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ADAPTATION AND SENSITIZATION TO PROTEOTOXIC STRESS

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□ Although severe stress can elicit toxicity, mild stress often elicits adaptations. Here we review the literature on stress-induced adaptations versus stress sensitization in models of neurodegenerative diseases. We also describe our recent findings that chronic proteotoxic stress can elicit adaptations if the dose is low but that high-dose proteotoxic stress sensitizes cells to subsequent challenges. In these experiments, long-term, low-dose proteasome inhibition elicited protection in a superoxide dismutase-dependent manner. In contrast, acute, high-dose proteotoxic stress sensitized cells to subsequent proteotoxic challenges by eliciting catastrophic loss of glutathione. However, even in the latter model of synergistic toxicity, several defensive proteins were upregulated by severe proteotoxicity. This led us to wonder whether high-dose proteotoxic stress can elicit protection against subsequent challenges in astrocytes, a cell type well known for their resilience. In support of this new hypothesis, we found that the astrocytes that survived severe proteotoxicity became harder to kill. The adaptive mechanism was glutathione dependent. If these findings can be generalized to the human brain, similar endogenous adaptations may help explain why neurodegenerative diseases are so delayed in appearance and so slow to progress. In contrast, sensitization to severe stress may explain why defenses eventually collapse in vulnerable neurons.

Key words: dual hit; two hit; Parkinson's disease, Alzheimer's disease, preconditioning, hormesis, U-shaped

INTRODUCTION

It has long been observed that organisms can adapt to mild stress but are weakened by exposure to severe stress. Many decades ago, the father of toxicology, Phillipus von Hohenheim (also known as Paracelsus), observed that the dose makes the poison (Ottoboni 1997). Despite the dearth of scientific data in the 1500s, Paracelsus suggested that anything can be toxic in high doses and, conversely, that many poisons are not toxic in low doses. Hans Selye, who described biological responses to stress in the first half of the 20th century, also categorized stress into two forms: eustress and distress (Selye 1975). Eustress was defined as mild stressors that improve behavioral function and could be contrasted with severe stressors that exert a negative impact on adaptive behavior. Selye's seminal studies demonstrated that the response to stress is largely duration-dependent. Stress initially elicits a phase of resistance in animals,

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whereas chronic stress that is unyielding results in distress. Both of these pioneers, Paracelsus and Selye, therefore described two features that strongly influence the direction of the response to stress: dose and duration. A plethora of more recent studies have largely confirmed their initial suspicions and also identified underlying mechanisms.

Our recent mechanistic studies on adaptations as well as sensitizations to stress are the subject of the present review. It will become evident from our studies that even long duration stress can elicit adaptations in some cellular models provided the stressor dose is low, and that even high dose stress can elicit adaptations in some cell types. However, in other cellular models, severe stress elicits synergistic toxicity when combined with a second challenge. Thus, the response to stress is not unidirectional and is probably highly dependent on stressor dose, stressor duration, cell type, brain region, prosurvival protein profile, organismal age, and many other factors. Our models of stress all revolve around proteotoxicity, stress caused by protein misfolding and aggregations. Proteotoxic stress is a hallmark pathology of neurodegenerative diseases (Walker and LeVine 2000; Walker *et al.* 2006; Morimoto 2008; Dickson 2009; Jellinger 2009; Uversky 2009; Angot *et al.* 2010; Gundersen 2010; Morimoto 2011). Many neurodegenerative conditions, including the more common Alzheimer's and Parkinson's diseases, are therefore known as proteinopathies. Each of these diseases is characterized by signature protein aggregations or inclusions in specific brain regions. Not surprisingly, there are also reductions in proteasome activity in both Parkinson's and Alzheimer's disease (Keller *et al.* 2000; McNaught *et al.* 2003; McNaught 2004). The barrel-shaped ubiquitin-proteasome system degrades misfolded proteins that are tagged with a polyubiquitin tail. Although proteasome inhibition cannot mimic the full extent of the pathologies in neurodegenerative diseases, inhibition of proteasome activity with pharmacological tools elicits some of the salient features of these disorders, such as the formation of protein aggregations and cell death (Rideout *et al.* 2001; Rideout and Stefanis 2002; Sawada *et al.* 2004; Rideout *et al.* 2005; Sun *et al.* 2006; Xie *et al.* 2010). Another important caveat of current models of neurodegeneration is that they fail to mimic the decades-long pathophysiology of chronic neurodegenerative diseases. This is a difficult obstacle to overcome for the entire field and can be attributed to our lack of knowledge of the primary cause of these disorders, the short rodent lifespan, and the acute nature of insults applied to *in vitro* models of neurodegeneration. On the other hand, studies on genetic or familial forms of Alzheimer's and Parkinson's disease strongly support the hypothesis that abnormally shaped proteins are causally linked to neurodegeneration. Despite this significant advance, our lack of knowledge of the original stimulus that first precipitates protein misfolding in sporadic forms of Alzheimer's and Parkinson's disease has stalled the identification of curative therapies and

hindered the development of animal and cellular models. Until we identify the reason for protein aggregation in sporadic forms of these diseases, eliciting proteotoxicity with proteasome inhibition in cellular models or by direct infusion into the brain appears to be a reasonable and practical model of protein misfolding stress (Fornai *et al.* 2006; Pan *et al.* 2008; Vernon *et al.* 2010; Zhang *et al.* 2012). Another form of proteotoxicity in neurodegenerative disorders is the presence of autophagic stress (Nixon and Yang 2012; Son *et al.* 2012; Salminen *et al.* 2013). Autophagy by the lysosome is an alternative means to clear cellular debris such as misfolded proteins, and can be mobilized in self-defense when the proteasome is inhibited (Iwata *et al.* 2005; Ding *et al.* 2007; Rubinsztein *et al.* 2007; Janen *et al.* 2010; Wong and Cuervo 2010). MG132, the toxin that we have used to inhibit the proteasome, also inhibits lysosomal cathepsins (Lee and Goldberg 1998). Thus, treatment with MG132 mimics both the proteasomal and the autophagic stress of proteinopathies. In this respect it is similar to lactacystin, a proteasome inhibitor that also inhibits cathepsin A (Lee and Goldberg 1998; Aikawa *et al.* 2006).

In this review, we use the term "mild" to describe stressors that are short enough in duration or low enough in dose to be sublethal. We use the term "severe" to describe stressors that are long enough in duration or high enough in dose to be lethal to some fraction of the cellular population. We envision that the response to stress in the human brain may switch from enhanced resistance to increased vulnerability with a shift in either stressor dose or duration. Our earlier work was based on the hypothesis that adaptations to mild cellular stress may partly explain the delayed onset and protracted nature of neurodegenerative conditions (Leak *et al.* 2006; Leak and Zigmond 2007; Leak *et al.* 2008). We speculated that endogenous defenses against stress may keep full-blown neurodegenerative illnesses at bay in young individuals and may also brake disease progression in those who do finally develop Parkinson's or Alzheimer's disease in old age. Such favorable reactions elicited by low dose stressors or eustress are defined as hormetic responses and are well established in the toxicology literature (Calabrese 2008c; Giordano *et al.* 2008; Mattson 2008; Calabrese 2010). Hormesis can be viewed as the prototypical homeostatic reaction to environmental fluctuations, in accordance with the original definition of homeostasis by Claude Bernard and Walter Cannon. The ability to respond dynamically to challenges that threaten internal homeostasis is also described as plasticity and is more evident in some cell types than others. With higher levels of stress, however, adaptive responses may fail and the response to subsequent challenges can be compromised. In the latter situation, pre-stressed cells become sensitized to a second hit instead of protected. This toxic type of response may predominate in vulnerable brain regions or in aged animals (Boger *et al.* 2010). The biphasic nature of the response to stress is

reflected in the U-shaped, hormetic dose-response curves that are often reported in toxicology. With some exceptions (Calabrese *et al.* 2007; Calabrese 2008b), discussions of U-shaped dose response curves and hormesis do not always include reference to a second hit. However, adaptation or sensitization to stress can be quantitatively measured by the response of pre-stressed cells to a second challenge, as will be discussed further below. If previously stressed cells are protected against a second challenge relative to naïve cells, they are said to be preconditioned or in a state of tolerance. On the other hand, if pre-stressed cells respond to two hits with synergistic toxicity they are said to be sensitized to the second challenge. In contrast, if two toxic hits are additive and not synergistic, neither adaptation nor sensitization is at work and the first hit is, in a sense, neutral, because it leaves behind cells that are as vulnerable to the second hit as naïve cells. In short, a two hit protocol is extremely useful to gauge both the direction and magnitude of the stress response.

