD-95). PSD-95 connects NMDA receptors to signaling pathways necessary for the excitotoxic cascade and inhibiting these circuits reduces stroke volume in primates (Cook et al., 2012). A recently published prospective, randomized, double-blind controlled trial demonstrated safety and improved neurologic outcome and fewer acute infarcts in patients undergoing endovascular intracranial aneurysm repair who received a PSD-95 inhibitor (Hill et al., 2012). As barriers to accurately mimic clinical practice in the laboratory are reduced and the ability to manipulate multiple recovery pathways are improved, more effective neuroprotective therapies can be developed.

Cell-Based Therapies

Stem cell therapy is an exciting area of research that has entered the clinical arena with multiple ongoing trials. Stem cells are pluripotent or multipotent cells that have the ability to transform into multiple cell types and are self-perpetuating. Endogenous therapeutic strategies focus on increasing mobilization, longevity, and production of neural stem cells in the SVZ and dentate gyrus. Exogenous stem cell treatments refer to transplanted cells from another source into a patient. Exogenous stem cells have been delivered to the brain via the blood stream or direct transplantation and have shown great promise in animal models to enhance stroke recovery.

Endogenous Stem Cells—Neural progenitor cells (NPCs), which in health migrate along the rostral migratory system to the olfactory lobe and other brain regions, traverse to injured areas of the brain in the setting of neurological insult (Goings et al., 2004). Brain ischemia results in upregulation of endogenous NPCs and sometimes differentiation into the predominant cell type of the injured region (Arvidsson et al., 2002; Parent et al., 2002). Therapeutic approaches have focused on augmenting the brain's normal endogenous reaction to injury. Multiple pathways induce neurogenesis, including those triggered by numerous neurotrophic and growth factors such as GDNF, BDNF, granulocyte colony-stimulating factor (G-CSF), and insulin growth factor (IGF-1) (Kobayashi et al., 2006;Dempsey et al., 2003). Alternative mechanisms of increasing endogenous NPC proliferation include anti-inflammatory drugs like indomethacin, non-coding RNA, and hormones such as erythropoietin (Hoehn et al., 2005; Schouten et al., 2012; Wang et al., 2004). Delivering G-CSF and IGF-1 to alter key survival pathways such as the phosphoinositide 3-kinase-Akt

pathway are able to reduce NPC death (Lee et al., 2006). Current clinical trials are investigating the ability of G-CSF to mobilize endogenous bone marrow cells as well as utilizing its neuroprotective effects to determine efficacy in stroke recovery (Dunac et al., 2007; Kawada et al., 2006).

Increasing the numbers of migrating endogenous stem cells can be achieved using various chemokine receptors such as stromal derived factor1 and integrin β1 (Ohab et al., 2006; Yan et al., 2007), and manipulation of ECM components or electrical fields to guide endogenous NPCs has been investigated (Lee et al., 2006; Babona-Pilipos et al., 2011). However, currently this research has been limited to the preclinical arena.

Exogenous Stem Cells—Exogenous stem cells are typically divided into three categories: (1) immortalized cell lines, (2) NPCs or neural stem cells, and (3) bone marrow-derived hematopoietic/endothelial progenitors and stromal cells (Bliss et al., 2010). Immortalized cell lines have been developed from tumor cells or from manipulation with oncogenes (such as myc in the human fetal neural cell line ReN001 of ReNeuron). The NT2N cells, derived from teratocarcinoma, differentiate into post-mitotic neuron-like cells with the addition of retinoic acid and mitotic inhibitors (Andrews et al., 1984; Pleasure and Lee, 1993) and have been shown to improve outcome in several ischemic models (Saporta et al., 1999). ReNeuron's cells have shown dose-dependent recovery in stroke rodent models (Stroemer et al., 2009) and creatively have been engineered to be immortalized only in the presence of tamoxifen to reduce the risk of tumor formation (Stroemer et al., 2008).

