

CARBOXY TERMINUS OF HEAT SHOCK COGNATE 70-INTERACTING PROTEIN (CHIP)
FOLLOWING CELLULAR STRESS

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ABSTRACT

LAUREN GHISLAINE ANDERSON: Carboxy terminus of Heat shock cognate 70 Interacting Protein (CHIP) following Cellular Stress
(Under the direction of David Y. Huang, M.D., Ph.D. and Rick B. Meeker, Ph.D.)

Carboxy terminus of Hsc70 Interacting Protein (CHIP) is thought to be a cytoprotective protein with roles in protein quality control in neurodegenerative diseases and myocardial ischemia. This study examined CHIP expression in normal mouse brain and in primary cultures of cortical neurons following heat stress (HS) and oxygen-glucose deprivation (OGD). CHIP was highly expressed throughout the brain, predominantly in neurons where the staining pattern was primarily cytoplasmic. More intense nuclear staining was observed in primary cultured cells than in brain sections. Nuclear accumulation of CHIP occurred very rapidly after 5-10 minutes of HS and decreased at or below baseline by 30-60 minutes. Increased durations of HS gave rise to sharp increases in delayed cell death and were inversely correlated with the loss of nuclear CHIP. While no changes in cytoplasmic CHIP were observed immediately following OGD, nuclear levels of CHIP increased slightly in response to OGD by 30 minutes and remained increased through 240 minutes. Increased CHIP levels did not decrease immediately following extended durations of OGD, but rather decreased during recovery following OGD. Nuclear CHIP decreased earlier in recovery following 120 minutes of OGD (4 hours) than 30 minutes of OGD (12 hours). Significant cell death first appeared between 12 and 24 hours after OGD again suggesting that delayed cell death follows closely behind the disappearance of nuclear CHIP. Cell viability in heterozygous and homozygous hippocampal slice cultures from transgenic mice lacking CHIP was impaired following OGD. Transgenic cultures displayed increased delayed cell death following 30 minutes of OGD compared to wildtype. Following 120 minutes of OGD,

cell death in all cultures was greater than baseline but cell death in the transgenic samples was no longer significantly greater when compared to wildtype. A yeast two-hybrid screen to identify proteins that interact with full-length recombinant CHIP yielded 19 sequences from known binding partners heat shock protein 70 and heat shock protein 90. Together the results support the idea that the ability of CHIP to translocate to and accumulate in the nucleus may be a limiting variable that determines how effectively cells respond to external stressors to facilitate cell survival.

This dissertation is dedicated to my parents:

James and Jacqueline Anderson,
*for all those who have invested their pride in me,
and the memory of those who inspired me.*

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PREFACE

I have prepared my dissertation in accordance with the guidelines set for by The Graduate School of The University of North Carolina at Chapel Hill. The dissertation consists of a general introduction, four chapters of original data, and a conclusion chapter. Each data chapter includes an introduction, results, discussion, and materials and methods section. A complete list of the literature cited throughout the dissertation has been appended to the end of the dissertation. References are listed in citation order and follow the format of the journal, *Neurology*. Chapters one and two include original data that was included in a submission for publication.

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LIST OF ABBREVIATIONS

2DG	2-deoxy-D-glucose
aCSF	artificial Cerebral Spinal Fluid
AD	Alzheimer's Disease
ADP	adenosine diphosphate
ATP	adenosine triphosphate
Ca ²⁺	calcium ion
CHIP	Carboxy terminus of Heat shock cognate 70 Interacting Protein
CM67	Conditioned Medium Mouse-anti-CHIP clone 67
CNS	Central Nervous System
CT	cross-tolerance
ER	Endoplasmic Reticulum
FCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
gfMEM	glucose-free Minimum Essential Medium
GFP	green fluorescent protein
Grp78	glucose-regulated protein 78
HIF	Hypoxia-inducible factor 1
HS	Heat Shock
HSC70	Heat Shock Cognate 70
HSF1	Heat Shock Factor 1
HSPs	Heat Shock Proteins
HSP70	Heat Shock Protein 70
IT	ischemic tolerance
LBD	Lewy Body Dementia
NLS	nuclear localization sequence
NMDA	N-methyl-D-aspartate
mM	milliMolar (10 ⁻³)
MEM	Minimum Essential Medium

O ₂	oxygen gas
OGD	Oxygen-Glucose Deprivation
PBS	Phosphate-Buffered Saline
PC	pre-conditioning
PD	Parkinson's Disease
PI	propidium iodide
RanGTP	Ras-related nuclear protein guanosine-5'-triphosphate
tPA	tissue plasminogen activator
TPR	tetratricopeptide
Ub	ubiquitin
UPR	Unfolded Protein Response
UPS	Ubiquitin-Proteasome System

INTRODUCTION

Etiology of Stroke

Stroke is the third leading cause of death and the leading cause of long term disability in the United States.¹ Over eighty percent of all strokes are ischemic, defined as cellular injury due to a loss of cerebrovascular blood flow caused by vessel blockage. An ischemic stroke is identified by a lesion composed of two main regions: a core infarct where perfusion is lost completely and a much larger penumbral region that surrounds the core and receives varying levels of perfusion.²

Cells in the infarcted core experience a complete loss of glucose and oxygen. Under these conditions, ATP is depleted by 50% within one minute, which dramatically affects ATP-dependent pumps most notably the Na⁺ pump.³ With oxygen deprivation, glucose metabolism changes to anaerobic glycolysis, leading to lactate accumulation and tissue acidosis.^{2,4} Deficits in energy production and prolonged acidosis contribute to anoxic depolarization and the subsequent collapse of the sodium gradient.^{3,5} As the electrochemical gradient reverses, calcium ions may be pumped into cells by the reverse operation of the Na⁺/Ca²⁺ exchanger.³ Without the sodium ion gradient, neurons depolarize and release neurotransmitters, the most abundant of which is glutamate. Increases in extracellular glutamate lead to over-activation of the N-methyl-D-aspartate (NMDA) receptor channel. The subsequent influx of sodium and calcium ions increases the osmotic gradient set up by ion influx, and the neurons swell. Glutamatergic over-activation and secondary osmotic swelling is characteristic of necrotic cell death.

In the ischemic penumbra, blood flow is below the level needed to maintain electrical activity but above that required to maintain cellular ionic gradients.⁶ At the expense of further

energy depletion, cells are able to re-polarize. Small elevations in intra-mitochondrial Ca^{2+} increase the efficiency of several mitochondrial enzymes.⁷ Continued depolarization gives rise to high concentrations of intracellular Ca^{2+} resulting in the activation of numerous degradative enzymes and continued loss of mitochondrial function.^{3,8} The loss of mitochondrial function results in the release of mediators of apoptosis, including cytochrome c, an early activator of the caspase cascade and apoptotic cell death.⁸ The gradual decline in neuronal function can take days or weeks, during which time the life or death of the cell depends heavily on the ability of the cell to maintain basic homeostatic functions.^{6,9} Any event that tips the balance in favor of physiological stability and survival could have a substantial beneficial impact on the large population of cells within the penumbra.

Acute Stroke Treatments

Current acute stroke treatments focus on re-establishing perfusion using proteolytic enzymes like tissue plasminogen activator (tPA). These enzymes convert the blood protein, plasminogen, to plasmin.¹⁰ Plasmin dissolves fibrin in the insoluble matrix of clots.¹⁰ As such, a fibrin thrombus can be cleared by plasminogen activators.¹⁰ tPA gained approval from the Federal Drug Administration for acute stroke after the NINDS tPA trial showed that patients receiving intravenous tPA within three (3) hours of stroke onset were at least 30% more likely to have minimal or no disability at 3 months compared to placebo.¹¹ Unfortunately, tPA use in stroke is extremely limited, with less than 5% of eligible ischemic stroke patients receiving tPA, primarily due to the narrow therapeutic time window (<3 hr from the onset of symptoms) and strict eligibility requirements, and the high risk for severe brain hemorrhage.¹²

The relatively low rate of use of thrombolytic drugs has necessitated the exploration of additional agents as potential treatments for acute stroke.^{10,13-15} A limitation of drug therapy is the requirement of adequate blood flow to deliver drug to the injured site.

Restoration of blood flow or increased circulation could have detrimental effects if blood vessels are damaged or neurons are in a toxic environment prior to or following an occlusion. Since the penumbra has some partial blood flow which may be increased by thrombolytic agents, it is a viable target for therapy. Indeed, in preclinical studies, many neuroprotective drugs have shown promise in extending the therapeutic time window and promoting neuronal survival of cells in the penumbra.¹⁶⁻¹⁹

To develop potential therapeutic treatments, surgical models have been generated experimentally to mimic stroke injury. Of these models, middle cerebral artery occlusion (MCAO) is the most utilized for cerebral ischemia. Injury in this model targets the striatum and adjacent cortex. This mimics blockage in the MCA or lenticulostriate arteries in humans, the most common type of ischemic stroke. With this *in vivo* model, the similar carotid tie-off model of global ischemia, and other parallel *in vitro* models, researchers have been able to study a number of cellular mechanisms and agents (**Table 0-1**) implicated in ischemic cell death and cell survival.⁸

A number of pharmacologic treatments have been studied²⁰ (**Table 0-2**), many of which target final molecular stages that precede cell death.²¹ Fewer compounds intervene at early stages of neuronal dysfunction in response to the ischemic stress. For example, NMDA receptor antagonists have been heavily explored to prevent the influx of calcium ions and attenuate depolarization.^{8,22-25} Numerous studies have implicated NMDA receptor overactivation in the mechanisms of neuronal death, generally referred to as the “excitotoxicity” hypothesis.²⁵ However, depolarization due to loss of ion flux regulation and subsequent NMDA receptor overactivation occurs rapidly and may model conditions within the ischemic core better than the surrounding tissue in the penumbra.⁸ Thus, such intervention would be too late to significantly affect destruction of viable tissue. Failures of this approach have led some to question the merits of this pharmacologic strategy.²⁶⁻²⁸ Other strategies have been designed to inhibit calcium entry²³, prevent oxidative damage

due to free radicals⁷, indirectly modulate glutamate receptor activity²⁴, and more recently to inhibit reverse exchange operation of the sodium-calcium exchanger²⁹⁻³². Each of these approaches is designed to intervene at later stages of cellular pathogenesis to block the immediate cause of damage or death. If the cellular environment is already volatile as a result of a compound or precipitating disease state, these mechanisms could already be compromised, thus making it difficult to successfully intervene.

Cellular Stress Response

In the ischemic penumbra the hypoxic, transiently energized environment challenges the homeostatic mechanisms of the cell to produce the proteins necessary for survival. The endoplasmic reticulum (ER) is the intracellular site of newly synthesized proteins. The molecular system that monitors and responds to changes in the ER protein processing environment thereby maintaining cellular homeostasis in spite of stress has been termed the unfolded protein response (UPR).³³ At least three pathways integrate to upregulate proteins to respond to the stress, maintain membrane stability, and continue to process proteins.^{34,35} The UPR monitors the protein-folding capacity of the ER and signals cell responses to maintain capacity and prevent the accumulation of unproductive and potentially toxic protein products.³⁴ Additionally, the release of calcium by the ER in response to more severe stress provides additional challenge to the mitochondrial microenvironment^{3,34}. As such, the UPR is a commonly used, early indicator, of cell response and viability.^{34,36}

An alternative approach to re-establishing perfusion in stroke treatment is to protect cells by making them more tolerant to the stresses of ischemia. This concept, known as ischemic tolerance (IT), comes from numerous studies that have shown sub-lethal stress affords substantial long-term protection from subsequent challenges. A common explanation for ischemic tolerance is that the mobilization of cellular mechanisms that maintain homeostasis provides an extra level of protection in the face of a subsequent

challenge.^{9,37,38} Thus, any procedure that facilitates these processes has the potential to extend the therapeutic time window for thrombolytics as well as enhance the intrinsic level of neuroprotection. Such procedures may also be useful as prophylactic measures to minimize stress-related damage.

Endogenous Neuroprotection

Pre-conditioning

Research on inducing IT has centered on two experimental paradigms; ischemic pre-conditioning (PC) and cross tolerance (CT). The fundamental basis for both are observations that, in most living organisms, repeated exposure to a sub-lethal environmental stress often leads to transient tolerance to an otherwise lethal stress.^{9,16} Although it is very unlikely that the current techniques used to induce IT can be used as clinical treatment (e.g. manually inducing transient ischemic attacks via MCAO), the information gained from these studies have given researchers insights into the endogenous protective mechanisms in neurons.

For well over a decade, studies in numerous model systems have identified cellular mechanisms implicated in PC (**Table 0-3**). The first observation of ischemic tolerance was made in heart.^{9,39} The protection produced by PC (for periods ranging from 2.5 to 10 minutes) nearly doubled the duration of permanent ischemia needed for infarction to occur with a subsequent challenge.⁴⁰ This brief onset and duration suggest a metabolic mechanism of tolerance. In contrast, the induction of ischemic tolerance in brain occurs over a longer time frame, persists over days in brain, and is dependent on protein synthesis.⁴⁰⁻⁴² Biochemically, these mechanisms stabilize membrane potentials, activate protective pathways, and increase the capacity of endogenous stress response pathways following ischemia-reperfusion.⁴³ Sub-lethal chronic stress often leads to accommodation by cells which over time can cause cell death or protect cells. The conditions which favor

neuroprotection are not fully understood. However, much of the protection may stem from increases in heat shock proteins (HSPs).

Heat Shock Proteins

The heat shock family of proteins has emerged as one of the most ubiquitous and evolutionarily conserved inducible mechanisms of endogenous neuroprotection. There are a number of HSPs, typically grouped by molecular weight (**Table 0-4**). HSPs along with co-chaperones facilitate assembly, transport, folding, removal, and activity of functional proteins generated by the cell. HSPs have been widely studied in experimental ischemia and are strongly up regulated in the context of stroke and IT.^{42,44-46} In particular, studies of the 70-kDa family of HSPs indicate that these proteins are essential for recovery following stroke.⁴⁷⁻

⁵¹ This family includes heat shock cognate 70 (HSC70, constitutively expressed form, also known as HSP73) and HSP70 (inducible form, also known as HSP72). HSP70 is synthesized in especially high levels in the central nervous system (CNS) in response to ischemia, and appears to be highly neuroprotective.^{42,47,52}

Induction of HSP70 was seen in models of IT. Attempts to inhibit HSP70 lead to partial inhibition of IT.¹⁶ Gene therapy with HSP72 was neuroprotective in rat models of stroke.⁵³ Transgenic mice over-expressing HSP70 were protected against cerebral infarction in models of focal ischemia.^{48,54} Conversely, reduced HSP70 gene expression led to increased cellular damage after focal ischemia in HSP70 knockout mice.⁵⁵ *In vitro*, survival was correlated to the amount of HSP70 induction after thermal or ischemic stress and not with the similarity of subsequent stress.^{56,57}

Among other effects, HSP70 may help to preserve mitochondrial function.⁵⁸ ATP is rapidly depleted during an ischemic event and preservation of mitochondrial function is a potential early intervention to facilitate cell survival.³ The importance of HSP70 for preservation of mitochondrial function is highlighted in several studies.^{58,59} Expression of

HSP70 correlated with mitochondrial protection against oxidative injury in immortalized cell lines.⁶⁰ HSP70 exhibits late anti-apoptotic protection, responding to increases in caspase-3 expression.⁶¹ In mice over expressing HSP-70, early mitochondrial release of cytochrome c is suppressed.^{59,62} In MCAO models of cerebral ischemia, HSP70 induction was greatest in areas of partial perfusion and increased expression was seen in cortical penumbra neurons.⁶³

As a chaperone molecule, HSP70 works in conjunction with many other proteins. Not surprisingly, HSF1 (heat shock factor 1, an HSP transcription factor) is activated by many of the same triggers known to activate HSP70, including increases in intracellular concentrations of misfolded proteins.

HSPs and the Ubiquitin-Proteasome System (UPS)

In normal conditions, HSPs are the major components of the molecular chaperone and protein folding systems.⁶⁴ During cellular stress, HSPs are involved in the refolding of denatured proteins.⁶⁵ HSPs have also been shown to contribute to the ubiquitination of damaged proteins, thus targeting these proteins for degradation via the ubiquitin/proteasome pathway.⁶⁶ Under conditions of stress, survival of the cell depends heavily on the ability to make competent and essential proteins⁶⁷ as well as the elimination of defective proteins⁴. The folding of proteins requires energy and the maintenance of high intraluminal calcium concentrations in the endoplasmic reticulum. As energy stores fail it becomes increasingly difficult to maintain proper folding and the number of misfolded proteins increases. These misfolded proteins can aggregate and become quite toxic to cells. It has been suggested that the regulation of protein degradation may actually be the crucial role for HSPs following cellular stress.⁶⁸

The UPS is a major evolutionarily conserved method of protein degradation. UPS involves a three-enzyme (E1, E2, and E3), ATP-dependent process to tag proteins with

ubiquitin (Ub) for recognition by the 26S proteasome. E1-ubiquitin activating enzymes prepare ubiquitin to be associated with E2-conjugating enzymes.⁶⁹ E2 proteins associated with Ub bind to E3-ubiquitin ligase proteins and misfolded or target proteins to transfer ubiquitin moieties to the misfolded proteins for degradation.⁶⁹ In addition to their role in the cytosol, many HSPs, including HSP70 and HSP90, are present in the nucleus suggesting binding partners of the HSPs will also have a nuclear presence and function.⁷⁰

Brief hypoxia does not impair proteasome activity; however, prolonged periods of hypoxia initiates a shift from the Ub-ATP-dependent mechanisms of the 26S proteasome to the Ub-ATP-independent mechanisms of the 20S proteasome, a subunit of the 26S proteasome.⁷¹ In the absence of a functional UPS, protein degradation proceeds via lysosomal pathways.⁷¹ These events are correlated with the end stages of the stress response and may be a critical turning point in the ability of the cell to withstand the damage induced by the stress.

Cellular Stress Response

Evolutionarily conserved strategies of protection in the mammalian nervous system have provided valuable information on endogenous neuroprotective mechanisms. Under normal circumstances a variety of chaperone proteins insure proper protein folding.^{35,65} In the endoplasmic ER these functions are highly dependent on intraluminal Ca^{2+} and ATP.^{43,72} During ischemia, the rapid loss of energy production disables the protein folding mechanisms which results in the appearance of unfolded or misfolded proteins. This triggers the unfolded protein response (UPR). Proteins including transcription factors are shuttled from the ER and cytoplasm to the nucleus to initiate the production of proteins necessary for survival at the expense of other proteins. The dynamic balance between normal protein folding and an unmanaged aggregation of unfolded proteins is highly regulated and only partially understood. The regulation of proteins in the unfolded protein

response will ultimately play a role in determining whether apoptotic pathways are activated and the cell dies; or normal function is restored and the cell recovers.⁴³

Of the transcription factors activated during ischemia, HSF1 is activated by a rate-limiting trimerization and is then translocated from the cytoplasm to the nucleus.⁷³ Once in the nucleus, the HSF oligomers are phosphorylated at serine residues before binding to the heat response element (HRE) for transcription of HSP genes, in particular HSP70.^{74,75} Of note, increased DNA binding of HSF1 occurs prior to inhibition of O₂-dependent metabolic pathways and ATP depletion.⁷⁶ Nuclear and subnuclear movements of HSF1 within minutes of exposure to ischemic challenge prepare the cell to upregulate heat shock proteins for survival at the very earliest stages of the stress response.⁷³ It is likely that decisions of cell fate are being made at this time. Thus, a better understanding of these early activation and nuclear translocation of HSPs is needed to appreciate fully the neuroprotective role of HSP induction.

While the movement of transcription factors into the nucleus has obvious implications, several studies have demonstrated the nuclear translocation of other HSPs. For example, HSP60, typically localized to the mitochondria, is released into the cytoplasm and is capable of nuclear localization in response to mitochondrial destabilization.⁷⁷ Metabolic acute cell injury in the form of hypoxia is also associated with gene induction and translocation of the HSP transcription factor, hypoxia-inducible factor 1 (HIF1).⁷⁸ HSC70 has been shown to translocate from the cytoplasm to the nucleus following *in vivo* heat stress.⁷⁹ The time course of HSC70 translocation is within 15 minutes and initially does not involve HSP70.⁷⁹ When the synthesis of HSPs increase to a level proportional to the appearance of unfolded proteins, HSP70 and other chaperones re-localize to the nucleus and bind to the HSF1 transcriptional transactivation domain, thereby repressing transcription of heat shock genes.⁷⁴ The translocation of HSPs to the nucleus is perhaps the least well understood

response to stress and more studies are needed to evaluate the extent and significance of this process.