Our long term goal is to characterize in detail the protein profile of stressed, but adapted neuronal and glial cells and to contrast this profile with cells that are sensitized to subsequent challenges. A better understanding of this "adaptive proteome" in the brain and how it responds to homeostatic challenges might hasten the development of CNS pharmacotherapies or identify dietary/lifestyle changes that mimic these stress-responses without causing any harm. Estimates place 15% of highly conserved proteins in the stress responsive category (Kultz 2005); their abundance and phylogenetic conservation can be viewed as a testament to their importance in homeostasis. Stress responsive proteins include (1) sensors to recognize perturbations, (2) transducers to amplify and integrate signals and (3) effectors to counteract stress (Kultz 2005; Babar *et al.* 2008). The effector proteins that battle challenges to homeostasis include the antioxidant enzyme systems and folding chaperone machinery as well as the prosurvival signaling cascades. Although these prosurvival effectors have often been observed to be lower in postmortem tissue from Alzheimer's and Parkinson's victims, the occasions on which they are higher in disease states are instructive. Well-established examples of antioxidant defenses that are lowered early in the course of Parkinson's and Alzheimer's disease include loss of the essential tripeptide glutathione (Sofic *et al.* 1992; Sian *et al.* 1994; Calabrese *et al.* 2006; Baldeiras *et al.* 2008; Zeevalk *et al.* 2008; Lloret *et al.* 2009; Martin and Teismann 2009; Johnson *et al.* 2012). Based on our two hit model of proteasome inhibition in N2a cells (discussed below), catastrophic drops in glutathione may reflect synergistic proteotoxicity within vulnerable cell types. One should note that these prodeath cellular changes are not necessarily maladaptive to the organism as a whole. It may benefit the organism to clear dysfunctional cells that are damaged beyond repair. Such removal may decrease mutagenesis risk and preserve energy for other cel-

lular systems that can be salvaged. In other words, even toxic responses to stress may play an evolutionarily adaptive role under some circumstances.

In contrast to the loss in glutathione, some prosurvival molecules are raised in the brains of victims of neurodegenerative diseases. Some of the more enlightening examples of these changes will be discussed here. One might speculate that increases in prosurvival molecules reflect those remaining cells that have either successfully battled proteotoxic stress or are able to prolong their lives by delaying the harmful sequelae of proteotoxicity. It is also important to characterize regional variations in the adaptive proteome in this context, as differences in endogenous defense strategies across brain regions may underlie the topographic nature of neurodegenerative disorders, where all neurons are not equally vulnerable to inclusion formation and cell death (Mattson *et al.* 1989; Mattson and Kater 1989; Braak *et al.* 2000; Braak *et al.* 2003; Posimo *et al.* 2013). An example of regional differences in glutathione content has been published by Mythri and colleagues (Mythri *et al.* 2011). Although glutathione levels in the nigra are reduced in Parkinson's disease, glutathione levels are raised in the less vulnerable frontal cortex, caudate, and putamen (Mythri *et al.* 2011). This increase in glutathione was accompanied by a decrease in the activity of gamma glutamyl transpeptidase, the enzyme that breaks down glutathione. Glutathione peroxidase activity levels were also raised in the caudate and putamen, supporting the hypothesis that more resilient brain regions are protected from oxidative damage in Parkinson's disease (Mythri *et al.* 2011). Another instructive study of glutathione peroxidase 4 expression within neuromelanin-containing cells in the nigra reveals an important caveat of these types of experiments (Bellinger *et al.* 2011). In the Bellinger study, total glutathione peroxidase 4 immunoreactivity was decreased in the substantia nigra of Parkinson's victims. However, when glutathione peroxidase 4 immunoreactivity was expressed relative to cell density, there was an upregulation of glutathione peroxidase 4 levels within the remaining nigral neurons in Parkinson's brains. Thus, some previous studies that have reported losses in prosurvival systems in neurodegenerative disorders may actually reflect an overall loss in neuron number. Some studies have dealt with this caveat by quantifying immunostaining intensities within remaining neurons. One example of this type of study shows that the ferroxidase ceruloplasmin is higher in the remaining CA1 hippocampal neurons in Alzheimer's brains (Loeffler *et al.* 2001). Ceruloplasmin is a serum copper chaperone, but recent studies show that it also plays a protective role in the central nervous system (Kaneko *et al.* 2008; Hineno *et al.* 2011; Texel *et al.* 2011). Ceruloplasmin concentrations in the brain are increased in Alzheimer's and Parkinson's disease (Loeffler *et al.* 1996). Given its ability to rise with stress in other human conditions, the rise in ceruloplasmin in neurodegenerative diseases is not

surprising and may reflect the endogenous defense capacities of the human brain (Mezzetti *et al.* 1996; Mezzetti *et al.* 1998; Louro *et al.* 2000; Taysi *et al.* 2002; Memisogullari and Bakan 2004; Chacko and Cheluvappa 2010).

Other defensive proteins that are upregulated in neurodegenerative diseases include the well-studied heat shock family of proteins. The heat shock response to stress is a primordial defense against denatured proteins (Verbeke *et al.* 2001). Heat shock proteins battle apoptosis, refold misfolded proteins, and escort damaged proteins to the proteasome or lysosome for degradation (Kalia *et al.* 2010; Lanneau *et al.* 2010; Aridon *et al.* 2011). One might therefore speculate that an endogenous rise in heat shock proteins is a self-defense mechanism that slows down proteotoxicity in proteinopathies. Heat shock protein 90 is raised in Parkinson's disease and is colocalized with α-synuclein in Lewy bodies (Uryu *et al.* 2006). Furthermore, heme oxygenase 1 (also known as heat shock protein 32) is raised in hippocampal and cortical tissue in Alzheimer's disease (Schipper 2000; Schipper *et al.* 2006) and is also increased in astrocytes in Parkinson's disease (Schipper *et al.* 1998). In mild cognitive impairment, a possible precursor to dementia, heat shock proteins 70 and 27 are both increased in the inferior parietal lobule (Di Domenico *et al.* 2010). Furthermore, heat shock protein 27 is raised in the nigrostriatal pathway in Parkinson's disease (Zhang *et al.* 2005). The major risk factor for neurodegenerative diseases is aging. Although the induction of heat shock proteins is impaired with aging, chaperones in general are increased with aging (Fargnoli *et al.* 1990; Maiello *et al.* 1998; Soti and Csermely 2000; Schultz *et al.* 2001; Walters *et al.* 2001; Gupte *et al.* 2010). For example, heat shock proteins 40, 27, 60, 70, and constitutive heat shock protein 70 are known to increase in the aged central nervous system (Lee *et al.* 2000; Lu *et al.* 2004). Clearly, different aspects of endogenous defenses can be impaired or increased in individuals at risk for neurodegenerative diseases or with full-blown disorders. Thus, both types of responses can coexist in the same human brain, perhaps not the least because of striking regional variations in vulnerability (Mattson *et al.* 1989; Mattson and Kater 1989; Braak *et al.* 2000; Braak *et al.* 2003; Posimo *et al.* 2013).

