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Cerebroprotection for Acute Ischemic Stroke: Looking Ahead

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Abstract

We search for ischemic stroke treatment knowing we have failed—intensely and often—to translate mechanistic knowledge into treatments that alleviate our patients' functional impairments. Lessons can be derived from our shared failures that may point to new directions and new strategies. First, the principle criticisms of both preclinical and clinical assessments are summarized. Next, previous efforts to develop single-mechanism treatments are reviewed. Finally, new definitions, novel approaches, and different directions are presented. In previous development efforts, the basic science and preclinical assessment of candidate treatments often lacked rigor and sufficiency; the clinical trials may have lacked power, rigor, or rectitude; or most likely both preclinical and clinical investigations were flawed. Single-target agents directed against specific molecular mechanisms proved unsuccessful. The term neuroprotection should be replaced as it has become ambiguous: protection of the entire neurovascular unit may be called cerebral cytoprotection or cerebroprotection. Success in developing cerebroprotection—either as an adjunct to recanalization or as stand-alone treatment—will require new definitions that recognize the importance of differential vulnerability in the neurovascular unit. Recent focus on pleiotropic multi-target agents that act via multiple mechanisms of action to interrupt ischemia at multiple steps may be more fruitful. Examples of pleiotropic treatments include therapeutic hypothermia and 3K3A-APC. Alternatively, the single-target drug NA-1 triggers multiple downstream signaling events. Renewed commitment to scientific rigor is essential, and funding agencies and journals may enforce quality principles of rigor in preclinical science. Appropriate animal models should be selected that are suited to the purpose of the investigation. Prior to clinical trials, preclinical assessment could include subjects that are aged, of both sexes, and harbor co-morbid conditions such as diabetes or hypertension. With these new definitions, novel approaches, and renewed attention to rigor, the prospect for successful cerebroprotective therapy should improve.

The search for stroke treatments began with the earliest physicians, seeking to alleviate functional impairments in patients with 'apoplexy'[1]. Remedies described in Greek, Roman, Persian, and medieval texts included manipulations of diet, herbs, and some surgeries[2].

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These treatments—which may seem naïve or whimsical to us now—were devised to address the known mechanisms of apoplexy: a lack of balance among the four humors. For example, bloodletting and purging gained popularity along with cranial cauterization to allow release of the bad humor. The search for stroke treatment as we know it began in earnest after two critical mechanistic discoveries: the ischemic penumbra and the excitotoxic hypothesis[3, 4]. Then, with the advent of recanalization therapies—thrombolysis and thrombectomy—the search for stroke treatment shifted from neuroprotection to treating reperfusion injury, including mechanisms related to free radical generation[5].

Yet the real story of neuroprotection lies not in the successful elucidation of the molecular mechanisms underlying ischemia/reperfusion, leading to the rational design of effective treatments. Rather, we now search for ischemic stroke treatment knowing we have failed—intensely and often—to translate mechanistic knowledge into treatments that alleviate our patients' functional impairments. No doubt, two millennia from now physicians will look back on our notions of ischemia and treatment with the same awe and bemusement we hold for Galen, Aristotle, and Avicenna.

What then are we to do next? Given our extraordinary track record (of failure), how do we re-focus and re-organize our search for ischemic stroke treatment? Sifting through the flotsam and jetsam of innumerable—really: too many to count—failed trials, can we learn anything that might guide future research? Perhaps lessons can be derived from our shared failures that may point to new directions and new strategies.

PRECLINICAL ℃ CLINICAL FAILURE

Several prior authors thoroughly documented the magnitude of our collective failure to find effective treatment besides recanalization for acute ischemic stroke[6–12]. A plethora of putative protective treatments emerged from laboratories; many qualified in Phase 1 and Phase 2 trials; and some proceeded to Phase 3 definitive trials where all disappointed. A cottage industry emerged to explain all these failures and the literature here is vast. To simplify: either the basic science and preclinical assessment of the candidate treatment lacked rigor and sufficiency, or the clinical trial design lacked power, rigor, or rectitude. Likely both are true: we probably neglected to properly assess candidate treatments at the preclinical stage, and we probably lacked the optimal approach to definitive clinical trials.

The principle criticisms of both preclinical and clinical assessments can be summarized (Table). Difficulties in selecting an appropriate animal model for qualifying a candidate treatment of stroke are reviewed elsewhere[6, 13–15]. Some limiting features of animal models are well known: studies typically include only young male rodents free of any co-morbid diseases while stroke patients tend to be older, with co-existing diseases that moderate stroke outcome, e.g., diabetes and hypertension. Although changing, there has been a tendency among preclinical investigators to test their candidate drugs early after stroke onset, while in clinical practice, patients present hours or many hours after stroke onset. Pressure to publish causes a positive publication bias, which has been well described and quantified[16]. Recanalization with thrombectomy can be easily modeled with transient occlusion of the middle cerebral artery (MCAo)[17].

Considerable confusion remains associated with the choice of endpoints, both in clinical and preclinical assessments (Table). Regulatory agencies require a demonstration of "substantial evidence to support claims of effectiveness for new drugs" (21CFR314.126 (a)). It is understood that effectiveness must be shown in terms of something the patient understands, e.g., survival or improvement in functional capacity. Usually, clinical trial protocols use the modified Rankin score as their primary endpoint because the mRS describes the patient's ability to care for themselves and accomplish activities of daily living[18]. The Rankin is widely accepted and understood, is easy to administer, can be administered by telephone, and performs well in clinimetric analyses[18–20]. Other outcome scales, however, could serve as well if they were widely accepted in the stroke community and quantified how the patient feels, functions, or survives[21, 22]. Functional rating instruments, such as the Rankin score, are mis-understood, and frequently criticized for being insensitive[21]. In fact, the modified Rankin holds great power to detect meaningful differences between treated groups in a clinical trial. On the other hand, volume of infarction—or its inverse, the volume of remaining intact brain—is intuitively and obviously important to the subject, and easier to measure quantitatively. In preclinical assessment, infarct volume is usually the primary outcome measure, although increasingly investigators are including behavioral endpoints in preclinical assessment studies[23]. To date, infarct volume has not been used as a primary outcome measure in Phase 3 clinical trials intended for regulatory licensure.