Human NPCs are derived from embryonic and fetal tissue and have the ability to produce astrocytes, neurons, and oligodendrocytes (Gage, 2000). In stroke models, NPCs are able to migrate to the injured regions and improve recovery (Zhang et al., 2001; Reubinoff et al., 2001; Kelly et al., 2004). NPCs sometimes integrate into the host tissue and differentiate and can demonstrate neuronal characteristics, including expression of synaptic proteins, synapse formation, and electrophysiological properties (Bühnemann et al., 2006; Daadi et al., 2009a, 2009b).

Progenitor cells derived from bone marrow, umbilical cord blood and adipose tissue have all been shown to improve recovery in stroke models (Shen et al., 2007). Many of these sources are already used for the treatment of other disorders clinically such as malignancy and can be obtained from autologous harvesting. Although numerous cell types are included in each of these sources and it appears that the mononuclear or marrow stromal cell component mediates recovery, it is not clear which subtype is responsible for improving functional outcomes. Multiple trials have been performed or are ongoing using these exogenous stem cells (Table 2).

Induced Stem Cells—The discovery of induced pluripotent stem (iPS) cells created a paradigm shift in cell therapy. The ability to transform host somatic cells such as fibroblasts into pluripotent stem cells bypassed many of the concerns of traditional stem cell therapy, such as ethical discussions, supply limitations, and the possible requirement of immunosuppression (Meissner et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). Further development has led to vector- and transgene-free techniques to derive iPS

cells that improve functional outcome after brain ischemia (Mohamad et al., 2013). Recently, it has been possible to generate neural cells directly from mouse or human fibroblasts using transcription factors, without passing through a pluripotent phase, which may ultimately have clinical relevance (Pang et al., 2011).

Stem Cell Mechanism—The precise mechanism of action of stem cell therapeutics remains elusive. Until mechanisms are better understood, more intelligent design of trials and applications will be limited. The ability to fabricate and secrete trophic factors is common to all stem cell types and may create the optimum environment for stroke recovery (Bliss et al., 2010). Enhanced recovery most strongly was associated with a reduction in apoptosis in a recent meta-analysis evaluating preclinical stem cell studies (Janowski et al., 2010). Stem cells' role as local or systemic immunoregulators also may contribute to their ability to improve stroke recovery by decreasing inflammatory effects (Horie et al., 2011). NPCs and bone marrow-derived stem cells augment post-stroke plasticity through upregulation of synapse formation, dendritic branching, and axonal connections (Liu et al., 2008; Andres et al., 2011). Stem cells also enhance angiogenesis and blood-brain barrier repair, which have also shown to improve recovery (Chen et al., 2003; Horie et al., 2011). Although small numbers of transplanted cells may integrate into tissue, the extent of behavioral improvement does not appear to correlate with the number of cells. Additionally, the timing of synapse formation does not always correlate with functional improvement (Song et al., 2002; Englund et al., 2002). Given the complex pathophysiology of stroke, the importance of timing on the effects of factors, and the balance of signals in the pathways of recovery as described above (Figure 2), it is essential to better understand the mechanisms of improvement following stem cell therapy in order to translate these discoveries to clinical applications.

Delivery—While intravenous and intra-arterial techniques likely rely on inflammatory modulation or paracrine effects of the cells on the post-ischemic brain, invasive transplantation of stem cells provides a more direct route for cell-to-cell interactions as well as for the stem cells' trophic effects. More advanced delivery methods are also being developed, including bioengineered polymers to enhance stem cell survival and efficacy. Inert polymer matrices, such as hydrogels and particles, were first described for stem cell delivery (Teng et al., 2002; Zhong et al., 2010). The next step will be developing interactive polymers that are capable of communicating with stem cells in their transplanted environment to enhance recovery.