Although it seems reasonable to speculate that rises in prosurvival molecules in human neurodegenerative disorders slow down disease progression, this is not known for certain, as the human studies are correlational and do not establish causation. Furthermore, the analysis of postmortem brain tissue is a cross-sectional snapshot of one moment in time. As a result, we do not know whether changes in prosurvival proteins within individual neurons are long term or transient in nature. Finally, it is also not known how long stress-induced protection or sensitization can linger in the human brain. Most of the answers to these questions come from experimental model systems. The best way to experimentally

address whether a response to stress is adaptive or toxic is to challenge cells with a second hit and to quantify the degree of protection or sensitization. As mentioned earlier, sublethal stress-induced protection against subsequent challenges is known as preconditioning. The mechanisms underlying preconditioning have been well defined in studies of ischemia but are often neglected in the study of neurodegenerative diseases. Only a small number of studies have considered the possibility of preconditioning in models of neurodegenerative diseases and some of these will be described here. First, Mark Mattson has proposed that dietary and behavioral manipulations such as exercise and food restriction may protect against models of neurodegeneration by activating stress-responsive pathways (Duan and Mattson 1999; Guo *et al.* 2000; Mattson *et al.* 2004; Son *et al.* 2008). For example, dietary restriction increases levels of brain derived neurotrophic factor, neurogenesis, and heat shock proteins (Mattson *et al.* 2004). Mattson has further proposed that dietary phytochemicals ingested from plants can precondition against multiple diseases, including Parkinson's and Alzheimer's disease (Son *et al.* 2008). From an evolutionary point of view, phytochemicals may activate stress-responsive pathways because they are designed to repel insects, molds, and even mammals. Two examples are nicotine and caffeine, both of which have been associated with reduced risk for neurodegeneration in epidemiological studies (Tan *et al.* 2003; Powers *et al.* 2008). Calabrese and colleagues have argued that hormetic phytochemicals work through vitagenes, genes which encode for heat shock proteins, sirtuin, and thioredoxin (Calabrese *et al.* 2012). If phytochemicals continue to have these effects for the long term, chronic adaptation may be possible in humans. Second, dietary habits such as moderate alcohol consumption are also associated with lower risks of Alzheimer's disease and may also be effective over the long term (Peters *et al.* 2008; Anstey *et al.* 2009). In support of this notion, ethanol can precondition against models of Alzheimer's disease (Mitchell *et al.* 2009; Collins *et al.* 2010). Other natural dietary compounds, such as the green tea polyphenol epigallocatechin-3-gallate and the red wine ingredient resveratrol have also been proposed as preconditioning agents in Alzheimer's and Parkinson's disease models (Raval *et al.* 2008; Tai and Truong 2010; Tang *et al.* 2011; Wu *et al.* 2012). Resveratrol is thought to protect cells in a sirtuin-dependent manner (Farghali *et al.* 2012; Wu *et al.* 2012). Third, anesthetics induce tolerance against subsequent challenges in Alzheimer's disease models and raise levels of phosphorylated tau (Wei and Xie 2009; Tang *et al.* 2011). Even β-amyloid itself can be used as a preconditioning tool against subsequent challenges, such as glutamate excitotoxicity, by promoting endocytosis of the NMDA receptor (Goto *et al.* 2006).

Another example of long term preconditioning in humans may be the lifelong benefits of exercise. Exercise can be viewed as a natural, mild

stress. Exercise is known to raise free radical content and can precondition against ischemia (Frasier *et al.* 2011; Powers *et al.* 2011; Zhang *et al.* 2011). Many studies have supported the long-term benefits of exercise in humans, even in Alzheimer's and Parkinson's patients (Chen *et al.* 2005; Xu *et al.* 2010; Erickson *et al.* 2012; Mayeux and Stern 2012; Fisher *et al.* 2013; Intlekofer and Cotman 2013; Winchester *et al.* 2013). Animal studies have also shown convincingly that exercise is protective in experimental models of neurodegeneration (for some examples, see Adlard *et al.* 2005; Nichol *et al.* 2009; Pothakos *et al.* 2009; Zigmond *et al.* 2009; Gerecke *et al.* 2010; Vuckovic *et al.* 2010; Intlekofer and Cotman 2013; Souza *et al.* 2013). The studies on the benefits of long term exercise, dietary phytochemicals such as nicotine and caffeine, and moderate alcohol consumption all suggest that chronic adaptation to stress may be achievable in humans. Furthermore, exercise is known to generate an adaptive proteome. For example, we have observed that treadmill exercise raises ceruloplasmin in primates and that levels of physical activity are positively correlated with ceruloplasmin levels (Leak *et al.* 2012). Despite its stress-responsive nature, ceruloplasmin has not been extensively explored in connection with severe proteotoxic stress and brain neuroprotection. Further studies on this protein are therefore warranted.

In a cellular Parkinson's disease model, we have shown that sublethal oxidative stress from 6-hydroxydopamine can precondition dopaminergic cells against subsequent lethal exposures to higher concentrations of the same toxin (Leak *et al.* 2006). The protection in this model was kinase dependent, as inhibitors of ERK1/2, Akt, and JNK activation all attenuated the preconditioning-induced protection. Besides sublethal 6-hydroxydopamine, another means of eliciting protection against lethal doses of 6-hydroxydopamine is by thrombin pretreatment *in vivo* (Cannon *et al.* 2005). Dopaminergic terminal loss in the striatum and ventricular enlargement were both attenuated by thrombin preconditioning. Third, hyperoxia preconditioning can protect animals against the behavioral symptoms of 6-hydroxydopamine toxicity, such as apomorphine-induced rotations and motor performance on the rotarod (Hamidi *et al.* 2012). Fourth, preconditioning can also be elicited by the bacterial endotoxin lipopolysaccharide. Lipopolysaccharide is well known to elicit inflammatory responses but, in low concentrations, can precondition organotypic midbrain cultures against subsequent lipopolysaccharide challenges (Ding and Li 2008). Lipopolysaccharide preconditioning protected against dopamine neuron loss as well as lactate dehydrogenase release in this organotypic slice model. Lipopolysaccharide preconditioning also prevented the microglial activation and tumor necrosis factor-α release in response to the second, higher concentration of lipopolysaccaride. In addition to these inflammation-suppressing functions of preconditioning, homeostatic crosstalk between endoplasmic reticulum stress and

autophagy may also mediate the benefits of preconditioning in *Drosophila* and mouse Parkinson's disease models (Fouillet *et al.* 2012; Matus *et al.* 2012). In these studies, inhibition of autophagy was found to impair endoplasmic reticulum stress-induced protection. Matus and colleagues have recently reviewed these hormetic responses to protein-misfolding stress (Matus *et al.* 2012). Fifth, activation of the antioxidant response element by endoplasmic reticulum stress inducers can also precondition against 6-hydroxydopamine toxicity (Hara *et al.* 2011). Sixth, low dose methamphetamine challenges can protect dopaminergic cells against 6-hydroxydopamine toxicity (El Ayadi and Zigmond 2011). Finally, *in vitro* studies also provide evidence that heat shock can precondition against 1-methyl-4-phenylpyridinium (MPP^+), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), another well-established model of Parkinson's disease (Quigney *et al.* 2003; Fan *et al.* 2005). These studies, while not numerous, reveal that preconditioning can indeed occur in models of neurodegenerative diseases.

