

## Review

# Importance of Preclinical Research in the Development of Neuroprotective Strategies for Ischemic Stroke

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**IMPORTANCE** Preclinical stroke research has had a remarkably low translational success rate, and the clinical need for novel neuroprotective therapeutics has gone largely unmet, especially in light of the severe underuse of thrombolysis in acute ischemic stroke.

**OBJECTIVE** In this review, we aim to provide a brief overview of the commonly used stroke models, their merits and shortcomings, and how these have contributed to translational failures. We review some recent developments in preclinical stroke, providing examples of how improved study quality and the use of novel methods can facilitate translation into the clinical setting.

**EVIDENCE REVIEW** This is a narrative review of ischemic stroke neuroprotection based on electronic database searches, references of previous publications, and personal libraries.

**FINDINGS** The stroke research community has not been complacent in its response to criticism: preclinical stroke studies now demonstrate considerable rigor, standardization, and emphasis on minimization of experimenter bias. In addition, numerous innovative methods and strategies are providing novel avenues for investigating neuroprotection, as well as more extensive characterization of established models.

**CONCLUSIONS AND RELEVANCE** The improvements in preclinical stroke models and methods will make stroke research a good example for preclinical medicine, in general, and will hopefully instill greater confidence in the clinical community regarding which compounds are worthy of further investigation in a clinical setting.

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Stroke is a leading cause of morbidity and the fourth leading cause of mortality in the Western world.<sup>1</sup> The majority of strokes are ischemic in nature (85%), leading to infarction of tissue supplied by the occluded vessel: the core of the infarct suffers extensive irreversible damage, whereas the penumbra—characterized by diminished cerebral blood flow in the absence of detectable tissue damage—may be salvageable following reperfusion.<sup>2</sup>

One of the major goals in clinical neuroscience has been to develop neuroprotectants that would reduce or delay ischemic damage, thereby increasing the time available for imaging and thrombolysis. With the noteworthy exception of recombinant tissue plasminogen activator<sup>3</sup> (administered within 4.5-6 hours of stroke onset<sup>4</sup>), little preclinical research has translated into effective stroke therapies,<sup>5</sup> despite numerous conceptual advances from preclinical models (Figure).<sup>2,3,6-19</sup> This is perhaps surprising, given that, unlike many other neurological disorders (including Alzheimer disease or amyotrophic lateral sclerosis), the fundamental cause of pathology—diminished cerebral blood flow and resultant nutrient deprivation of tissues—is obvious in stroke and easily replicated in

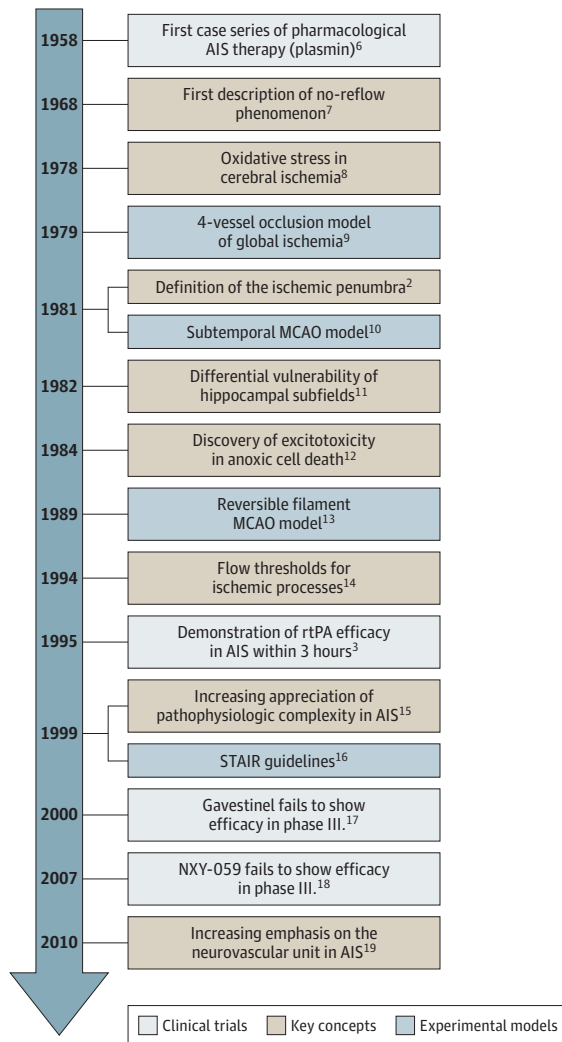
an experimental setting. Moreover, the protective effects of hypothermia in stroke can be seen as a physiological proof of concept for pharmacological neuroprotection.<sup>20</sup> Clearly, there are a number of factors that make it less straightforward to draw clinically relevant conclusions from preclinical research. Nonetheless, this is not universally accounted for by biological differences between patients and preclinical models, but is also substantially dependent on shortcomings in methodology, particularly the discrepancies between clinical and preclinical trial design,<sup>21</sup> which are amenable to improvement. This review aims to outline the general problems that have plagued the field of neuroprotection in stroke, while emphasizing the immense potential of preclinical stroke research and some recent advances that make achieving these goals more realistic.