Clinical Trials—Apart from efficacy, safety is an important consideration to move forward with cell transplant therapy. Careful classification and understanding of the biology will be critical for reducing any predilection for tumor formation and adverse effects (Jandial and Snyder, 2009). The immortalized cell lines (NT2N) were the first human cells to be used in a clinical Phase I stroke trial and were implanted into the infarcted region of 12 patients, 6 months to 6 years after a basal ganglia stroke (Kondziolka et al., 2000). No significant adverse events occurred and functional improvement was seen in this small group of patients (p = 0.046). A subsequent Phase II trial with NT2N cells implanted into the peri-infarct or peri-hemorrhagic cavity showed no increase in adverse events (Kondziolka et al., 2005). An

open-label, single-blinded randomized trial using mesenchymal stem cells (MSCs) showed significant improvement in functional outcome based on the modified Rankin scale (a functional outcome scale with 0–3 being able to walk with varying degrees of disability) in the treatment group without a difference in adverse events, and multiple other trials have shown safety and feasibility (Lee et al., 2010; Bhasin et al., 2011, 2013). In addition, trials using bone marrow mononuclear cells (BMMNCs) have shown safety and feasibility in the acute and chronic phases of recovery (Friedrich et al., 2012; Savitz et al., 2011; Moniche et al., 2012). A phase 1/2A study that transplanted human modified bone marrow-derived stromal cells showed safety and feasibility of direct intracerebral transplantation 6 months to 5 years post-stroke, with improvement in neurological outcomes (Steinberg et al., 2014). The first neural stem cell trial for ischemic stroke (PISCES) has been completed with results showing promise. In this open-label, dose-escalation study, no adverse events have been observed among the preliminary results in 11 patients with follow up between 9 to 24 months, and functional outcomes were improved after transplantation (D. Kalladka et al., 2014, European Stroke Conference).

Multiple questions have been raised about translating cell therapy to clinical applications. Thus far, tumorgenecity of cell therapies has not been shown to be problematic. Demonstration of efficacy in randomized, double-blinded trials is needed, but numerous clinical trials are underway (Table 2) to determine whether cell-based therapy will become the next modality of restorative stroke therapeutics.

Modulating Circuits to Increase Stroke Recovery

A shift in the excitatory-inhibitory balance in neural networks across the brain occurs after ischemia. In the setting of a long-term depression of inhibitory signals mediated by gamma-aminobutyric acid (GABA) receptors in bilateral hemispheres, cortical hyperexcitability peaks several weeks after stroke and can persist for months (Buchkremer-Ratzmann et al., 1996; Schiene et al., 1996). Sustained increase in glutamate transmission for 4-weeks post-stroke also contributes to greater excitatory signals (Centonze et al., 2007). Modulation of the tonic inhibition regulated by GABA(A) receptors improves functional recovery in animal models (Clarkson et al., 2010). The unaffected hemisphere also can influence the excitatory state of the damaged hemisphere altering recovery (Murase et al., 2004). Further regulation and understanding of the excitatory-inhibitory balance may prove critical when designing therapeutic approaches for stroke recovery.

Stimulation Techniques—Cortical stimulation is an exciting area of research aimed at restoring this excitatory-inhibitory balance of the damaged brain and reorganizing neural circuitry to improve stroke recovery. It is another method targeting multiple signaling pathways as electrical fields are applied across large areas of neural tissue. Noninvasive methods (i.e., repetitive transcranial magnetic stimulation [rTMS] and transcranial direct current stimulation [tDCS]) and invasive methods (i.e., implantable epidural electrodes) exist. Initially, animal models showed functional improvement after stimulation of motor areas and later stimulation of connected but separate pathways have shown efficacy (Kleim et al., 2003; Machado et al., 2009). Utilizing the fact that high-frequency rTMS increases cortical excitability and low-frequency stimulation decreases excitability, ipsilateral or