In contrast to the small numbers of studies on preconditioning in neurodegeneration, several seminal papers on short duration ischemic episodes initiated a flood of investigations on preconditioning in stroke models (Murry *et al.* 1986; Kitagawa *et al.* 1990; Kirino *et al.* 1991; Liu *et al.* 1992; Kirino 2002; Dirnagl *et al.* 2003). These studies consistently showed that short, sublethal ischemic episodes elicit tolerance of subsequent, longer duration ischemic attacks that would otherwise be lethal. As argued by Valina Dawson, ischemic preconditioning offers a way to "mine for survival genes" (Dawson and Dawson 2006) and has been a productive field of research for many decades. Ischemic preconditioning also has translational potential; remote ischemic preconditioning of an arm or leg with a tightened blood pressure cuff may protect distant organs from ischemic events such as stroke and cardiac bypass surgery (Fairbanks and Brambrink 2010; Candilio *et al.* 2011). The state of our knowledge on ischemic preconditioning has been discussed in many recent reviews (Dirnagl and Meisel 2008; Della-Morte *et al.* 2012; Kitagawa 2012; Prabhakar and Semenza 2012; Thompson *et al.* 2012) and will not be described further here.

As mentioned above, if the stressor is severe, it can exacerbate the toxic response to future insults and result in greater than additive cell loss. For example, previous exposures to thromboembolic events can combine with a subsequent ischemic insult to produce larger areas of ischemic injury (Dietrich *et al.* 1999; Danton *et al.* 2002). In the field of neurodegeneration, the synergistic toxicity of multiple challenges is the subject of the "two hit" or "dual hit" hypothesis (Zhu *et al.* 2004; Carvey *et al.* 2006; Manning-Bog and Langston 2007; Sulzer 2007; Zhu *et al.* 2007; Weidong *et al.* 2009; Boger *et al.* 2010; Gao and Hong 2011; Unnithan *et al.* 2012). A few reports of dual-hit insults in Parkinson's and Alzheimer's

disease will be described below. First, Carvey and Di Monte have discussed the dual hit concept with reference to Parkinson's disease in that sufficient cell loss to elicit symptoms may require multiple stress exposures (Di Monte *et al.* 2002; Ling *et al.* 2004b). For example, toxic environmental agents might interact with endogenous factors such as α-synuclein and aging. Indeed, gene-environment interactions are particularly relevant to the two hit hypothesis (Gao and Hong 2011) and can be studied by fusing animal models (Manning-Bog and Langston 2007). Genetic disruptions that result in fewer dopamine neurons at birth may also result in Parkinsonian symptoms when combined with age-related neuronal attrition (Weidong *et al.* 2009). Other models of the two hit hypothesis have examined loss in trophic factors such as glial cell derived neurotrophic factor (GDNF) and its impact on the response to aging and to methamphetamine challenges (Boger *et al.* 2010). As expected, genetic reductions in GDNF exacerbate age-related changes in dopaminergic systems and increase vulnerability to methamphetamine. Several studies have examined the two hit hypothesis in the context of inflammatory changes to the brain. The authors of these studies consistently report that the pesticide rotenone or the neurotoxin MPTP can both combine with the inflammogen lipopolysaccharide to elicit synergistic neurotoxicity in dopamine neurons (Gao *et al.* 2003a; Gao *et al.* 2003b; Ling *et al.* 2004a). Dopamine oxidation and mitochondrial dysfunction have also been suggested to combine with loss of function gene mutations or autophagic self-degradation to underlie cell death in Parkinson's disease (Sulzer 2007). Smith and colleagues have put forth a two hit hypothesis for Alzheimer's disease in that oxidative stress and mitogenic dysregulation may combine to increase risk for Alzheimer's pathology (Zhu *et al.* 2001; Zhu *et al.* 2004; Zhu *et al.* 2007). In their model, oxidative stress and abnormalities in mitotic signaling can both initiate pathology, but both must be present to propagate the full extent of the pathology. A multiple hit model of changes in tau function has also been suggested to promote tau assembly (DeTure *et al.* 2006). Furthermore, traumatic brain injury has been hypothesized to predispose individuals to both Parkinson's and Alzheimer's disease (Kiraly and Kiraly 2007). Finally, a two hit study from the stroke literature is particularly edifying (Qiao *et al.* 2009). In this study by Tuor and colleagues, a 40 min stroke resulting in focal necrosis was combined with a 60 min stroke three days later. Proximal to the ischemic core, where loss of blood flow was the most severe, the damage exceeded that of the first insult, whereas distally, there was tolerance to the insult. These findings reveal that adaptation and sensitization can occur within the same brain and show elegantly that the direction of the response depends on the magnitude of the insult.

The two hit terminology has not generally been applied to studies of preconditioning although preconditioning protocols also apply two

sequential stressors. The word 'hit' is not typically used in reference to sublethal stress even though it elicits transient damage (for an example, see Dembinski *et al.* 2006). Sublethal preconditioning stimuli also increase reactive oxygen species and activate the caspase cascade (McLaughlin *et al.* 2003; Thompson *et al.* 2012). Without sublethal injury, there would be no stress response because the sensors would not recognize any perturbations. Before setting semantic issues aside, we argue here that the two hit hypothesis should, by definition, encompass any protocol that involves two hits, be they sublethal or lethal, and that the hypothesis must account for the biphasic nature of stress responses. Therefore, we propose that the response to two hits can involve the following: 1) preconditioning-style adaptations following sublethal stressor hits, 2) additive toxic responses to two severe stressor hits in which the first hit does not change the response to the second hit, 3) synergistic toxic responses to two severe stressor hits in which the first hit magnifies the response to the second hit, and 4) adaptive responses to severe stress so that the impact of a second hit is blunted in the cells that manage to survive the first hit. To explore some of these possibilities, below we describe our recent work investigating responses to proteotoxicity in various two hit cellular models. We begin with a description of adaptations to chronic low dose proteotoxic challenges. A high-throughput two hit model of synergistic neurodegeneration is also presented. Finally, we summarize our recent findings that astrocytes, a glial cell type well known for stress-induced plasticity, can adapt to proteotoxic stress delivered at a high enough concentration to kill half the population. This was the first demonstration that the glial survivors of severe proteotoxic stress are more resistant than naïve cells. The mechanisms underlying the adaptations and sensitization are also presented.

EVEN LONG-TERM STRESS CAN ELICIT ADAPTATIONS

As mentioned above, neurodegenerative conditions are characterized by inhibition of the normal role of the proteasome. Indeed, the chronic nature of neurodegenerative conditions raises the possibility that diseased brains are exposed to proteotoxic stress for an extended timeframe. Of course, Selye had argued that chronic stress weakens defenses. Nonetheless, we wondered if cells are able to adapt to chronic stress if the dose is low enough. If this was true, mild stress-induced plasticity might explain why neurodegenerative conditions are so slow to progress despite evidence of long-term proteotoxicity.