HSPs, CHIP, and Ubiquitin

HSP functions are modulated by co-chaperones. The ability of HSPs to ubiquitinate proteins and promote protein degradation comes from interactions with a large family of E3-ubiquitin ligases. The role of the E2-ligases is unique considering only two E1-activating enzymes have been identified. However, tens of E2-conjugating enzymes are presumed. The E3-ligating enzymes likely number in the thousands and are hypothesized to confer specificity in the ubiquitinating process. One of the better studied E3 enzymes is the co-chaperone CHIP (Carboxy terminus of Hsc70 Interacting Protein), first cloned in the Patterson lab.⁸⁰⁻⁸² CHIP has three functional domains: the tetratricopeptide (TPR) domain, a charged region, and a RING-finger like U-box domain in addition to a nuclear localization sequence.^{81,83} The TPR domain allows CHIP to bind to HSPs. The charged region has been implicated in dimerization of CHIP.⁸⁴ When bound to HSPs, CHIP functions as a chaperone dependent ubiquitin ligase via its U-box region.^{85,86} Two lysine rich, nuclear localization sequences are present in advance of the charged region (KKKR) and in advance of the U-box domain (KRKKR).⁸¹

The three amino-terminus TPR domains are similar to domains in other proteins that interact with HSP, namely Hip, protein phosphatase 5, Hop, and CyP-40.⁸¹ Chaperone binding is accomplished through an electrostatic interaction between the EEVD sequence of the TPR domain and a carboxylate anchor on the chaperone.⁸⁷ Unlike Hip and Hop, CHIP required a charged region adjacent to the EEVD domains for Hsp interaction.⁸¹

The U-box domain is structurally similar to the non-catalytic ubiquitin ligating RING (Really Interesting New Gene) finger domains, but lacks the characteristic cysteines and a histidine that would cause the structure to be stabilized by coordinating zinc ions. Instead,

the U-box is stabilized through strongly conserved charged and polar residues.⁸³ The U-box activates E2s through an allosteric aromatic stacking interaction to transfer ubiquitin to the target substrates. CHIP binds two E2 enzymes: UbcH5, which mediates autoubiquitination, and Ubc13-Uev1a, which mediates polyubiquitination of chaperone bound target proteins.⁸⁸

Proteins in the active site of the CHIP-HSP70 complex are ubiquitinated and directed down the proteasomal degradation pathway. Other co-chaperones work with HSPs to refold proteins. The balance between folding and degradation is likely to be critical to cellular homeostasis. *In vitro* studies suggest at baseline the HSP machinery favors protein folding. However, even small increases in CHIP appear to reconfigure the HSP machinery to favor the ubiquitination pathway. Previous data has shown that CHIP-mediated ubiquitination of misfolded proteins requires interactions between CHIP and E2/ubiquitin conjugating proteins and that CHIP on its own does not directly interact with the proteins to be ubiquitinated.^{89,90} Thus, in situations of severe cellular stress, the CHIP-mediated degradation pathway may predominate when the refolding pathway is overwhelmed.

Functions of the transcription factor HSF1 are also modulated by co-chaperones. The ability of HSF1 to trimerize is directly proportional to CHIP levels, and the accumulation of active HSF1 complexes persists for at least 48 hours.⁹¹ The other HSF1 activators are self-terminating, however it has been suggested that CHIP induced active complexes are possibly resistant to attenuation and self-terminating effects are absent.⁹¹ This is important if CHIP levels are modulated by stress. HSF1 is not ubiquitinated by CHIP but is phosphorylated coincident with trimerization.⁹¹ Activation of HSF1 by CHIP coincident with ubiquitination of chaperone substrates supports CHIP as a ubiquitin ligase.⁹¹ This further suggests CHIP has a dual cytoprotective role during cellular stress, increasing the folding capacity by activating HSF1 and promoting degradation as a ubiquitin ligase.⁹¹ How and if these two functions are related is unknown.

Ubiquitin in neuroprotection and chronic neurodegeneration

Ubiquitin is also a stress-response protein that is up-regulated following ischemia. A number of studies support a role for ubiquitin in protection from stroke. Following transient global ischemia, free ubiquitin immunoreactivity disappeared in all regions of the hippocampus. Ubiquitin immunoreactivity subsequently recovered in CA3 and the dentate gyrus, but did not return in CA1 neurons—the population most susceptible to delayed neuronal death.⁹²⁻⁹⁴ Ubiquitin was likely incorporated into ubiquitin-protein conjugates. However, when animals underwent induction of tolerance prior to the ischemic insult, fewer neurons in CA1 died, and ubiquitin immunoreactivity recovered in the CA1 region. Recovery of ubiquitin was likely due to protein recycling as well as new synthesis.⁹⁵ Ubiquitin-immunoreactive protein aggregates were also seen in dying CA1 hippocampal neurons following global ischemia.⁹⁶ Aggregates were not seen in neurons surviving the ischemic stress. These observations support the notions that efficient ubiquitination and proteolysis are crucial parts of the ischemic stress response and that protein aggregation is an early indication of failure of the degradation machinery. It is expected that the increase in misfolded proteins caused by ischemic stress will increase ubiquitination of proteins by CHIP.

Accumulations of misfolded proteins are hallmarks of a number of chronic neurodegenerative diseases. Ubiquitin aggregates are found in senile plaques and neurofibrillary tangles of Alzheimer's Disease, Lewy bodies and Parkinson's Disease and Lewy body dementia, and in nuclear inclusions of numerous CAG repeat disorders like Huntington's chorea.⁹⁷

CHIP in chronic neurodegeneration, ischemic stress, and cellular protection

CHIP has been implicated in the pathophysiology of Parkinson's disease⁹⁸, Alzheimer's disease (AD)^{99,100}, amyotrophic lateral sclerosis¹⁰¹, cystic fibrosis⁸², and

myocardial ischemia¹⁰². CHIP immunoreactivity was found in *in vitro* models of Lewy bodies and co-localized with alpha-synuclein and HSP70.⁹⁸ CHIP immunoreactivity was found in lesions of several tauopathies, including AD. CHIP was also shown to promote the ubiquitination of tau *in vitro* and *in vivo*.¹⁰⁰ Phosphorylation of tau appeared to be a recognition requirement for subsequent HSC70-binding dependent ubiquitination. CHIP could rescue cultured cells from phosphorylated tau-induced cell death and the authors suggested the HSC70-CHIP complex might provide a new therapeutic target for the tauopathies.¹⁰⁰ Since these studies support a protective role for CHIP in chronic, neurodegenerative diseases, we hypothesize that CHIP also plays a neuroprotective role in acute stresses, in particular, cerebral ischemia.

In addition, Ballinger *et al.* showed CHIP has protective properties in other organ systems following heat stress.⁸¹ The Patterson lab generated a CHIP (-/-) mouse strain that appeared to develop normally but was temperature sensitive and developed apoptosis in multiple organ systems in response to thermal challenge.⁸¹ Apoptotic changes were rare in thermally-challenged wild-type mice.⁸¹ The heat-stress sensitivity of the CHIP (-/-) phenotype appears to be much more severe relative to phenotypes of mice lacking various HSP70 isoforms.^{91,102} Similar findings were documented by the same lab in *in vivo* models of myocardial ischemia and reperfusion.¹⁰² CHIP(-/-) mice had greater infarcted area, impaired upregulation of HSP70 in reperfusion, decreased survival, greater incidence of arrhythmias, and were more prone to apoptosis *in situ*. These studies strongly support a central role for CHIP in the ischemic stress response.

CHIP in Acute Stress

At baseline the molecular chaperone system favors protein folding, however in the presence of stress CHIP mediates protein ubiquitination and HSF1 activation. CHIP forms an active homodimer that binds both a co-chaperone-target protein complex, and binds an

E2-conjugating enzyme.^{84,103} This complex facilitates the transfer of Ub moieties from the E2 to the target protein. An additional function has been illustrated for this dimer as a binding partner of HSF1. HSC70-bound CHIP dimer along with HSP90 and HSP70 hold HSF1 monomers in an inactive state.¹⁰⁴ In the presence of stress HSP90 and HSP70 are released and the CHIP-HSC70 complexes hold HSF1 in its trimerized active state.¹⁰⁴ So, in the presence of stress CHIP serves two functions: 1) ubiquitin ligase to promote proteasomal degradation of ubiquitinated proteins, and 2) HSF1 activator thereby promoting gene transcription of HSPs (**Figure 0-1**).

However, if the stress is severe, protein aggregates will form. These aggregates contain unfolded or misfolded proteins of unknown identity and are often also immunoreactive for chaperone proteins and ubiquitin.^{96,105} More recently, these aggregates have been shown to also be immunoreactive for CHIP.¹⁰⁶ Current hypotheses suggest that these aggregates cannot be broken down by the proteasome, thereby promoting further aggregation and possibly cell death. The possibility remains that these two processes (degradation and aggregation) are part of a continuum: aggregates form because the degradative process cannot keep up; or proteins typically associated with aggregates (i.e. tau, α -synuclein, ubiquitin, or parkin) or novel targets reach local concentrations that initiate the aggregate process.

Open Opportunities

The current descriptions of acute stress focus on durations of stress that cause significant injury, but are not chronic in nature. The fields of myocardial and cerebral ischemia have shown that short durations, typically those used for pre-conditioning, are capable of causing long-lasting changes in cells. Thus interventions in acute stress may offer protection earlier than current processes which target the hours, days, and occasionally weeks following an ischemic event. In hours to days, transcription of new

proteins has occurred, protein aggregates have formed, and by days to weeks some cells may have undergone apoptosis. These points are well after the removal of the precipitating stressor. More thoroughly understanding the processes that come within minutes or hours of the presentation of stress could reveal new points of intervention, some of which could be targeted prior to a catastrophic event.

As has been suggested, the acute cellular response to stress remains somewhat of a mystery. What is clear is that cells are capable of responding to stress quickly and that perhaps even the most modest of environmental perturbations may initiate a biological response. The acute processes are highly energy-dependent, and involve components of protein folding, gene transcription, protein degradation, and the toxic effects of protein aggregation. Understanding how early and acute cellular stress response processes interface with the chronic and degenerative manifestation of neurologic disease may help elucidate the role of this system in cell survival.

The previous descriptions of acute stress do not firmly characterize the presence of molecular chaperones and their relative importance in the acute time frame, given the possibility that they may contribute to the disease process in two seemingly opposing ways: 1) by facilitating neuroprotection when the recovery system works and 2) by driving delayed cell death when the system fails. Current data shows that CHIP is a key modulator of HSP molecular chaperones. To date there has been no analysis of the CNS localization of CHIP in neither models of ischemia nor examination of changes to CHIP that occurs in response to stress *in vivo* and *in vitro*. These opportunities form the basis of the experiments outlined in the following chapters. We have extended the analysis of CHIP to an *in vitro* system that has suggested the importance of CHIP as a key factor in the intracellular movement of molecular co-chaperones during the acute stress response. The results further support the hypothesis that CHIP is an important modulator of the cellular stress response, making a significant contribution to cell survival following ischemic injuries like stroke.

Table 0-1: Perpetrators of and Agents that Mitigate Cell Death

Agents and cellular processes that have been studied in connection with pro-death or pro-survival pathways in ischemic cell death as reviewed in Lipton (1999)⁸. Of note, components of apoptosis (i.e. caspase-3 inhibitors) and excitotoxicity (i.e. NMDA antagonists, increases in intracellular Ca^{2+}) are universal points of intervention for lessening the toxic effects on a cell subjected to stress.

Table 1: Perpetrators of and Agents that Mitigate Cell Death	
<i>Pro-Death</i>	<i>Pro-Survival</i>
Increases in intracellular Ca^{2+}	Caspase-3 inhibitors
Increases in Nitric Oxide	Matrix Metalloproteinase inhibitors
Increases in Free Radicals	Antileukocyte adhesion agents
Increases in platelet-activating factor	CD95 ligand activity
Decreased ATP	CDP-choline
Peroxyntirite	NDAPH oxidase knockout
Protease Activity—Calpain	nNOS inhibitors
Proteolysis	PARP inhibitors
Changes in proteins, phospholipids, and DNA	COX-2 inhibitors
Phospholipase Activity	PAF antagonist
Phospholipid changes	Ca^{2+} channel antagonists
Poly-ADPribose polymerase	Calmodulin antagonist
	NMDA antagonists
	AMPA/kainite antagonist
	Hypoglycemia
	Interleukin-1 antagonism
	TNF- α antagonism
	Polyamine oxidase blockage
	S20 proteasome inhibition

Table 0-2: Compounds Tested in the Treatment of Stroke Injury

Compounds that have been investigated for the treatment of stroke injury, as included in the database by the Internet Stoke Center at Washington University²¹ and reviewed in Lipton (1999)⁸ Signaling cascades, ion regulation, and environmental factors are among those interventions pursued to no avail.

Table 0-2: Compounds Tested in the Treatment of Stroke Injury	
NMDA Receptor Antagonists MK-801, CGS 19755 (Selfotel), Dextrorphan, Dextromethorphan, Aptiganel (Crestat), NPS1506, Remacemide, 7-chlorokynurenate, ACEA1021, GV 150526, NR2B antagonists (ifenprodil)	JNK inhibitors DOPS cyclohexyl ester 17 β -estradiol enantiomer Tumor Necrosis Factor (TNF) Microglial conditioned medium IL-6
Sodium Channel Blockers Fosphenytoin, Lubeluzole, 619C89	Cyclosporin A Piracetam GABA antagonist (muscimol, clomethiazole)
Calcium Channel Antagonists Memantine, Nimodipine, Flunarizine	S-100 β Uric acid Fenamates Prenanolone derivatives
Glutamate Antagonists AMPA receptor antagonists (NBQX, YM90K, MPQX, GYKI 52466, YM872) Mg ²⁺	Erythropoietin Selegiline NOS antagonists Clenbuterol Dihydroergocryptine Propofol GMCSF
Other Sigma receptor ligands AMPA receptor antagonists (YM872, MPQX) K ⁺ channel antagonist (BMS-204352) CDP-choline (citicoline) Fosphenytoin FGF, EGF, TGF- β 1 PGI ₂ (prostacyclin) Phenyl-t-butyl-nitrone Free radical scavengers (tirilozide, ebselen) HSP-72 Lipoxygenase inhibitors Chemokine inhibitors (NR58-3.14.3) Tetrodotoxin (TTX) Geldanamycin Isoflurane Caspase inhibitors	Oxygen regulated protein mGluR1 antagonists Cortico-Releasing Factor (CRF) Repinotan ONO-2506 (2-propyloctanoic acid)

Table 0-3: Cellular Mechanisms Implicated in Pre-conditioning

The benefits of cross tolerance are closely related to the mechanisms of endogenous neuroprotection. The idea being that the brain has an innate way of protecting itself from ischemic injury. While many mechanisms exists from ion regulation to gene transcription, the actions of the heat-shock family of proteins is a main mechanism of interest. *Adapted from Kirino T (2002)⁹ and Kaufman (1999)³³.*

Table 0-3: Inducers and Mechanisms of Ischemic Tolerance	
<u>Ischemic Preconditioning</u>	<u>Proposed Mechanisms of Protection</u>
Sublethal global ischemia Brief transient ischemia	Membrane stabilization and inhibition of excitability Inhibition of apoptosis Unfolded Protein Response (UPR) Induction of Heat Shock Proteins
<u>Cross-Tolerance</u>	
Hyper/Hypo-thermia Spreading Depression Epilepsy Inhibition of oxidative phosphorylation Lipopolysaccharide Traumatic Brain Injury	

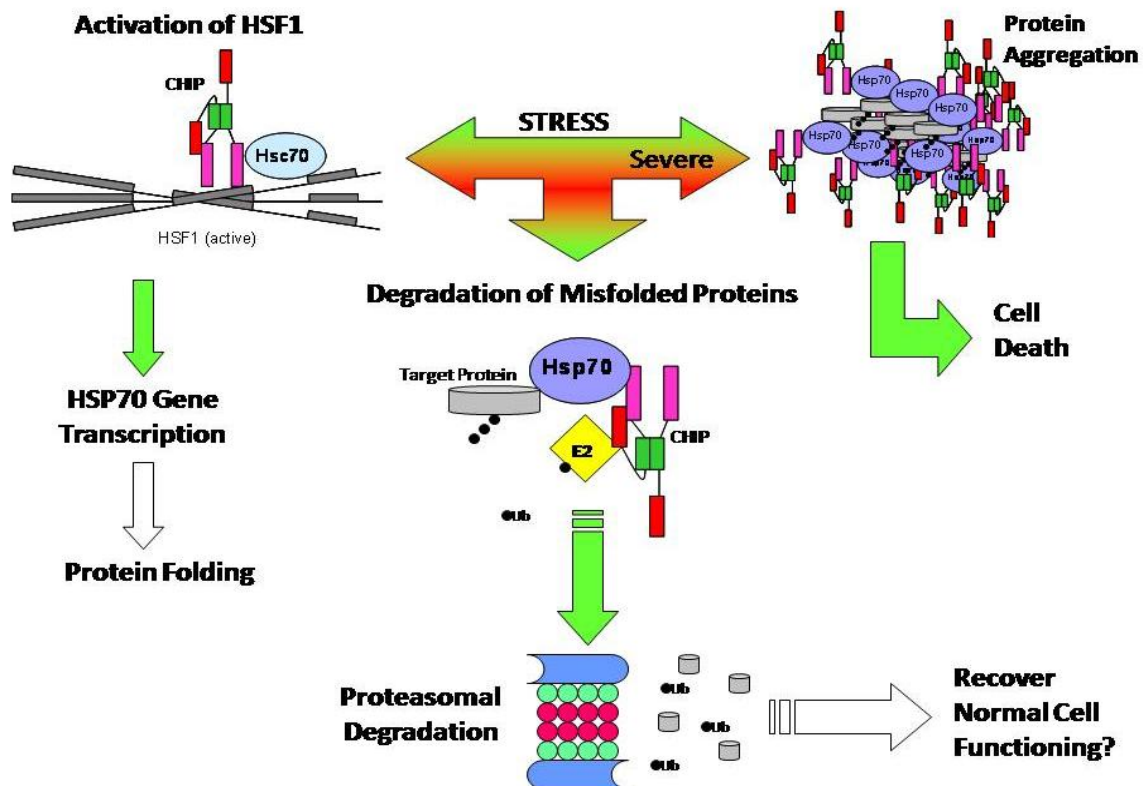
Table 0-4: Heat Shock Family of Proteins

The heat shock proteins (HSPs) represent a large number of proteins actively involved in the normal maintenance and stress response of cells. The chaperone proteins typically function in bound complexes (i.e. HSP70-HSP40, HSP60-HSP10) to complete a given function. The 70-kDa HSPs have been widely studied for their dual role in normal cell function and neuroprotection.

HSP Family	Localization	Function
HSP10	Mitochondria	Substrate release with HSP60
Small HSPs	Cytoplasm	F-actin assembly, molecular chaperone
HSP40	Cytoplasm, Nucleus	Protein folding, Collagen binding and transport, HSP70 co-chaperone
HSP60	Mitochondria, Cytoplasm, Nucleus	Polypeptide assembly, membrane transport, accelerate protein folding/unfolding
HSP70	Cytoplasm (ER), Mitochondria, Nucleus	Molecular chaperone, Protein assembly/transport/folding/unfolding/removal, ATPase activity, neuroprotection
HSP90	Cytoplasm (ER), Nucleus	Specific polypeptide and receptor binding (signal transduction)
Ubiquitin	Cytoplasm, Nucleus	Target proteins for proteasomal degradation, transcription regulation, signal transduction, marker for cell damage

Figure 0-1: CHIP Function Summary Diagram

Upon activation CHIP dimerizes and binds to an E2-ubiquitin conjugating enzyme and HSP co-chaperone to poly ubiquitinate target proteins for degradation by the proteasome. In addition, the CHIP-HSP complex facilitates and stabilizes the active, trimerized state the HSF1 transcription factor. Once active the subsequent HSP gene transcription increases HSP70 in the cell, primarily for protein folding. This potentially facilitates cell recovery. In the presence of severe stress the CHIP-HSP-target protein complex is capable of irreversible aggregation, thereby preventing proteasomal degradation of the target protein. Aggregate accumulation is neurotoxic and has been associated with cell death.



