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Ischemic damage and neuronal stress responses: Towards a systematic approach with implications for therapeutic treatments

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Abstract

Based on Lipton's (1999) suggestion that ischemic brain injury shares formal features with dynamic systems theory, we explore algorithms that may aid in further systematizing ischemic injury with emphasis on therapeutic interventions. Present therapeutic alternatives include decreasing upstream damage or methods to effectively reduce ischemia. We suggest that neuronal stress responses provide an intermediate

layer between damage mechanisms and cell death pathways. We discuss schemes for mapping damage mechanisms to activation of neuronal stress responses. We develop the notion that ischemia, as a continuous input variable, leads to discreet output domains or phenotypes of post-ischemic neurons. The output domains are defined by the ensemble of stress response pathways activated and include homeostatic, preconditioning, delayed neuronal death and necrotic output domains. Any given stress response pathway can be further broken down into initiation, maintenance and termination phases. Together, the ideas of domains and phases of stress response pathways, provides a general framework for assessing the functional significance of any empirically observed stress response in the ischemic brain. Importantly, these notions provide a scheme to prioritize such changes in terms of their contribution to functional outcome in any specific domain. These general notions are illustrated by organizing empiric observations of the heat shock response following brain ischemia and reperfusion.

Abbreviations used

AMP kinase, adenosine monophosphate-activated protein kinase; CA, Cornu Ammonis; DND, delayed neuronal death; eIF2 α , alpha subunit of the eukaryotic initiation factor 2; HSF, heat shock transcription factors; HSP, heat shock protein; HSR, heat shock response; I/R, ischemia and reperfusion; PA, protein aggregate; ROS, reactive oxygen species; TPA, tissue plasminogen activator; UPR, unfolded protein response.

Introduction

In a comprehensive review of ischemic brain injury, Peter Lipton (1999) [1] suggested that a dynamical formalism will aid in the understanding of underlying mechanisms. To quote his major points on this issue:

“There are three aspects of ischemic cell death which suggest that a formalism that is applied to dynamical systems, in which attractors constitute stable states in a multidimensional space, will provide a useful framework. The first is that there is a very clear insult threshold. ... The second ... is that there is an early profound damage...If the insult is subthreshold, cells recover, whereas if it is superthreshold, they do not. ... The third ...is that the end stages of ischemic damage are metastable states that are very different from the normal state of the cell.

These properties suggest that ischemic cell death can be treated semi-formally, based on the formalism used for describing stable and unstable states of dynamical systems in terms of attractors...in which the emergent properties of networks are manifested.”

To our knowledge, no elaboration of this suggestion has been presented. But it is a powerful insight, and well worth exploring the utility of taking such an approach. The purpose of the present article is to use this suggestion as a point of departure. However, our goal is not to use any specific dynamical method to conceptualize ischemia damage, for our empirical understanding is not developed enough to allow application of specific quantitative dynamical methods. Instead, it is our purpose to begin the attempt to outline systematic algorithms or logical structures that may underlie ischemic brain injury, attempting to generalize key observations, such as those cited by Lipton, especially the notion of “thresholds”.

Additionally, we constrain our thinking to be of relevance to the development of therapeutic approaches. By no means do we feel the present work to be anything but a start in the direction suggested by Lipton. However, we do hope to illustrate that there is indeed utility in conceptualizing ischemic brain injury within a more formalistic framework. At bare minimum, the attempt helps to generalize and systemize the myriad of ostensibly unrelated empirical observations that presently dominate the field. Such generalizations can of themselves provide hypotheses for further empirical work. Given the rise of “-omic” technologies (genomics, proteomics, ribonomics, lipidomic, etc) that begin to allow empiric observation of complex multi-dimensional data-sets, there will be a growing need for increasingly rigorous treatments.

Conceptualizing the problem for therapeutic purposes

Much research in brain ischemia and reperfusion (I/R) injury has focused on identifying the mechanisms of cellular damage. The importance of this endeavor is self-evident. However, human clinical trials based on therapies directed against single specific damage mechanisms have failed [2,3]. In part this is due to the fact that ischemia triggers many upstream damage mechanisms, and so directing treatment at single mechanisms obviously does not prevent other damage mechanisms [4]. Methodological issues in translating laboratory findings to the clinic have also contributed to this failure [5]. However, we suggest a broader issue is at play here: how we formally conceptualize ischemic injury at the cellular level.

Current therapeutic approaches have operated under the perhaps implicit assumption that the damage mechanisms directly activate cell death pathways [6,7]. This assumption can be stated simply as follows:

Damage mechanisms → Activation of cell death pathways

This assumption leads to the idea that inhibiting the damage mechanism will halt activation of the cell death pathway.

However, this view fails to take into account the well-established fact that all cells, including neurons, have evolved means for coping with exogenous stressors [8,9,10]. Collectively, these coping mechanisms make up the repertoire of intracellular stress responses. So, a more accurate view of the neuronal response to I/R injury may be something along the lines of the following branching diagram:

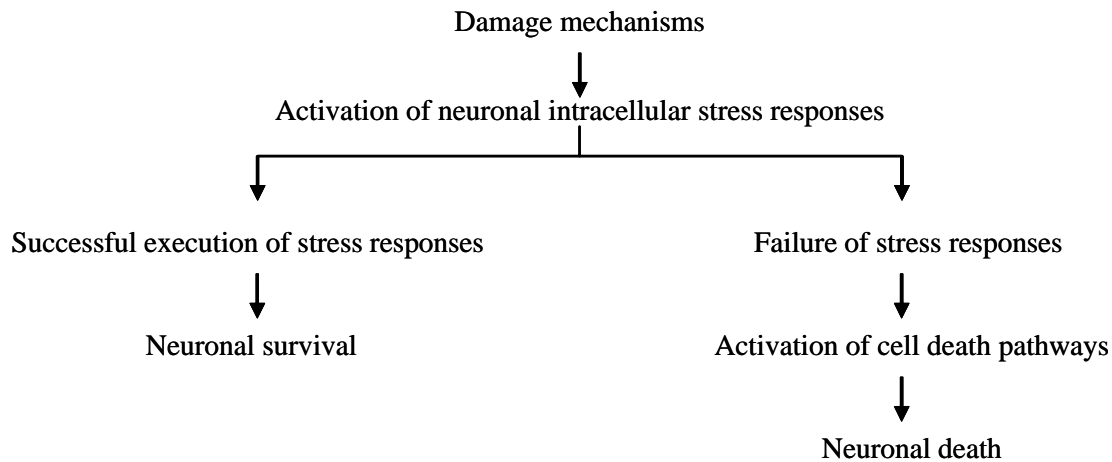


Figure 1. A model to conceptualize the relationship between ischemia-induced damage mechanisms and the role of neuronal stress responses in outcome.

In this view, we add an additional layer of regulation: neuronal responses to stress. Note that “responses” is plural; neurons respond in more than one fashion to ischemic injury. The idea is that failure of one or more neuronal stress responses activates cell death pathways, not the damaging stimuli. In this model, the damaging stimuli serve to activate intracellular stress responses. There is very strong evidence that successful execution of neuronal stress responses correlates with neuronal survival following I/R and that conversely, their failure is associated with delayed neuronal death (DND) [11,12]. The advantage of incorporating the idea of cellular stress responses into our view of potential I/R therapies is that it offers two major points of intervention where therapies should be of value: (1) inhibiting upstream damaging mechanisms, and (2) augmenting neuronal stress responses.