## State of Ischemic Neuroprotection

### Stroke Models

Using the correct model is paramount for translational applicability. There are several commonplace strategies used to model stroke

**Figure. Time Line of Selected Historical Events and Conceptual Advances in Stroke Neuroprotection**



AIS indicates acute ischemic stroke; MCAO, middle cerebral artery occlusion; rtPA, recombinant human tissue plasminogen activator; and STAIR, Stroke Academic Industry Roundtable.

in vitro and in vivo (Table 1). In vitro models are more suitable for mechanistic studies—to elucidate the roles of individual cell types (neuronal, glial, vascular, or immune) or for target validation at the molecular level—owing to our ability to control the conditions, intervene more extensively, and use a greater variety of tools than would be possible in vivo. In these models, either cell cultures or organotypic preparations are deprived of oxygen and glucose, or exposed to excitotoxic agents (eg, glutamatergic agonists) to mimic ischemic conditions. The reductionist nature of these models comes at the cost of similarity to actual strokes: although simple to perform, the influence of different physiological and homeostatic factors on cell death cannot be evaluated.

In vivo models, blood supply to the brain is interrupted, leading to either global or focal ischemia (Table 1). Both 4-vessel occlusion and 2-vessel occlusion with hypotension models of global forebrain ischemia cause neuronal death in vulnerable brain areas (eg,

**Table 1. A Brief Overview of Experimental Models of Cerebral Ischemia Commonly Used for Neuroprotection Studies<sup>a</sup>**

Category of Model and Common Examples	Advantages and Disadvantages
In vitro models (cell cultures or organotypic slices): oxygen-glucose deprivation, metabolic-mitochondrial toxins, excitotoxicity (eg, glutamatergic agonists [NMDA])	Excellent for mechanistic investigations owing to the variety of tools that are available (eg, more amenable to knockdown/overexpression methods), reductionist in nature (enable study of cell-specific responses more conveniently than selective knockout animals), impossible to evaluate effects of physiology
In vivo models (global ischemia): 4-vessel occlusion (carotid and vertebral arteries), cardiac arrest, 2-vessel occlusion (carotid arteries) with hypotension	Very severe ischemia; allow examination of selective vulnerability in hippocampus, cortex, and cerebellum; replicate clinical cardiac arrest or strangulation rather than stroke
In vivo models (focal ischemia): intraluminal filament MCAO, direct MCA ligation/cauterization, photothrombosis, endothelin-1 injection, thromboembolism	Closest models of clinical stroke (particularly the permanent models), reliable and reproducible infarcts, transient or permanent, varying extent of surgical invasiveness (minimal for filament MCAO, more severe for direct approaches)

Abbreviations: MCA, middle cerebral artery; MCAO, MCA occlusion; NMDA, N-methyl-D-aspartate.

<sup>a</sup> See Traystman<sup>22</sup> and Macrae<sup>23</sup> for in-depth reviews.

CA1 of the hippocampus<sup>11,24</sup>) while sparing resistant areas (eg, the dentate gyrus and CA3). This differential response allows us to investigate the molecular determinants of either resistance or vulnerability in a given region, thus potentially uncovering endogenous neuroprotective strategies.<sup>24</sup> The main caveat of global ischemia is that its extent, severity, and duration do not match clinical ischemic stroke; instead, it replicates cardiac arrest.<sup>22</sup> Focal ischemia models typically involve the occlusion of one of the major cerebral arteries, typically the middle cerebral artery, using mechanical means, vasoconstrictors, or thrombi.<sup>23</sup> The most popular variant is the reversible intraluminal filament model,<sup>13</sup> in which middle cerebral artery occlusion is achieved using a remotely inserted filament that can be retracted to reperfuse the tissue.