For the purpose of looking ahead to the next generation of stroke treatments, endpoints chosen for preclinical assessment must come into concordance with human clinical trial design. Almost certainly, the optimal approach will include both behavioral and histomorphometric measurements.

SINGLE-AGENT SINGLE-TARGET

In 1983 the term "Ischemic Cascade" appeared in print for the first time (that I could find) in the Neurologic Clinics, Volume 1, Number 1, although in that same year the word 'cascade' appeared in numerous symposium reports and review papers addressing cerebral ischemia and reperfusion injury[24]. For the next 20 years, countless drawings of the ischemic cascade appeared in print, always drawn with reverent arrows connecting disparate observations as if to imply a causal, orderly sequence. One such example appears in Figure 1, although in this version there was no attempt to communicate a causal sequence. Students and investigators came to believe in a cascade that had a beginning, middle, and an end. The search for stroke treatment turned entirely toward finding the 'master switch': the single molecular step that would control the ischemic cascade.

Study sections and journal reviewers insisted that putative stroke treatments should have a known mechanism of action, which should involve only one step or action. Drugs that harbored multiple mechanisms were derided as 'dirty drugs'.

The prototypical single-mechanism target may be the glutamate receptor, as in Figure 1. Recognizing that ischemia produced a flood of pre-synaptic glutamate release, it was determined that excess glutamate stimulation and neuronal depolarization led to elevated intra-cellular calcium that activated a variety of calcium dependent toxic enzymes[4, 25].

Receptor specific glutamate antagonists were found, or re-purposed, and rushed into clinical trials that failed[26–28]. Knowing that activation at the GABA receptor caused hyperpolarization that blocked depolarization-gated calcium influx, we showed that GABA agonists were as effective in animals models as glutamate antagonists[29, 30]. Again, however, clinical trials in patients failed[31–33].

Many investigators began to question the single-target, single-agent approach to stroke therapy. In an early stab at pleiotropic neuroprotection, we showed the combination of glutamate antagonists and GABA agonists could be used synergistically, although demonstrating true synergism required an attentive experimental design[34–36]. Others assembled ‘cocktails’ or combinatorial therapies, most of which looked promising in experimental models[37–39]. Acceptance of multi-targeted approaches lagged, however, at funding and regulatory agencies.

As the current investigative focus shifts from single-target to pleiotropic stroke treatments, a few obvious conclusions emerge from nearly 3 decades of preclinical and clinical stroke drug development. Firstly, the role that dogma played in limiting investigation must be admitted. Dramatic, beneficial results arising from combinatorial approaches were ignored or belittled in peer review and labeled as violations of agreed-upon convention: scientists must be driven by an understanding of the molecular and cellular mechanism of any candidate treatment. In contrast, many have pointed out that the most effective neuroprotectant in animal stroke models, therapeutic hypothermia, works by many mechansims[40–43].

The second important lesson from the single-target drug development years emphasizes scientific rigor. Most studies failed to consider key issues: randomization, blinding, sample size, and using appropriate statistics. Stroke treatment development has suffered greatly due to the failure to adhere to principles of rigorous design[13, 16, 44–46]. Important new initiatives are underway to correct this failure, and to require standards of rigor at journals and at grant review[47–49].

NEW DIRECTIONS

The graphic artist M. C. Escher specialized in complex drawings that appear banal until suddenly the viewer solves the optical illusion, and the drawing inverts into a completely distinct perspective. Similarly, we who search for stroke treatment require a novel perspective, a new viewpoint that will allow us to find a way past previous translational failure. We have learned much in our failures and we have progressed significantly. Although much of what we propose today will tomorrow go the way of bloodletting and purging bad humors, yet we can formulate some new directions and ideas, at least enough to get us started.

Nothing speaks to new perspectives so well as re-defining terms. In a separate publication, a STAIR (Stroke Treatment Academic Industry Roundtable) workshop on neuroprotection has proposed new terminology (Lyden et al, in press). The term neuroprotection, having outlived its usefulness, is proposed to be replaced with terms more specific and more appropriate. At

the earlier STAIR X, workshop participants proposed to rename the process of protecting the entire brain during stroke “cerebral cytoprotection”[50]. The term “neurovascular unit” was proposed to indicate the brain consists of several different cell types, each playing a unique role[51, 52]. In the NVU, consisting of neurons, astrocytes, endothelial cells, pericytes and other glia subtypes, each element plays a different role and there is considerable cross-cell communication[53, 54]. At STAIR XI, participants proposed to define cerebral cytoprotection in terms of the NVU. Preclinical and clinical research targeting neurons would be called “Neuronoprotection”; that targeting astrocytes “glioprotection”, and that targeting the blood brain barrier (BBB), “vasculoprotection”. Cerebral cytoprotection or just cerebroprotection connotes treatment designed to benefit the entire brain and presumably neurological function. But what good are new definitions if they do not influence or assist preclinical investigation? New investigative directions have opened, and novel insights gained, in response to our new understanding of the NVU. Three such insights include: our understanding of reperfusion injury, our understanding of NVU response to injury, and ‘help-me’ signaling in the NVU.