Inhibiting upstream damage mechanisms

When discussing inhibition of upstream damage mechanisms, there are two broad approaches: (1) pharmacologic inhibition of ischemia-induced cellular damage and (2) ways to effectively decrease ischemia.

Pharmacologic therapies directed towards inhibiting damage mechanisms possess the limitations discussed above, what could be called the “hydra

principle”. Cut off the head of a hydra and two more grow in its place. Inhibit only one damage mechanism, and the others will kill the cell. In principle, we could administer a set of drugs targeting all known damage mechanisms. However, this leads to serious problems: drug interaction issues, routes of administration, time of onset of effects, blood brain barrier issues; metabolic by-product effects, clearance issues and so on. Perhaps the primary weakness of this approach is that it is based on the unlikely assertion that we have identified all ischemia-initiated damage mechanisms. When seen in this light, the failure of single drug clinical trials directed against one specific form of ischemia-induced damage is not especially surprising.

The second approach to inhibiting damage is to effectively reduce the amount of ischemia experienced by the brain. This is the approach of TPA, the only clinically approved therapy for stroke [13]. TPA does not target specific intracellular damage mechanisms induced by ischemia. TPA effectively reduces the degree of ischemia by chemical recanalization [14]. Hypothermia may, in part, operate similarly. By lowering temperature, reaction rates are proportionally lowered, including the reaction rates of damage mechanisms, thereby effectively decreasing the amount of ischemia experienced by the brain. This view of hypothermia of course does not preclude the idea that hypothermia itself acts as a stimulus triggering endogenous cellular response pathways, akin perhaps to the hibernation state [15]. Also, resuscitation methods that decrease cardiac arrest time [16] or result in greater brain blood flow during cardiac arrest, such as active compression-decompression cardiopulmonary resuscitation [17], also effectively reduce ischemia. With respect to the latter, technologies aimed at improving CPR efficiency, and thereby increasing brain blood flow during the cardiac arrest period [18], are proving to be particularly effective at ameliorating resuscitation brain damage, particularly when coupled with hypothermia [19]. Thus, targeting ischemia reduction will prove important in the arsenal of technologies brought to bear on reducing reperfusion brain injury.

Augmenting neuronal stress responses

The second point of intervention: augmenting neuronal stress responses is most relevant in situations where targeting ischemia reduction is not clinically feasible, for example in cases of prolonged out-of-hospital cardiac arrest, or in penumbral neurons following stroke. The utility of bolstering intrinsic neuronal defenses is contingent upon our understanding of the intimate details of neuronal stress response, and how these play out following brain I/R. Our knowledge along these lines at present is incomplete. We know that post-ischemic neurons express stress responses, and that differences in stress responses correlate with selective vulnerability, but a full characterization is a work in progress.

Augmentation of neuronal stress responses perhaps underlies the effectiveness of neurotrophin treatments in animal models that show improved survival [20]. Growth factors will alter intracellular signaling pathways that are expected to impinge on intrinsic neuronal stress response pathways [21], possibly increasing their effectiveness [22]. Strategies aimed at taking advantage of preconditioning fall under this category as well. A preconditioning treatment will not reduce subsequent ischemia. Instead, it primes the neurons to better cope with the subsequent ischemia [23]. Similar to growth factor treatment, preconditioning alters intracellular signaling pathways and gene expression [24]. Again, these changes have by no means been fully characterized. However, one of the main working models in this area is that preconditioning serves to activate intracellular stress responses so that, when the subsequent “lethal” or “test” ischemia is applied, neurons have increased their capacity to cope with the damage induced by the “lethal” ischemia insult [25]. Some issues with a preconditioning approach to clinical therapeutics include identifying target populations, and developing effective methods for application of a preconditioning stimulus prior to a lethal brain ischemia episode. The “cross-induction” of preconditioning stimuli [23] suggests that pharmacologics may serve in this capacity, although no such drugs presently exist for humans.

Therefore, the notion of augmenting endogenous neuronal stress responses is not new to the field. The intent of the remainder of this article is to attempt to generalize aspects of current work in this area, and offer suggestions and considerations that may aid in the further systemization of our understanding of neuronal stress responses following brain I/R. This attempt is predicated on the assumption that a clearer understanding of these processes will lead to increased effectiveness in designing therapeutic strategies.

Neuronal responses to I/R injury

It is not our intention here to lay out all the complex molecular details known of post-ischemic neuronal stress responses. Many current reviews detail these and will be cited as appropriate. It is our intention here to generalize these separate aspects together into a larger picture, to provide a framework for understanding how targeting neuronal stress responses may be a useful therapeutic strategy.

A logical outline for conceptualizing the relevant issues is perhaps:

1. Identification of ischemia-induced damage mechanisms.
2. Identification of ischemia-induced neuronal stress responses:
 - a. Mapping of damage mechanisms to stress responses.
 - b. Distinguishing acute and chronic stress responses:
 - i Cellular effects of acute responses.

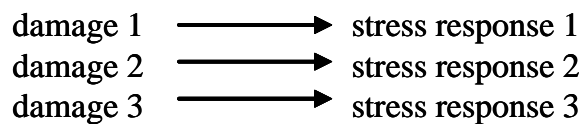
- ii Cellular effects of chronic responses:
 1. Transcriptional aspects of stress responses.
 2. Translation aspects of stress responses.
 3. Roles of induced stress proteins in abating damage mechanisms.
- c. Determine the causality of activated stress pathways in relation to outcome.

In principle, the intention here is straight-forward. Define the damage induced by I/R injury, how these activate the relevant intracellular stress responses, and characterize the intracellular stress responses in terms of short-term acute cellular changes, and longer-term changes requiring gene transcription and translation. The therapeutic relevance hinges on point c: determining which of the myriad changes causally contributes to post-ischemic cell death. Simple in principle, but the devil is in the details.

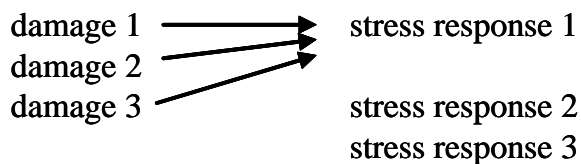
Mapping damage to stress response induction

Figure 1 leads to the hypothesis that the set of damage mechanisms will act as stimuli to activate corresponding stress response pathways. Therefore, we ask how the damage stimuli map to activation of stress responses. We can approach this mapping from a logical perspective (Figure 2). In a one-to-one

A. One-to-one mapping



B. Many-to-one mapping



C. One-to-many mapping

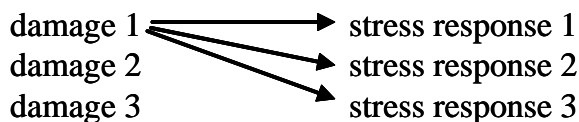


Figure 2. Logical possibilities for mapping I/R induced stresses and activation of intracellular stress responses.

mapping scenario, for every specific damage mechanism, the cell has a specific compensatory response. In a many-to one scenario, several damage mechanisms will converge to the activation of the same damage pathway. In a one-to many scenario, one damage mechanism will diverge and result in activation of more than one stress response. These logical possibilities, while providing a classification scheme, more importantly focus our attention on the nature of neuronal stress responses.