Although Parkinson's disease involves degeneration of multiple brain regions, such as noradrenergic (Forno 1996; Gesi *et al.* 2000), serotonergic (Halliday *et al.* 1990; Gai *et al.* 1992; Politis *et al.* 2012), and hypocretin/orexin systems (Fronczek *et al.* 2007; Thannickal *et al.* 2007), the motor deficits are largely attributed to massive dopaminergic cell loss in

the nigrostriatal tract. Parkinson's disease is also characterized by its late age of onset and progressive nature, suggesting that endogenous adaptations may be hard at work. In order to test the hypothesis that cells can adapt to chronic stress in a dopaminergic model, PC12 cells were exposed to long durations (14 days - 6 months) of the proteasome inhibitor MG132 (Leak *et al.* 2008). In our model, MG132 (0.1 μM) effectively reduced chymotrypsin proteasome activity by 47%. Furthermore, there was a statistical trend towards higher levels of ubiquitin-conjugated proteins with MG132 treatment. These findings suggest that MG132 was proteotoxic in this model. However, no impact of chronic MG132 on overt morphology or tyrosine hydroxylase expression was observed. Tyrosine hydroxylase is the rate-limiting enzyme for dopamine biosynthesis. We also measured viability at 4-5 day intervals for two weeks after initiation of MG132 treatment and found no change. MG132 (0.1 μM) was left in the media at all times for up to 6 months. Viability was measured within two days after plating cells throughout this entire procedure. We discovered that chronic pretreatment with 0.1 μM MG132 did indeed protect against subsequent challenges. Fourteen days or longer exposure to sublethal MG132 protected against either 6-hydroxydopamine or higher-dose MG132 (40 μM). Protection in this system was verified by two independent assays for cell viability: the Cell Titer Glo assay for ATP and counts of Hoechst-stained nuclei. We typically perform at least two viability assays in order to reduce the likelihood of false positives and to measure protection of both cell numbers and metabolic activity. This helps us ascertain the impact of treatments on cellular structures as well as their function. However, a potential confound of our interpretation that chronic MG132 was protective would be if MG132 decreased activity of the dopamine transporter that shuttles 6-hydroxydopamine into the cytoplasm from the extracellular medium. In other words, we were concerned that the rise in viability in chronically stressed cells might be an artifact of reduced influx of the 6-hydroxydopamine toxin. In contrast to this expectation, we observed a 36% *rise* in dopamine transporter activity after chronic treatment with MG132, not a fall. This suggested that the pre-stressed cells were protected despite slightly greater exposure to 6-hydroxydopamine. Notably, when MG132 was removed from the media for more than two weeks, the protection disappeared, suggesting that the stress had to be continuous to elicit an adaptive response. Conversely, this low concentration of MG132 was not sufficient to elicit protection if administered for less than two weeks. Taken together, all of these observations are consistent with the hypothesis that dopaminergic cells have the capacity to adapt to chronic sublethal stress but that the protection disappears when the stressful stimulus is removed.

Next we proceeded to examine the mechanism underlying long term adaptive defenses. First, we scrutizined the role of the ubiquitous thiol

glutathione in this model. Thiol defenses are so important to cells that glutathione is present in millimolar concentrations in most tissues (Cooper and Kristal 1997; Wilson 1997; Dringen 2000; Pompella *et al.* 2003; Pocernich and Butterfield 2011). Proteotoxicity and oxidative stress are inextricably intertwined in neurodegenerative conditions because oxidized proteins can become misfolded and must be degraded by clearance systems such as the proteasome. We initially speculated that glutathione would stave off the negative impact of chronic proteotoxic stress. Thus, we hypothesized that inhibiting glutathione synthesis would abolish or attenuate the stress-induced protection against 6-hydroxydopamine. In contrast to this expectation, inhibition of glutathione synthesis with buthionine sulfoximine exacerbated 6-hydroxydopamine toxicity to the same degree in naïve cells and cells treated with chronic MG132 and did not attenuate the MG132-induced protection at all. Chronic MG132 also did not raise glutathione levels. This suggested that glutathione defenses were not responsible for the adaptation to long term MG132.

As a result of these negative findings, we proceeded to examine levels of other antioxidant molecules and folding chaperones. We found an increase in CuZn superoxide dismutase enzymatic activity and protein levels with chronic MG132. In contrast, a small rise in Mn superoxide dismutase protein levels was not accompanied by a parallel rise in enzyme activity. Superoxide dismutases catalyze the dismutation of superoxide into hydrogen peroxide and oxygen. Catalase and heat shock protein 70 levels were also raised by chronic MG132. Catalase aids the breakdown of hydrogen peroxide and thus may act in conjunction with the superoxide dismutase enzymes. Heat shock protein 70 actively battles apoptosis in addition to its chaperone functions and is thought to be protective against neurodegeneration (Koren *et al.* 2009; Nagai *et al.* 2010; Witt 2010; Aridon *et al.* 2011; Silver and Noble 2012). We decided to focus on the change in CuZn superoxide dismutase because previous investigations had shown that CuZn superoxide dismutase overexpression is protective in models of Parkinson's disease whereas deficiencies in this protein exacerbate dopaminergic neurodegeneration (Przedborski *et al.* 1992; Asanuma *et al.* 1998; Barkats *et al.* 2002; Sturtz and Culotta 2002; Van Remmen *et al.* 2004; Barkats *et al.* 2006; Wang *et al.* 2006). Notably, CuZnSOD levels are lowered in Parkinson's and Alzheimer's patients (Boll *et al.* 2008; Torsdottir *et al.* 2010), and CuZnSOD is found in Lewy bodies (Nishiyama *et al.* 1995). We therefore hypothesized that knockdown of CuZn superoxide dismutase with RNA interference would attenuate the MG132-induced protection against 6-hydroxydopamine. As expected, CuZn superoxide dismutase knockdown with either of two short interfering RNA (siRNA) sequences attenuated MG132-induced protection. Using two independent siRNA sequences in place of one

sequence alone reduced the likelihood of false positive results from nonspecific effects of RNA interference. Taken together, these findings revealed multiple adaptive changes in stressed cells and showed that CuZn superoxide dismutase was responsible for long term stress-induced protection against oxidative toxicity.

In summary, these studies describe the protective nature of chronic but mild proteotoxic stress. The protection lasted for at least 6 months (the latest timepoint we tested), but removal of the stimulus caused loss of defenses. Adaptive proteins such as anti-apoptotic chaperones and antioxidant enzymes were raised by chronic stress and CuZn superoxide dismutase mediated protection of dopaminergic cells against oxidative toxicity. It must be acknowledged here that our definition of chronic falls short of the decade-long march of neurodegeneration in Parkinson's and Alzheimer's disease. The only conceivable model systems in which decades-long insults might be applied are the nonhuman primates. Whether mild, low dose proteotoxic stress can protect the primate brain for the truly long term remains to be seen. Second, although they express tyrosine hydroxylase, PC12 cells are not always predictive of dopaminergic neurons because PC12 cells originate from the adrenal gland and not the brain. Although PC12 cells can be differentiated to a neuronal phenotype, differentiated PC12 cells are unsuitable for the long term studies conducted here because they begin to die. For our next high-throughput cellular model, we switched to a neuroblastoma cell line, N2a, which originates from the mouse spinal cord. Although N2a cells are not dopaminergic, Parkinson's disease is now well known to extend beyond the ventral midbrain, as mentioned earlier. Many extranigral brain regions, including the spinal cord, are affected with synuclein inclusions (Braak *et al.* 2002; Braak *et al.* 2003; Del Tredici and Braak 2012). In the studies discussed below, we examined the N2a response to acute, severe proteotoxic stress in a cellular model of synergistic neurodegeneration.