The first insight arising from a new appreciation of the NVU concerns the effect of reperfusion after a prolonged period of ischemia. Reperfusion affects all elements of the NVU and causes a new set of pathological mechanisms not found during ischemia without reperfusion (Figure 2)[5]. If the ischemia lasts long enough, then with reperfusion several deleterious effects result: astrocyte swelling, pericyte contraction, and platelet accumulation on the abluminal wall of the dysfunctional endothelial cell. Eventually, leukocytes adhere, clotting factors activate, and micro-clotting begins. Post-ischemic microcirculatory failure was identified in the 1960’s as the “no-reflow” phenomenon[55, 56], but in contemporary parlance we call it reperfusion failure. Using the Thrombolysis in Cerebral Infarction (TICI) scale, the extent of recanalization can be described[57]. After successful recanalization, any score worse than TICI-3 may include areas of poor capillary perfusion, which is an imaging approximation of no-reflow, i.e., failure to reperfuse the microcirculation downstream of the recanalized vessel. It is crucial in discussing these issues, by the way, to clearly define recanalization as the opening of a large, feeding artery; we define reperfusion as the opening of the microcirculation allowing blood to reach the tissue supplied by that feeding artery. This distinction is often confused in practice because microcirculatory reperfusion depends mainly on successful upstream recanalization. Augmentation of collateral flow is another way to improve perfusion, and does not require recanalization, so therapies targeting recanalization should remain distinct from those targeting reperfusion.

The availability of mechanical thrombectomy in clinical practice has not only saved thousands of patients but allowed investigators to define and understand the role played by reperfusion in mediating brain injury. Prior to the clinical deployment of thrombectomy, cerebroprotective therapies probably failed to enter the ischemic brain in large quantities. Collateral flow might carry some amount of the test agent into ischemic brain, but only recanalization allows proper delivery of the test agent in enough amounts to influence outcome. The first clinical trial of stroke treatment to enroll patients after mechanical thrombectomy was actually ongoing when thrombectomy received regulatory approval – the study was amended part way through[58]. Subsequently, contemporary clinical trial design allows—if not requires—enrollment of patients after documented recanalization.

An argument can be made that some cerebral cytoprotectants, by virtue of their mechanism of action, could preserve brain, pending recanalization. Therapeutic hypothermia, or specifically head cooling with local cooling devices[59, 60], lowers cerebral metabolic demand, and allows brain to survive pending recanalization or augmentation of collateral flow. So far therapeutic hypothermia has not succeeded in clinical trials[61, 62]. Transcranial near-infrared light therapy was another treatment proposed for ischemic stroke patients without recanalization, by providing light energy directly to mitochondria, thus preserving metabolic function until recanalization or augmentation of collateral flow[63]. Despite early promise, this treatment failed in a large, pivotal trial[64]. Undoubtedly, future, novel cerebral cytoprotectants will emerge, and some may likely prove useful, but for the foreseeable future, it seems prudent to require documented recanalization in the context of a clinical trial of a putative cerebral cytoprotectant.

A second insight following the definition of the NVU arose out of an attempt to understand the differences among the elements comprising the NVU. The different elements—neurons, astrocytes, pericytes, endothelial cells—differ markedly in their tolerance for ischemia[65]. The notion that some areas of brain, and some cell types, are selectively vulnerable dates back a few decades[66–68]. Direct comparisons of the various NVU cell types was accomplished only recently, and the mechanism for this differential vulnerability remains unclear[65]. The mechanism of regional selective vulnerability relates, perhaps, to differences in the ratio of excitotoxic versus inhibitory transmitter efflux during ischemia, called the excitotoxic index[66]. Regional selective vulnerability would depend on intrinsic differences in the tolerance to ischemia of each NVU cell type; on the regional variation in cerebral blood flow; and on the variation in the distribution of glutamatergic versus GABAergic receptors subtypes. In contrast, differential vulnerability depends solely on the innate resistance to ischemia in each cell type[65].

Regional selective vulnerability in the brain and differential vulnerability among elements of the NVU together imply many cautions while developing treatments for acute ischemic stroke. There is no doubt, for example, that some treatments targeting the BBB, i.e., vasculoprotectants, may impact neurons very differently[69]. It would be predicted that treatment dose and duration of treatment would differ[70]. Further, the time window in which treatment might be predicted to remain effective should differ considerably among different NVU elements.

Knowledge of the differential vulnerability among elements in the NVU yields a powerful tool that can be used to target therapies at different NVU elements. For example, if one were targeting neurons with a novel compound that was solely neuronoprotective, then using a lower dose very early after ischemia onset would preferentially benefit neurons. In contrast, vasculoprotective therapy might be given hours or days later, when post-ischemic BBB damage was evolving. Such targeting could avoid side effects that result from excessive dosing or excessive treatment duration[65].

A third insight to emerge from our new understanding of the NVU is the ‘help-me’ signaling concept. It is now very apparent that cell-cell communication occurs among the different elements in the NVU. Some of this communication proceeds via canonical synaptic release

of neurotransmitters. In the previous decade, non-canonical calcium flux among astrocytes was discovered and related to regional control of cerebral blood flow[71, 72]. Most recently, however, non-cell autonomous or paracrine communication among NVU elements has been demonstrated. In one direction, neurons seek assistance during ischemia or other insult, and in another direction, glia appear to secrete protective factors (Fig. 3).