Clearly, the cell intrinsically partitions its stress responses. This partitioning may take the form of: (1) responses to final common forms of damage, or macromolecular-based response systems, and (2) organelle specific responses. However, the set of known neuronal stress responses is clearly less than the set of all potential damage mechanisms, suggesting that convergence is important, and that, generally, the one-to-one scenario is uneconomical.

Separate categories of stress response have been identified for specific macromolecular damage: DNA [26], proteins [11,81], mRNA [27]. These would represent many-to-one convergences in which many damage mechanisms converge to the same result. For example, heat stress, loss of ATP [28], free radical damage [29], altered pH [30], heavy metal toxicity [31], and ionic imbalances [32] all result in damage to intracellular protein complexes as a final common pathway, and all of these damage stimuli activate response systems related to maintaining protein homeostasis. The heat shock response (HSR) [31], the unfolded protein response (UPR) [33], and increased activity of the proteasome [34] are some of the intracellular programs that have been identified to cope with intracellular protein damage.

Stress responses also exist based on organelle compartment. Damage to cytoplasmic proteins more specifically triggers the HSR [31] and upregulation of the proteasome [34]. Endoplasmic reticulum stress triggers the UPR and the endoplasmic reticulum overload response [35]. Specific roles for mitochondrial in stress responses have been identified [36]. Damage to nuclear DNA (e.g. UV light, free radicals, etc.) activates DNA repair programs [37]. Organelle-compartmentalized stress responses would also represent many-to-one mappings: different mechanisms of damage converge to the organelle, resulting in activation of stereotyped organelle stress responses.

In spite of the obvious importance of the apparent generality of many-to-one convergences, some damage mechanisms trigger compensatory responses in a more one-to-one fashion. Osmotic stress triggers specific osmo-regulatory mechanisms in the cell [38]. Free radical damage activates transcription of antioxidant genes [39].

Therefore, while providing a classification scheme, the attempt to logically map damage to intracellular stress response activation is complex, and ultimately not highly informative. What is lacking in this view is a basis by which to assign functional significance with regard to outcome. A more

functionally geared scheme is required. Considering both stress input magnitude and the magnitude of the stress response pathway output get us closer to this goal.

Ischemic stress and neuronal response thresholds

We need to increase our precision as to the definitions of terms such as “stress” and “damage mechanism” when we invoke these terms. Clearly “stress” occurs along a continuum involving relatively harmless perturbations away from homeostasis at one end, to stressors resulting in lethal damage at the other end of the spectrum [40].

Consider first homeostatic perturbations. Low blood glucose activates an endogenous hormonal stress response involving glucagon, ACTH, epinephrine and cortisol release, but this system acts analogous to a *process control device* to maintain glucose homeostasis [41]. Similarly, the AMP kinase pathway appears to be an intracellular homeostatic process control system analogous to the whole organism endocrine glucose response [42]. Decreased energy charge activates AMP kinase, which in turn activates pathways to enhance ATP synthesis and decrease ATP usage [42]. Both the hormonal and cellular systems operate continuously, under nonlethal conditions where the change in the control variable (glucose or ATP level) is not necessarily lethal to the organism. However, prolonged glucose or ATP deprivation is obviously lethal.

Similarly, ion gradients are perturbed away from steady-state during ischemia. Below a certain threshold of ischemia, ion gradients rapidly recover with recovery of energy charge during reperfusion [43,44], and so might also be considered homeostatic responses in that a simple resetting back to steady-state occurs. However, over a certain threshold of ischemia, disturbance of steady-state ion gradients leads to osmotic effects that overwhelm the cells capacity to respond, contributing to cell lysis and necrotic death [43,45]. Therefore, both of these examples illustrate the importance of two concepts: (1) the *magnitude* of the stress, and (2) the *threshold of response* to a given magnitude of stress.

If we consider a complete ischemic insult which can be of a variable duration (something routinely generated in laboratories), then clearly the *quantity* of ischemia will be a graded and continuous input variable. The quantity of the global ischemia will simply be a function of its duration. However, the neuronal *response* to this graded input contains major discontinuities. While Lipton’s quote above talks about *a* threshold, in fact, at least three significant thresholds have been identified (Figure 3).

The first threshold is simply a perturbation away from steady-state and occurs in brain with very short ischemic durations (~1 min) [46,47]. Here, essential metabolites are momentarily decreased, and with reperfusion, rapidly

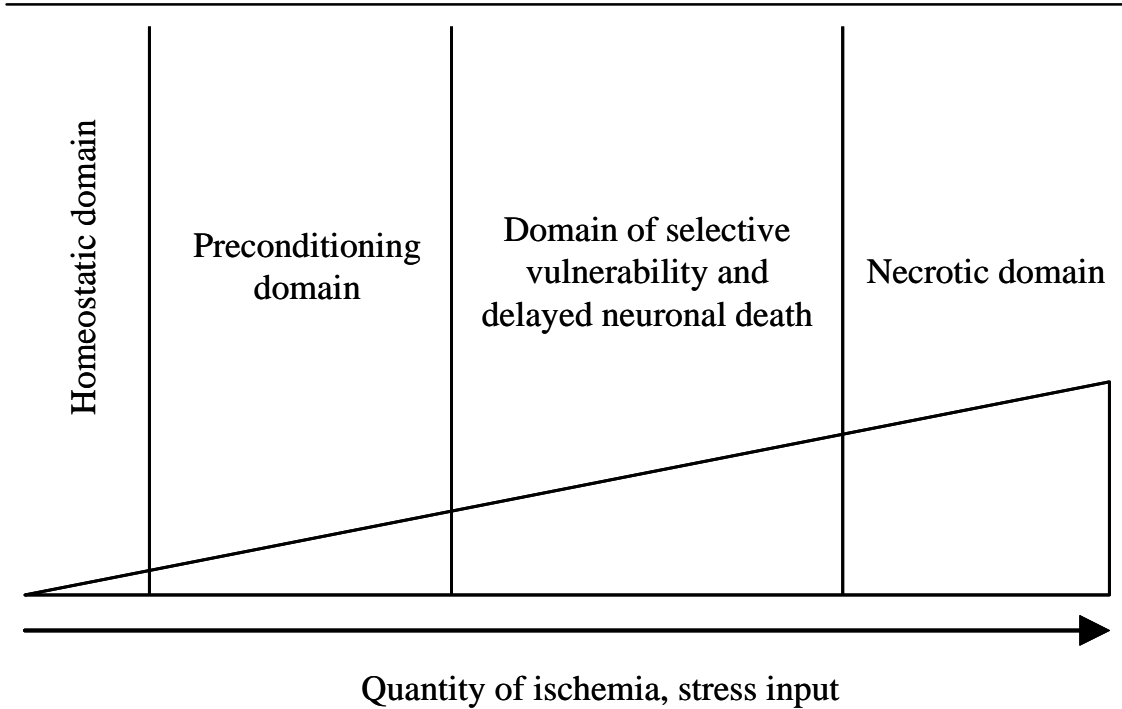


Figure 3. Although ischemic stress is continuous and graded, the response of neurons to the stress is discretely divided into regimes or domains with different functional outcomes.

restored. Responses below this threshold would constitute a purely homeostatic response. Below the second threshold, sublethal ischemia (< 5 min, dependent upon the vascular architecture) puts the cell in a protected “preconditioned” state [23]. The ischemia is intense enough to activate additional responses that appear to deem the input stimulus lethal. However, the stimulus is not in fact lethal, and the cell is left in a state where it is buffered for a time against further damage. Above the third threshold, the ischemic stimulus overwhelms the neuron’s compensatory responses, and the neurons die rapidly by necrosis as seen with the prolonged ischemia characteristic of stroke, resulting in a necrotic core [48]. Between the 2nd and 3rd thresholds is a regime in which selective vulnerability and DND occur. Within this regime, some neurons survive, but others eventually succumb to the ischemic input, giving rise to selective neuronal vulnerability [49,50,51].