### Reasons Behind Past Failures

Despite more than 1000 published preclinical studies and more than 100 clinical trials, previous successes in conferring preclinical neuroprotection have failed to translate into efficacious therapies,<sup>5</sup> an attrition rate that is probably further worsened by publication bias against negative results.<sup>25</sup> This reflects caveats in the design and conduct of both clinical and preclinical studies (Table 2). In particular, clinical studies have used dosing and treatment time windows not supported by preclinical studies.<sup>20,21</sup> Preclinical studies themselves have suffered from small sample sizes, insufficient statistical power calculations, and lack of randomization and blinding, all potentially leading to false-positive results.<sup>25</sup> The use of young, mostly male rodents of a similar strain does not encapsulate the heterogeneity of stroke patients, who are usually elderly with comorbidities severely impacting outcome. Critically, preclinical studies have relied mostly on histological end points demonstrating protection as reduction in infarct volume.<sup>21</sup> Even behavioral tests, predominantly used at early time points following ischemia, are not necessarily representative of clinical outcome measures of disability or dependence at 90 days after a stroke.<sup>21</sup>

**Table 2. A Comparison of Clinical and Experimental Stroke, With Suggested Modifications to Increase Clinical Relevance of Animal Models<sup>a</sup>**

Clinical Stroke	Preclinical Stroke	Potential Improvements	Caveats of Improving the Models
Typically >65 y	Typically young animals	Use of aged animals	Increased cost, time, mortality
Often extensively comorbid: hypertension, diabetes, hepatic disease, renal disease, cardiovascular disease	Typically healthy at time of stroke inducement	Use of comorbid animals: hypertensive rat strains, chemically induced diabetes	Increased cost, time, mortality; comorbidity models not necessarily accurate representations of clinical disorders
Great variation in site, duration, and extent of ischemia	Highly consistent areas of ischemia, targeting MCA in vast majority of experiments	Use of thromboembolic models, stroke-prone strains or transgenics (particularly in conjunction with comorbidities)	Current models are extremely well documented and widely used throughout the field, making conclusions more generalizable; targeting other arteries would be surgically more invasive.
Outcome measured in terms of mortality and functional impairment, on a chronic timescale	Outcome measured primarily in terms of histological or MRI changes (particularly in rodents), on an acute/subacute timescale	More extensive behavioral testing using clinically relevant tasks, longer survival periods following ischemia	Procedural confounders can complicate analysis: presence of neck and head wounds, ligation of arteries supplying cranial muscles, nerve damage
Dose and delivery optimization of putative neuroprotectants is limited; ethical concerns; patient availability	Wide scope for optimization of dose and delivery	Clinically relevant administration methods, based on ADME and toxicity data, need to be established preclinically.	Even if clinically inappropriate, experiments using large doses and pretreatment can provide insight into pathological processes and reveal further, potentially more accessible targets
Pretreatment is impossible (or, at best, very challenging and costly), except in very high-risk patient subsets	Pretreatment is widely used	Drug administration must be limited to clinically relevant time window (ie, $\geq 1$ h from occlusion)	

Abbreviations: ADME, absorption, distribution, metabolism, and excretion; MCA, middle cerebral artery; MRI, magnetic resonance imaging.

<sup>a</sup> Key characteristics of stroke patients are compared with in vivo models.

Another widespread caveat of preclinical studies is ignoring physiological variables that directly affect ischemia outcome. For example, the glutamatergic *N*-methyl-D-aspartate receptor antagonist MK801 mediates its protective effect by lowering body temperature rather than suppressing excitotoxicity, which is feasible in small animals but less so in humans.<sup>20,26</sup> Similarly, compounds that indirectly affect and improve cerebral blood flow (again including MK801) can confer protection by reducing the severity of the insult rather than exhibiting their purported pharmacological mechanism of action on the brain parenchyma.<sup>27,28</sup> There are also pharmacokinetic differences that need to be taken into account when translating findings to the clinical setting. This can be highlighted by the very promising neuroprotective free radical scavenger, NXY-059; although developed with a preclinical design that addressed many previous limitations, its ability to cross the blood-brain barrier (BBB) and exert its effects in the brain was never clearly demonstrated in humans.<sup>29</sup>

### Importance of Improved Preclinical Research

The failures described have been tremendously costly to the credibility of the field, but they also serve as guidelines for improvements in preclinical and clinical stroke research. It is essential that both preclinical and clinical research communities interact and coordinate greater methodological cohesion. Two notable ways in which preclinical research can be improved include (1) greater emphasis on good study design (eg, by adherence to Stroke Academic Industry Roundtable [STAIR]<sup>30</sup> guidelines) and (2) performing meta-analyses of existing data to reveal an overall drug effect.