CELLS CAN BE SENSITIZED TO INJURY BY SEVERE STRESS

Although stressors that potentiate the response to subsequent challenges may occur decades prior to the second hit, two hits may also occur in rapid succession, such as exposure to toxicants in careers in agriculture or industry. Many agricultural workers, for example, are exposed to pesticides and herbicides on a daily basis. Some of these pulsatile challenges to humans may elicit oxidative damage and protein misfolding. In support of this notion, pesticide and herbicide exposures increase the risk for Parkinson's disease and cause protein aggregations and cell death in animal models (Liou *et al.* 1997; Betarbet *et al.* 2000; Alam and Schmidt 2002; Manning-Bog *et al.* 2002; McCormack *et al.* 2002; Franco *et al.* 2010; Tanner *et al.* 2011). We hypothesized that synergistic responses to dual proteotoxic challenges should be dose dependent and only elicited by

severe stress, such as high concentrations of MG132. In support of this hypothesis, lethal, but not sublethal concentrations of MG132 were found to synergize in their negative impact on N2a viability when administered 24 hours apart (Unnithan *et al.* 2012). Three independent and unbiased viability assays, conducted on the third day, illustrated this effect. For assaying viability in a high-throughput manner, we stained the nucleus and cytoplasm with a combination of two infrared stains (DRAQ5 and Sapphire) and measured levels of the cytoskeletal protein α-tubulin with immunocytochemistry. The third viability assay, Cell Titer Glo, measured ATP levels. Interestingly, the Cell Titer Glo assay demonstrated that sublethal concentrations of MG132 raised ATP without a parallel change in cell numbers. This favorable metabolic reaction to low level proteotoxic stress may allow slightly stressed cells to battle sublethal injury more effectively and is an example of hormesis. As hypothesized, higher concentrations of MG132 elicited cell loss and increased the toxic response to a second MG132 hit by all three viability assays. Low or subtoxic concentrations of MG132 did not elicit this synergistic response. Toxic, but not subtoxic concentrations of MG132 greatly raised ubiquitin-conjugated proteins in this model, suggesting that higher concentrations of MG132 effectively hindered the clearance of misfolded proteins (Fig. 1).

Adaptive responses to low level stress in our dopaminergic model of the previous section included rises in several antioxidant proteins and the anti-apoptotic folding chaperone heat shock protein 70. Conversely, we expected toxic responses to severe stress to involve loss of antioxidant and chaperone defenses. Contrary to these hypotheses, we found no loss in heat shock protein 70 with toxic MG132 concentrations. In addition, toxic, but not subtoxic MG132 elicited a rise in ceruloplasmin, not a loss (Fig. 1). Our data showing an MG132-induced rise in ceruloplasmin in neuronal cells is consistent with previous studies that it increases with stress (Mezzetti *et al.* 1996; Mezzetti *et al.* 1998; Louro *et al.* 2000; Taysi *et al.* 2002; Memisogullari and Bakan 2004; Chacko and Cheluvappa 2010) and demonstrate that this response can be elicited by proteotoxicity even when the stress is severe.

Next we examined whether toxic MG132 would elicit loss of autophagic markers, because concomitant failure of autophagic and proteasome defenses might underlie the synergistic toxicity of two MG132 hits. However, toxic concentrations of MG132 raised proteins involved in chaperone-mediated autophagy such as heat shock cognate 70 (Hsc70) and the lysosome-associated membrane protein type-2a (LAMP2a). These responses again reflect adaptive responses to severe proteotoxic stress, not toxic responses to stress (Fig. 1). The notion that the chaperone-mediate autophagy markers reflect cellular engagement in self-defense was supported by the finding that ammonium chloride, an inhibitor of autophagic protease activity (Kaushik and Cuervo 2009), increased the

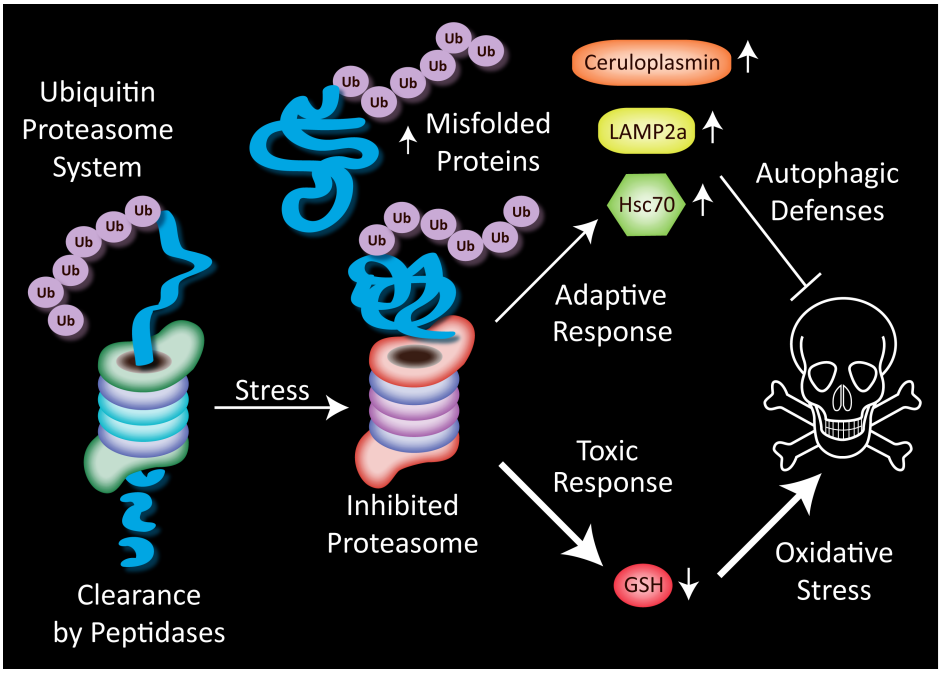

FIGURE 1. Misfolded proteins are tagged with an ubiquitin tail, linearized, and fed through one end of the barrel-shaped proteasome. Peptides exit the other end of the proteasome and are further degraded by cytoplasmic peptidases into amino acids for recycling into fresh proteins. Stress on the proteasome, such as that induced by proteasome inhibitors, causes the buildup of misfolded proteins that can no longer be degraded. In N2a cells treated with high concentrations of the proteasome inhibitor MG132, two types of responses are elicited in response to the reduced clearance of damaged proteins. A rise in the antioxidant ceruloplasmin and the chaperone-mediated autophagy proteins lysosome-associated membrane protein type-2a (LAMP2a) and heat shock cognate 70 (Hsc70) may serve to defend the cell. Inhibiting autophagic defenses increases the toxicity of both single and dual MG132 hits in this model, suggesting that cells use autophagy as an alternative clearance mechanism when the proteasome is inhibited. Cells also respond to dual hits of severe proteotoxicity with a synergistic loss in glutathione (GSH) defenses. This loss of GSH may increase oxidative toxicity and enhance cell death.

toxicity of both single and dual hits of MG132 in our model. Thus, cells exposed to toxic concentrations of proteasome inhibitors may rely on autophagy as an alternative mechanism to clear cellular debris. Of course, adaptive responses such as rises in ceruloplasmin or autophagic markers failed to explain why two toxic MG132 hits were synergistic in nature. We therefore proceeded to test the hypothesis that loss of thiol defenses underlay the toxic impact of two hits.

In contrast to the rises in autophagic proteins and ceruloplasmin, we found that two hits elicited a synergistic loss of glutathione. The response of glutathione to two hits therefore paralleled the synergistic loss of viability and supported the hypothesis that loss of thiol defenses might underlie the toxicity of two hits (Fig. 1). As a result of these findings, we

examined whether raising glutathione levels would protect the N2a cells against two toxic hits of MG132. The glutathione precursor N-acetyl cysteine prevented glutathione loss and almost completely abolished the toxic response to two MG132 hits by all three viability assays. N-acetyl cysteine is an over-the-counter supplement well known to be protective in animal models (for some examples, see Perry *et al.* 1985; Martinez Banaclocha 2000; Pocernich *et al.* 2000; Farr *et al.* 2003; Fu *et al.* 2006; Tucker *et al.* 2006; Sharma *et al.* 2007; Clark *et al.* 2010). It has even been shown to benefit cognitive status in Alzheimer's patients (Adair *et al.* 2001). N-acetyl cysteine is therefore currently being tested in clinical trials of Parkinson's disease (Clinicaltrials.gov ID: NCT01470027). Our findings are consistent with a protective effect of N-acetyl cysteine against proteinopathies, even when the proteotoxic stress is high in concentration and unremitting in nature.