Therefore, the four major regimes, or domains, illustrated in Figure 3 – homeostatic, preconditioned, DND, and necrotic – constitute the meta-stable attractor states of neurons in response to brain I/R injury. In more biological terms, these are discreet phenotypes that result from the graded ischemic input. As Lipton stated, this quantization of neuronal response is clear evidence for the operation of dynamical attractor systems in the post-ischemic brain.

Implications of response domains

The idea that the neuronal response to a graded ischemic input is discreetly divided into domains with different functional outcomes possesses implications for how we assign significance to specific pathways activated following brain I/R.

A first implication of thresholds of response is that, *within a given domain*, the set of pathways activated in that domain is expected to show graded responses. For example, in the nonlethal homeostatic domain, the amount to which ATP decreases is proportional to the degree to which ATP recovery and conservation processes are activated so as to restore steady-state ATP levels. The very definition of homeostasis is to maintain a steady-state. Therefore, stress responses in this domain in particular are expected to function exclusively in a process control fashion, similar to a thermostat [52]. Graded changes have been observed in the other domains as well. It has been shown that, up to thresholds of ischemia resulting in both preconditioning and DND, the degree of protein synthesis inhibition during reperfusion is a function of ischemia duration, as is the time it takes for translation to fully recover [53]. Also, the effectiveness of a preconditioning stimulus at subsequent neuroprotection forms a graded optimization curve as a function of the duration of the preconditioning ischemia and the time between the application of the preconditioning and lethal ischemia [54]. Thus, while the thresholds in Figure 3 set global discontinuities in the neuronal responses, within a given domain or regime, continuous variables have been observed, and this “within domain” continuity may apply generally.

Recognizing that, within a given domain, responses (outputs) are continuous and graded up to specific thresholds implies the importance of taking a quantitative approach to I/R studies, something that is not always rigorously pursued. Oftentimes, empirical readouts (e.g. caspase activation, specific transcription products, etc, etc) are assigned binary measures: they either occurred or did not occur. The future development of dynamical approaches to I/R injury will require a knowledge of the quantitative relation between input and output variables. More importantly perhaps, failure to capture a graded response when it occurs may lead to misleading conclusions regarding the relevance to outcome of the measured output. That is, it is possible that below a threshold the output has one functional consequence, and a different functional consequence above the threshold.

Second, we offer here the conjecture that, although there are discreet regimes of neuronal response, that the number of response pathways activated in any specific regime is cumulative. That is, the observed responses are those specific to that regime plus all of the previous regimes as well. Specifically, the homeostatic regime consists only of its specific process control pathways. The preconditioning domain will include pathways activated in the homeostatic

regime, plus the activation of new pathways that specifically define the preconditioning domain. The DND regime would then consist of DND-specific, preconditioning and homeostatic pathways, superimposed. By definition, in the necrotic regime, the cumulative neuronal responses don't matter because they are overwhelmed and the cells die.

This conjecture is the crux of assigning functional significance to any specific pathway activated following I/R. For each of the four domains is associated with highly specific net outcomes. It is to be presumed that any pathway specific to a given domain contributes to the net function of that domain. Therefore, this notion is of potentially great significance in that it provides a basis to disentangle the functional significance of any specific pathways activated following application a specific quantity of ischemia, by associating that pathway with specific contributions to outcome. Thus, a more natural type of mapping between the damage mechanisms and the activation of neuronal stress responses would entail: (1) a list of perturbations (or damage) associated with each domain, and (2) a list of response pathways activated within each of domain, and (3) an understanding of how a specific response pathway contributes to the net function of its domain.

An obvious and immediate question to arise from the conjecture of cumulative activation of response pathways is: how does a pathway act after the transition from its domain of origin into the next domain? On one hand, the pathway output may continue in a graded fashion right through a given threshold. On the other hand, however, carry over of a pathway from a previous to a subsequent domain may modify its function, and unexpected consequences, or emergent properties, may ensue.

For example, in the homeostatic regime, acute compensatory mechanisms will be activated to recover ATP levels. If ischemia duration drives the brain into the preconditioning domain, the ATP recovery pathways activated in the homeostatic domain may simply be more strongly activated and thereby confer additional ATP synthesis and conservation capacity, providing some of the protective characteristics of the preconditioning state. This may be particularly relevant to the short-term preconditioning characteristic of heart, as opposed to the delayed type preconditioning that is more prominent in brain [23,25]. This example illustrates how the function of a pathway could be unmodified across a threshold, and that its magnitude simply increases with increasing ischemia input.

A second example illustrates how a pathway's function may actually change across thresholds. It has been shown that brain preconditioning alters genetic programs such that, following the lethal stimulus, a genotype different from that observed with only the application of a single lethal stimulus occurs [56,57,58]. More specifically, results of microarray studies comparing brain gene expression following the "lethal ischemia only" condition with "lethal

ischemia after a preconditioning stimulus” show that preconditioning down-regulates some gene expression that occurs following a single lethal ischemic insult [56,57,58]. Mechanistically, this is explained by the activation of transcriptional repressors following preconditioning ischemia that inhibit gene expression following the lethal ischemia [56].

One possible interpretation of this proposed mechanism is that the induction of transcriptional inhibitors following preconditioning ischemia represents a transcription termination phase in the preconditioning domain. These transcription terminators however carry over into the DND regime following application of lethal ischemia, and result in repression of transcription in that phase as well, resulting in improved outcome, not teleologically, but perhaps accidentally, by suppressing transcription that may be injurious following lethal ischemia. This particular example is complex because the ischemia application is not continuous, it is discreet, but it serves to illustrate how the function of a pathways activated in a prior regime (preconditioning in this example) may change when carried over to a subsequent regime (the lethal ischemia, in this example).

Other variables affecting outcome

Although ischemia can be construed as a simple continuous input variable, other considerations muddy this view. There are clearly other variables that are expected to affect outcome following an I/R insult. First, as alluded to above, there is the possibility of different temporal patterns of ischemia, something particularly relevant when considering “real world” clinical ischemia [59,60]. Preconditioning does not functionally manifest unless a second “lethal” or “test” ischemia is discreetly applied subsequently, within a specific time window [23]. Additional complexity is involved when we link the quantity of ischemia to brain blood flow rates. That the term “ischemia” is applied to both very low blood flow (incomplete ischemia), and no blood flow at all (complete ischemia), is perhaps misleading, as the two conditions set up very different parameters impinging on the neurons (e.g. flow rate gradients and all that follow from them in terms of extra- and intracellular chemical concentrations) [61]. And of course, neurons do not exist in vacuo. The paracrine and other influences of neuronal synaptic contacts, and interactions with the glia and vasculature are currently areas of intensive research. Additionally, at the whole animal level, various forms of environmental enrichment alter post-ischemic behavioral outcome [62].