contralateral stimulation has shown to increase functional improvement of the affected extremity for intermediate periods (Khedr et al., 2005; Kirton et al., 2008; Conforto et al., 2012). The role of the contralesional hemisphere after stroke is an area of continued interest and may have beneficial as well as detrimental effects on stroke recovery. The contralesional hemisphere likely can be recruited to improve recovery, but it also imposes increased inhibition on the affected hemisphere especially in the primary motor strip (Murase et al., 2004). This suppressive effect may be beneficial at reducing complications such as seizures, but may prevent plasticity and functional recovery. The majority of therapies aim to restore the excitatory balance between the two hemispheres in order to improve recovery. Stimulation with tDCS has found similar outcomes with improvement after stroke during therapies and for short durations after stimulation (Hummel et al., 2005). Recent Cochrane reviews of rTMS and tDCS both conclude that further studies are required to determine these techniques' role in stroke recovery (Hao et al., 2013; Elsner et al., 2013). Longer term efficacy is currently being studied with randomized, double-blinded trials to evaluate the utility of these treatment paradigms (Plow et al., 2013).

Invasive cortical stimulation offers the advantage of stimulus patterns of greater duration and at a more stable position. Given that multiple sessions of non-invasive stimulation produce longer sustained improvement, implantable electrodes provide a unique method of delivering more continuous or frequent stimulation (Khedr et al., 2005). Upper-extremity recovery is a significant limitation following stroke with only one-fifth of patients obtaining full recovery at 6 months (Kwakkel et al., 2003). Animal and pilot human studies have demonstrated improved recovery and safety with invasive stimulation techniques (Kleim et al., 2003; Levy et al., 2008). Based on these preliminary studies, the Everest trial was initiated using cortical stimulation combined with rehabilitation to improve upper-extremity recovery after ischemia (Harvey et al., 2009). Unfortunately, this study was discontinued prematurely by the company (Northstar Neuroscience). As we await the results of ongoing clinical trials, further pre-clinical research is underway to help determine the most efficacious locations and patterns for stimulation to improve stroke recovery (Cheng et al., 2014). A better understanding of the proper stimulation sites and paradigms should enable translation of this technique to the clinical arena.

Optogenetics—With the growth in the field of optogenetics, circuits in the brain can be more easily adjusted to tease apart mechanisms of recovery. Because stimulation can be targeted more precisely, the underlying circuits can be more carefully evaluated. Using optogenetic technology, it was discovered that even small ischemic injuries and depression in excitability could lead to relatively large effects on motor circuits (Anenberg et al., 2014). In addition to contributing to our understanding of the circuitry of the brain in the post-stroke environment, optogenetics also has potential as a therapeutic modality. Optogenetic techniques have been used to mitigate seizures and similar strategies could be utilized to alter neural excitability post-stroke (Paz et al., 2013). With the ability to stimulate specific circuits, optogenetics serves as another useful tool for stimulating defined neural pathways in particular brain regions to improve recovery. A recent study demonstrated that utilizing optogenetics to selectively stimulate ipsilesional primary motor cortex neurons after stroke improved functional outcomes in a rodent model, as well as modulated neurotrophic factors

in the contralesional cortex (Cheng et al., 2014). A greater understanding of the exact regions that are critical for recovery will allow stimulation to be more effectively tailored to augment recovery. The requirement of gene alteration for optogenetics currently limits it clinical applications, but as clinical gene therapy advances, the use of optogenetics to modulate recovery pathways will be more easily translated to help patients.