Neurons seek assistance during ischemia by secreting activating substances that act on adjacent astrocytes (Fig. 3)[73]. The serine protease prothrombin is released from ischemic neurons and promotes astrocyte activation[74]. The resulting astrocyte activation stimulates gene expression changes consistent with the so-called protective astroglial phenotype, but also some genes associated with the toxic astrocyte phenotype. Activated astrocytes then secrete protective factors—that remain to be delineated—that protect neurons from ischemia[74]. Another help-me signal recently identified is estrogen[75]. Like prothrombin, under conditions of ischemia, neurons appear to secrete estrogen that activate adjacent astrocytes in a paracrine manner. Finally, the peptide lipocalin-2 was shown to activate neurons and microglia in a paracrine fashion[76]. Undoubtably other signaling molecules exist and future effort will be required to determine which are fully functional and relevant in human stroke or cardiac arrest patients suffering brain ischemia.

In response to neuronal ‘help-me’ signals, astrocytes respond with a protective response. Likely other elements of the NVU—notably microglia—also participate. The key components of the astrocyte protective response remain undefined and provide a rich opportunity for future pharmacological development. In a highly novel experiment, Lo and colleagues demonstrated transfer of mitochondria from astrocytes to injured neurons, with resultant salvage[77]. This truly remarkable observation will require further delineation, but potentially opens a considerable therapeutic opportunity. In response to ischemia, astrocytes also secrete a variety of peptides that are known to function as cytoprotectants, including growth factors. Likely, protection from injury utilizes the same functions that astrocytes serve during normal neuronal growth, survival, and synaptogenesis. Neurotoxic astroglial responses do occur as well, however, and astrocytes may contribute to the death of adjacent neurons[78]. In another recent, stunning observation, astrocytes were documented to phagocytose neurons in the adult hippocampus as part of activity-related pruning[79].

Clearly the role of astrocytes in protecting neurons requires considerable further exploration and definition. One immediate implication of these recent developments is that cerebroprotectant development must proceed cautiously, because treatments designed to benefit one element of the NVU may alter or even impede protective responses from other NVU elements. To demonstrate this pitfall, therapeutic hypothermia was tested for effects on help-me signaling and the astrocyte protective response[65]. Hypothermia is one of the most effective cerebroprotectant treatments ever studied in preclinical models of stroke and cardiac arrest[40, 80], yet clinical benefit in patients has been difficult to prove. Hypothermia significantly impaired the astrocyte protective response after neuronal help-me signaling[65]. This finding demonstrates the principle that neuronoprotective and glioprotective treatments may clash—thoughtful approaches are required to dosing and timing of new, candidate cerebroprotectants.

PRECLINICAL ASSESSMENT OF STROKE TREATMENTS

The search for effective cerebroprotectants requires an efficient and valid experimental paradigm[81]. Candidate treatments may emerge from an understanding of mechanisms—e.g., understanding the molecular and cellular pathophysiology of ischemia—or from agnostic pharmacological screening. By whatever route, investigators must show evidence that the candidate treatment shows efficacy. Much has been written about preclinical disease models, and stroke models in particular[6, 49, 82, 83]. Mostly, examining the models leads to more questions than answers, but a few key hypotheses are available for testing.

The first and most relevant question to ask concerns the purpose of the planned investigation. If an investigator wishes to test a hypothesis about the molecular mechanism underlying an aspect of ischemia, then models using OGD in cell culture or with brain slices are appropriate. Each element of the NVU can be studied in monocellular cultures, or together in co-cultures or transwells. The OGD model mimics ischemia sufficiently well that candidate treatments can be screened in these in vitro models. A key misinterpretation of this approach is that treatment of monocellular culture cells predicts the results of the assembled NVU in the whole brain. Rather, studies involving OGD reveal the isolated behavior of each cell type of the NVU. Ultimately, in vivo studies must be done to complement and confirm such in vitro work. Another key limitation of OGD in monocellular cultures is that the conditions resemble those of the ischemic core and not penumbra; in the penumbra residual blood flow may support cell survival and this aspect is difficult to model in vitro.

Despite the limitations of OGD models, they are simple (relative to an in vivo model) and rapid. Thus, OGD models allow high throughput screening of candidate treatments. A candidate treatment that benefits neurons during OGD would emerge as a neuronoprotectant, one that benefits astrocytes in monocellular culture a glioprotectant, and so on. Such studies allow for the determination of cell-type differences in dose, duration and timing of administration.

Whole animal stroke models employing young, disease-free animals can provide appropriate, efficient, and valid test environments, if the molecular process under study is known to act similarly with aging, sex differences, or in the face of co-morbidities. Here the investigator should choose thoughtfully. A variety of animal stroke models have been proposed over the years to simulate focal or global ischemia[82]. There are two main approaches: occluding the middle cerebral artery (MCAo) using a mechanical approach or using a thrombo-embolic approach. While a thrombo-embolic model may seem more ‘natural’ or in some way replicate human stroke more faithfully, in practice such models are highly variable and difficult. For studies of thrombolytic drugs, a thromboembolic model may be ideal[84]. On the other hand, if the investigative purpose is to demonstrate benefit of a candidate cerebroprotective therapy using as few subjects as possible, then a mechanical MCAo model is more appropriate; it has been suggested that the nylon filament MCAo model[85, 86] faithfully replicates the sudden, total recanalization seen during mechanical thrombectomy in stroke patients[5].