The STAIR criteria are guidelines for preclinical stroke studies to improve the translational potential of neuroprotectants.<sup>16,30</sup> They address numerous shortcomings in categories such as blinding, randomization, exclusion criteria, sample size calculations, and trans-

parent reporting, and they propose ways in which these categories could be improved on. Despite widespread acclaim of such initiatives, most drugs that undergo clinical testing do not satisfy many of these criteria.<sup>31</sup> It must be noted that fulfilling all of these criteria does not necessarily translate into clinical efficacy, as no compound evaluated with STAIR criteria has thus far successfully translated into the clinical setting, most notably in the case of NXY-059.<sup>18,31</sup> This does not indicate that such guidelines are worthless but, rather, emphasizes the need for improved study designs and quality criteria. The STAIR criteria also refer to the choice of animal model: using aged or comorbid animals or determining outcome with standardized functional tests is thought to approximate clinical stroke more closely, although any animal model will remain an imperfect representation of stroke patients. These strategies have confounders of their own and are hampered by increased research costs, time constraints, and animal mortality (Table 2), but in the long term, high-profile phase III failures will be immeasurably more costly in terms of funding, lost time, and confidence in the concept of neuroprotection as a whole. In parallel, information can be gleaned even from models that fall short of the ideal: owing to the wealth of preclinical data and numerous studies on a multitude of compounds, a meta-analysis of multiple independent studies (thus representing a data set with fundamental heterogeneity and procedural differences) provides greater insight into whether a drug works reliably in a preclinical setting and, therefore, whether it should be taken forward into clinical trials.<sup>5</sup>

### The Road Ahead

Despite the skepticism that surrounds neuroprotection in acute ischemic stroke, there are nonetheless a number of promising candi-

date strategies that have incorporated many of the practices already described. In addition, the development of powerful new methods, such as proteomics and novel imaging modalities, have increased our understanding of the pathophysiology of stroke and have helped us to uncover putative target mechanisms that warrant further investigation.

### PSD-95

NA-1 (Tat-NR2B9c) is a peptide disruptor of interactions between glutamate *N*-methyl-D-aspartate receptor subunits, PSD-95, and neuronal nitric oxide synthase. NA-1 reduces overproduction of nitric oxide during ischemic excitotoxicity, thereby targeting a mechanism further downstream of the failed antiexcitotoxic compounds.<sup>32</sup> Following demonstrations of its neuroprotective effects *in vitro* and against middle cerebral artery occlusion in rodents, NA-1 was shown to reduce ischemic damage in nonhuman primate models.<sup>33</sup> A recent phase II clinical trial for patients undergoing aneurysm coiling (ie, at considerable risk of iatrogenic embolic strokes) demonstrated the safety of NA-1 and provided limited evidence for its efficacy.<sup>34</sup> Currently, there are no phase III trial data that would provide definitive conclusions regarding its clinical efficacy. Despite this, NA-1 is an excellent example of the rigor that experimental ischemic neuroprotection has adopted since the high-profile failures of the recent past, in its use of multiple *in vitro* and *in vivo* models, adherence to STAIR criteria, and use of trials featuring tightly controlled patient populations.

### IL-1

In addition to the ischemic cascade, there is an increasing appreciation of the stroke-induced inflammatory response contributing to tissue damage. Although these processes may also have implications for long-term remodeling, the main emphasis of experimental interventions targeting inflammation has been on suppression of the response. The best-understood example of this is IL-1 $\beta$ , a proinflammatory cytokine associated with tissue damage via both inflammatory and noninflammatory genomic responses in neurons, glia, and the vasculature.<sup>35</sup> Antagonism of IL-1 with neutralizing antibodies (IL-1ra, anakinra) or by targeting the signaling pathway downstream of the receptor has shown considerable promise in rodent models.<sup>35</sup> A phase II trial has also suggested that the compound is safe and that clinical outcome may be improved by use of IL-1ra.<sup>36</sup> Like PSD-95, the case for IL-1ra is strengthened by the multifaceted approach and adherence to STAIR criteria during its development, but phase III trials have not yet been conducted.

### Hamartin

A recent analysis of proteomic changes following 4-vessel occlusion in rats indicated that the selective induction of hamartin (TSC1) in CA3 cells was associated with their resistive properties to global ischemia and reperfusion.<sup>24</sup> Hamartin upregulation was also associated with protection achieved through preconditioning of the otherwise vulnerable CA1 cells.<sup>24</sup> Hamartin associates with tuberin to form the tuberous sclerosis complex, which acts as a tumor suppressor by inhibiting the mammalian target of rapamycin (mTOR) via its GTPase-activating protein activity toward Rheb.<sup>37</sup> A possible mechanism by which hamartin upregulation may afford neuroprotection is recycling of proteins through productive autophagy.<sup>24</sup> Hamartin is an example of a novel conceptual approach to neuropro-

tection—rather than targeting one or, at best, a few points in the ischemic cascade, it may be more efficient to upregulate multimodal endogenous neuroprotective mechanisms. However, therapeutic applications of this concept are not yet available, and its clinical feasibility is unknown.