Altering Connections—With scientists focusing on the connectome and the complicated neural circuits that integrate in the brain to enable function, technologies that can alter these connections may eventually offer therapeutic applications. One such technology that is being investigated for multiple neurological diseases, including essential tremor, is magnetic resonance-guided focused ultrasound (MRgFUS) (Lipsman et al., 2014). MRgFUS combines advanced MRI and ultrasound technology to accurately focus ultrasonic energy to specific locations in the brain. Several advances make this technology possible, including (1) high-resolution brain and temperature mapping with MRI, (2) merging of software and a phased array, ultrasound transducer helmet to compensate for skull distortion, and (3) technology enabling precise focusing of the ultrasonic energy. MRgFUS allows for controlled thermal ablation of specific brain regions (Clement and Hynynen, 2002). As our understanding of the remodeling of circuits after ischemic injury (some beneficial and some maladaptive) improves, we will be able to apply technology such as MRgFUS to enhance functional recovery. Additionally, MRgFUS has been show to disturb the blood-brain barrier, which allows for therapeutic agents to more easily reach an infarcted area (Hynynen et al., 2001). It may also be possible to modulate circuits after stroke using stereotactic radiosurgery techniques, including CyberKnife, Gamma Knife, linear accelerator (LINAC) and cyclotron, which use focused radiation to place tiny lesions in brain structures. These therapies have already improved outcomes in select patients with Parkinson's disease, essential tremor, chronic pain, epilepsy, and mood disorders (Romanelli et al., 2013; Kim and Lee, 2008; Lad et al., 2007; Ohye et al., 2012). An innovative, non-invasive stimulation method utilizing vibrotactile skin stimulation is currently being planned for patients with epilepsy and may be applicable for stroke patients as well (D'Alonzo and Cipriani, 2012). While these technologies remain in their early stages with regards to clinical applications, their use can provide a method to determine how different brain connections affect recovery and ultimately begin to study their applications as stroke treatments.

Brain-Computer Interface—Given the elaborate networks connecting motor control regions of the brain to our muscles, several methods of stroke recovery look to bypass damaged areas of the brain and focus on healthy central or peripheral circuits. Because ischemia is usually an isolated event as opposed to an ongoing neurodegenerative process, many of the neural networks not affected by the infarct remain unharmed. Utilizing technology to harness intact circuits in the brain has led to the field of brain-computer interfaces. Through the study of extra-cellular potentials, researchers have deciphered cortical representation of motor movements (Moran and Schwartz, 1999). Often movements are controlled by interpreting cortical activity to induce movements in primates (Churchland et al., 2010), and more recently, cortical signals recorded through high-density microelectrode arrays or electrocorticography grids allowed paralyzed patients the ability to control robotic limbs and computer cursors (Hochberg et al., 2012; Collinger et al., 2013).

Alternative approaches stimulated pathways in the spinal cord to induce movements for walking and hand function (Moritz et al., 2007). Activation of spinal circuits has the benefit of triggering multiple muscle groups required to perform a certain task. Closed-loop systems have begun to explore the ability for primates to control limb function by utilizing cortical signals to stimulate spinal circuits to induce upper limb movements (Zimmermann and Jackson, 2014). In patients whose primary cortical areas have been damaged, alternative areas must be trained, or remaining electromyography (EMG) activity has also been used to trigger spinal cord circuits for the performance of certain tasks (Amsuss et al., 2014; Zimmer-mann and Jackson, 2014). Non-invasive methods such as electroencephalography (EEG)-based systems have also been implemented in neurorehabilitation programs, and as this technology is developed further, it may replace implantable arrays (Ang et al., 2014). These methods are still limited by complications in long-term tissue/electronic interfaces and our ability to accurately decipher integrated neural outputs of the cortex. As our ability to interpret cortical signals and robotics continue to advance, brain-computer interfaces offer exciting potential to restore function to patients with hemiplegia or language impairment from stroke.