From a dynamic systems point of view, these variables represent the “boundary conditions” impinging on the neurons. Thus, a clear prerequisite to developing a systematic understanding of the neuronal response to ischemia must include not only a concept of the “quantity” of ischemia, but the temporal pattern of its application to the brain, the mode of ischemia, whether complete

or incomplete, a knowledge of how other cell interactions play into neuronal responses, and the effect of total brain (e.g. behavioral) activity. For a dynamical systems approach to bare fruit, a complete list of the different sets of boundary conditions is required. Clearly, at present, this would be a task of immense empirical complexity. Nonetheless, we already empirically know how the story ends: the four domains –homeostatic, preconditioning, DND and necrotic– are the final outcomes. Although complex, this array of boundary conditions must be constrained by and contribute to the outcomes we already know occur.

Summary: Stress and responses

Demonstrations of causality in brain ischemia research have proven elusive, to the detriment of therapeutic developments. The foregoing discussion highlights several considerations relevant to furthering the potential to treat ischemic brain injury. First is the recognition that specific response pathways are associated with specific regimes, and that the specific regimes have functional or phenotypic outcomes. Clearly, the homeostatic and preconditioning regimes are not lethal to neurons. Therefore response pathways associated with these regimes are not expected to be causal in neuronal death mechanisms, unless their function becomes modified when carried into the subsequent DND domain following a continuous ischemia period. By definition, the necrotic regime results in cell death no matter what the neuronal response. The only logical alternative to cope with the necrotic domain is to prevent neurons from entering it, which would entail effectively reducing ischemia, as discussed above.

When discussing the idea of mapping damage mechanisms to the intrinsic neuronal responses, the idea of regimes, domains, or phenotypes of response provides a template to organize empirical observations and to disentangle the multitude of changes that have been documented to occur in post-ischemic neurons. Thus, while a number of different ways to categorize the neuron's response to “stress” were discussed above, the idea of thresholds and regimes is perhaps most fruitful in providing a framework to prioritize any stress-activated pathways in terms of outcome.

Short-term & long-term stress responses

In the previous section, we spoke generally of “neuronal stress responses”. In this section we would like to further develop ideas related to the intracellular stress responses. There are two classes of neuronal responses that could potentially be activated: acute, short-term intracellular changes, and long-term chronic changes. The former is characterized by reversible post-translational modifications of already existing intracellular components, such

as enzymes, structural elements, or binding partner interactions. The later is characterized by changes in gene transcription and translation, and the long-term effect is due to changes initiated by the de novo synthesis of mRNA and subsequently proteins. These do not necessarily form nonoverlapping sets. Many acute post-translational modifications are upstream components of pathways that alter gene expression. Some acute post-translational modifications may even diverge and carry out in parallel both acute and chronic functional changes. An example of the later is phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2 α). Acutely eIF2(α P) inhibits translation initiation, but it also sets in motion cascades that alter gene expression in a set of processes known as the integrated stress response or ISR [33].

Therefore, the traditional way of characterizing intracellular stress response pathways in terms of acute and chronic, while critical for understanding the specific cascades involved, is not necessarily all that informative in general for understanding the contribution to outcome. We therefore now develop ideas that link pathway activation, whether acute or chronic, to outcome.

Formal structure of intracellular stress responses

We recently suggested that the inhibition of protein synthesis that accompanies specific intracellular stress response pathways can be subdivided into initiation, maintenance and termination phases [63]. We think it is fruitful to expand this generalization to stress response pathways as a whole. We do not here detail all the empirical evidence to support this suggestion, but instead point out that most responses in cells are: (1) initiated by some specific set of circumstances, (2) carry out a specific function for a duration in response to the initiating circumstances, and (3) are self-limiting, usually such that accumulation of a reaction product will terminate both the initiating circumstances and the response pathway itself.

Such logic clearly applies to stress responses. A damage stimulus activates upstream stress response effectors. The effectors induce biochemical cascades, resulting in reaction products that effect abatement of the damaging stimuli and/or effect cellular repair, and (3) with cessation of the damaging stimuli, there is usually a self-limiting mechanism that terminates the stress response. Importantly, it is becoming increasingly appreciated that unsuccessful execution of a stress response will activate pro-death pathways [64,65,66,67], again reinforcing the notion that death pathways are not necessarily directly activated by upstream damage mechanisms. As with the idea of outcome regimes or domains, the value of this idea is that, as an organizing framework, it allows us to assign significance to specific pathways. In short, we simply

propose to formalize the obvious: that stress response pathways have a beginning, middle and an end. Although obvious, this insight provides a simple yet powerful framework for hypothesizing about functional roles of specific pathways.

Ensembles of stress responses

The above concepts allow us to revisit Figure 1. In Figure 4, we have refined Figure 1 to: (1) account for the outcome regimes associated with increasing intensities of ischemia, and (2) added the fact that the stress responses possess initiation, maintenance and termination phases. This diagram indicates that a specific intensity of ischemia will drive the system into a specific regime. The regime in turn is defined by an enumerable set (e.g. a finite list) of response pathways that can be tagged as activated in the current or prior regimes. This enumerable set constitutes the *ensemble* of pathways activated by any given intensity of ischemia.

Each member of the set of activated stress pathways will then undergo sequential initiation, maintenance and termination phases. These are depicted as simply yes/no branch points. If all members of the activated stress pathways undergo completion of all three phases, then the given stress pathway has successfully run its course, and contributes to neuronal survival. However, what this diagram makes plain is the logical possibility that any given stress pathway could undergo disruption at any of its three phases, precluding the transition to the next phase.

A further branch point indicates the unsuccessful completion of a stress response pathway could either: (1) serve as a stimulus that activates a cell death pathway, or (2) predisposes the cell towards death simply for being incapable of carrying out its damage abatement and/or repair function. Presumably then, a balance is set between successful and unsuccessful pathways such that the cumulative failure of several stress pathways may tip the balance and lead to cell death. Whether incomplete expression of a stress response pathway activates a specific death mechanism or simply predisposes the cell towards death is something that needs to be decided by experiment for each relevant stress pathway.

Again, the value of this line of thinking is it allows us to assign significance in terms of outcome to a specific pathway and its phases. For example, in the DND regime, the enumerable list will include homeostatic, preconditioning and DND-specific pathways. Some of these can be excluded as significant to outcome because of their functions. Or alternatively, some of the prior regime pathways may be functionally modified so that they contribute, either positively (in the case of resistant neurons) or negatively (in the case of vulnerable neurons), to the outcome of the DND regime. Further, for each specific pathway, it can be empirically determined if they run their complete