In summary, the response of neuronal cells to two MG132 hits reveals an exquisite dose-sensitivity of synergistic effects. Low concentrations of MG132 did not elicit synergistic toxicity; only severely toxic concentrations of MG132 potentiated the response to the second hit. Despite the toxic effects of high concentrations of MG132, highly stressed cells nonetheless appeared to raise adaptive defenses in the form of autophagic markers and ceruloplasmin (Fig. 1). One might speculate that stressed cells would be even worse off without such defenses. This speculation is supported by our observation that the toxicity of MG132 was increased with an autophagy inhibitor. These studies showed for the first time that the two hit neurodegenerative phenomenon can be extended to protein-misfolding stress from proteasome inhibition. Furthermore, the data on glutathione support the classic notion that oxidative and proteotoxic stressors propel and propagate each other. Oxidative stress has been associated with neurodegenerative proteinopathies for many decades ever since Denham Harman drew attention to free radicals in aging in the 1950s (Harman 1956; Floyd and Hensley 2002; Harman 2006, 2009).

A few caveats of our neuroblastoma studies are worth mentioning here. Our N2a studies of two proteotoxic hits were extremely short in duration compared to the decades-long exposure to stress in human neurodegenerative conditions. Our protocol is better suited to model the toxic impact of rapid, successive hits of proteotoxic stress. Second, the protein profile and concentration of reactive oxygen species in tumor cells is often different from that of normal cells, especially when contrasted to differentiated cells that have exited the cell cycle some time ago. Studies on immortalized lines cannot fully recapitulate the highly heterogenous and differentiated pool of neurons in the brain. Primary cultures are therefore probably more predictive of *in vivo* brain function and were used in the next series of experiments. Because we had observed a number of defensive responses to severe proteotoxic stress in

N2a cells, we wondered whether primary astrocytes might adapt to high dose proteotoxic stress. A previous study by Friedman and colleagues provides precedence to examine the protective impact of severe stress. In that study, exposure to a moderately toxic hit of glutamate (eliciting 30% cell loss) protected hippocampal neurons against exposure to a lethal glutamate challenge 7 days later (Friedman and Segal 2010).

ASTROCYTES CAN ADAPT TO SEVERE STRESS

Thus far we had shown that dopaminergic cells can adapt to low level proteotoxic stress in a CuZn superoxide dismutase-dependent and glutathione-independent manner but that neuronal cells cannot survive multiple bouts of high level proteotoxic stress because of catastrophic glutathione loss. Notably, highly stressed neuronal cells still responded to toxic MG132 with some adaptations, such as a rise in autophagic markers and ceruloplasmin. All of these findings led us to wonder whether severe proteotoxicity would elicit adaptations in cells known for their stress resistance, astrocytes (Shao and McCarthy 1994). Astrocytes are well known to interact with neighboring neurons, providing them with trophic support and metabolic precursors such as lactate (Westergaard *et al.* 1995; Rathbone *et al.* 1999; Benarroch 2005; Barres 2008). Furthermore, astrocytes probably serve as sentinels, as they express many types of neurotransmitter receptors (Fuller *et al.* 2010; Verkhratsky *et al.* 2012). Astrocytes are also critical for the production of glutathione in the brain (Dringen *et al.* 2000). In Parkinson's disease, astrocytes in the amygdala, septum, cortex, thalamus, and striatum become immunoreactive for the neuronal protein α-synuclein (Wakabayashi *et al.* 2000; Braak *et al.* 2007). These findings support the hypothesis that astrocytes engulf α-synuclein from the extracellular space through endocytosis to protect neighboring neurons (Lee *et al.* 2010). Furthermore, astrocytes are also known to engulf extracellular β-amyloid (Wyss-Coray *et al.* 2003). Because of their plasticity and critical roles in maintaining neuronal viability, astrocytes have been proposed to define homeostasis in the central nervous system (Parpura *et al.* 2012).

Given the plasticity of astrocytes, we wondered whether adaptive astrocytic responses could be elicited even with LC_{50} concentrations of MG132 that were lethal to half the cellular population. To our knowledge this question had not been answered in astrocytes before, although previous studies had shown that astrocytes can adapt to *sublethal* stressors (Rajapakse *et al.* 2003; Calabrese 2008a; Chu *et al.* 2010; Du *et al.* 2010; Du *et al.* 2011; Johnsen and Murphy 2011). In our model, we delivered two hits of toxic concentrations of MG132 one day apart to primary cortical astrocytes (Titler *et al.* 2013). Cells were assayed for viability on the third day by counting the remaining Hoechst-stained nuclei and measuring ATP. Toxic MG132 concentrations that killed approximately half the pop-

ulation of astrocytes did render the remaining cells resistant to a second MG132 hit. In other words, the toxicity of two hits was neither additive nor synergistic and the response to the second hit was blocked. Both cell count and ATP level viability assays verified that pre-stressed astrocytes were protected against a second hit. Although the cell count data confirmed the lethal nature of the first hit, the ATP assay actually revealed a slightly different pattern than cell counts. That is, the first hit did not elicit any ATP loss as it had loss of cell numbers; ATP output per cell had risen instead. Second, the first hit completely prevented the usual ATP loss in response to the second, higher dose challenge. The ATP dose-response curve therefore looked very similar to a traditional preconditioning curve with sublethal stress protecting against a second, otherwise lethal challenge. Because the pre-stressed cells were protected against ATP loss in response to a second MG132 challenge, we concluded that astrocytes exhibit active metabolic adaptations in response to severe proteotoxic stress. The rise in ATP output with the first hit may help fuel anti-apoptotic signaling cascades and preserve homeostasis.

As a potential confound to our interpretations on active astrocytic adaptations, we initially wondered whether the first MG132 hit was simply leaving behind astrocytes that were refractory to the toxin and therefore also unresponsive to a second hit. If this was the case, we could not claim that astrocytes mounted any adaptations to MG132. Alternatively, a second MG132 hit could continue to have an impact on the proteasome in pre-stressed cells even though it did not lead to additional cell death. We tried to distinguish between these two possibilities by assaying for ubiquitin-conjugated proteins. We found that two hits of MG132 caused a synergistic rise in ubiquitin-conjugated proteins (Fig. 2). This finding was incompatible with the hypothesis that the remaining cells were simply refractory to MG132. Even though the downstream impact of proteotoxic stress on cell viability itself was abrogated, these data demonstrate that the stress on the proteasome itself was not prevented. The potentiation of this proteasomal response verifies the continued impact of MG132 in the survivors of the first hit and also reflects the severity of the proteotoxicity.