CHAPTER 1:

Characterization of CHIP in Neurons

INTRODUCTION

Proper protein management is a key component of cellular homeostasis.⁵² The balance between protein folding and degradation that is mediated through the molecular chaperones ensures the proteins needed for normal cell functioning are available and dysfunctional proteins are renewed. The decision between folding and degradation has been termed “protein triage” though the factors that influence this triage are not completely understood. Stress⁶⁸, disease^{105,107}, and age¹⁰⁸ have been shown to affect the balance of protein degradation and protein folding. By evaluating the molecular chaperones and the factors that influence chaperone function we should be able to postulate which chaperone functions are important for protein management and cell survival.

The carboxy terminus of HSC70 interacting protein (CHIP) is a negative regulator of molecular chaperones.^{80,81,89,109} CHIP promotes protein ubiquitination which leads to protein degradation.^{81,85,86} CHIP mRNA was shown to be widely distributed throughout the human body.⁸¹ mRNA levels were highest in the striated muscles of the heart and skeletal muscles and to a lesser extent in the pancreas and brain.⁸¹ CHIP mRNA levels were also reported in human cell lines and primary cultures.⁸¹ To date, similar analysis of CHIP protein levels in primary cultures has yet to be presented.

Murata et al. cited a high protein level of CHIP in the brain, but have not presented evidence of CHIP protein expression.^{85,110} Functionally, such an observation encourages the hypothesis that CHIP is found at high levels in cells that have a high energy consumption and protein turnover. With increased activity and protein production the

possibility for error would also increase making the appearance of misfolded proteins more frequent.¹¹¹ As a link between the chaperone (folding) and proteasome (degradation) systems, CHIP-mediated proteasomal degradation of damaged and misfolded proteins may play a key role in demanding organ systems, such as the brain. The presence of mis-folded proteins prompts the activity of the unfolded protein response (UPR) in the ER as well as the ubiquitin-proteasome response. The ubiquitin-proteasome system is considered to play a key role in protein homeostasis by promoting the immediate degradation of misfolded or impaired proteins. Investigation into the degradation of hyper-phosphorylated tau and α -synuclein in Alzheimer's disease (AD)^{100,112} and Parkinson's disease (PD)^{90,98} respectively have illustrated, at least in isolated contexts, the ability of CHIP to function as an ubiquitin ligase to degrade these proteins. Further, the presence of components of the system in the cytoplasm and nucleus suggests the proteasomal system is capable of both routine cellular maintenance and an early response to the generation of misfolded proteins.

In an organ system like the brain where great variety is found in cell types, energy consumption, and disease susceptibility proteins important to basic homeostasis could vary. None of the previous studies examined the cellular and regional distribution of CHIP in the brain. To further characterize CHIP, we documented the localization in the mouse brain *in vivo* and in primary mixed cultures of neurons and astrocytes *in vitro*. Additional observations were made on the characteristics of sub-cellular localization. To clarify the stability of CHIP in the cell culture model system, the stability of CHIP was evaluated.

RESULTS

CM67 Antibody Specificity:

To study CHIP, anti-CHIP monoclonal antibodies were produced in our lab against a purified GST-CHIP fusion protein. Product from hybridoma clone 67 (CM67) was selected for high immunoreactivity against CHIP on ELISA and Western blot screens. On Western blot,

CM67 recognized a single band of approximately 35kDa molecular weight present in fibroblast whole cell lysate (WCL) from wildtype (+/+) mice. Specificity was confirmed by the absence of immunoreactivity in lysates from CHIP (-/-) mice (**Figure 1-1**).

CHIP Protein Localization in Mouse Brain Sections:

To determine expression and localization of CHIP *in vivo*, sections of adult mouse brain were stained using the lab-generated monoclonal antibody against CHIP. Representative examples of CHIP staining from the brain of a 10-week old Sv129 mouse are shown in **Figure 1-2**. Immunoreactivity was observed throughout the brain including the cortex (1-2A), hippocampus (1-2B), hypothalamus (1-2C), Purkinje neurons (1-2D), choroid plexus and ependymal cells (1-2E), and thalamus (1-2F). The staining pattern was primarily cytoplasmic, demonstrated by double staining sections for CHIP (1-2G) and a nuclear stain, bisbenzamide (1-2H) and the two images combined (1-2I) to show co-localization. Higher magnification of 1-2G-I is shown in the panel Figure 1-2J to highlight perinuclear localization of CHIP *in vivo*.

CHIP Protein Localization in Cultured Cortical Neurons:

To verify the presence of CHIP in dissociated cultures of primary cortical/hippocampal cells, representative examples of staining are shown in **Figure 1-3**. Dissociated cortical cultures from fetal rat brains showed abundant expression of CHIP (1-3A). Neurons in the cultures, identified by immunoreactivity for MAP-2 (1-3B) showed the greatest immunoreactivity for CHIP (1-3A, arrow). There was lower expression in astrocytes (1-3A, arrowhead). A nuclear counterstain with bisbenzimidazole (1-3C) was also applied to the cultures. A combined image of CHIP, MAP-2 and bisbenzimidazole (1-3D) illustrated CHIP immunoreactivity was both nuclear and cytoplasmic, but primarily cytoplasmic with enhanced intensity in the perinuclear

region. The combined image also illustrated the presence of CHIP in both neurons and astrocytes in primary dissociated cortical cultures.

CHIP expression in different culture conditions:

Primary cortical cultures enriched in neurons were prepared normally and kept in MEM for the first 3 days and then switched to Neurobasal culture medium for the last 7 days. In these cultures, neurons accounted for at least 80% of cells with astrocytes making up most of the remaining cells. To determine if an enriched culture preparation would be appropriate for future studies, cortical neurons from fetal rats were kept in culture for 10 days. Control cultures were kept in MEM for all 10 days. Equal amounts of protein were analyzed by Western blot (**Figure 1-4**). CHIP was present in both the cytoplasmic and nuclear fractions in both MEM and Neurobasal preparations. Greater immunoreactivity was observed in the nuclear fraction of cultures kept in Neurobasal versus the nuclear fraction of cultures kept in MEM (i.e. less stress in complete MEM conditions).

Density of Neurons in Cultures:

MEM was chosen as the standard culture condition. Representative examples of dissociated cortical mixed cultures of neurons and astrocytes are shown in **Figure 1-5**. After 10 days in vitro, cultures consisted of neurons with astro-glial support. Increased cell density resulted in the more frequent appearance of clusters of cells and more elaborate networks of connections among the neurons.

CHIP stability profile

To determine the presence of CHIP throughout the ischemic time course, cultures were exposed to increasing durations of OGD with or without cycloheximide (CHX). Cultures exposed to 120 minutes of OGD were allowed to recover for 24 hours in the presence of

CHX. Immediately following OGD CHIP in the cytoplasm and nucleus remained unchanged (**Figure 1-6A**, for Western see Figure 2-5A). CHIP in whole cell lysate from cultures exposed to increasing durations of OGD with CHX, again remained relatively unchanged, with an estimated $t_{1/2}$ of 111.36 hours (**Figure 1-6B**). CHIP levels in whole cell lysate did decrease during recovery when CHX was present generating a $t_{1/2}$ of 71.33 hours (**Figure 1-6C**). This suggests that 1) CHIP is stable during OGD, 2) Stable CHIP levels are not the result of new synthesis during OGD, and 3) During a 24 hour recovery in the absence of new synthesis CHIP decreased slowly suggesting gradual consumption.

DISCUSSION

In the present study we examined CHIP expression and stability in neurons. Previously, CHIP expression had been demonstrated in immortalized cells and in numerous organ systems^{81,113}, but not the brain. In COS7 cells, GFP-CHIP fusion proteins first demonstrated CHIP expression in the cytoplasm⁸¹ and later indirect immunoreactivity in the cytoplasm was also observed⁸². Cytoplasmic CHIP was also identified in neuroblastoma SH-SY5Y cells⁹⁰, hamster lung fibroblasts (O23)¹¹⁴, and Chinese hamster ovary (CHO) cells¹¹⁵. Our work showed CHIP expression *in vivo* in sections of mouse brain that was primarily cytoplasmic with perinuclear intensity (Figure 1-2J). *In vitro*, CHIP immunoreactivity in primary cortical cultures was both cytoplasmic and nuclear with perinuclear intensity (Figure 1-3). Unlike previous reports in cell lines^{82,91,114-116}, nuclear localization of CHIP in primary neurons was observed without intentional perturbation of the cell culture. These data demonstrate endogenous CHIP expression that is primarily cytoplasmic with the possibility of dense localization in the perinuclear region. In cultured cells CHIP may also be localized in the nucleus. It is possible this is an adaptive measure to ensure cell survival in culture. Primary cortical cultures consisted of both neurons and astrocytes from E17 rat fetuses.

Cultured cells also displayed CHIP immunoreactivity in astrocytes. Previous reports have stated astrocytes produce HSPs for secretion into the extracellular space.^{38,117} Whether astrocyte activity is part of the adaptive measure that increases nuclear CHIP expression in culture remains to be evaluated.

Under the assumption that nuclear translocation is a sign of stress, the primary cultures described in this report were maintained as to provide a minimum level of nuclear CHIP. Though nuclear levels were not absent as *in vivo*, maintaining cultures in MEM provided an *in vitro* system in which nuclear CHIP responses could be manipulated.

CHIP stability in these cultures was demonstrated by testing CHIP levels in the presence or absence of the protein synthesis inhibitor, cycloheximide (CHX). CHX is also a proteotoxic substance. In response to the addition of CHX localization of CHIP expression in cultures fluctuated (Figure 1-6A). In differentiated NT2 cells displaying the neural phenotype (NT2-N), indirect CHIP immunoreactivity was observed in the cytoplasm and nucleus.¹¹⁶ There may be initiation of the stress response during early and acute exposure of cells to CHX. Nuclear CHIP levels increased over time in culture (data not shown). When CHX is present in cultures exposed to increasing durations of OGD CHIP levels did not significantly decrease, indicating that CHIP is likely not synthesized or consumed during OGD. Additionally when CHX is added after OGD for the duration of recovery, CHIP levels in whole cell lysate again decreased slowly.

These data suggest that CHIP is present at levels comparable to baseline throughout OGD and during recovery. Further, our data suggests that CHIP localization is primarily in the cytoplasm of the cell body with some nuclear localization in culture. Additionally, nuclear CHIP localization could be manipulated by different culture conditions. Other studies have shown that in the presence of an HSP90 inhibitor and synthetic glucocorticoid steroid, dexamethasone, CHIP immunoreactivity was localized to fragmented globules in neurites.¹¹⁶ Similar results were observed in HEK293 cells exposed to heat stress.¹¹⁵ CHIP also

localized to neurofibrillary tangles containing mostly four-repeat tau in cells of supranuclear palsy-affected brain.¹¹² Yet this study provided convincing evidence that even with a presumed stress in pathological tangles, CHIP may remain active.¹¹²

MATERIALS AND METHODS

Cell culture

Fetuses were removed from euthanized pregnant rats at 17 days gestation. Brains were removed from the cranium in a sterile hood and rinsed 3X in fresh sterile HEPES Buffered Salt Solution (in mM: 1.25 NaCl, 5.36 KCl, 0.49 MgCl₂·6H₂O, 0.40 MgSO₄, 138.1 NaCl, 1.0 D-glucose, 19.97 HEPES, pH 7.4, Osmolality 290±3). The cortex-hippocampus was dissected from the brain and placed in calcium-magnesium free HBSS (CMF-HBSS, same as HBSS except add 141.52mM NaCl and omit calcium and magnesium salts). The tissue was then, minced, transferred to a 15ml tube containing 5ml CMF-HBSS containing 2.5U/ml dispase + 2U/ml DNase I and incubated for 15-20 minutes at 37°C. Cells were dissociated by trituration and the suspended cells were transferred to a 50ml culture tube containing 25mls of complete medium (MEM + 10% Fetal Bovine Serum (FBS, Hyclone) + 20µg/ml gentamicin (Gibco, 15750-060)). After several rounds of trituration the final tube of cells was counted and the cells seeded onto poly-D-lysine (0.1mg/mL, Sigma, P1024) coated coverslips at a density 40,000 cells/cm². 100mm dishes and 48-well plates were coated with poly-D-lysine and cells seeded at 100,000 cells/cm². The resulting cultures were grown from 7-10 days in vitro and contained a mixed population of neurons, astrocytes, and microglia. For comparison, after 4 days in vitro (DIV) some cultures were maintained in Neurobasal (Gibco, 21103) supplemented with B27 (1:50, Gibco, 17504-044) + 20µg/ml gentamicin (Gibco, 15750-060) for an additional 7 days. Culture medium was changed every 2-3 days. Cells were used at least 48 hours after the most recent medium change.

Mouse brain preparation

The brains from 10 week-old Sv129 mice were perfused with 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS: in mM: 9.4 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 12.1 $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 140 NaCl, pH 7.4-7.5) and harvested. Brains were paraffin embedded and cut into 5 μm coronal sections (UNC-CH Mouse Histopathology) for immunohistochemistry.

Immunostaining

The localization of CHIP was determined by immunostaining. *Mouse Brain* Sections were unmasked in heated citrate buffer (2% $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$ + 9.1% $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$), permeabilized with 0.1% Triton (Sigma, T-9284) in PBS, blocked with horse serum (1:100) then incubated with antibody. Representative coronal sections were stained using a polyclonal rabbit anti-CHIP antibody (Affinity Bioreagents, PA1-015) diluted 1:100 in PBS and visualized with an Alexa-594 conjugated donkey anti-mouse secondary (Invitrogen, A21207) diluted 1:100.

Cultured Cortical Neurons Coverslips of cultured cortical neurons were fixed in 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton in PBS, blocked with horse serum then incubated with the polyclonal CHIP antibody followed by directly conjugated anti-rabbit-Alexa 594 conjugate (Molecular Probes, 21207) for visualization. Sections and cultures were counterstained with goat anti-MAP2 (Santa Cruz, sc-12012) diluted 1:100 and visualized using Alexa 488 conjugated donkey anti-goat (Invitrogen, A11055) diluted 1:100. Nuclear counterstains were performed with bisbenzimidazole (Hoechst 33258, 1 μM , Sigma, B1155). Sections and coverslips were mounted using Fluoromount-G (SouthernBiotech, 0100-01).

Western blot

Cells grown at a density of 10^5 cells/ cm^2 on 100 mm plates were washed three times to replace MEM with artificial cerebral spinal fluid (aCSF: concentrations in mM: 137 NaCl, 5.0

KCl, 2.3 CaCl₂, 1.3 MgCl₂, 10 HEPES, 20 Glucose, pH 7.37-7.40, Osmolality 290), immediately placed on ice and scraped and transferred to 15mL conical tubes after 4 minutes. Cytoplasmic and nuclear fractions were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, 78833) per kit instructions and protein concentrations were determined using the BCA Assay (Bio-Rad). Equal amounts of lysate were mixed with 4X sodium dodecyl sulfate (SDS) sample buffer (40% glycerol, 240mM 1M Tris-Cl, pH 6.8, 8% SDS, 0.1% bromophenol blue) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to nitrocellulose membranes (Bio-Rad, 162-0115). Western blotting was performed using the in-house mouse-anti-CHIP monoclonal antibody, conditioned medium clone 67 (CM67) undiluted followed by goat anti-mouse-horseradish peroxidase (HRP)-conjugated secondary antibody (Calbiochem, 402335) diluted 1:10,000. Membranes were reacted with Enhanced Chemiluminescence (ECL) reagent (Amersham, RPN2109) and exposed to film.

CHIP degradation

100mm dishes were cultured at 10⁵ cells/cm² and maintained 10 DIV. Cultures were transferred to MEM with cycloheximide (100µg/mL, Sigma, C-1988) or MEM with cycloheximide and proteasomal inhibitor, MG132 (20 µM, Peptides International, I2L-3175-V) for durations between 0 to 480 minutes. Control cultures were not exposed to cycloheximide or MG132. For CHIP degradation in recovery studies, cultures were maintained in MEM with cycloheximide or MEM with cycloheximide and proteasomal inhibitor in recovery for durations between 0 to 24 hours. After exposure to OGD, cells were immediately harvested as whole cell lysate using RIPA buffer (1X0 TBS (20mM Tris pH 8.0, 137mM NaCl), 1% NP-40, 10% glycerol, protease inhibitors (1µMm, Sigma, P8340)) or placed on ice for 4 minutes and cytoplasmic and nuclear fractions were extracted using the NE-PER Cytoplasmic and Nuclear Extraction Kit (Pierce, 78833) per kit instructions. Protein

concentrations were determined using the BCA Assay (Pierce, Reagent A: 23223, Reagent B: 23224) and equal protein amounts were separated by SDS-PAGE and analyzed by Western blot for CHIP.

FIGURE LEGENDS

Figure 1-1:

CM67 recognized a single band of approximately 35kDa molecular weight present in fibroblast whole cell lysate (WCL) from wildtype (+/+) mice. Specificity was confirmed by the absence of immunoreactivity in lysates from mice lacking CHIP (-/-).

Figure 1-2:

Positive staining for CHIP in sections from adult Sv129 mice. Immunoreactivity was observed in the cortex (**1-2A**), hippocampus (**1-2B**), hypothalamus (**1-2C**), Purkinje neurons (**1-2D**), choroid plexus and ependymal cells (**1-2E**), and thalamus (**1-2F**). Cortical CHIP staining (**1-2G**) and nuclear counterstain (**1-2H**), demonstrate co-localization (**1-2I**). Higher magnification images from 1-2G-I are shown in panel **1-2J**.

Figure 1-3:

CHIP immunocytochemical staining of cultured neurons showed reactivity in both neurons and astrocytes. Cultured neurons (10 DIV) were stained for CHIP (**1-3A**), MAP-2 (**1-3B**) and bisbenzimidazole (**1-3C**). The three images were combined (**1-3D**) to illustrate CHIP immunoreactivity in neurons (arrow) and astrocytes (arrowhead) and localization in the cytoplasm and nucleus.

Figure 1-4:

CHIP sub-cellular localization in cultured neurons showed greater nuclear intensity in cultures kept in neurobasal compared to MEM.

Figure 1-5:

Primary cultures of dissociated cortical neurons and astrocytes. Examples of dissociated cortical cultures at 45,000 cells/cm², 80,000 cells/cm², and 100,000 cells/cm².

Figure 1-6:

CHIP is stable in culture following OGD and recovery. **A** CHIP levels in cytoplasmic and nuclear fractions following increasing durations of OGD. **B** CHIP levels in whole cell lysate following increasing durations of OGD in the presence of cycloheximide. Image from the Western blot is inset in the graph. **C** CHIP levels in whole cell lysate following 2 hours of OGD and during 4-24 hours of recovery with cycloheximide present during recovery. Image of the Western blot is inset in the graph.

ACKNOWLEDGEMENTS

Anti-CHIP monoclonal production

Anti-CHIP monoclonal antibodies were produced in our lab against a purified GST-CHIP fusion protein.⁸¹ Product from hybridoma clone 67 (CM67) was selected for high immunoreactivity against CHIP in ELISA and Western blot screens. On Western blot, CM67 recognized a single band of approximately 35kDa molecular weight present in fibroblast whole cell lysate (WCL) from wildtype (+/+) mice. Specificity was confirmed by the absence of immunoreactivity in lysates from CHIP -/- mice (Figure 1-1).

Courtesy of David Y. Huang, M.D., Ph.D. and Laboratory of Dr. E-S Huang.

Figure 1-1: CM67 Antibody Specificity

CM67 recognized a single band of approximately 35kDa molecular weight present in fibroblast whole cell lysate (WCL) from wildtype (+/+) mice. Specificity was confirmed by the absence of immunoreactivity in lysates from mice lacking CHIP (-/-).