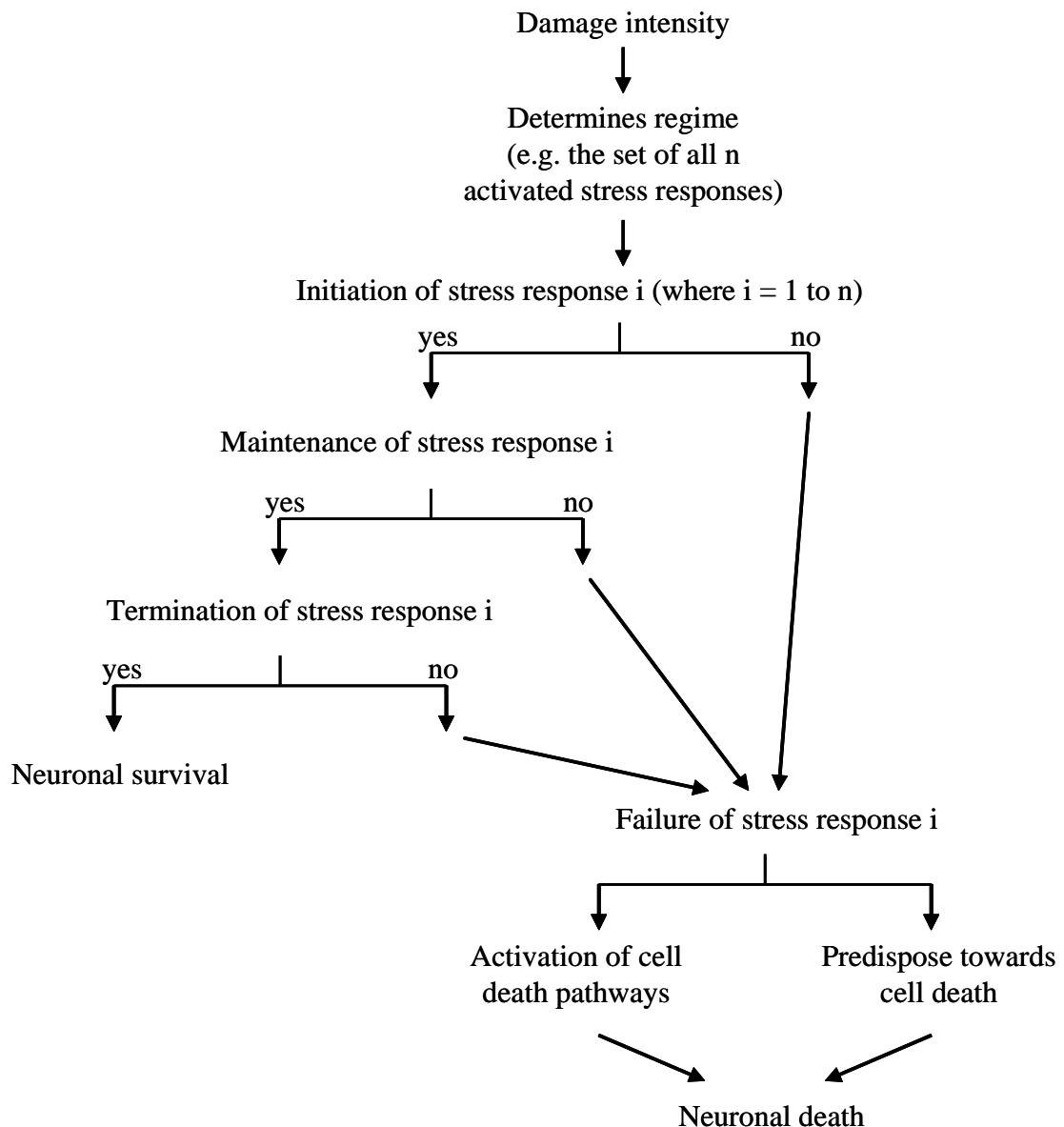


Figure 4. A more refined model to conceptualize the relationship between ischemia-induced damage mechanisms and the role of neuronal stress responses in outcome, modified to take into account the phases of stress responses and the idea that any of the phases could fail and push post-ischemic neurons towards cell death. Note that the sequence shown is intended to apply to each specific stress response in the fashion: from $i = 1$ to n , where n is the total number of activated stress responses, and i is any specific stress response.

course or if they do not. If they do not, then the phase at which a pathway halts can be identified, and the causes and consequence of that dysfunction can be identified. Ultimately, once specific pathways are identified in this fashion, they can serve as targets for therapeutic interventions, keeping clearly in mind

the intended effect on outcome. Hence, the model presented here implies a general method of targeted therapeutic design.

Of course, given the empirical complexity of the post-ischemic brain, by today's standards, such thinking may seem "pie in the sky". However, again, given the rapid growth and refinement of "-omic" technologies, coupled to the use of bioinformatics and network computational approaches [68], what today seems fantastically complex may tomorrow be mainstream. Hence, the value of the present approach is that it anticipates the day when such complexity will be empirically manageable.

Example: The heat shock response

We close out this article with an example intended to illustrate the notions developed above. We now turn attention to the expression of the HSR following brain I/R. We choose the HSR for the following reasons: (1) it is a clear-cut neuronal stress response activated by I/R brain damage, (2) the HSR shows significant differences depending up which regime the brain is driven into via the ischemic input, (3) in the DND regime there are large difference in how the HSR runs its course in ischemia resistant and vulnerable neurons that are relevant to cell death mechanisms, (4) this example has direct relevance in terms of therapeutic intervention and outcome management, and (5) the complexity of the post-ischemic HSR has been well documented, and we hope to illustrate that our approach provides increased ability to theoretically manage some of this complexity.

We begin by applying the general ideas of Figure 4 to a characterization of our current knowledge of the HSR as an algorithm. We then use this algorithm to organize the present body of data regarding expression of the HSR in post-ischemic brain, and illustrate that identification of the various phases of the HSR can serve as a basis for new empirical questions, and provide a platform that suggests therapeutic opportunities.

The heat shock response in algorithmic form

We now appreciate that initiation of the HSR involves nuclear translocation of the heat shock transcription factors (HSF) HSFs1-4 [69], which induces transcription of HSR genes [70]. In vertebrates, HSF1 mediates stress induced upregulation of heat shock protein (HSP) genes [71]. Molecular chaperones, including HSP90, HSP70, and Hdj1 maintain HSF1 in an inactive monomeric state; with accumulation of denatured cytoplasmic proteins, mass action causes the dissociation of HSF1-bound HSPs, resulting in nuclear translocation of HSF1 [72]. This is a precise example of cellular damage activating upstream stress response effectors.

Transcription and nuclear export of HSR-induced mRNAs then occurs. It is emerging that there is a complex regulation of HSR-induced mRNAs in the cytoplasm. First, shut of off translation [73] and selective sequestration and silencing of non-HSR mRNAs (e.g. housekeeping mRNAs) occurs [74]. This set of processes is linked to the selective translation of the HSR-induced mRNAs; the structures that sequester non-HRS mRNAs are thought to exclude HSR-induced mRNAs [74,75]. Once translated, HSPs function to ameliorate protein misfolding. Additionally, evidence suggests that the HSPs serve to: (1) inhibit their own transcription, thereby abating the HSR at the transcriptional level [76], and (2) also serve to recover generalized protein synthesis [75,77]. Finally, the HSR is known to induce cell death signals leading either to apoptosis or necrosis [78,79], illustrating the idea that it is not the damage mechanism that initiates the cell death pathway, but the stress response itself.

A sequence of the HSR in terms of initiation, maintenance and termination is illustrated in Figure 5. Note that within the greater HSR, we have depicted translation arrest itself as undergoing initiation, maintenance and termination phases as we previously described [63]. Several damage stimuli relevant to I/R injury are depicted, which will be discussed in the next section. The damage stimuli converge to a common end product: denatured or misfolded cytoplasmic proteins, which in turn activate the initiation phase of the HSR. The end product (or output) of the initiation phase of the HSR is the accumulation of HSR-induced mRNAs, and an acute inhibition of translation. The maintenance phase of the HSR is characterized by accumulation of HSPs, the expression of their biochemical functions and the maintenance of a general suppression of housekeeping translation. The latter we depict in terms of sequestration of housekeeping mRNAs in structures such as stress granules (although this needs to be empirically determined) [75]. The termination phase of the HSR follows from self-limiting mechanisms in which the HSPs shut off transcription of the HSR-induced mRNAs and induce recovery of general protein synthesis. Additionally, damaged proteins and the HSPs themselves are eliminated from the cell via increased proteosomal activity. The end result is elimination of the damaged cytoplasmic proteins and a resetting of the cell to its initial state before the damage.