If the purpose of the planned investigation is to demonstrate efficacy, and perhaps safety, of a treatment candidate prior to a clinical trial, then additional considerations enter into choosing an animal model. In the ideal scenario, we would like a preclinical assessment that can reliably predict the outcome of human clinical trials. If we screen dozens of candidate treatments in a preclinical assessment, we would like to know which ones are most likely to succeed in a clinical development program that includes Phase 2 dose-finding and definitive Phase 3 trials in humans. This ideal scenario may be asking too much of preclinical modeling; we may find that preclinical assessment tools can establish efficacy, and perhaps safety, of a candidate treatment, but only the actual human clinical trials can establish benefit in human patients. At the very least, we would like a preclinical assessment that biases our selection of candidate treatments towards success in human clinical stroke trials, a process sometimes called de-risking.

To strengthen the preclinical assessment of candidate cerebroprotectants, many authors recommend essential changes to the traditional paradigm[13, 16, 44, 81, 87]. Age must be accounted for at some point in the development process, as there is no proof that cerebroprotectants that function well in young animals will also function well in older humans. Sex has proven to be a problem as well. For example, the cerebroprotectant drug tirilazad—after a long and expensive preclinical assessment—was found in human clinical trials to require different dosing in females[88]. Other drugs likewise may require different dosing in males compared to females[89, 90].

Some attention must also be given to assessing candidate cerebroprotectants in the setting of comorbid conditions such as diabetes or chronic hypertension. As yet, there is no consensus on the optimal approach to modeling the role of age, sex, and co-morbid conditions on the preclinical assessment, but the hypothesis is that such enhanced models will provide a superior approach. This hypothesis remains to be tested.

The National Institute of Neurological Disorders and Stroke (NINDS) issued a call (RFA-NS-18–033 and RFA-NS-18-034) for investigators to propose a multi-site network, called the Stroke Preclinical Assessment Network, or SPAN. The call followed an NINDS sponsored symposium that gathered a large number of experienced investigators who surveyed past failures and recommended future directions[48, 49]. The bulk of the innovations to be included in SPAN concern rigor and the reduction of bias. For example, SPAN will include centralized subject randomization, masking of the test compounds when they are administered, and blinded outcome measurement. Most importantly, SPAN includes 6 study sites, all doing the same stroke models and studying the same candidate cerebroprotectants. This network approach will avoid several sources of bias and harness the power of heterogeneity across sites to identify effective treatments[87]. It remains to be determined whether such rigor and heterogeneity proves effective in selecting candidate treatments for eventual success in clinical trials, i.e., de-risking.

PLEIOTROPIC AGENTS

Currently, a small number of candidate cerebroprotectants are under study in human trials[91]. Interestingly, in a recent review the authors included non-pharmacological treatments such

as remote ischemic conditioning and transcranial electrical stimulation, again testifying to the interest in pleiotropic agents that act via multiple—or even unknown—mechanisms[91]. The efficacy of conditioning for acute ischemic stroke has been reviewed[92]. While no evidence has yet emerged that ischemic conditioning benefits patients, significant gaps in our knowledge base prevent firm conclusions; further clinical trials are underway.

The ultimate example of a pleiotropic candidate cerebroprotectant is therapeutic hypothermia, which acts to interrupt a large number of death pathways in ischemia[41]. Although hypothermia has not succeeded in planned, clinical trials[61, 62], this was due to failure to recruit enough subjects and fear that prolonged, whole-body hypothermia might prove deleterious. Currently, focal cooling via the embolectomy catheter is under study[93]. Certainly more work will be needed to optimize the delivery of therapeutic hypothermia to stroke patients[65].

An example of a pleiotropic effect based on a single molecule concerns the effect of thrombin, a naturally occurring, blood circulating serine protease also called activated Factor II in the coagulation cascade. In addition to cleaving fibrinogen to fibrin, thrombin acts on the G protein-coupled receptor PAR (protease activated receptor), of which there are four main subtypes[94, 95]. PARs are found on neurons, astrocytes, and endothelial cells, although the effects on each cell type differ[69]. Thrombin activation of PAR1 leads to cytotoxic effects[96, 97]. In preclinical assessment, it was shown that the direct thrombin inhibitor, argatroban, powerfully ameliorated infarction and behavioral deficits after MCAo in animals[98]. A clinical trial of argatroban (NCT03735979) is underway in the StrokeNet. In a striking example of biased agonism, PAR1 activation by other serine proteases, e.g., activated protein C (APC) results in cytoprotective rather than cytotoxic effects[99]. Several laboratories have demonstrated significant and powerful benefit after MCAo using APC analogues, and a large, Phase III clinical trial testing the drug 3K3A-APC, which acts protectively on PAR1 has been proposed[95, 100–102].

In contrast to agents with pleiotropic effects, a single molecular target with multiple downstream effects is the post-synaptic density protein PSD-95. Specific agents that decouple this protein from its effector molecules were shown in preclinical assessments to be neuronoprotective[103]. Preclinical assessment of an agent targeting PSD-95 included a study in a gyrencephalic animal model[104]. Although a pivotal clinical trial that was properly powered failed to show benefit of the PSD-95 targeting agent nerinitide, follow up studies are planned that will target a potentially more appropriate subgroup of patients[105].

Interventions targeting neuroinflammation tend to mimic a pleiotropic agent due to the multiple feedback, feed forward, and cross talk loops in the neuroinflammatory response to ischemia[106]. Early efforts such as corticosteroids appeared to fail, although it must be said that steroids were tested prior to the advent of modern clinical trial design[107–109]. A biological agent targeting the ICAM-1 receptor should have prevented neutrophil entry into brain and reduce stroke related injury, but instead, patients appeared to worsen after anti-ICAM-1 treatment[110]. Other targets in the neuroinflammatory response to ischemia include IL-1, the IL-1 receptor, and IL-6[111, 112]. Inasmuch as a large number of receptors in neuroinflammatory pathways are tyrosine kinases, another example of a single-molecule

target with pleiotropic effects are the tyrosine kinase inhibitors[113]. Several inhibitors of tyrosine kinase are in clinical use for cancer treatment and many are being explored as possible cerebroprotectants. Cell based therapies, using a variety of engineered progenitor-like cells, or exosomes derived from such cells, illustrate another approach that may act on many targets[114–116].