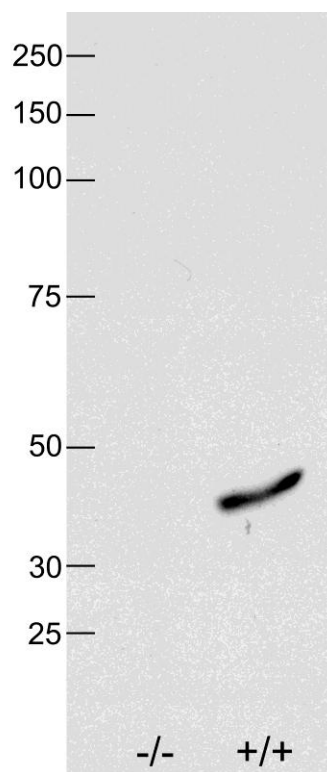


Figure 1-2: CHIP Protein Localization in Mouse Brain Sections

Positive staining for CHIP in sections from adult Sv129 mice. Immunoreactivity was observed in the cortex (**1-2A**), hippocampus (**1-2B**), hypothalamus (**1-2C**), Purkinje neurons (**1-2D**), choroid plexus and ependymal cells (**1-2E**), and thalamus (**1-2F**). Cortical CHIP staining (**1-2G**) and nuclear counterstain (**1-2H**), demonstrate co-localization (**1-2I**). Higher magnification images from 1-2G-I are shown in panel **1-2J**.

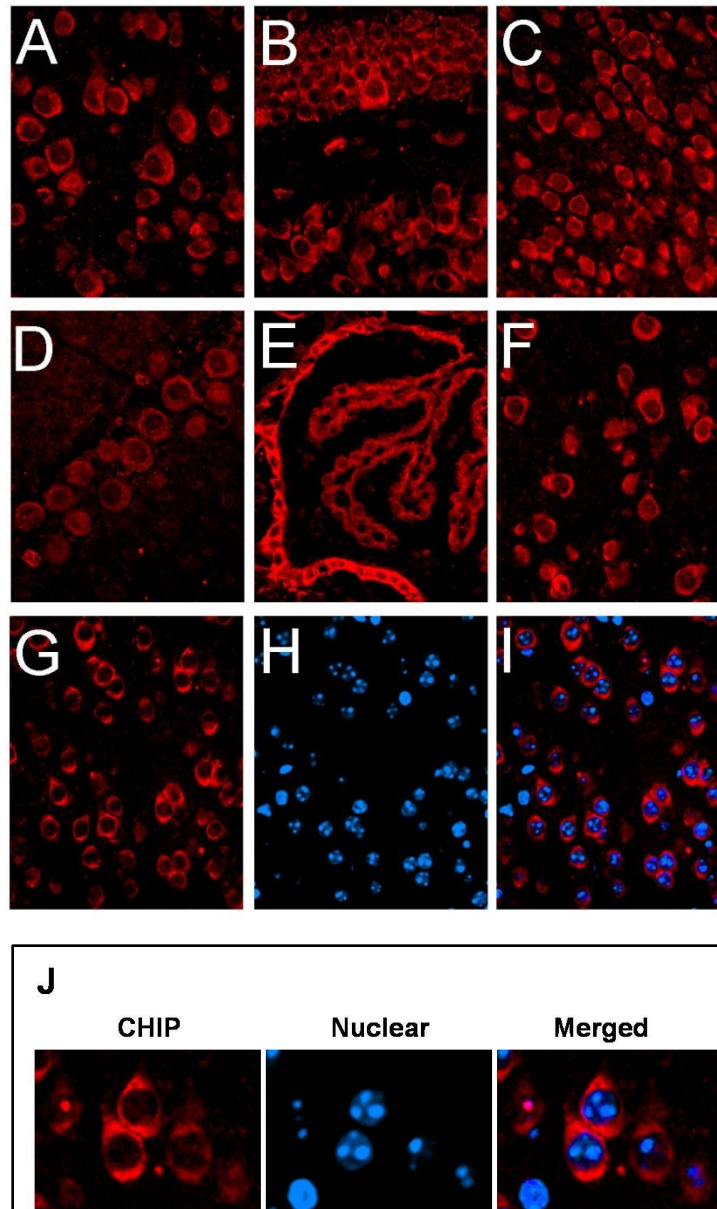


Figure 1-3: CHIP Protein Localization in Cultured Cortical Neurons

CHIP immunocytochemical staining of cultured neurons showed reactivity in both neurons and astrocytes. Cultured neurons (10 DIV) were stained for CHIP (1-3A), MAP-2 (1-3B) and a nuclear Hoechst stain (1-3C). The three images were combined (1-3D) to illustrate CHIP immunoreactivity in neurons (arrows) and astrocytes (arrowheads) and localization in the cytoplasm and nucleus.

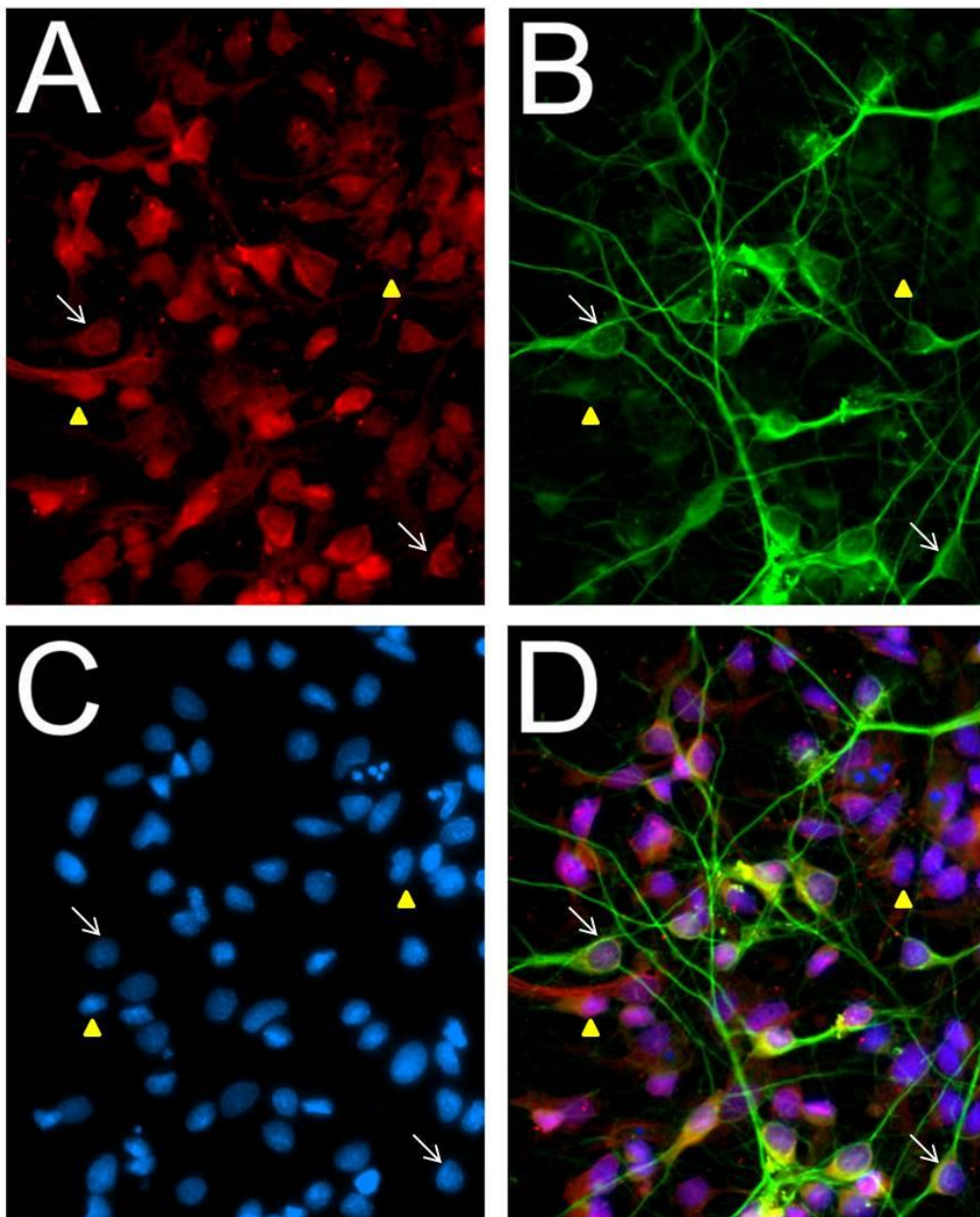


Figure 1-4: CHIP Sub-Cellular Localization in Baseline Culture Conditions

CHIP sub-cellular localization in cultured neurons showed greater nuclear intensity in cultures kept in neurobasal compared to MEM.

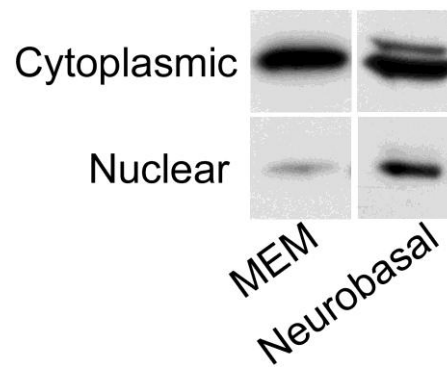


Figure 1-5: Density of Neurons in Culture

Primary cultures of dissociated cortical neurons and astrocytes. Examples of dissociated cortical cultures at 40,000 cells/cm², 80,000 cells/cm², and 100,000 cells/cm².

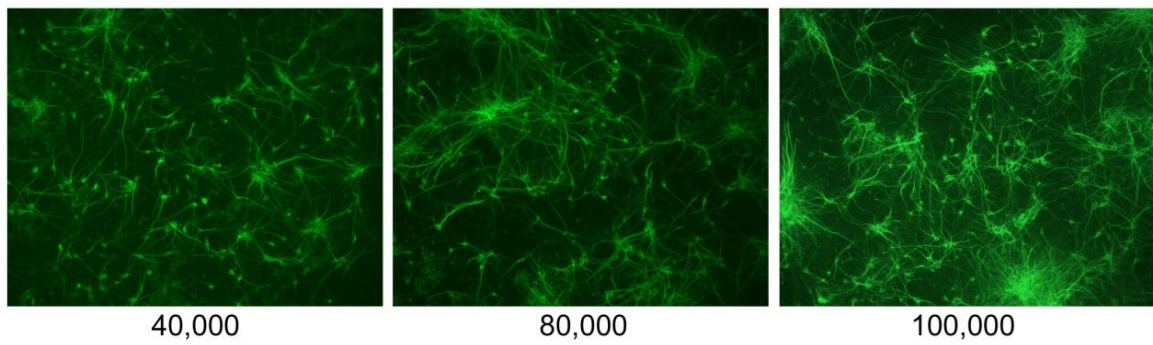
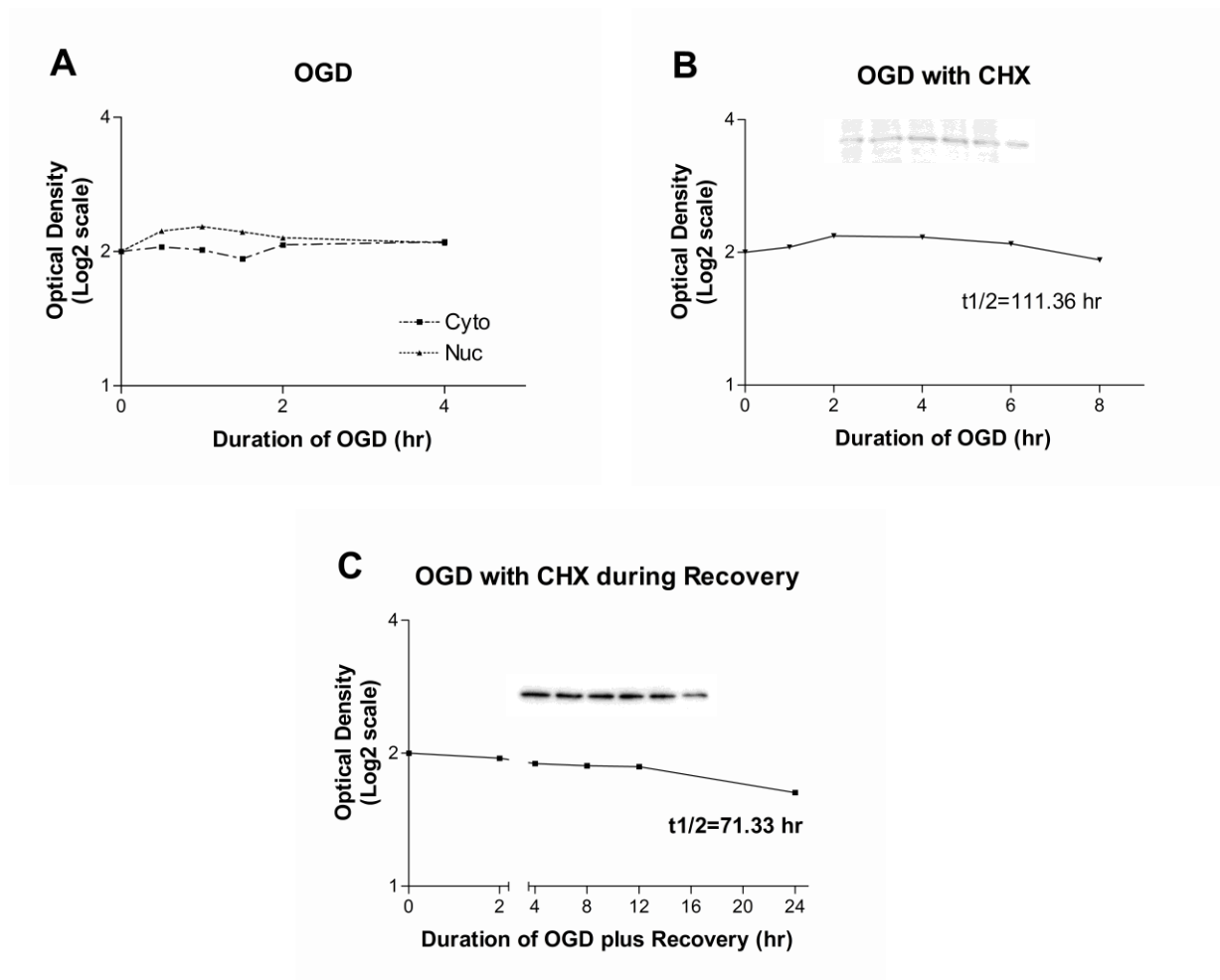


Figure 1-6: CHIP Stability in Cell Culture

CHIP is stable in culture following OGD and decreases during recovery. **A** CHIP levels in cytoplasmic and nuclear fractions following increasing durations of OGD. **B** CHIP levels in whole cell lysate following increasing durations of OGD in the presence of cycloheximide. Image from the Western blot is inset in the graph. **C** CHIP levels in whole cell lysate following 2 hours of OGD and during 4-24 hours of recovery with cycloheximide present during recovery. Image of the Western blot is inset in the graph.



CHAPTER 2

Nuclear Localization of CHIP Following Acute Cellular Stress

INTRODUCTION

Survival of cells following stress depends heavily on the balance of quality control mechanisms that both make functional proteins and eliminate defective proteins. During ischemic stress, the rapid loss of energy production disables protein folding mechanisms, resulting in unfolded or misfolded proteins, thus triggering the unfolded protein response (UPR). The UPR up-regulates HSPs, activates HSP transcription factors, and promotes protein refolding and degradation¹¹⁸

In the brain, the 70-kDa family of HSPs has been widely studied in experimental models of cerebral ischemia.^{54,56,59,63} HSP70 is synthesized in especially high levels in the central nervous system (CNS) in response to ischemia and appears to be highly neuroprotective.^{47,52,119} Transgenic mice overexpressing HSP70 were protected against cerebral infarction in models of focal ischemia.⁵⁴ Conversely, reduced HSP70 expression led to increased cellular damage after focal ischemia in HSP70 knockout mice.⁵⁵ CHIP also appears to play a protective role in the cellular stress response, as CHIP(-/-) mice and cells derived from these mice undergo temperature-sensitive apoptosis in response to thermal and proteotoxic stress.⁸¹

Key to the UPR is the translocation of transcription factors and other HSPs from the ER to the nucleus to both initiate and suppress protein transcription.^{43,118} Translocation to the nucleus and sub-nuclear movements of HSF1, within minutes of exposure to ischemic

challenge, prepares the cell to upregulate HSPs for survival at the very earliest stages of the stress response. Constitutively active HSC70 also translocates from the cytoplasm to the nucleus within minutes of *in vivo* heat stress.⁷⁹ The time course of this translocation was within 15 minutes and initially did not involve the inducible HSP70.⁷⁹ HSP70 and other chaperones are involved in repressing transcription of heat shock genes.⁹¹ CHIP is part of the activating complex for HSF1⁹¹, but the role of CHIP in the complex is unknown. Inclusion of CHIP in an HSF1 complex suggests an early role for CHIP in the UPR, which may set the stage for a survival-promoting response.

Rodent models of ischemia have established significant, reproducible injury from as little as 20 minutes of global^{106,107}, and more commonly, 2 hours of focal ischemia^{105,120}, while clinical intervention from the time of onset is almost exclusively limited to a 3 hour window¹², beyond which significant impairment results. Traditional *in vitro* ischemia models require 4 or more hours of ischemia to generate significant delayed cell death, possibly due to residual O₂ and glucose present in the cultures. However, primary neuronal cultures subjected to oxygen-glucose deprivation using argon displacement and 2-deoxy-D-glucose (2DG) were sensitive to the absence of serum¹²¹ and demonstrated significant delayed cell death after as little as 30 minutes of *in vitro* ischemia.^{122,123}

Since studies also support a protective role for CHIP in chronic neurodegenerative diseases⁹⁸⁻¹⁰⁰, we hypothesized that CHIP also plays a neuroprotective role following acute stresses such as cerebral ischemia. We therefore undertook studies to determine changes in expression and localization patterns of CHIP in response to acute cellular stressors. Presented is evidence for rapid translocation of CHIP to the nucleus in response to both heat stress and oxygen-glucose deprivation (OGD) in *in vitro* models.

RESULTS

Immediate and Delayed Cell Death following Heat Stress

Because we are interested in acute cellular responses to stress we needed to determine what durations of heat stress resulted in cell death. Identifying the minimum duration of stress required to generate significant cell death would also identify the point at which heat stress damaged the majority of cells in the culture. Cultured neurons were stained with Sytox Green (1 μ M) and measured for fluorescence using a plate reader. No significant cell death was observed during or immediately after exposure to heat stress (data not shown). When measured after a 24 hour recovery period in normal culture conditions following heat stress, significant cell death was observed following 30 minutes of heat stress (298.33% \pm 4.89 of baseline) and increasing exponentially to 60 minutes (835.41% \pm 76.84 of baseline) (* p <.001, n =47) (**Figure 2-1**).

CHIP in Cultured Cortical Neurons following Heat Stress:

Our previous data suggested we could be able to increase nuclear localization of CHIP with the application of stress. Primary mixed cultures were heat stressed and stained for CHIP. Images of CHIP (red) and with a nuclear counterstain (blue) and images merged to show co-localization (**Figure 2-2**). Unstressed cells (Figure 2-2A, 0 min) presented immunoreactivity patterns as was seen previously (Figure 1-3A) with both cytoplasmic and nuclear CHIP expression in a granular pattern and a dense perinuclear staining (Figure 2-2A, higher magnification 2-2B, yellow arrowhead). A fragmented apoptotic nucleus (Figure 2-2A, white arrows), also in the field, had notably less CHIP immunoreactivity compared to other cells in the field. After 5 minutes of heat stress the CHIP staining pattern was more uniform throughout the cytoplasm and nucleus, but again with a denser perinuclear region (Figure 2-2A, higher magnification 2-2B, yellow arrowhead). Following 30 minutes of heat stress the CHIP staining pattern was again granular in appearance, and consistent with dense perinuclear staining (higher magnification 2-2B, yellow arrowhead). Also at 30 minutes, a condensed nucleus (Figure 2-2A, white arrow) had notably less CHIP

immunoreactivity compared to other cells in the field and had a pattern similar to the apoptotic cell in the 0 minute field.