Conclusions

Ischemic brain injury is a complicated disease affecting a variety of brain regions, resulting in disruption of numerous neural circuits and involving complex injury response. As we look to the horizon for stroke recovery and therapeutics, a more holistic approach may be required. Utilizing treatments that alter multiple cell injury pathways is likely needed to achieve clinically relevant improvements. Currently, cell therapies and stimulation techniques appear closest to clinical application, but many exciting therapeutic approaches are being developed. Advances in genomics, proteomics, and metabolomics will help to better evaluate patients and their individual response to treatments. A focus on modulating circuits that mediate recovery will become increasingly important, and the burgeoning field of brain-computer interface has the potential to have a major impact. As medicine becomes more personalized with a better understanding of genetic implications on therapeutics, stroke treatments will become more tailored to the individual patient and specific stroke types. More sophisticated clinical outcome scales to characterize patient deficits will allow for better evaluation of treatments translated from the lab. As is most often the case, new biomedical discoveries will continue to advance the field and elucidate mechanisms of injury to guide novel therapeutics.

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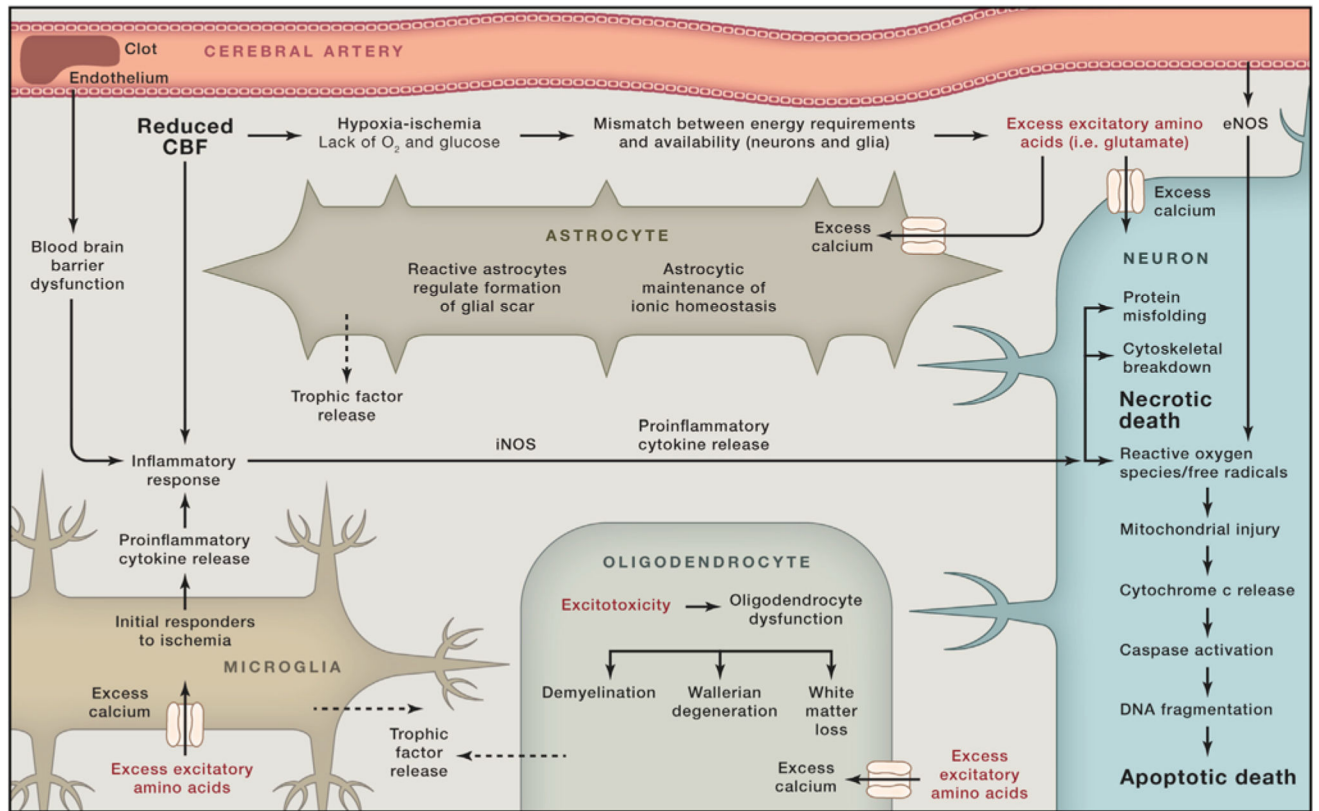
Figure 1.
Pathophysiology of Stroke
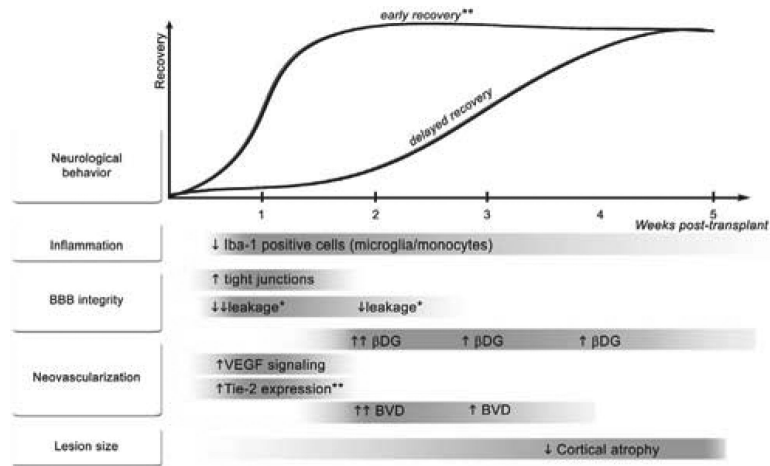
Figure 2. Temporal Profile of Changes Induced by Neural Stem Cell Enhanced Stroke Recovery
This figure was courtesy of Horie et al., 2011. Neurological behavior indicates motor recovery. The asterisk indicates that Avastin affected all parameters except for the ones marked; two asterisks indicate the inconclusive effects of Avastin. BBB, blood-brain barrier; βDG, β-dystroglycan; BVD, blood vessel density; Iba-1, ionized calcium binding adaptor molecule 1; Tie-2, a receptor tyrosine kinase; VEGF, vascular endothelial growth factor.