Early studies that are pursuing mitochondrial transfer are based on the extraordinary finding that astrocytes and neurons exchange mitochondria[77]. The initial step in cell death during ischemia is the deprivation of glucose and oxygen, resulting in mitochondrial failure to generate energy[117]. Thus, it would make sense to target energy failure in stroke treatment, although energy failure occurs so early after blood flow interruption it may prove an unwieldy target. An initial preclinical study showed extraordinary success in salvaging neurons with astrocyte mitochondria[77]. A treatment using laser light to attempt to deliver energy (photons) to impaired mitochondria failed[64].

Pleiotropic biological therapies for stroke include the use of exosomes and microRNAs. Exosomes are an example of an extracellular vesical produced by exocytosis to transfer material between cells. Traditionally defined as 40–100 nm in diameter, exosomes generated from brain cells may contain peptides, lipids, RNA, or other undefined material. Although neuronoprotective and cerebroprotective effects of exosomes can be demonstrated, this treatment modality will require further development[114]. A huge number of microRNAs have been tested in stroke models, with wildly mixed results[118].

CONCLUSIONS

A generation of stroke researchers has grown up watching large clinical trials of cerebroprotective treatments fail, while simultaneously celebrating the success of recanalization therapies, thrombolysis and thrombectomy. Looking ahead, success in developing cerebroprotection—either as an adjunct to recanalization or as stand-alone treatment—will require new definitions that recognize the importance of differential vulnerability in the NVU. Success will require new focus on pleiotropic agents that act via multiple mechanisms of action. Renewed commitment to scientific rigor is essential to success, as embodied in the new SPAN effort as well as resolve among grant agencies and journals to enforce principles of quality in preclinical science. With these new definitions, novel approaches, and renewed attention to rigor, the prospect for successful cerebroprotective should improve.

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Non-standard abbreviations and acronyms

APC	Activated protein C
BBB	Blood brain barrier
GABA	gamma amino butyric acid
ICAM	Intercellular adhesion molecule
LD_{50}	50% lethal dose
MCAo	Middle cerebral artery occlusion
NVU	Neurovascular Unit
NINDS	National Institute of Neurological Disorders and Stroke
OGD	Oxygen glucose deprivation
PAR	Protease activated receptor
PSD-95	Post synaptic density protein 95
STAIR	Stroke Treatment Academic Industry Roundtable
TICI	Thrombolysis in Cerebral Infarction

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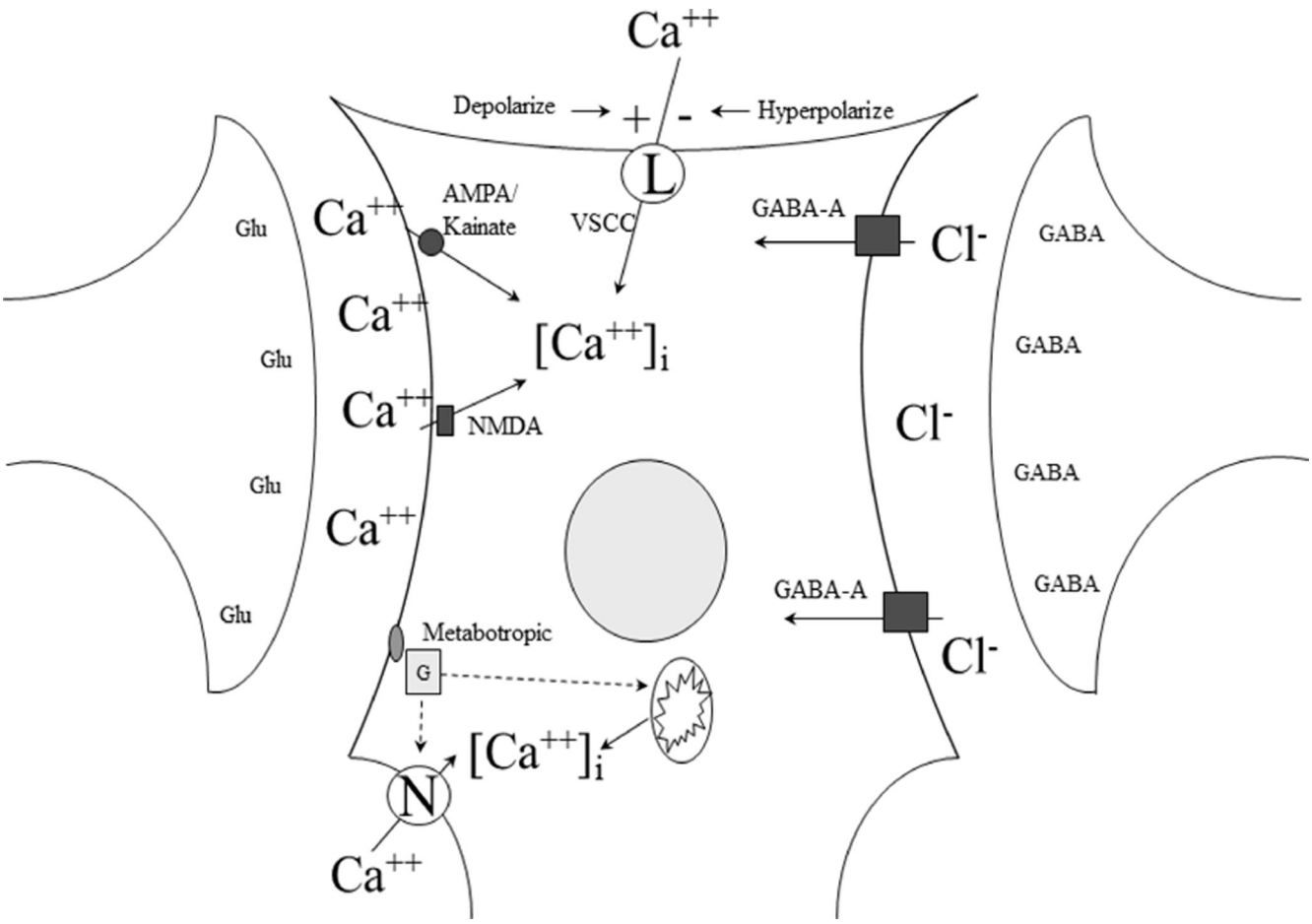