CHIP Sub-cellular Localization following Heat Stress

To determine if the absolute changes observed with staining were representative of relative changes in CHIP localization; cultures were heat stressed and fractionated by differential salt buffer lysis to isolate the cytoplasmic and nuclear fractions for analysis. Equal protein concentrations were probed with anti-CHIP, anti-HSC70, or anti-HSP70 antibodies (**Figure 2-3**). CHIP immunoreactivity was observed in cytoplasmic and nuclear fractions at baseline (0). With increasing durations of heat stress the relative amount of CHIP in the cytoplasm decreased at 30 and 60 minutes to $57.43\% \pm 12.52$ and $53.71\% \pm 14.50$ of baseline, respectively. In the nuclear fraction, CHIP peaked at 10 minutes ($102.23\% \pm 26.54$) then decreased below baseline by 30 ($84.62\% \pm 22.24$) and 60 ($69.11\% \pm 20.56$) minutes. The relative amount of the CHIP co-chaperone HSC70 in the cytoplasm slightly decreased with increasing durations of heat stress, to $83.96\% \pm 20.49$ at 60 minutes. However, a slight increase was observed with HSC70 in the nuclear fraction with increasing durations of heat stress to $124.77\% \pm 26.87$ by 60 minutes. The inducible molecular chaperone, HSP70 increased early in the cytoplasmic fraction, peaked at 10 minutes ($270.57\% \pm 208.92$), and decreased to near baseline levels by 30 ($72.86\% \pm 20.75$) to 60 ($100.97\% \pm 53.49$) minutes. In the nuclear fraction, HSP70 again increased, peaking at 10 minutes ($909.92\% \pm 852.29$) before decreasing to near baseline levels at 30 ($156.98\% \pm 113.66$) to 60 ($204.08\% \pm 177.21$) minutes.

To this point, our *in vitro* model of ischemia has been HS. The HS model is well known to be a severe stress that is more closely related to excitotoxicity than *in vivo* ischemia. Our lab adopted a model of *in vitro* OGD to better model *in vivo* ischemia previously described by the Hossmann group.^{122,123} Fundamental objectives in adopting an

alternative model were to 1) evaluate increases in nuclear CHIP, 2) model a time course for delayed cell death similar to that observed *in vivo*, 3) establish a time frame for the transition from injury to cell death. Human and *in vivo* rodent studies clearly illustrate a 2-hour ischemic threshold before the appearance of significant delayed cell death. It is expected that changes within this early time window will predict the delayed cell death seen *in vitro* and *in vivo*. A therapeutic time window might then be established that accounts for the correction of critical deficits.

Immediate and Delayed Cell Death following OGD

Cultured neurons were stained with Sytox Green (1 μ M) and measured for fluorescence using a plate reader. No significant cell death was observed during or immediately after exposure to OGD (data not shown). When measured after a 24 hour recovery period in normal culture conditions following OGD (**Figure 2-4A**), significant cell death was observed following 90 (221.87% \pm 8.97) and 120 minutes (229.68% \pm 7.08) of OGD. Significance was lost by 240 minutes (181.62% \pm 6.33), which was attributed to the loss of dead cells that were no longer adhered to the 48 well-plate.

Cell Death Counts

To determine if the percentage increase in dead cell staining was indicative of an actual increase in cell number and not an increased staining intensity, cells from cultures exposed to OGD and 24 hours recovery were stained with Sytox Green and imaged under 20X oil objective (Metamorph field calibration=0.2493mm²) (**Figure 2-4B**). 10 images per condition were captured for analysis from 48-well plates (well size=0.95cm²). Dead cell percentage was calculated as: (((dead cell count x well size)/field calibration)/cells per well) x 100. Baseline cell death (Figure 2-4B, 0) was calculated as 4.11% \pm 1.84. 30 and 120 minutes were 28.75% \pm 11.55 and 38.07% \pm 8.78 respectively.

CHIP localization following OGD

To evaluate change in relative CHIP levels, immunoreactivity was observed in cytoplasmic and nuclear fractions analyzed by Western blot at baseline (**Figure 2-5A**, 0). With increasing durations of OGD the relative amount of CHIP in the cytoplasm remained constant (**Figure 2-5A**). In the nuclear fraction, a slight increase in CHIP (to 113% of baseline) was observed following 30 minutes of OGD which remained slightly less elevated following increasing durations (to 104% at 4 hours).

Compared to HS, OGD is a mild, metabolic stress where misfolded proteins develop over time. Since CHIP likely responds to the presence of misfolded proteins we hypothesized that 1) changes in CHIP localization were likely to occur during the recovery period following OGD and 2) a difference would exist between mild durations of stress where minimal increases in delayed cell death occurred and severe stress where significant increases in delayed cell death were observed.

CHIP Localization following Mild and Severe OGD plus Recovery

Following a mild stress (30 minutes of OGD) (**Figure 2-5B**) CHIP in the cytoplasmic fraction remained unchanged until a decrease to $69.54\% \pm 16.41$ of baseline at 24 hours of recovery. In the nuclear fraction following mild stress (**Figure 2-5B**, right), CHIP increased acutely ($125.27\% \pm 14.95$) following OGD and steadily decreased through 12 hours of recovery before decreasing below baseline at 24 hours of recovery ($38.59\% \pm 15.04$). Following a more severe stress (120 minutes of OGD) (**Figure 2-5B**) CHIP was increased immediately following the stress in the cytoplasmic fraction ($105.28\% \pm 15.12$) (**Figure 2-5B**, 120 min, 0 hr), as was observed previously (**Figure 2-5A**), but the increases were not sustained and decreased to baseline or lower throughout the later recovery time points.

CHIP co-chaperones, HSC70 and HSP70 elevated insignificantly at 8 hours of recovery to $106.21\% \pm 64.68$ and $158.93\% \pm 30.30$ respectively in the cytoplasmic fraction following mild OGD (30 minutes) (Figure 2-5B). In the nuclear fraction HSC70 increased acutely ($126.15\% \pm 38.20$) but remained steady at or near baseline levels throughout the recovery timeframe. Comparatively, the slight acute increase in nuclear HSP70 immediately after OGD ($128.67\% \pm 58.54$) decreased throughout recovery following mild OGD.

However, following a more severe duration of OGD (120 minutes) (Figure 2-5B), the CHIP co-chaperone, HSC70 remained unchanged in the cytoplasmic fraction throughout recovery. The inducible HSP70 demonstrated also remained relatively unchanged from baseline in the cytoplasmic fraction immediately after OGD (120 min, 0 hr) through 12 and 24 hours of recovery.

In the nuclear fraction, following severe OGD ((Figure 2-5B), early increases CHIP ($135.54\% \pm 40.04$) (120 min, 0 hr) were not sustained throughout the later recovery time points. Nuclear HSC70 remained largely unchanged in response to OGD and throughout recovery. Nuclear levels of HSP70 also did not significantly change in response to OGD or during recovery.

DISCUSSION

Perhaps the most important observation from these studies was the rapid movement of CHIP to the nucleus under conditions of stress. Under normal conditions, the pattern of CHIP localization in our cortical/hippocampal primary cultures was primarily cytoplasmic, but nuclear staining was greater than seen *in vivo*. This may be an adaptation that allows survival of the neurons under culture conditions. Substantial additional increases in nuclear CHIP are seen with a potent stressor such as HS. Increases in nuclear CHIP following HS in our experiments have been confirmed by others.^{82,91,114-116} At longer durations of stress the quality of the staining was more granular in appearance. This observation was

consistent with reports of granular staining patterns of ubiquitin¹²⁴⁻¹²⁶ and HSP70^{38,127}, supporting the hypothesis that protein-chaperone complexes accumulate and aggregate with persistent or severe stress.^{34,128}

Nuclear accumulation as early as 5 minutes after the introduction of HS indicated that CHIP can be rapidly mobilized to the nucleus. Nuclear CHIP was reported previously in the context of heat stress, though was not a focus.⁹¹ This was faster than the nuclear accumulation in previous reports. Accumulation of CHIP in the nuclear fraction of COS7 cells following 90 minutes of 42°C HS.⁹¹ and Similar observations were made by Tripathi et al. (2007) in KB cells.¹²⁹ We attribute the difference in sensitivity, in part, to our use of primary cortical and hippocampal cells instead of immortalized cell lines. At 30 and 60 minutes of HS nuclear CHIP was nearly undetectable. Durations of stress that depleted CHIP were also associated with delayed cell death. After a 24 hour recovery period significant delayed cell death following 30 minutes of heat stress was 298.33%±12.14 of baseline and following 90 minutes of OGD significant cell death was 221.87%±8.97 of baseline.

As with HS the loss of CHIP appears just prior to the beginning of cell death. A similar pattern of results was seen following OGD albeit with a different time course. Decreased nuclear CHIP begins at approximately 4 hours of recovery following 120 minutes of OGD and 12 hours following 30 minutes of OGD. During recovery these levels were maintained without synthesis or degradation. Under these conditions nuclear HSC70 remains elevated in recovery following severe OGD even when levels of activated HSF1 are known to have decreased.⁹¹ Of the HSPs likely to be involved, CHIP is the only protein studied to decrease in the nucleus during recovery.

CHIP in protein aggregates

In very limited models of cerebral ischemia, CHIP was observed in ribosomal aggregates that accumulated in the cytoplasm following ischemia *in vivo*.¹⁰⁶ Such aggregates formed after as short an ischemic duration as 7 minutes and increased aggregation was observed following longer durations.¹²⁵ Of note, proteins that accumulated early in these aggregates did not recover after HSP induction in the post-ischemic recovery phase.¹²⁰ The timeframe for the loss of nuclear CHIP *in vivo* is similar to the time in which the folding co-chaperone HSP40 accumulates in irreversible protein aggregates in the cytoplasm.^{106,120} The similarities in timing suggest that the development of aggregates could be a factor that restricts chaperone availability. The Hu lab promotes the hypothesis that irreversible aggregates are an important indicator in cells destined to die.⁹⁶ They found protein aggregates during recovery periods of 4 hours or greater.⁹⁶

CHIP as a chaperone

As with aggregation, CHIP movement is probably tied to its binding with other proteins. In particular HSC70 and HSP70 have been shown to accumulate in the nucleus after heat shock and oxidative stress.¹³⁰ Others have proposed that CHIP translocates to the nucleus in complex with HSF1⁹¹ as part of the mechanism that upregulates the heat shock response¹⁰⁴. Alternatively, CHIP may translocate with other proteins as a direct chaperone. Rosser et al. (2007) have demonstrated the ability of CHIP to act as a chaperone is likely the result of its TPR domain.¹³¹ Tripathi et al. (2007) have shown that CHIP and p53 were present together on the DNA binding sites of the p21 and p53 promoters.¹²⁹

Since CHIP is a negative regulator of HSP-mediated protein folding, it is unlikely that increased protein folding is occurring in the nucleus in relation to CHIP. More likely, CHIP is interacting with both HSC70 and HSF1 to promote HSP transcription. The delayed increase

we observed in nuclear HSP70 during the recovery phase is consistent with the hypothesis that HSP70 is part of a second phase of the stress response.

The role of CHIP in the nucleus and the consequences for neuroprotection are poorly understood. Our results and the observations of other investigators raise the possibility that E3 ligases, like CHIP, participate in the nuclear regulation of the stress response. Very little is known about these potential interactions and significant investigation is required to understand the potential role of CHIP in neuroprotection. Our finding of acute changes in sub-cellular localization of CHIP in response to cellular stress suggests that changes that occur shortly after exposure to stress ultimately impact on whether or not a cell has the capacity and capability to recovery.

MATERIALS AND METHODS

***In vitro* stress models**

Heat Stress (HS) Cultures maintained 10 days in vitro (DIV) in MEM were subjected to heat stress: 42°C water bath for durations between 0 and 60 minutes in aCSF.

Oxygen-Glucose Deprivation (OGD) Cultures maintained 10 DIV in MEM were subject to glucose-free MEM salt solution (gfMEM, in mM: 1.3 CaCl₂·2H₂O, 5.3 KCl, 0.81 MgSO₄, 116.4 NaCl, 26.2 NaHCO₃, 1.0 NaH₂PO₄·H₂O) + 2-deoxy-D-glucose (5 mM, Sigma, D8375) + 1X Amino Acid Solution (Gibco) + 1X Vitamin Solution (Gibco)) in an anaerobic chamber (Modular Incubator Chamber, Billups-Rothenberg, Del Mar, California) gassed with 95% Argon/5% CO₂ (National Welders, Durham, NC), purged for 7 minutes and sealed for durations between 0 and 240 minutes. Cells were returned to normal cell culture conditions for durations between 0 and 24 hours following OGD to assess recovery.

Oxygen displacement using argon was used to reduce partial oxygen in the medium within minutes of exposure¹²³ instead of mitochondrial inhibitors like FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone). Also, as an inert gas argon does not

presumably interact with the culture and generate reactive oxygen species, unlike nitrogen gas.

Immunostaining

Sub-cellular localization of CHIP following HS was determined by immunostaining.

Coverslips of cultured cortical neurons exposed to durations of HS were placed on ice for 4 minutes then fixed in 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton in PBS, blocked with horse serum then incubated with the monoclonal mouse-anti-CHIP antibody that was directly conjugated to the red fluorescent dye, Cy3, for visualization. Sections and cultures were counterstained with bisbenzimidazole (1 μ M, Sigma, B1155) to visualize the nuclei. Coverslips were mounted using Fluoromount-G (SouthernBiotech, 0100-01).

Western blot

Stressed and control cells grown at a density of 10^5 cells/cm² on 100 mm plates were washed three times in aCSF, placed on ice and harvested after 4 minutes immediately or following recovery intervals of 4-24 h in normal culture conditions. Cytoplasmic and nuclear fractions were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, 78833) per kit instructions and protein concentrations were determined using the BCA Assay (Pierce, Reagent A: 23223, Reagent B: 23224). Whole cell lysates from CHIP transgenic mice were gifts from the Patterson lab. Equal amounts of lysate were mixed with 4X sodium dodecyl sulfate sample buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed with the appropriate antibodies, developed with ECL reagent (Amersham, RPN2109) and exposed to film.

Delayed Cell Death

For cell death experiments, cell cultures seeded at 10^5 cells/cm² in poly-d-lysine coated 48-well plates were subjected to stress and allowed to recover for 24 h in normal cell culture conditions. The cells were then stained with 1 μ M Sytox Green (Molecular Probes, S-7020) to stain nuclei of dead cells and the fluorescence intensity measured on a plate reader.

Antibodies

The following primary antibodies were used for immunoblotting: mouse monoclonal mouse anti-CHIP conditioned medium, undiluted (CM67), goat anti-HSC70 diluted 1:1000 (Santa Cruz, sc-1059), goat anti-HSP70 diluted 1: 1,000 (Stressgen, SPA-812), and mouse-anti- β -actin diluted 1:5,000 (Sigma A5441). Peroxidase conjugated secondary antibodies diluted 1: 10,000, for Westerns were all from Calbiochem: rabbit anti-mouse (402335), goat anti-rabbit (401315), and rabbit anti-goat (401515). Primary antibody incubations were performed overnight and secondary antibody incubations for no more than 1 h.

FIGURE LEGENDS

Figure 2-1:

Cell death after prolonged heat stress was increased after a 24 hour recovery period.

Cultures were subjected to durations of heat stress between 0 and 60 minutes, recovered in normal culture conditions for 24 hours and stained with Sytox Green (1 μ M). Sytox Green intensity was measured by a Wallac Victor² fluorescent plate reader. Sytox Green intensity was analyzed as % of baseline \pm SEM 24 hours after 0 (100% \pm 4.89), 5 (155.45% \pm 3.51), 10 (174.85% \pm 6.35) 15 (215.78% \pm 8.36), 30 (298.3% \pm 12.14) and 60 minutes (835.41% \pm 76.84) of heat stress. Measurements following 30 and 60 minutes were significant. (* p <.001, n =47).

Figure 2-2:

Increased nuclear staining of CHIP in cultured neurons exposed to heat stress. **A** Representative images from cells subjected to 0, 5, or 30 minutes of heat stress are shown. CHIP (red) staining is merged with a nuclear Hoechst (blue) stain to show co-localization. Dead cells (white arrow) display decreased or absent CHIP immunoreactivity. Isolated cells (white box) are shown at higher magnification in panel B. **B** Higher magnification images show CHIP immunoreactivity (red) and nuclear counterstain (blue). Increased CHIP immunoreactivity in the perinuclear region (yellow arrowhead) is shown at 0 min. Nuclear CHIP increases at 5 min. Granular CHIP staining occurs following 30 min of heat stress

Figure 2-3:

Nuclear CHIP decreases following extended durations of heat stress. Equal protein concentrations from sub-cellular fractions of primary mixed cultures exposed to heat stress were probed for CHIP, HSC70, and HSP70 on Western blot. β -actin is shown as a loading control. Comparisons were made within each fraction, between cytoplasmic and nuclear fractions, and between CHIP, HSC70, and HSP70 across time. (n(CHIP)=8-10, n(HSC70)=8-9, n(HSP70)=2)

Figure 2-4:

Cell death after prolonged OGD was increased after a 24 hour recovery period. Cultures were subjected to durations of OGD between 0 and 240 minutes, recovered in normal culture conditions for 24 hours and stained with Sytox Green (1 μ M). Sytox Green intensity was measured by plate reader. **A** Sytox Green intensity was analyzed as % of baseline \pm SEM 24 hours after 0 (100% \pm 1.85), 30 (161.86% \pm 3.37), 60 (204.38% \pm 5.63) 90 (221.87% \pm 8.97), 120 (229.68% \pm 7.08) and 240 minutes (181.62% \pm 6.33) of OGD. Measurements following 90 and 120 minutes were significant. (* p <.001, n=47). **B**

Representative images from 0, 30, and 120 minutes are shown. Individual stained nuclei were counted per field and total cell death counts per culture were calculated at $4.11\% \pm 1.84$ at 0 minutes. 30 and 120 minutes were $28.75\% \pm 11.55$ and $38.07\% \pm 8.78$ respectively (n=10).

Figure 2.5:

A Relative amounts of sub-cellular CHIP are slightly altered immediately following OGD.

Equal protein concentrations from sub-cellular fractions of primary mixed cultures exposed to OGD were probed for CHIP on Western blot. No changes were observed in the cytoplasmic fraction with increasing duration of OGD. In the nuclear fraction, a slight increase was observed following 30 minutes of OGD and were sustained, but not otherwise altered following increasing durations of OGD.

B Elevated levels of nuclear CHIP were maintained during recovery after 30min OGD. Equal protein concentrations from sub-cellular fractions of primary mixed cultures exposed to mild (30 min) and severe (120 min) OGD were probed for CHIP, HSC70, and HSP70 on Western blot. Comparisons were made within each fraction, between cytoplasmic and nuclear fractions, between mild and severe stress and between CHIP, HSC70, and HSP70 across time. (n(CHIP)=5-7, n(HSC70)=2-3, n(HSP70)=3-4)

ACKNOWLEDGEMENTS

Cell Culture

Cultures were produced and maintained in large part by Cynthia Rundle and Winona Poulton.

Figure 2-1: Delayed Cell Death following Heat Stress

Cell death after prolonged heat stress was increased after a 24 hour recovery period.

Cultures were subjected to durations of heat stress between 0 and 60 minutes, recovered in normal culture conditions for 24 hours and stained with Sytox Green (1 μ M). Sytox Green intensity was measured by a Wallac Victor² plate reader. Sytox Green intensity was analyzed as % of baseline \pm SEM 24 hours after 0 (100% \pm 4.89), 5 (155.45% \pm 3.51), 10 (174.85% \pm 6.35) 15 (215.78% \pm 8.36), 30 (298.3% \pm 12.14) and 60 minutes (835.41% \pm 76.84) of heat stress. Measurements following 30 and 60 minutes were significant. (* p <.001, n =47).