### Beyond the Neuron

For many years, a neurocentric view of cerebral ischemia-induced brain damage dominated the field. However, the involvement of BBB (dys)function during ischemia is increasingly seen as crucially important. At the molecular level, the BBB is composed of brain endothelial cells interconnected by tight junction proteins, allowing for selective movement or transport of molecules into the parenchyma, as opposed to unrestricted diffusion. In addition to brain endothelial cells, other components of the neurovascular unit—pericytes, astrocytes, and microglia—play an essential role in the integrity of the BBB.<sup>38</sup> Under ischemic conditions, the integrity of the BBB is disrupted, leading to an increase in capillary permeability, extravasation of plasma components, and ultimately vasogenic edema. These conceptual advances have yielded novel targets such as matrix metalloproteinases, the inhibition of which can prevent barrier breakdown and improve outcome in models of focal ischemia.<sup>39</sup>

### Repair and Rehabilitation

An alternative approach to neuroprotection is neurorepair, in which the endogenous reparative mechanisms of the brain are stimulated. In response to brain injury, the brain attempts to self-heal by, at least partly, initiating neurogenesis, as well as production of protective mediators such as growth factors.<sup>40</sup> Both pharmacological and cellular strategies, or encouraging neurogenesis, have demonstrated reduced infarct volume and improved functional recovery in animal models of stroke when given 24 hours or more after ischemia.<sup>40</sup> However, the role of adult neurogenesis is much less clear in humans, raising doubts about the translational potential of such strategies.

### Novel Methods

In addition to these promising target mechanisms, the field of experimental stroke has also experienced a renaissance in novel methods that improve our understanding of the pathophysiology of stroke and our ability to evaluate neuroprotective drugs. Multiple magnetic resonance imaging modalities are applicable to stroke, in particular T2-, diffusion-, and perfusion-weighted imaging for determination of infarct size, location, and penumbra over time.<sup>41</sup> More specialized techniques, such as magnetic resonance spectroscopy for metabolic studies and diffusion tensor imaging for evaluation of white matter damage, allow for specific aspects of ischemic damage and neuroprotection to be evaluated *in vivo*.<sup>41</sup> The resolution of magnetic resonance imaging is relatively poor, but *in vivo* 2-photon microscopy has enabled us to examine individual cell and vessel responses to ischemia, as well as provided the means to occlude single arterioles and thereby model very small ischemic strokes.<sup>42</sup> On a molecular level, transcriptomic, proteomic, and metabolomic approaches have recently been applied in preclinical ischemic stroke to identify either stroke-specific biomarkers or molecular pathways relevant to diagnosis, prognosis, and therapy.<sup>24,43</sup> These can potentially provide an indication of how accurately pre-

clinical models correspond to the clinical picture, given that similar studies can be performed on peripheral blood and other samples from stroke patients.

Innovation continues to occur not only in the methods of investigation but also in the fundamental principles of preclinical study design. To bridge the gap between independent preclinical animal studies and clinical trials, multicenter phase III animal trials have been proposed.<sup>44</sup> These trials are not intended to replace the single-center preclinical efficacy and mechanistic studies, but to confirm the safety and efficacy of the most promising neuroprotective treatments. There are some legitimate concerns about this proposal—in particular, ethical issues relating to the large number of animals required and data handling questions—but it is nonetheless an exciting strategy to improve the translational success rate in stroke.<sup>44</sup> Ultimately, large data sets from preclinical phase III trials, meta-analyses of smaller trials, or experimental systematic reviews will inform us as to what might be working; however, mechanistic, small-scale proof-of-concept studies are needed to tell us the why and how.

Understanding the pharmacological and physiological mechanisms through which a compound exerts its effects before proceeding with clinical trials is just as crucial as meta-analysis of the preclinical literature if we are to avoid the same pitfalls that have proven so problematic in stroke research thus far.

## Conclusions

To a large extent, the problems discussed in this review were evident more than a decade ago<sup>21,45</sup> and have been reinforced by subsequent failures. It is nonetheless clear that these criticisms have not gone unheeded, and there is a notably increased emphasis on study quality within the ischemic stroke field. This, in conjunction with greatly improved tools and methods for investigating ischemic changes, raises the hopes for greater translational success rates in stroke, a disorder for which there remains an immense need for clinical innovation.

### ARTICLE INFORMATION

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