Notice that this depiction of the HSR shows it essentially as a linear algorithm with two main parallel branches: transcription, translation and functioning of HSPs run concurrently with translational regulation; the points of interaction of these two branches (e.g. selective translation of HSPs, or the role of HSPs in translation recovery) are omitted for simplicity.

Heat shock response after brain I/R

The complexity of the post-ischemic HSR has been noted in terms of its dependences on ischemia duration, brain region, species dependence, which

HSPs are expressed in a given cell type, and whether or not HSR transcripts are translated [80,81]. We will not here attempt to address all of these aspects of post-ischemic HSR complexity. However, using Figure 5 as a template allows us to manage at least some of this noted complexity. First, we focus only on neurons, since these are the cells that succumb in the DND regime. Second, the reliance on ischemia duration and the differential response of brain regions both speak to the intensity of the insult and its role in the initiation of the HSR. Third, the well known fact that vulnerable neurons do not translate the HSR-induced transcripts can be understood as a failure of the maintenance phases of the HSR. Finally, for simplicity, and because these have been well-characterized, we use HSP70 transcription and translation as our main marker of HSR expression in the reperfused brain. Additionally, we assume that if HSP70 is translated, then its chaperone activity is functional. Admittedly, each of our starting assumptions has problems, but our justification for using them is that we have to start somewhere.

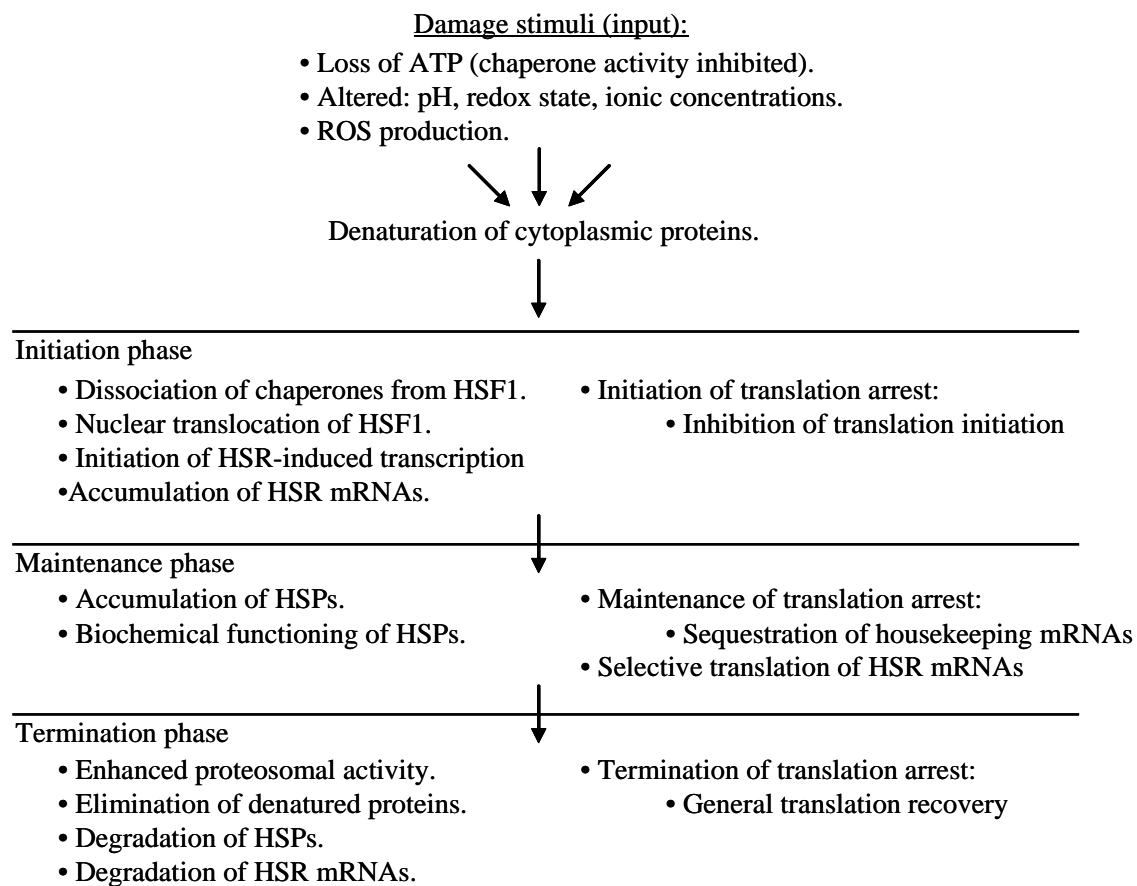


Figure 5. Schematic outline of the HSR in terms of (left) initiation, maintenance and termination phases. Right branch shows the role of translation arrest as a component of the HSR, also in terms of initiation, maintenance and termination phases.

A. Damage stimuli

Figure 5 depicts several forms of identified I/R damage that can converge to and result in disruption of protein structure and function. Loss of ATP, the core feature of ischemia, will cause inhibition of ATP-dependent chaperone activity. As chaperones play a crucial role in the folding of nascent peptides, it is expected that loss of ATP will result in a wholesale disruption of nascent protein folding in ischemic neurons [82]. A question that is as yet unanswered is to what extent this damage mechanism carries over in the reperfusion phase. ATP rapidly recovers following ischemia durations in the DND regime [43], and it would be expected that there would be a corresponding recovery of chaperone activity coincident with recovery of ATP. It would be expected, based on the considerations discussed above, that the amount of improperly folded proteins would be a function of the duration (e.g. the intensity) of the ischemia and be a graded and continuous variable.

Other perturbations associated with ischemia will also alter protein function, and possible structure. Disruption of intracellular ion concentrations and pH will affect protein activity, as protein activity is optimized for very specific pH and ionic strength values. If these diverge far enough from the optimum, they can indeed cause loss of protein structural integrity [30,32]. Again, these factors are expected to be graded in both input (e.g. degree of changes in H^+ or other ion concentrations) and output (amount of affected proteins). Finally, there is very strong evidence for bursts of reactive oxygen species (ROS) during both the ischemia and reperfusion periods [83], and proteins are well known to be targets of ROS [84]. Therefore, this representative, but not necessarily complete, list of damage mechanisms illustrates a many-to-one convergence to a final common pathway of intracellular protein damage.

B. Protein damage products

As per Figure 1 we see that the result of the damage stimuli is to produce intracellular damage to which the neurons will respond. We now know that the damaged proteins take a specific form in the cell [82,85,86,87,88,89]. It was originally discovered that free ubiquitin is rapidly depleted during reperfusion [90,91,92]. Via a number of lines of evidence, it has been shown that denatured proteins form relatively large cytoplasmic aggregates of ubiquitinated protein complexes [93,94,95,96,82,85,86]. These structures have been termed ubiquitin-containing protein clusters, or ubi-protein clusters. These have been shown to form in all post-ischemic neurons when exposed to durations of ischemia related to both the preconditioning and DND domains. They are strictly reversible following preconditioning [87,91,92,96]. In the DND domain, they are reversible in resistant but not vulnerable neurons [82,93,94,95].