Figure 1. Excitatory and inhibitory influences on post-synaptic neurons.

In the development of neuronoprotective treatment, antagonists of the glutamate receptors succeeded in preclinical models. Antagonists targeting the NMDA, AMPA/kainite, and metabotropic receptors all failed in clinical trials. Agents acting on the voltage gated calcium, or L-type, channel are used in treating post-hemorrhage vasospasm, but did not succeed as cerebroprotectants. Agonists of the GABA-A receptor, though promising in preclinical assessment, failed in large, pivotal clinical trials. Figure from the author. Abbreviations: NMDA *N*-methyl-D-aspartate; AMPA α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid; GABA γ-aminobutyric acid

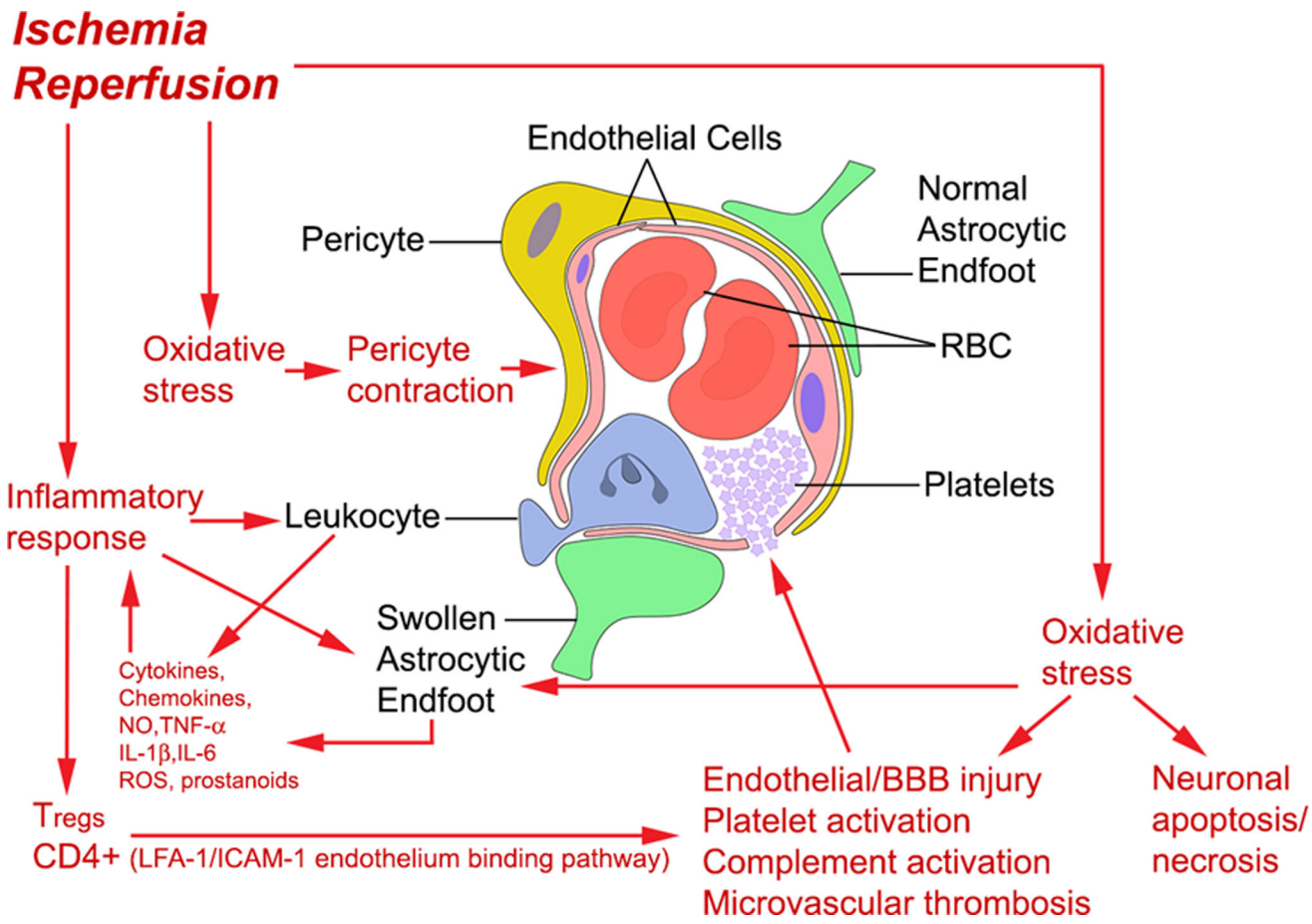

Figure 2. Reperfusion injury in the neurovascular unit.