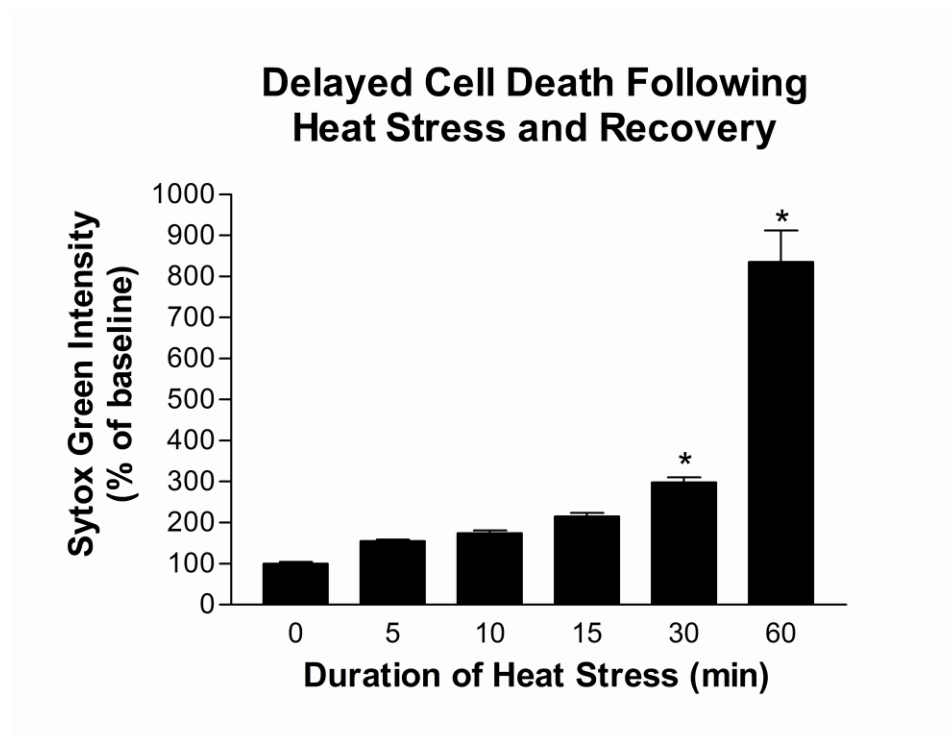


Figure 2-2: CHIP in cultured cortical neurons following heat stress.

Increased nuclear staining of CHIP in cultured neurons exposed to heat stress. **A**

Representative images from cells subjected to 0, 5, or 30 minutes of heat stress are shown.

CHIP (red) staining is merged with a nuclear Hoechst (blue) stain to show co-localization.

Dead cells (white arrow) display decreased or absent CHIP immunoreactivity. Isolated cells

(white box) are shown at higher magnification in panel B. **B** Higher magnification images

show CHIP immunoreactivity (red) and nuclear counterstain (blue). Increased CHIP

immunoreactivity in the perinuclear region (yellow arrowhead) is show at 0 min. Nuclear

CHIP increases at 5 min. Granular CHIP staining occurs following 30 min of heat stress.

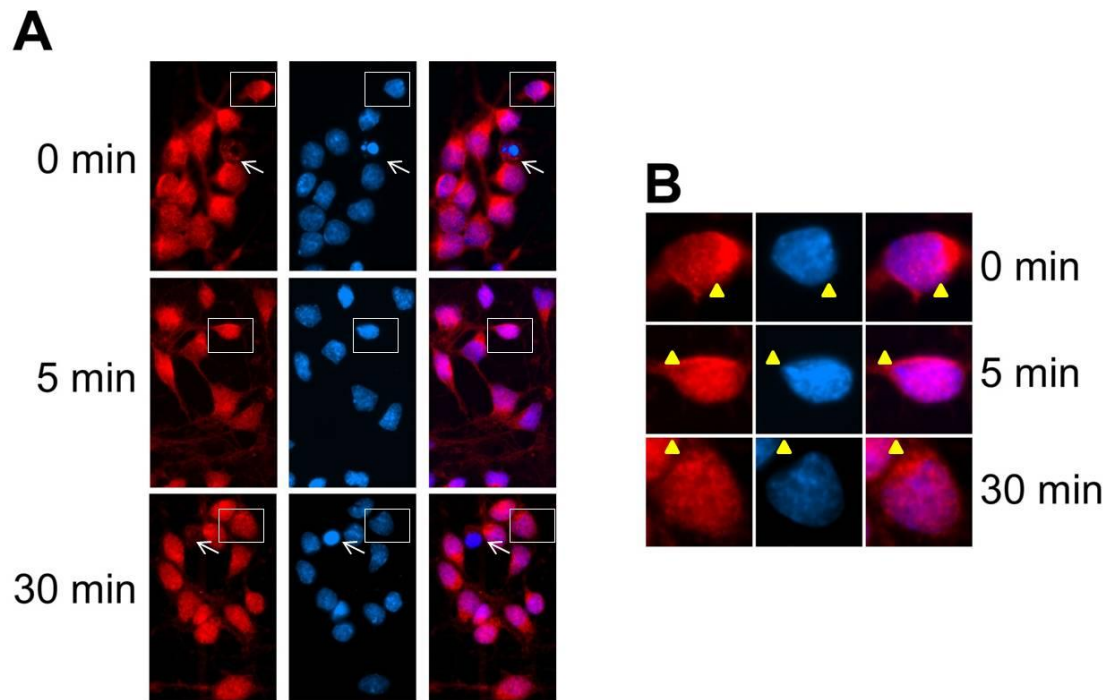


Figure 2-3: CHIP sub-cellular localization following heat stress

Nuclear CHIP decreases following extended durations of heat stress. Equal protein concentrations from sub-cellular fractions of primary mixed cultures exposed to heat stress were probed for CHIP, HSC70, and HSP70 on Western blot. β -actin is shown as a loading control. Comparisons were made within each fraction, between cytoplasmic and nuclear fractions, and between CHIP, HSC70, and HSP70 across time. (n(CHIP)=8-10, n(HSC70)=8-9, n(HSP70)=2)

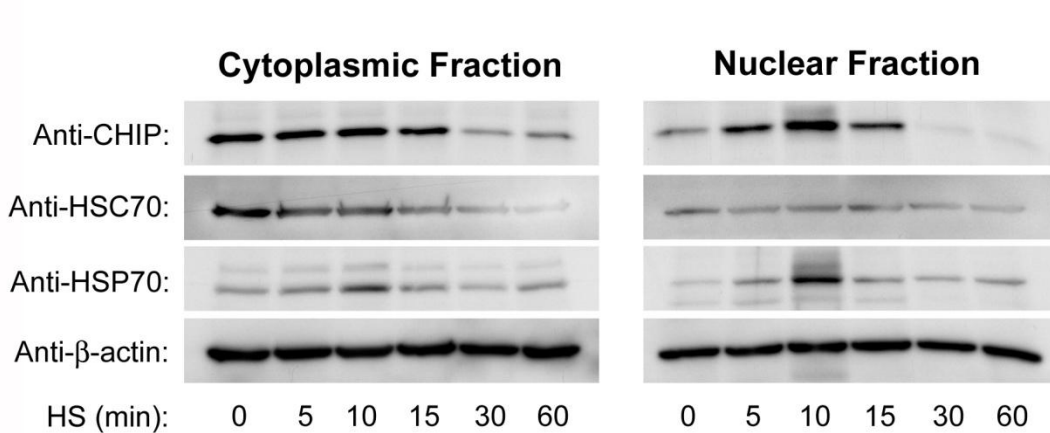
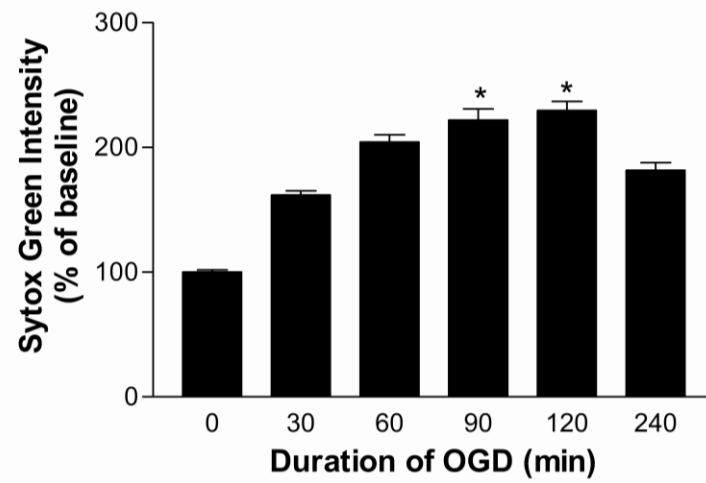


Figure 2-4: Delayed cell death following OGD

Cell death after prolonged OGD was increased after a 24 hour recovery period. Cultures were subjected to durations of OGD between 0 and 240 minutes, recovered in normal culture conditions for 24 hours and stained with Sytox Green (1 μ M). Sytox Green intensity was measured by plate reader. **A** Sytox Green intensity was analyzed as % of baseline \pm SEM 24 hours after 0 (100% \pm 1.85), 30 (161.86% \pm 3.37), 60 (204.38% \pm 5.63) 90 (221.87% \pm 8.97), 120 (229.68% \pm 7.08) and 240 minutes (181.62% \pm 6.33) of OGD. Measurements following 90 and 120 minutes were significant. (* p <.001, n =47). **B** Representative images from 0, 30, and 120 minutes are shown. Individual stained nuclei were counted per field and total cell death counts per culture were calculated at 4.11% \pm 1.84 at 0 minutes. 30 and 120 minutes were 28.75% \pm 11.55 and 38.07% \pm 8.78 respectively (n =10).

A

**Delayed Cell Death Following
OGD and Recovery**



B

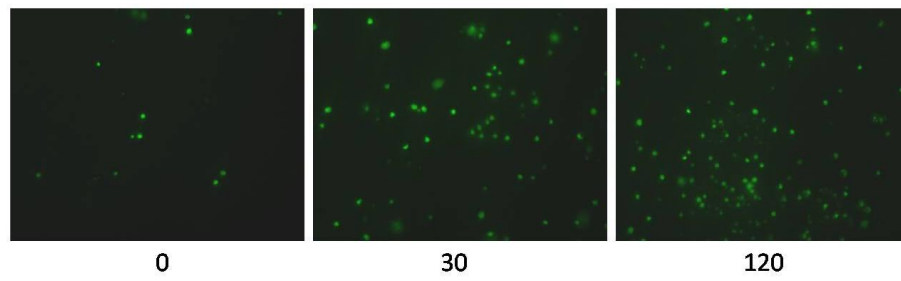


Figure 2-5: CHIP localization following OGD

A Relative amounts of sub-cellular CHIP are slightly altered immediately following OGD.

Equal protein concentrations from sub-cellular fractions of primary mixed cultures exposed to OGD were probed for CHIP on Western blot. No changes were observed in the

cytoplasmic fraction with increasing duration of OGD. In the nuclear fraction, a slight

increase was observed following 30 minutes of OGD and were sustained, but not otherwise

altered following increasing durations of OGD. **B** Elevated levels of nuclear CHIP were

maintained during recovery after 30min OGD. Equal protein concentrations from sub-

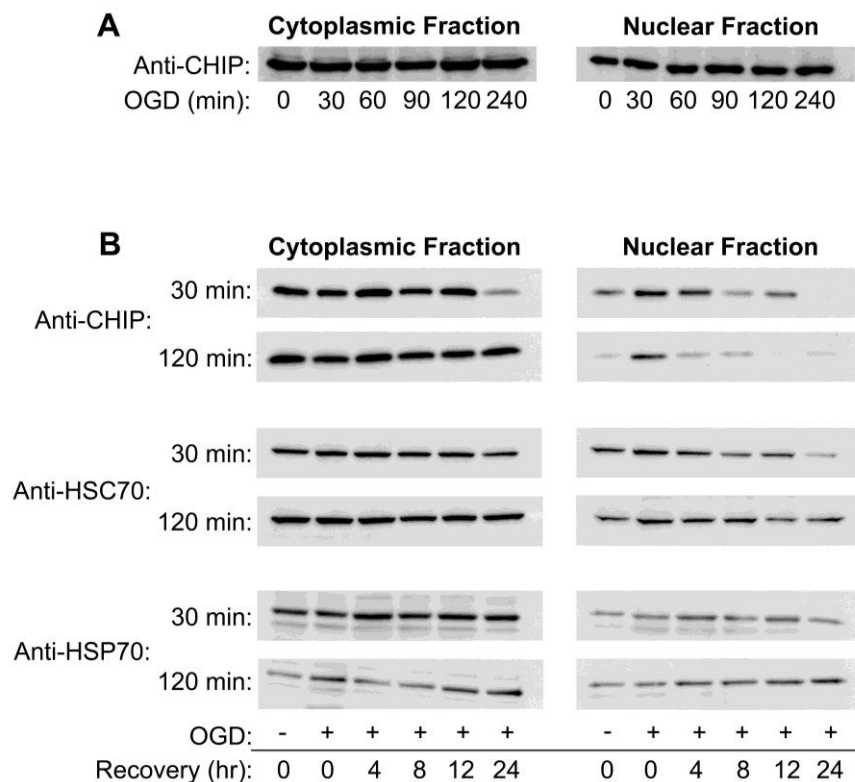
cellular fractions of primary mixed cultures exposed to mild (30 min) and severe (120 min)

OGD were probed for CHIP, HSC70, and HSP70 on Western blot. Comparisons were made

within each fraction, between cytoplasmic and nuclear fractions, between mild and severe

stress and between CHIP, HSC70, and HSP70 across time. (n(CHIP)=5-7, n(HSC70)=2-3,

n(HSP70)=3-4)



CHAPTER 3:

Effect of CHIP gene dose on cell survival following OGD

INTRODUCTION

Of the protective pathways induced following ischemia, significant data supports a role for heat shock proteins (HSPs), a major family of molecular chaperone proteins. Of the family of heat shock proteins, most experiments have focused on HSP70. HSP70 induction is a key component of cell survival following ischemia.^{54,55,61,124,132,133} Both overexpression of endogenous HSP70^{54,133,134} and induction of de novo synthesis of HSP70⁵⁶ have been shown to be neuroprotective. HSP70 and other HSPs attempt to maintain cell function in several ways. First, these proteins help to refold damaged proteins.^{124,125} If proteins cannot be repaired, the HSPs in conjunction with CHIP or other ubiquitin ligases facilitate protein ubiquitination and subsequent degradation.¹¹⁰ It is clear that the loss of proper protein folding and accumulation of misfolded proteins are deleterious to cells.^{65,99,105,120,135} The mechanism of protection is still largely unknown.

A frequent hypothesis in the mechanism behind HSP70-mediated neuroprotection is an increase in protein folding and protein degradation capacity. Indeed, protein folding is increased when HSP70 is overexpressed.⁵² However, when CHIP is overexpressed with HSP70, instead of an increase in protein degradation, rather an increase in protein folding was observed.¹¹⁴ These results were challenged by data from experiments that highlighted increased degradation of the androgen receptor¹³⁶, ataxin-1¹³⁷, and four-repeat tau¹¹² when CHIP was overexpressed. Presumably, the surrounding conditions and precipitating factors play a role in whether or not the protein chaperone system favors folding or degradation.⁴ It is likely that a proper balance between both processes is essential for cell survival following

an ischemic insult. Ubiquitin ligases play an essential role in this balance but are less well understood than the HSPs.

The protective role for CHIP has largely been investigated in models of chronic neurodegeneration. CHIP has been associated with aberrant proteins in Alzheimer's Disease (AD)^{99,100,112,135,138,139}, Parkinson's Disease (PD) and Lewy Body Dementia (LBD)^{90,98}, and Amyotrophic Lateral Sclerosis (ALS)¹⁰¹ among others. In each case, when presented with excessive amounts of misfolded or mutated protein CHIP has mediated ubiquitination and in most cases facilitated protein degradation. In the absence of CHIP, aggregates of misfolded and mutated proteins formed more rapidly.¹⁴⁰ Any compensatory mechanisms that were engaged in the absence of CHIP were insufficient to prevent this toxic aggregation. In cells that were unable to fold or degrade the aberrant proteins, cell death ultimately resulted.^{91,102,135} These results highlight the important role of CHIP in the removal of dysfunctional proteins and suggest that this process may be a rate limiting step in the struggle for cell survival.

Data also favor the idea that a cell's intrinsic capacity for protection can be increased. For example, ischemic tolerance is transient and develops quickly. The brief onset and duration of tolerance in the heart supports a metabolic mechanism, whereas tolerance in the brain persists over days.^{37,119} HSP mRNA induction in the brain occurs within 30-60 minutes of a mild ischemic insult while protein expression occurs several hours later.¹⁴¹ This temporal profile where HSP70 protein is increased from 24 hours to 5 days following a pre-conditioning stimulus is consistent with a role of HSPs in the generation of ischemic tolerance.¹¹⁹

Normally, HSPs, and HSP70 in particular are upregulated following ischemia-reperfusion injury in the heart.^{52,54,133} However, total protein levels of CHIP were unaffected by ischemia-reperfusion injury.¹⁰² HSPs and their co-chaperones are also abundant in cells that consume large amounts of energy. Considering ischemia is largely a failure of energy

stores and cellular metabolism we hypothesized that ATP-dependent, CHIP-mediated mechanisms might be compromised by ischemia. As such, in the absence of CHIP, increased cell death following ischemia would be observed.

CHIP appears to play a protective role in the cellular stress response, as CHIP(-/-) mice and cells derived from these mice undergo temperature sensitive apoptosis in response to thermal and proteotoxic stress.^{81,102} The region at risk in the left ventricle did not differ between wildtype and knockout animals under normal conditions and following 30 minutes occlusion but the infarcts of knockout mice were roughly 50% larger than wildtype.¹⁰² After 30 minutes of ischemia, percentage survival of CHIP knockout animals at 4 hours of reperfusion was 78%.¹⁰² Further, HSP70 induction was consistently lower after myocardial ischemia in CHIP(-/-) mice.¹⁰² A similar sensitivity in brain would indicate that neurons are also highly dependent on CHIP function in the face of an ischemic challenge. Results from Section 1 and 2 have demonstrated that CHIP is abundantly expressed in neurons, moves to the nucleus following stress and CHIP depletion is correlated with the onset of cell death. These events are on a time scale that is more rapid than those seen in chronic neurodegenerative diseases suggesting that CHIP availability is important from the earliest stages of dysfunction. Thus in the absence of CHIP injuries should both appear following shorter durations of stress and be larger compared to unstressed or wildtype samples.

RESULTS

Genotype Distribution from heterozygous matings

CHIP(-/-) mice are, in large colonies, birthed at a Mendelian ratio. However, per litter ratios differ considerably. To determine what could be expected in a smaller colony and to study the effects of gene dose on cell viability, a CHIP transgenic colony was established with 3 heterozygous female and 2 heterozygous breeding male Sv129 mice from the

Patterson Lab. The birth results are shown in **Table 3-1**. These results do not include 3 pups that died and were removed from the cages without viable tissue collection. In contrast to previous reports⁸¹, the birthrate for CHIP(-/-) mice was 4.3%.

Organotypic Slice Culture from CHIP transgenic mice

To maximize the data that could be generated from a single knockout mouse, organotypic hippocampal slice cultures were utilized. Unlike organotypic cortical cultures, hippocampal cultures generated a reasonably functional, intact system with variable susceptibility to ischemia, in a single culture.¹⁴²⁻¹⁴⁵ Further, a single postnatal day 7 (P7) brain could generate enough intact slices to culture three membranes (6-7 slices per membrane¹⁴⁵) which would allow three conditions (control, 30 minutes, 120 minutes) to be tested per animal.

Hippocampal sections were made at 100-150 microns and kept in culture for 10 days. Sections demonstrated similar cell outgrowth from the cultures (**Figure 3-1**) and good viability based on baseline propidium iodide (PI) staining (**Figure 3-2A-C**, 0 min) across all genotypes. Some sections did not sufficiently “flatten” thereby preventing small areas within a tissue section adequate access to medium which resulted in dry spots that stained brightly with Syto 24 (**Figure 3-2**, white arrows) and PI.

Effect of CHIP Gene Dose on Cell Viability

Cultures were subjected to zero, 30, or 120 minutes of OGD (Section 2 Materials and Methods) then returned to normal culture conditions for 24 hours. Cultures were then stained with Syto 24-a live cell nuclei stain, and propidium iodide-a dead cell nuclei stain. Images were taken with a 4X phase objective.

Representative images from cultures subjected to OGD are shown in **Figure 3-2A-C**. Images are shown with labels over regions of the hippocampus. Sections are generally

oriented so that the CA3 region is to the left, the dentate gyrus (DG) in the lower-middle, and the CA1 region to the upper-right. Qualitatively, we observed an increase in dead cells with increasing durations of OGD in cultures (Figure 3-2A-C), consistent across all genotypes.