In the DND domain, the ubi-protein clusters undergo divergent transformations in resistant and vulnerable neurons. They eventually disappear from resistant neurons, but they persist in a smaller, altered form known as protein aggregates (PAs) in vulnerable neurons [82,85,87]. The full extent of the difference in the composition of ubi-protein complexes and PAs is unclear. Our laboratory showed that ubi-protein complexes only weakly colocalize with the 40S protein S6 and the mRNA binding protein and stress granule component TIA-1, whereas PAs show a more substantial colocalization with S6 and TIA-1, within stress granules, at later reperfusion [97]. Clearly the ubi-protein complexes undergo some type of transformation in vulnerable neurons.

The discovery of both the ubi-protein complexes and PAs has substantial ramifications for understanding the HSR following brain I/R. That these are primarily cytoplasmic [63] indicates that they serve as stimuli to activate the HSR. The ubi-protein complexes form very rapidly in reperfusion [82,95], within the same time window as transcription of *hsp70* mRNA [98]. They additionally can serve as a marker for the effectiveness of the HSR insofar as an effective HSR outcome will lead to the elimination of these complexes. In cells known to translate HSP70 (e.g. dentate gyrus and CA3 neurons), the ubi-protein complexes disappear from the neurons [82] within the same time frame that high levels of HSP70 protein are found in the neurons [80,99,100]. Therefore, it is reasonable to presume that the ubi-protein clusters both activate the HSR and serve as the main substrate for HSR activity in the maintenance phase.

C. Initiation of the HSR

The main marker of the initiation phase of the HSR is accumulation of HSR mRNAs. Taking HSP70 as an important representative HSP, there are differences in accumulation of *hsp70* mRNA in the preconditioning and DND domains [80]. Following preconditioning, *hsp70* mRNA is most strongly expressed in vulnerable neurons, whereas its expression is minor or does not occur in resistant neurons [80,100]. Following DND levels of ischemia, there is copious expression of *hsp70* mRNA in both vulnerable and resistant neuron populations; however, the quantity of *hsp70* mRNA is ultimately much greater in vulnerable neurons [101]. From these data, one can conclude that the initiation phase of the HSR runs its course successfully in both the preconditioning and DND domains. This data is also consistent with the formation of ubi-protein clusters in both domains, serving as stimuli to initiate the HSR.

D. Maintenance of the HSR

The main marker of the maintenance phase of the HSR can be taken to be the translation of *hsp70* mRNA and accumulation of HSP70 protein. Inhibition

of general protein synthesis may also be a marker of this phase, but, because translation arrest is irreversible in vulnerable neurons, it is not suitable to be used as a marker of specifically this phase. In the preconditioning regime, HSP70 protein levels follow the mRNA levels. However, in the DND domain, a significantly different picture occurs. While both vulnerable and resistant neurons synthesize large amounts of *hsp70* mRNA, only the resistant neurons actually translate the mRNA in a time frame concurrent with appearance of *hsp70* mRNA [101]. In vulnerable neurons, *hsp70* mRNA continues to accumulate for roughly 24 hrs before there is synthesis of the protein in the rat [101], and the protein is never synthesized in vulnerable neurons of post-ischemic gerbil brain [98,99]. Thus, one can conclude that there is a defect in the HSR in vulnerable neurons in the maintenance phase of the HSR. We return to this point below.

E. Termination of the HSR

Markers of the termination phase of the HSR would be expected to include: (1) loss of ubi-protein clusters, (2) recovery of general translation, and (3) loss of HSP70 protein. In the preconditioning domain, these factors occur in vulnerable neurons [87,102,103], indicating that the HSR has run its course, and contributed to the survival of the vulnerable neurons after preconditioning durations of ischemia. In the DND domain, this same pattern holds true for resistant neurons. They show loss of ubi-protein clusters [82], large declines in the mRNA and protein levels of HSP70 [101], and a generalized recovery of translation [104], again, indicating the HSR has successfully run through all three of its phases and contributed to a survival phenotype. In the DND domain in vulnerable neurons, once HSP70 is translated late in reperfusion (e.g. in rat), there is indeed a decline in mRNA levels and corresponding increase in the protein [101]. However, PAs persist [82,97], presumably as a transformed product derived from the ubi-protein clusters. There is no translation recovery all the way to the point of vulnerable neuron demise [104]. Hence, although some features of HSR termination occur in vulnerable neurons, termination is incomplete. This most likely is a consequence of the fact that the maintenance phase had not successfully run its course.

Pinpointing the defect in the HSR in vulnerable neurons

This abbreviated characterization of the HSR following both preconditioning and DND durations of ischemia indicate that a defect lies in the maintenance phase of the HSR in vulnerable neurons in the DND regime. Specifically, the delay or lack of HSP70 translation can be construed as a defect of the maintenance phase. The take home message of the present analysis is the

need to focus specifically on the maintenance phase of the HSR and attempt to elucidate the cause of this defect.

Previously, it was thought that HSP70 was not translated because protein synthesis was inhibited [4]. However, our present knowledge clearly shows that HSP70 is translated while general protein synthesis is quiescent [73]. This occurs in resistant neurons in the DND regime: there is a period where these cells exclusively synthesize HSPs while general translation is shut-off [105]. That HSP70 itself is now recognized to play an important role in general translational recovery [75], indicates that invoking inhibition of translation as the cause of delayed synthesis of HSP70 is no longer a plausible idea.

However, other possibilities emerge. One possibility is that the same factors that suppress translation of housekeeping mRNAs also affect *hsp70* mRNA in vulnerable neurons. In other words, the defect may lie in some facet of mRNA regulation in the vulnerable compared to the resistant neurons. It is also possible that there is a rapid turnover of the *hsp70* mRNA in vulnerable neurons compared to resistant neurons, precluding its effective translation. In general, our analysis allows us to pinpoint a defect in the regulation of the *hsp70* mRNA, as opposed to a defect in the translation machinery, as underlying the lack of or delay in translation of HSP70 protein. In this regard, our example has provided new empirically testable hypotheses that may be of direct relevance to elucidating important factors that contribute to selective vulnerability and DND. The next stage after identifying the difference in *hsp70* mRNA regulation between vulnerable and resistant neurons, is to develop pharmacologic interventions to eliminate this difference, where the mechanism so allows.

Conclusion

Although it seems like a long and indirect walk to the conclusions presented above, by not attempting to impose some type of (semi-)formal order to the pathways invoked in neurons following I/R, one is left with no basis by which to prioritize which pathways are relevant to cell death mechanisms and which are not. Further, the idea of understanding the neuronal stress responses as having a beginning, middle and an end helps to organize seemingly disparate bytes of data into a coherent theoretical framework, again, allowing a prioritization of observations. In principle, the type of logic used in the example above can be applied to any pathway activated in the reperfused brain, providing a template to organize observations and make new predictions about causation with respect to outcome.

As stated at the beginning of the article, given the complexity of the empirical system (the reperfused brain), the myriad of cellular changes identified, and the ambiguity as to how these contribute to outcome, all that could be offered here are perhaps the most obvious and simplest of

generalizations. However, as both bench and information processing technologies progress, allowing a progressively better handle on the type of complexity we are dealing with, there is a strong hope that, in only a relatively few years, the notions presented here will seem as simple as they really are.

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