The NVU includes neurons, astrocytes, endothelial cells, pericytes, among other cell types. During reperfusion injury, several processes occur to impede microvascular reflow as well as open the blood brain barrier. During reperfusion, impaired mitochondria generate oxygen and nitrogen free radicals that mediate cell injury pathways throughout the NVU. Injury to endothelial cells triggers platelet aggregation and microthrombosis that can exacerbate perfusion failure. Figure reprinted[5] with permission of Sage Publications.

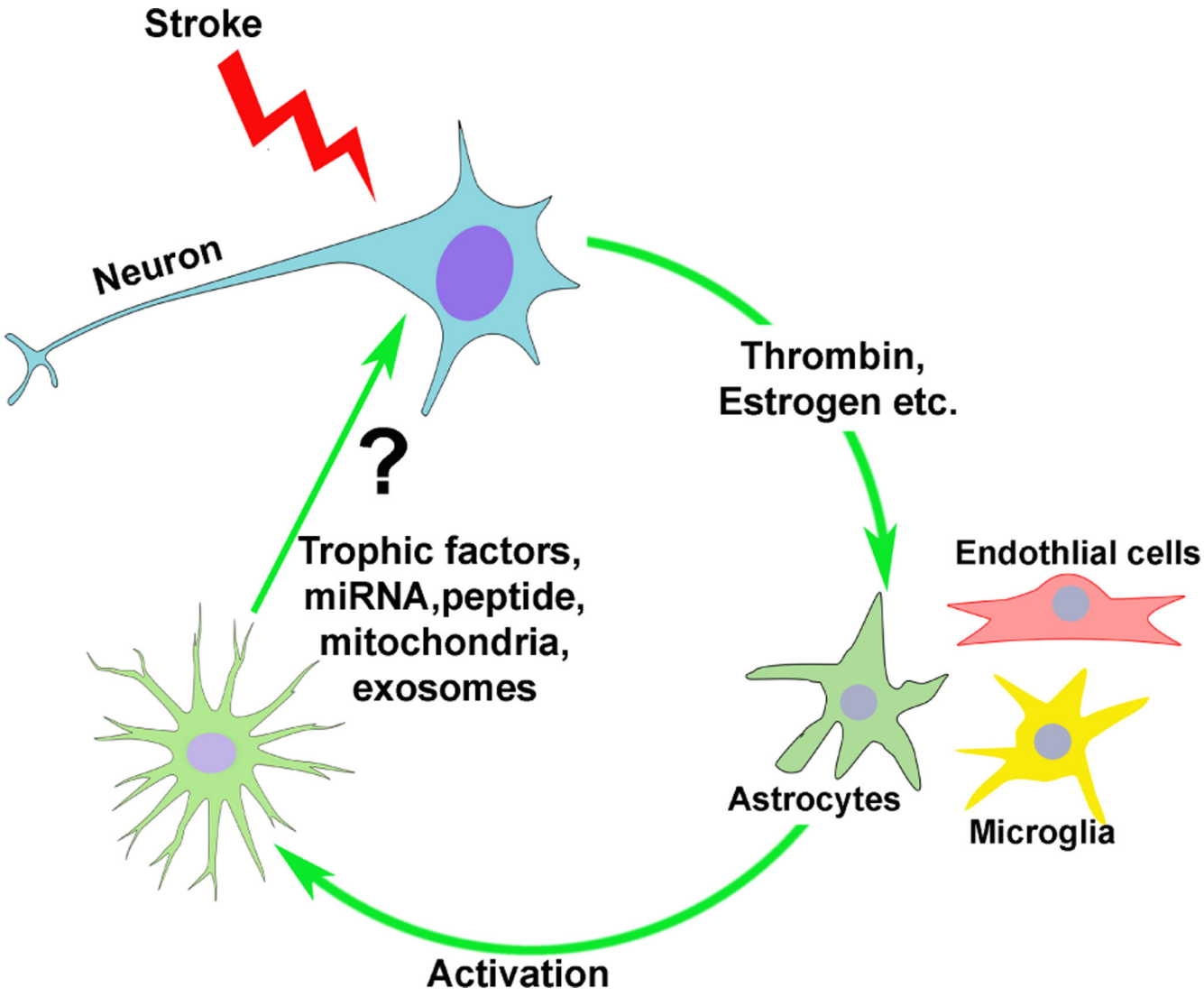

Figure 3. Help-me signaling in the neurovascular unit.

In response to injury, neurons generate paracrine signals that reach adjacent astrocytes and microglia, causing activation. Glial activation is pleiotropic, with some protective and some toxic responses. After activation, astrocytes generate paracrine factors that protect neurons from further injury, and promote regeneration. Figure from the author and Dr. Padmesh Rajput, PhD.

Table.

Summary of Issues in Preclinical and Clinical Assessment of Neuroprotectants.

The author's personal appraisal of the differences between typical preclinical and clinical investigations. These differences may partly help explain the failure to translate candidate cerebroprotectants from preclinical assessment to pivotal clinical trials.

Issue	Preclinical	Clinical
Time Window	Usually short	Usually long
Age	Usually young	Usually old
Sex	Usually male	Always both
Dose	Optimized	Limited by side effects
Validity	Publication bias towards positive results	Pre-specified clinical trial protocol
Reperfusion	Transient MCAo models	Endovascular therapy
Outcomes	Often lesion volume	Always behavior (modified Rankin)
Subjects	Usually homogeneous	Heterogeneous