Propidium iodide and Syto24 both stain for nuclei. To determine a relative size of injury a digital threshold of the images was taken to capture the stained nuclei that were in focus. The size of the injury is expressed as the percentage of the total stained area occupied by propidium iodide-stained dead cell nuclei (Figure 3-3). At baseline no significant differences in PI staining between wildtype ($4.89 \times 10^{-5} \pm 1.91\%$, n=9 animals (52 slices)), heterozygous ($1.22 \times 10^{-6} \pm 1.44\%$, n=5 animals (23 slices)) or knockout ($-3.50 \times 10^{-6} \pm 2.68\%$, n=2 animals (12 slices)) cultures was observed. Within genotypes compared to baseline, wildtype cultures following 30 minutes were $8.03 \pm 2.11\%$ (n=9 animals (50 slices)) greater than baseline and $39.26 \pm 3.46\%$ (n=8 animals (47 slices)) greater following 120 minutes of OGD. Heterozygous cultures were $22.65 \pm 3.19\%$ (n=5 animals (30 slices)) greater following 30 minutes and $38.94 \pm 5.08\%$ (n=5 animals (31 slices)) greater following 120 minutes. Knockout cultures were $30.77 \pm 6.59\%$ (n=2 animals (14 slices)) greater following 30 minutes and $50.59 \pm 6.48\%$ (n=2 animals (14 slices)) greater following 120 minutes. Across all genotypes the increases were significantly greater following 30 and 120 minutes compared to control (0 min). Increases following 120 minutes were also significantly greater than 30 minutes across all genotypes.

Compared to wildtype, no significant differences were observed when compared to heterozygous or knockout cultures at baseline. Following 30 minutes of OGD, both heterozygous and knockout cultures demonstrated significant increases in cell death compared to wildtype. Following 120 minutes heterozygous cultures were no longer significant compared to wildtype. Though injured area was larger in knockout cultures they were not significantly larger compared to wildtype.

DISCUSSION

Organotypic hippocampal cultures from animals lacking CHIP are more sensitive to OGD than wildtype cultures. Cell death patterns in cultures from animals lacking CHIP were more uniform and less region-specific within the hippocampus than what was observed in wildtype and heterozygous cultures. This is different from previous reports from myocardial ischemia experiments¹⁰² where cell death patterns were similar among genotypes, though varying susceptibility is not a known characteristic of cardiac tissue as it is in the brain. However, the size and pattern of injury observed in the knockout hippocampal cultures in the current experiments was smaller than what was previously reported in rat hippocampal slice cultures following OGD.¹⁴²

Previous reports by the Patterson lab showed injury in transgenic heart to be 50% larger in knockouts than wildtype using ischemic durations known to cause significant injury.¹⁰² Here we show that while patterns over time are similar across genotype, the degree of increased cell death varies. With the exception of control cultures, compared to their respective wildtype values, heterozygous and knockout cultures have larger injuries than wildtype at 30 minutes but by 120 minutes heterozygous cultures were no longer significant compared to wildtype. Significant differences across genotypes, while present, may indicate varying sensitivity by not only cell type but perhaps more importantly, organ system.

We were able to culture these slices with a relatively small amount of cell death, as identified by propidium iodide staining. The ability to generate a primary culture alleviated a concern because of the developmental sensitivity of fetuses lacking CHIP, some of which were absorbed in utero⁸¹. *Caenorhabditis elegans* lacking CHIP had impaired development and were heat sensitive.¹¹³ However, the nature of developmental sensitivity for CHIP transgenic animals and what role CHIP plays in development is largely unknown.

Compared to our dissociated cortical cultures, which are enriched in neurons with approximately 20-40% astrocytes, the organotypic hippocampal slice cultures have greater concentration of astrocytes (nearly 80% of the adult brain is comprised of glial cells). Thus, even small amounts of glial cell death could contribute significantly to total cell death in the slice. However, of the cells known to be astrocytes - in particular, those that grew out from the edge of the culture – very few stained with PI (stain for dead nuclei) under ischemic conditions where neuronal loss was significant. Thus, there appears to be little astrocyte death under these conditions. The pattern of PI staining also followed neuronal groups and did not show a random pattern which would be expected from astrocytes. Together these observations suggest that the majority of cell death in the explant cultures was from more vulnerable neurons. Thus, in Figure 3-2, the cell death is actually quite high due to the fact that the neurons are the majority of cells that die and the proportion takes into account all live cell nuclei.

It is possible that the modest injury seen in our culture model is due to the fact these cultures may have been pre-conditioned by the initial stress of culture before being subjected to OGD. From our dissociated culture experiments (Section 2, Figure 2-4) and other reports¹⁴⁶ the process of culturing and including antibiotics in culture medium are both sufficient to induce a stress response and cell death.^{147,148} Presumably our hippocampal mouse cultures undergo a similar neuronal death following our OGD paradigm.

The results of a lack of CHIP may be tempered further by the degree of redundancy in the molecular chaperone system, that in the absence of a component of the molecular chaperone system other proteins or a combination thereof completes a similar function.¹⁰² The sensitivity demonstrated by mice lacking a single copy of the CHIP gene could reflect the fact that in the complete absence of CHIP, HSP70 expression is impaired.⁹¹ Some but possibly not all of the necessary HSP70 is available and may be insufficient to return to a more normal state following stress. If 1) HSF1 activation is impaired and no other HSP

transcription factor fills the void created by a partial loss of CHIP, 2) HSC70 is insufficient to process misfolded proteins created by stress, or 3) no protein processes increased abnormal proteins in the absence of increased HSP70, then even if HSP70 is minimally present it would not be at a level high enough to maintain or restore normal cell function. The redundancy in the molecular chaperone system would be decreased by the presence of a single gene copy. While capable of maintaining viability, these compromised cultures may be functioning in a manner alternate to the assumed ER and HSP stress responses.

The likely redundancy of the molecular chaperone system may point further to a reason behind the different injury patterns in the slice cultures. The heart was one of the organs reported to express high levels of CHIP, along with skeletal muscle.⁸¹ The brain expressed high levels of CHIP⁸¹ also, however, not to the extent of heart muscle. The metabolic demand difference between the two organ systems may account for some of the difference in injury, such that energy demands in the brain differ from the energy demands in heart.⁴ Alternatively, if the stress response systems in the brain are already accustomed to the differing metabolic demands and priorities such differences injury size and location would be expected.

Increased sensitivity to the absence of CHIP may be reflected in the relative abundance of the protein. Whereas heart muscle expresses more CHIP than the brain⁸¹, the expectation is that the heart would be more sensitive to stress. Further, expression differences in this heat shock protein within the brain exist (Figure 1-2). Also, HSP70 is more readily induced in the CA1 region of the hippocampus compared to CA3 following ischemia.¹⁰⁵ It would be reasonable to expect similar induction differences would impact CHIP function and that such expression differences could differentially impact the survival of cells exposed to stress.

MATERIALS AND METHODS

Breeding and Genotyping

Generating mice lacking CHIP was previously described.^{91,102} *CHIP* (-/-) mice were produced on a Sv129 background backcrossed over 7 generations prior to the start of the current studies by the Patterson Lab. Heterozygous pairs of mice were mated at 8-12 weeks of age. Males were removed from the breeding cage when litters were born and another nursing female added to cage. At postnatal day 7 (P7) pups were removed from the cage for genotyping and slice culture. Tails were clipped for genotyping after animals were sacrificed.

Organotypic hippocampal slice culture

The organotypic slice culture protocol was modified slightly from Stoppini et al. (1991)¹⁴⁹ and Norberg et al. (1999)¹⁴⁵. Briefly, P7 Sv129 *CHIP*-transgenic mice pups were sacrificed and brains harvested. Tails were clipped and numbered for genotyping. Brains were rinsed once each in calcium-magnesium free HBSS (CMF-HBSS) and MEM. Brains were then chopped with a coronal orientation at 100-150µm using a McIlwain Tissue Chopper. Under a 4x dissecting microscope sections of hippocampus were isolated and trimmed. Hippocampal slices were placed on membrane inserts (Millicell) pre-wet with MEM. 6-7 slices were placed on a single membrane, and 3 membranes per animal were cultured. A minimum amount of MEM + 10% FBS + 20 µg/ml gentamicin was added below the membranes (1.2 mL) and sections placed in the incubator. Culture medium was replaced every 2-3 days.

Our culture preparation frequently resulted in “dry spots” of dead cells in cultures. Part of this could be eliminated by culturing at a younger age¹⁴⁸ or on collagen-coated coverslips¹⁵⁰. Cultures on collagen-coated coverslips are more difficult to use, but are far superior for antibody staining experiments. P5 slice cultures are not as developed so P7 slice cultures were used to examine cell outgrowth from the slice. Glial outgrowth on

collagen-coated coverslips was comparable to that seen on porous membranes (data not shown).

Organotypic Slice Culture Oxygen-Glucose Deprivation and Cell Viability Imaging

One culture per animal was subjected to 0, 30, or 120 minutes of OGD. Cultured membranes were placed in individual 35mm dishes with glucose-free MEM (gfMEM, in mM: 1.3 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5.3 KCl, 0.81 MgSO_4 , 116.4 NaCl, 26.2 NaHCO_3 , 1.0 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$). gfMEM was triple exchanged before cultures were placed in an anaerobic chamber, purged 7 minutes with 95% argon/5% carbon dioxide, and sealed. The cultures—in chamber—were returned to the incubator for the duration of OGD. After OGD, gfMEM was triple exchanged for complete MEM and membranes were returned to normal culture conditions for 24 hours. Cultures subjected to 0 minutes of OGD remained in normal culture conditions throughout. Following 24 hours of recovery 1 $\mu\text{g/mL}$ Propidium Iodide and 1 μM Syto 24 (Molecular Probes) were added to the culture medium to visualize dead cells and live cells, respectively. Culture medium was exchanged for HEPES-buffered aCSF and cultures were removed from the incubator for imaging. Cultures were imaged using an Olympus INT-2 inverted microscope and Metamorph™ Imaging System (Universal Imaging Corporation, West Chester, PA). PI and Syto 24 stained cultures were imaged with a 4X objective for a general survey of the cultures, and higher magnification images were digitally magnified from the 4X images or taken with a 10X objective. Cultures were then fixed with 4% paraformaldehyde and stored in 70% ethanol.

To compensate for staining variability across sections, each slice served as its own control. To make comparisons across genotypes, a threshold was set to capture the plane with the brightest stained cells which allowed us to evaluate the areas that visibly defined the injury. Wildtype and heterozygous cultures demonstrated a more diffuse stain; perhaps due to increased cell viability in the slice, whereas knockout cultures stained more clearly.

Organotypic slice culture analysis

Images from hippocampal slice cultures were inclusively thresholded for the positively stained cells that were in the image plane. Since brightness varied, ranges for each individual slice were different. Boundaries of the slice culture were identified based on live cell staining. Regions within each slice that dried, did not stain, or were otherwise abnormal were also identified. These abnormal areas were excluded from the analysis. Measurements for area, integrated intensity, threshold area based on propidium iodide or Syto24 stained nuclei, and percent of the culture area that was included in the threshold were automatically captured using Metamorph™ Imaging System (Universal Imaging Corporation, West Chester, PA).

FIGURE LEGENDS

Table 3-1:

CHIP(-/-) birthrate is less than predicted Mendelian ratio in small breeding colonies. The total birthrate from heterozygous mating pairs generated 138 animals. Of these 39 were wildtype, 48 were heterozygous, and 6 were CHIP(-/-). 45 animals were unidentified.

Figure 3-1:

Cell outgrowth from the cultured slice occurred in each genotype with similar distance from the original slice and cell density. The white line denotes relative boundary of the cultured slice and the white arrow marks the direction of cell outgrowth.

Figure 3-2:

Cell death in slice cultures from CHIP transgenic mice increased with increasing durations of OGD in each genotype. **A** Sample CHIP wildtype (+/+) cultures **B** Sample CHIP

heterozygous (+/-) cultures **C** Sample CHIP knockout (-/-) cultures stained with Syto 24 (green, live cells) and propidium iodide (red, dead cells). Dried areas within cultures are marked with white arrows.

Figure 3-3:

Size of injury due to OGD is expressed as the percentage of stained nuclei area occupied by propidium iodide normalized to the mean at 0 min. Significance was calculated compared to wildtype at each time point. (*p<.001)

ACKNOWLEDGEMENTS

CHIP transgenic breeding pairs were a generous donation from the Laboratory of Dr. Patterson (UNC-Chapel Hill). The transgenic mice were generated as previously described in Dai *et al.* (2003)⁹¹ and Zhang *et al.* (2005)¹⁰².

Genotyping was completed in large part by Pamela Lockyear.

Table 3-1: CHIP Genotype Distribution

CHIP(-/-) birthrate is less than predicted Mendelian ratio in small breeding colonies. The total birthrate from heterozygous mating pairs generated 138 animals. Of these 39 were wildtype, 48 were heterozygous, and 6 were CHIP(-/-). 45 animals were unidentified.

	Male	Female	Unknown (Slice Culture)	Percentage
Wildtype (+/+)	10	17	12	28.3%
Heterozygous (+/-)	18	18	12	34.8%
Knockout (-/-)	3	1	2	4.3%
Unknown	7	10	28	32.6%
Total (count)	38	46	54	100%

Figure 3-1: Cell outgrowth from slice cultures

Cell outgrowth from the cultured hippocampal slices occurred in each genotype with similar distance from the original slice and cell density. The white line denotes the relative boundary of the culture slice, while the arrow marks the direction of cell outgrowth.

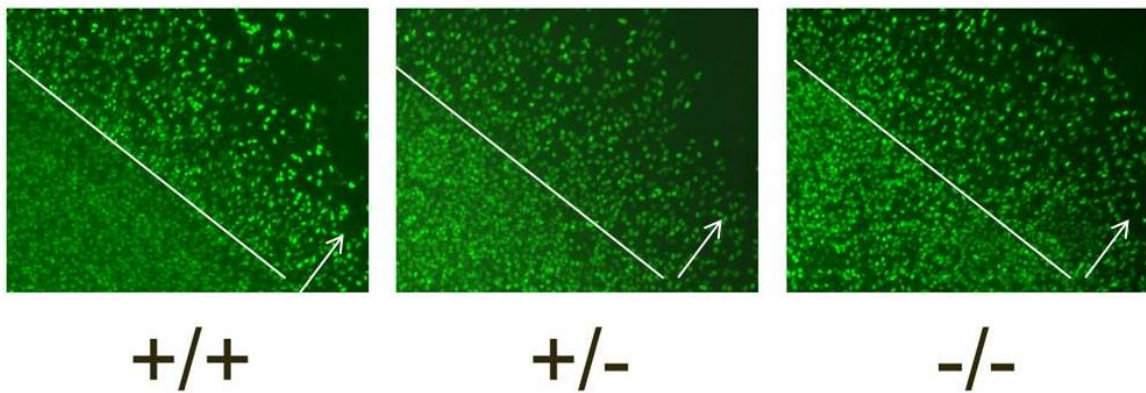
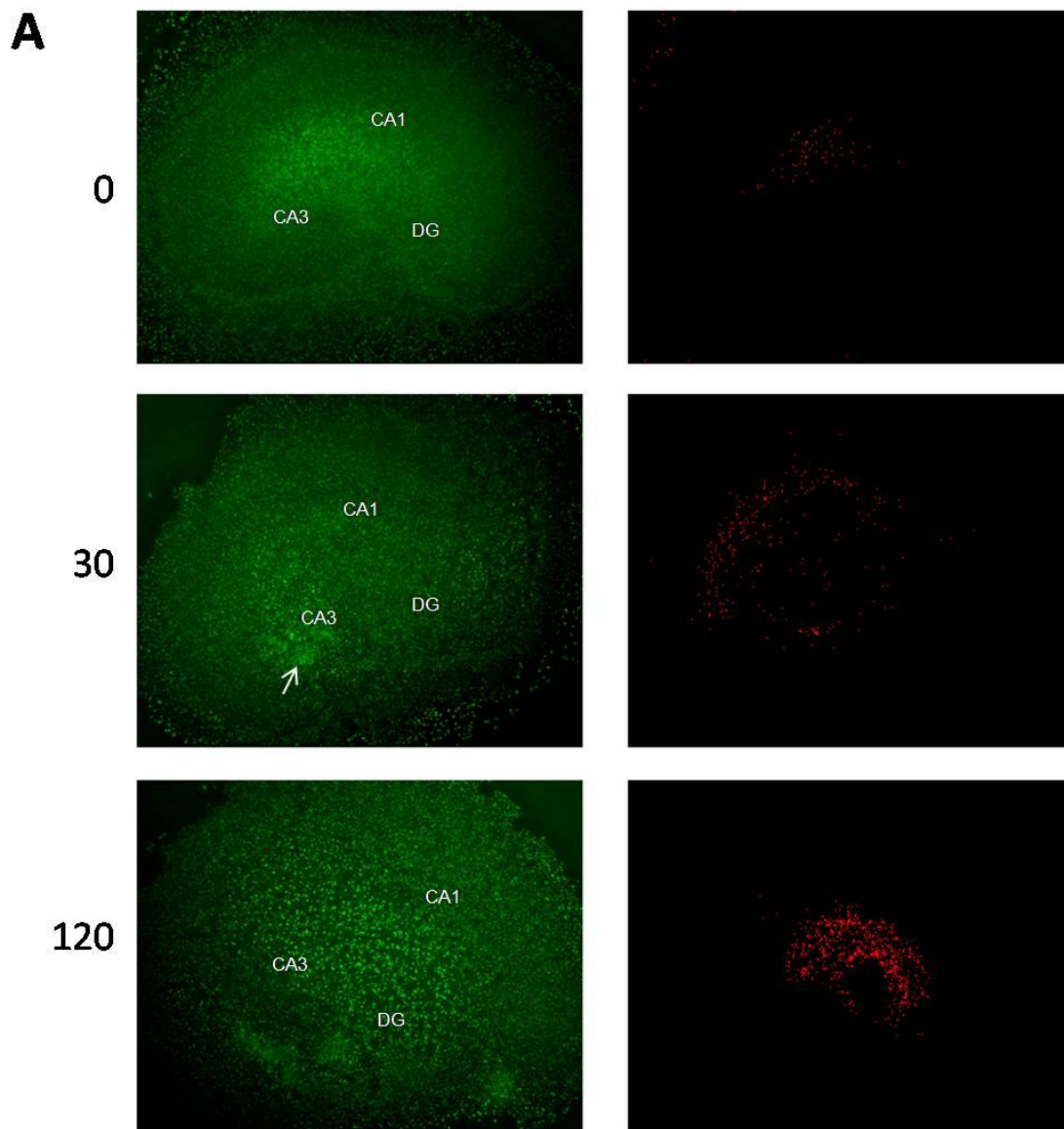
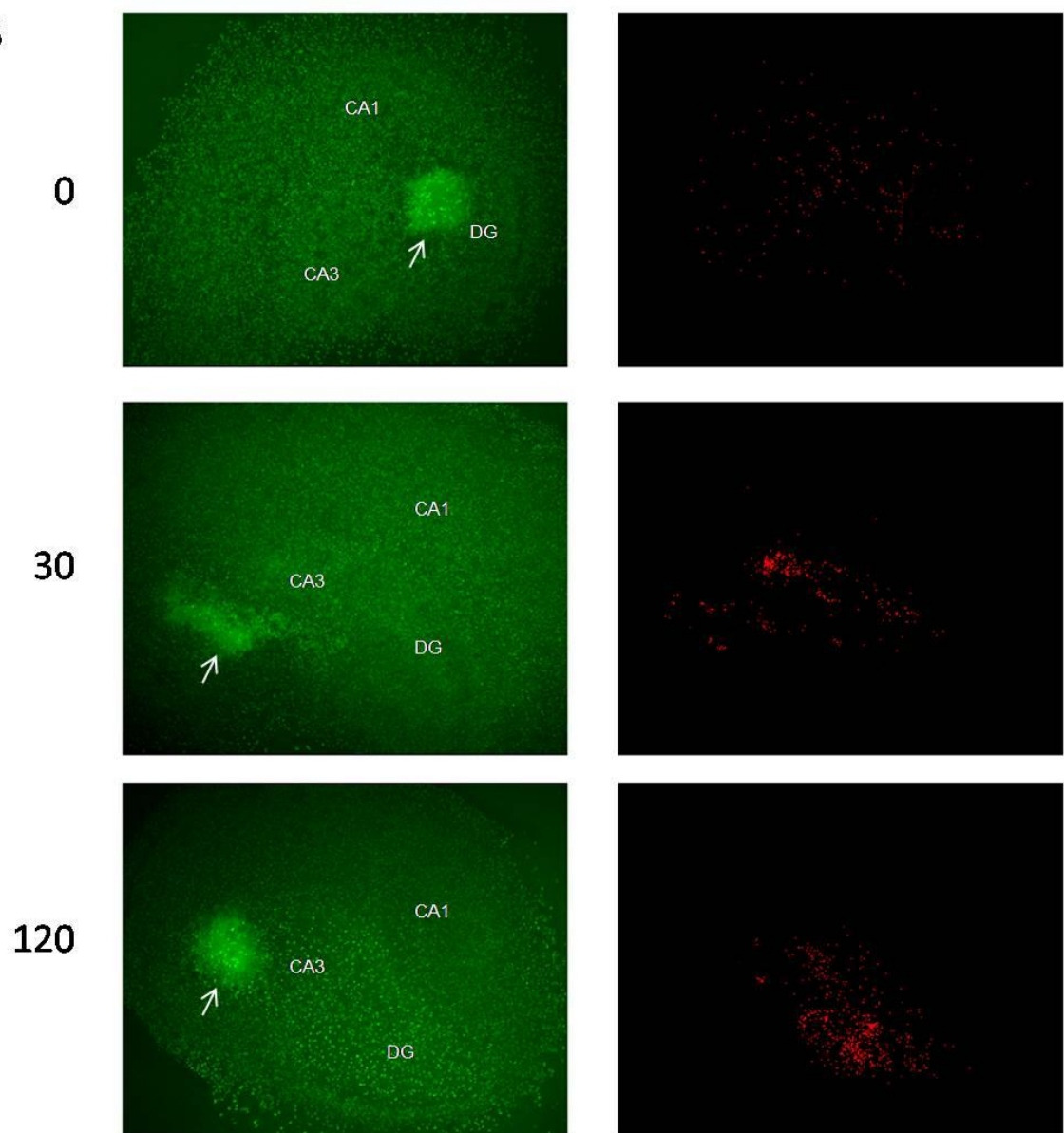


Figure 3-2: Effect of Gene Dose on Cell Viability

Cell death in slice cultures from CHIP transgenic mice increased with increasing durations of OGD in each genotype. **A** Sample CHIP wildtype (+/+) cultures **B** Sample CHIP heterozygous (+/-) cultures **C** Sample CHIP knockout (-/-) cultures stained with Sytox Green (green, live cells) and propidium iodide (red, dead cells). Dried areas within cultures are marked with white arrows.