In order to probe for adaptive rises in pro-survival molecules in stressed astrocytes, we measured heat shock protein 70 and heme oxygenase 1. Heme oxygenase 1 degrades heme into biliverdin and carbon monoxide (Grochot-Przeczek *et al.* 2012). Astrocytes express high levels of this protein, perhaps reflecting their inherent resilience (Dwyer *et al.* 1995). Both heat shock protein 70 and heme oxygenase 1 were raised by MG132, as expected from cells that are attempting to battle toxicity (Fig. 2). We also probed for glutathione levels in this model, and discovered that pre-stressed astrocytes failed to respond to the second hit with the usual glutathione loss, unlike naïve astrocytes challenged with high dose MG132. In other words, glutathione levels following the MG132 chal-

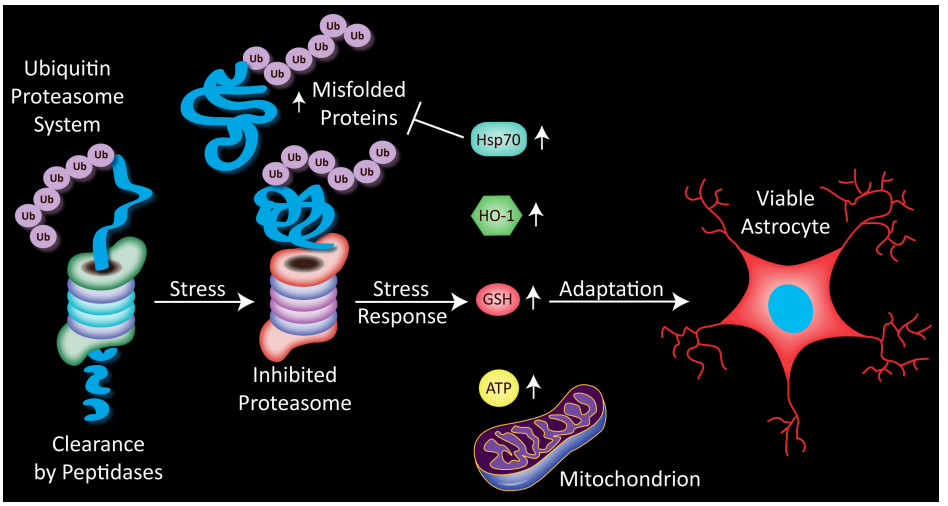

FIGURE 2. Stress on the proteasome, in the form on inhibition of its normal proteolytic function, increases the cellular burden of damaged proteins. Astrocytes respond to such proteasome inhibition with a rise in the anti-apoptotic heat shock protein 70 (Hsp70). Numerous studies reveal that Hsp70 refolds misfolded proteins or enhances their degradation by the proteasome. A parallel rise in heme oxygenase 1 (HO-1), a generally protective phase 2 enzyme, is also apparent. Astrocytes respond to severe proteotoxicity with glutathione (GSH) loss, unless they have been pre-stressed with MG132, in which case GSH levels are restored. This thiol adaptation serves to increase the number of viable astrocytes and is accompanied by a rise in ATP. Pre-stressed astrocytes are thus both structurally and functionally protected against further proteotoxicity.

lenge were higher in pre-stressed astrocytes than in naïve controls. This finding suggested that severely stressed astrocytes might use thiol defenses to protect themselves against future insults. Consistent with this hypothesis, depletion of glutathione stores with buthionine sulfoximine unmasked the cumulative impact of two hits; pretreated astrocytes now became vulnerable to the second MG132 hit and responded with additional cell loss. The unmasking of the vulnerability to two hits following glutathione depletion was also inconsistent with the notion that the first MG132 hit simply left behind cells that were unresponsive to the poison. As in the N2a two hit model, the findings reveal that antioxidant defenses help defend cells against proteotoxic stress. However, astrocytes and the neuronal N2a cells responded to severe proteotoxicity in opposite fashion. The response to the second proteotoxic hit was blocked in astrocytes but potentiated in N2a cells. We do not claim here that neuronal cells only respond to dual challenges with synergistic toxicity or that only astrocytes can adapt to severe stress. Instead, we have preliminary data in primary cortical cultures that neurons are able to adapt to severe oxidative stress from hydrogen peroxide pretreatment. Thus, whether severe stress elicits adaptations or exacerbates further insults may depend on the nature of the stress as well as the specific cell type, in addition to dose and duration.

In summary, astrocytes are a highly plastic cell type that can adapt to stress even when it is toxic enough to kill half the population. The astrocytes that survive the initial hit are less, not more, vulnerable to further proteotoxicity and have higher levels of glutathione upon the second hit (Fig. 2). We speculate that astrocytes that are exposed to similarly severe protein-misfolding stress in the human brain may fulfill their roles as neurosupportive cells better than if they had no such defenses. As mentioned above, astrocytes and neurons are well known to interact in the brain, probably in conjunction with oligodendrocytes (Amaral *et al.* 2013). It remains to be determined whether stressed astrocytes provide increased trophic, metabolic, or antioxidant support for neighboring neurons or whether stressed astrocytes engulf more misfolded proteins in proteotoxic conditions. Many have argued instead that activated astrocytes neglect their neurosupportive roles, particularly in the presence of chronic inflammation (Fuller *et al.* 2010). Thus, examinations of the protective or toxic impact of severe proteotoxic stress on neuronal-astrocytic interactions are highly warranted.

CONCLUSIONS

The mammalian brain enjoys manifold robust defenses. Even the simple observation that a large fraction of dopamine must be lost before movement deficits emerge reflects the impressive compensatory adaptations of the human brain (Hornykiewicz 1975; Zigmond *et al.* 1990; Hornykiewicz 1998). Another form of adaptation is the ability to raise anti-apoptotic proteins in response to stress. The studies detailed in this review, as well as many others not discussed here, have slowly begun to define this adaptive proteome. Our studies add to this body of work by specifically revealing that the ubiquitous tripeptide glutathione is responsible for adaptation against severe proteotoxicity. Conversely, when it is reduced in levels, a lack in glutathione is responsible for synergistic proteotoxicity.

As mentioned above, the long term goal of our studies is to characterize the adaptive proteome so that it can be mimicked with pharmacological tools. Clinical preconditioning with pharmacotherapies is not unprecedented or futuristic. Many FDA approved agents such as aspirin, isoflurane, and statins are already thought to precondition against ischemia (Gidday 2010). Defining the molecular targets of preconditioning and the effectors to counteract stress may also guide studies on lifestyle and dietary factors that elicit a naturally therapeutic protein profile. However, all the findings presented must be examined in further detail in whole animals and over longer timeframes. Even partial inhibition of the proteasome for many months does not recapitulate the full extent of the pathophysiology of neurodegenerative disorders. More chronic models than presented here are therefore required to rigorously

test the hypothesis that the cells that remain behind after neurodegeneration has commenced are either resistant or sensitized to further toxicity. Studies of this nature would build upon previous in vivo reports showing that repeated stressors can provide persistent protection against ischemia/reperfusion injury (Hoshida et al. 2002). Bearing these important gaps in our knowledge and the experimental caveats in mind, we predict that both neurons and glia in vivo will react to severe stress in a way that deeply affects their response to subsequent challenges, but that the response will depend on dose, duration, and perhaps brain region (Mattson et al. 1989; Mattson and Kater 1989; Braak et al. 2000; Braak et al. 2003; Posimo et al. 2013). More specifically, we propose that distinct adaptations and vulnerabilities to proteotoxic stress across different brain regions may underlie the signature topographies of protein inclusions in neurodegenerative diseases.

Finally, the two hit model is probably a simplification of the injuries that occur in neurodegenerative conditions because the diseased human brain may be exposed to a rolling landscape of hits, and not just two sequential stressors. Furthermore, some stressors may not even appear as a hit because they are not transient, but chronic in nature. Nevertheless, the two hit treatment protocol with MG132 is a useful tool to probe whether proteotoxic stress (the first hit) elicits adaptations or toxic responses. If more examples of adaptations can be collected *in vivo* in chronic proteotoxicity models, they would be consistent with the delayed onset and slowly progressive nature of neurodegenerative conditions. Conversely, toxic neuronal responses to severe proteotoxic stress may overwhelm defenses when the stress is unyielding, as originally postulated by Selye, and are consistent with the eventual collapse of vulnerable brain regions in Parkinson's and Alzheimer's disease.

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