Table 1

Current Approaches for Stroke Therapeutics

Restoration of Blood Flow (Acute)
Intra-arterial and intravenous tPA
Mechanical thrombectomy
Magnetic resonance-guided focused ultrasound
Neuroprotection (Acute)
Hypothermia
PSD-95
Cell Replacement Therapies (Recovery)
Endogenous stem cells
Exogenous stem cells
Induced stem cells
Modulation of Circuits (Recovery)
Transcranial direct current stimulation
Transcranial magnetic stimulation
Optogenetic stimulation
MR-guided focus ultrasound
Stereotactic radiotherapy
Brain-Machine Interface (Recovery)
Cortical signals to induce movement
Spinal cord signals to induce movement

Table 2

List of 35 Completed or Ongoing Trials Using Exogenous Stem or Progenitor Cells

Clinical Trial Identifier	Study Type	Cell Type	Planned Enrollment	Timing of Delivery	Delivery Route	Status/Results
NCT00473057	Ph1-NR-OL	BMMNC	15	3–90 days	IA or i.v.	Complete, no reported results
NCT02065778	Ph1-NR-OL	BMMNC	30	chronic	IT	Complete, no reported results
NCT01501773	Ph2-R-OL	BMMNC	11	7–30 days	i.v.	Safe, feasible
NCT01849887	Ph1/2-R-DB	BMMNC	40	1–3 days	i.v.	Not currently recruiting
NCT00859014	Ph1-NR-OL	BMMNC	10	1–3 days	i.v.	Safe, feasible
NCT02425670	Ph2-R-SB	BMMNC	120	7–30 days	i.v.	Safe, feasible, no efficacy benefit
NCT01832428	Ph1/2-NR-OL	BMMNC	50	chronic	IT	Recruiting
NCT02245698	Ph1-NR-OL	BMMNC	200	subacute/chronic	IT	Recruiting
NCT02290483	Ph2-R-OL	BMMNC	76	1–7 days	IA	Recruiting
India, 2011	Ph1/2-NR-OL	BMMNC	11	3–12 months	i.v.	Safe, feasible, improved neurologic outcomes
NCT01436487	Ph2-R-DB	multistem	126	1–2 days	i.v.	Safe, feasible, no efficacy benefit
NCT02117635	Ph2-NR-OL	CTX0E03, NSC	41	2–3 months	IC	Safe, improved neurologic outcomes
NCT01151124	Ph1-NR-OL	CTX0E03, NSC	12	6–60 months	IC	Not currently recruiting
NCT01453829	Ph1/2- NR-OL	ASC	10	subacute	IA	Not currently recruiting
NCT01091701	Ph1/2-R-DB	MSC	78	<10 days	i.v.	Not currently recruiting
South Korea, 2010	Ph1/2-R-OL-SB	MSC	85	5–7 weeks	i.v.	Safe, feasible, improved neurologic outcomes
NCT00875654	Ph2-R-OL	MSC	30	<6 weeks	i.v.	Not currently recruiting
NCT01297413	Ph1/2-NR-OL	MSC	35	>6 months	i.v.	Recruiting
NCT01678534	Ph1/2-R-DB	MSC	40	<14 days	i.v.	Not currently recruiting
Japan, 2011	Ph1-NR-OL		12	1–4 months		Safe, feasible, decreased infarct volume
NCT01714176	Ph1-NR-OL	MSC	30	3–60 months	IC	Recruiting
NCT01716481	Ph3-R-OL	MSC	60	<90 days	i.v.	recruiting
NCT0146172	Ph2-NR-OL	MSC	50	1week to 2months	i.v.	Not currently recruiting
NCT01922908	Ph1/2-R-DB	MSC	48	3–10 days	i.v.	Not currently recruiting
NCT01468064	Ph1/2-R-DB	MSC, EPC	90	5 weeks	i.v.	Recruiting
NCT00761982	Ph1/2-NR-SB	CD34+	20	5–9 days	IA	Safe, feasible, increased β-NGF
NCT00950521	Ph2-R-OL	CD34+	30	6–60 months	IC	Complete, no reported results
NCT00535197	Ph1/2-NR-OL	CD34+	5	7 days	IA	Safe, feasible, reduced infarct volume
NCT01518231	Ph1-R-OL	CD34+	40	<12 months	IA	Recruiting

Clinical Trial Identifier	Study Type	Cell Type	Planned Enrollment	Timing of Delivery	Delivery Route	Status/Results
NCT01438593	Ph1-NR-OL	CD34+	6	6–60 months	IC	Not currently recruiting
NCT01310114	Ph2-R-DB	PDC	44	acute	i.v.	Stopped by sponsor
NCT01327768	Ph1-R-SB	OEC	6	6–60 months	IC	Recruiting
NCT01287936	Ph1/2-NR-OL	SB623	18	6–36 months	IC	Safe, improved neurologic outcomes
BB-IND 7082	Ph2-R-OL-SB	NT2	18	1–5 years	IC	Safe, feasible, improved neurologic outcomes in secondary endpoints
BB-IND 7082	Ph1-NR-OL	NT2	12	6–72 months	IC	Safe, improved neurologic outcomes

ASC, adipose-derived stromal cells; EPC, endothelial progenitor cells; NSC, neural stem cells; OEC, olfactory ensheathing cells; PDC, placenta-derived stem cells; SB623, human mesenchymal stromal cells; NT2, tetracarcinoma cell-derived neurons; P1, Phase 1 trial; P2, Phase 2 trial; OL, open label; R, randomized; NR, nonrandomized; DB, double blind; SB, single blind; i.v., intravenous; IA, intra-arterial; IC, intracranial.