B



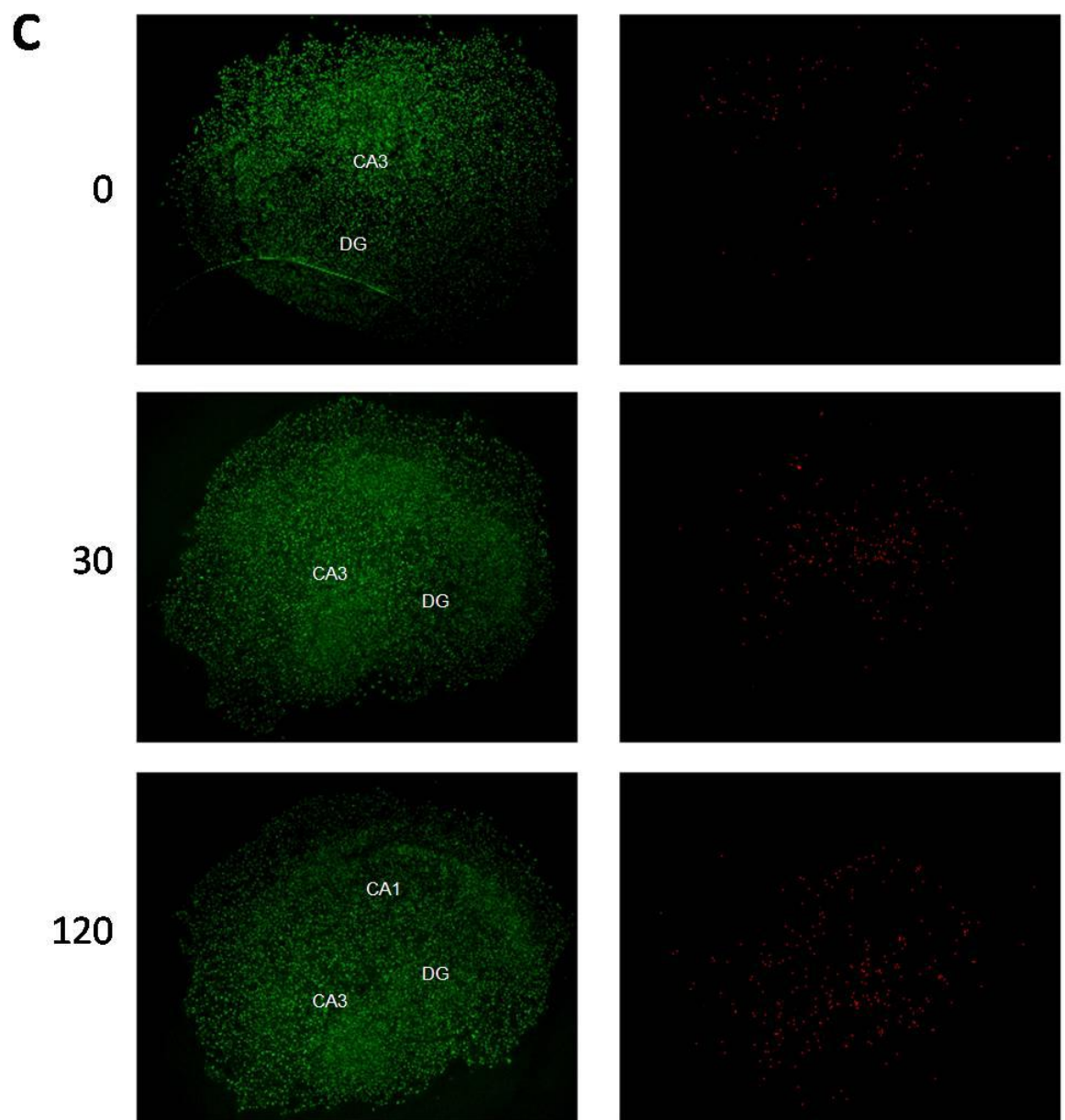
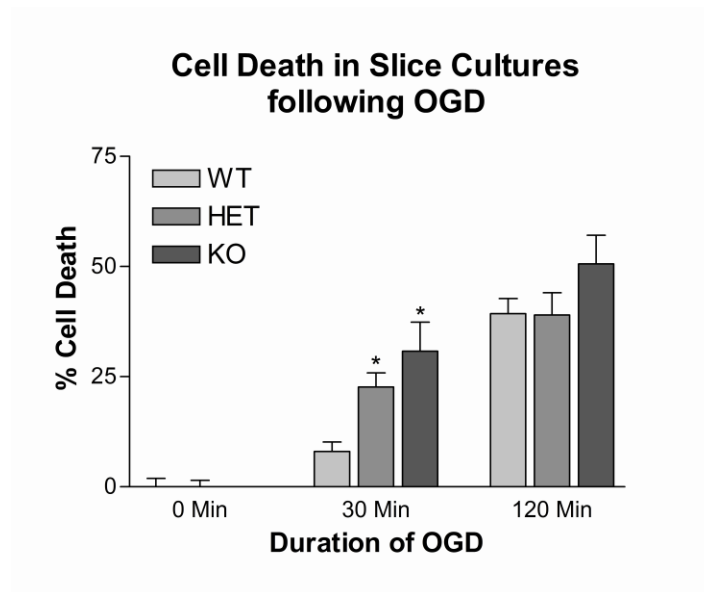


Figure 3-3: Injured Area in Slice Culture following OGD

Size of injury due to OGD is expressed as the percentage of stained nuclei area occupied by propidium iodide normalized to the mean at 0 min. Significance was calculated compared to wildtype at each time point (* $p < .001$).



CHAPTER 4:

CHIP Interacting Partners

INTRODUCTION:

Proteasomal degradation is mediated by ubiquitination. The process of labeling proteins for degradation involves activating, conjugating, and ligating multiple ubiquitin moieties to the target protein.^{69,97} Ubiquitin activation is mediated by one of two evolutionarily conserved proteins.⁶⁹ The process is amplified by families of ubiquitin conjugating enzymes, estimated to number in the hundreds.⁶⁹ Specificity for ubiquitination has been assigned to the ubiquitin ligases, whose number is estimated to be in the thousands.⁶⁹ The combination of any E2-conjugating enzyme with specific E3-ligating enzymes creates an enormous number of potential combinations.

Proteasomal degradation, and subsequently ubiquitination have largely been viewed as cytoplasmic phenomena. However, the recent demonstration of assembled proteasomal subunits in the nucleus¹²⁶ and monoubiquitination⁶⁹ of proteins in the nucleus clearly point to a nuclear function for this system. Many HSPs express nuclear localization sequences^{77,91,151,152} and are found in the nucleus following stress¹⁵³ (Section 2).

Since both CHIP and HSC70 show similar patterns of nuclear trafficking (Figures 2-3, 2-5), it is possible that these proteins work together in the nucleus to modify nuclear targets via ubiquitination. Further the decreased viability of cells lacking nuclear CHIP (Figure 3-3) suggests that CHIP translocation plays an important role in the cellular response to stress. Whether this role is through specific ubiquitination or chaperoning of proteins in the nucleus is unknown. To begin investigating the possibility of specific CHIP targets, we undertook two studies to identify potential target proteins using yeast-two-hybrid

screen and immunoprecipitation. Previous screens from cardiac samples suggested limited CHIP interaction⁸¹, while numerous immunoprecipitation experiments, primarily from cultured cells, showed ubiquitinated target proteins could be isolated from CHIP-containing complexes^{99,129,154-156}. The uniqueness of the brain in the development of tolerance^{2,9,37,42,132}, in chronic neurodegeneration^{82,97-101}, in energy consumption and metabolic mechanisms^{3,6} suggests potential targets of ubiquitination might not be identical to those found in other organ systems.

RESULTS:

Yeast-two hybrid screen for identification of CHIP interacting partners

Full length, recombinant human CHIP was probed against an adult human brain cDNA library. Nineteen (19) putative interacting partners were isolated for sequence analysis. Seventeen (17) of the interacting partners were identified as segments on the C-terminus of heat shock protein 70 (Accession number NM_006597.3). The two remaining interacting partners were sequenced and identified as segments on the C-terminus of the heat shock protein 90 (HSP90) alpha-subunit (Accession number NM_00107963.1). Figure 4-1 displays the positive results.

Table 4-1 summarizes the sequence results from this screen. Of the 17 segments from HSC70, 5 had greater than or equal to 90% coverage (interacting partners 1, 2, 7, 9 and 12). This included segments between nucleotides 659-2124 on HSC70. Segment 1064-2054 had 92% coverage (interacting partner 12). Notably, one of the key variations of STUB1 (NM_006597.3) was an “alternative splicing” of HSP70 (NM_153201.1) whose binding site is immediately above and includes an EEVD (glutamic acid-glutamic acid-valine-aspartic acid) nuclear localization sequence but does not include nucleotides 1469-1923 (residues 464-615) found in NM_006597.3. On the HSP90- α subunit, only 1 segment had 90% coverage between nucleotides 2012-3014, which is in the cytosolic domain.

Immunoprecipitation of CHIP-bound complexes

Subcellular fractions were prepared from cells subjected to either 30 or 120 minutes of oxygen-glucose deprivation (OGD) with or without 8 hours of recovery. Control samples were from unstressed cultures subjected to sub-cellular fractionation as well as whole cell lysates. Samples were immunoprecipitated using a polyclonal antibody against CHIP and subjected to denaturing SDS-PAGE and Coomassie staining (Figure 4-2). Our results indicated that in addition to the heavy chain and light chain of the anti-CHIP antibody, only 2 additional protein species were immunoprecipitated; a protein species with a molecular weight between 37-50kDa and another protein species with a molecular weight between 75-100kDa. These proteins could be a CHIP monomer that is ubiquitinated or otherwise modified, and a modified HSC/HSP70 respectively. The lower molecular weight species was not observed after 120 minutes OGD plus 8 hour recovery in the cytoplasmic fraction, nuclear fraction, or whole cell lysate. A parallel Western blot probed for poly-ubiquitin did not reveal any protein species and was non-specifically stained for the heavy and light chains of the antibody (data not shown).

DISCUSSION

Primary interacting partners with CHIP are the N-terminus region of HSC/HSP70 and the cytoplasmic domain of HSP90- α . Interestingly there was no similarity between the HSC70 and HSP90- α sequences.^{80,81} The lack of binding partners for CHIP other than HSC70/HSP70 and HSP90- α suggests that the target protein specificity might actually be a function of the CHIP-HSC70, CHIP-HSP70, or CHIP-HSP90 complex or alternatively, dictated by the structure of HSC/HSP70 or HSP90- α rather than by CHIP. In general, the limitations of the screen preclude the determination of protein interactions of the multimeric protein complexes. To take a different look at CHIP binding, constructs of CHIP that

exclude the TPR domain could potentially lead to the identification of other CHIP-bound proteins whose affinities are less than that of the known binding partners HSP70 and HSP90.

Previous experiments in cultured cells suggested a number of ubiquitinated species immunoprecipitate with CHIP⁸⁶ especially following heat stress⁸⁵. In our heat stress studies, we observed a few ubiquitinated species that immunoprecipitated with CHIP following heat stress in the nuclear fraction (data not shown). These ubiquitinated species were not observed in the cytoplasmic fraction suggesting specific protein targeting in the nucleus. Following OGD, target proteins were not isolated from the nucleus suggesting a possible loss of nuclear interactions. The inability to identify “target” proteins—proteins destined for ubiquitination and degradation—suggests a potential technical limitation in the study of CHIP target proteins. However, it should still be possible to isolate CHIP-bound complexes with the development of better techniques that preserve protein complexes.

Within minutes following a stress nuclear CHIP increases, HSF1 is activated^{76,104}, proteins are degraded and other proteins aggregate^{80,125,157}. The current results suggest that CHIP-mediated ubiquitinating complexes are transient. Previous reports of CHIP-containing complex immunoprecipitations have been performed primarily with cells expressing CHIP-tagged proteins.^{80,104,112,158} If studies of GFP-tagged HSP70¹⁵⁹ where transfection of the fluorescent tag induced HSP70 expression and mediated up regulation of COX-2 are any indication, such constructs may signal a stressful change and thereby alter the expression and folding of CHIP.

CHIP dimerization is needed for CHIP activity.^{160,161} The absence of this dimer in our Western blot experiments suggests that our isolation procedure could be eliminating the dimer. The presence of higher molecular weight species in Western blot from our whole cell lysate experiments (data not shown) would suggest that the sub-cellular fractioning protocol used might contribute to some dissociation. If this is the case we would expect

immunoprecipitations from whole cell lysates to generate results more in line with previous reports. However, in our HS and OGD experiments, whole cell lysate fractions failed to yield results that were different from the sub-cellular fractions (data not shown). Different protocols may be needed that cleanly isolate cytoplasmic and nuclear fractions, without eliminating the CHIP dimer. Such procedures would be more likely to immunoprecipitate CHIP-bound complexes and potential target proteins.

MATERIALS AND METHODS

Yeast two-hybrid screen

The protocol used by ProteinLinks, Inc. is modified from Gietz *et al.* (1992)¹⁶² and Golemis and Brent (1997)¹⁶³. Bait DNA (STUB1) was cloned into pLexA by gap repair homologous recombination in yeast Y338 by cotransformation of PCR product (with Pfu) and EcoRI-digested vector.

The primers used in the cloning are:

5'-5'CGCAACGGCGACTGGCTGgaattcatgaagggcaaggaggagaagg-3' and

5'-GCCATGGTCGACGGATCCCCGGtcagtagtcctccacccagcc-3'

The bait junction was sequenced to confirm the correct reading frame.

About 10 million independent Human Adult Brain Library clones (about 5 library coverage) were screened in the galactose medium lacking leucine, histidine, tryptophan, and uracil. All the 132 colonies that can grow in the selection media were picked and further tested for expression of LacZ gene. Interaction plasmids were prepared from 20 yeast colonies that can grow on the selection plates and are blue on the x-gal plates. Interaction plasmids were then transformed into KC8 *E.coli* cells, and amplified. All 20 plasmids were retransformed into EGY42 and tested for their interactions against bait STUB1 as well as the control bait RAS. Finally, 19 clones were verified. The results are shown in Figure 4-1.

CHIP immunoprecipitations

Neural cultures were prepared at 10^5 cells/cm² in 100mm dishes and maintained 10 DIV. Cultures were subjected to 30 or 120 minutes of OGD with or without 8 hours of recovery. Control cultures were not exposed to OGD. After exposure to OGD, cells were placed on ice for 4 minutes and cytoplasmic and nuclear fractions were extracted using the NE-PER Cytoplasmic and Nuclear Extraction kit (Pierce, 78833) per kit instructions. Extractions were validated cytoplasmic fractions that were not immunoreactive for Histone2B and nuclear fractions that were not immunoreactive for Grp78 on Western blot. Whole cell lysate from control cultures was immediately extracted using RIPA buffer (1X TBS (20mM Tris pH 8.0, 137mM NaCl), 1% NP-40, 10% glycerol, protease inhibitors (1μM, Sigma, P8349)). Protein concentrations were determined using the BCA Assay (Pierce) and equal amounts were separated by SDS-PAGE and either Coomassie stained or analyzed by Western blot for ubiquitin poly-ubiquitinated sequences.

FIGURE LEGENDS

Figure 4-1:

Yeast two-hybrid results from full-length human CHIP cDNA screened against an adult brain cDNA library yielded 19 positive results. Clones that grow faster or are bluer than others in x-Gal/Gal plate suggest possible stronger interactions. Control interacting proteins are Ras/Raf.

Table 4-1:

Summary of the 19 verified interacting clones from the yeast two-hybrid screen of bait STUB1 (NM_005861) against Human Adult brain library (ProteinLinks, Inc., San Diego, CA).

Figure 4-2:

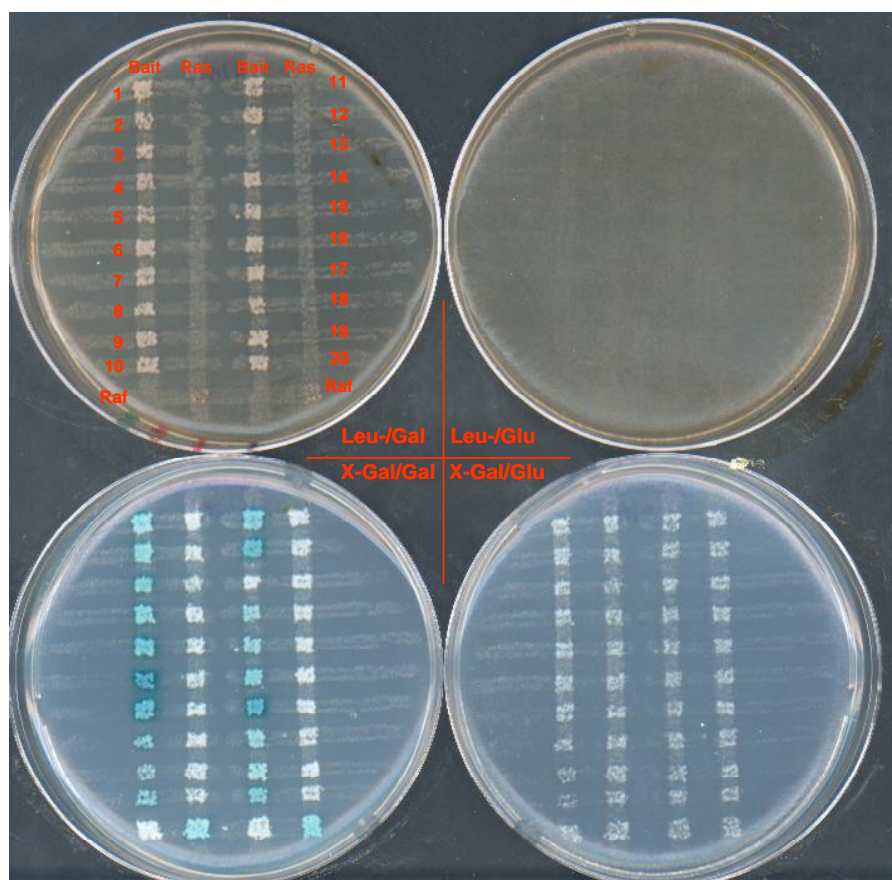
Cytoplasmic and nuclear fractions from cultures subjected to OGD with or without recovery and whole cell lysate (WCL) were immunoprecipitated with a polyclonal antibody against CHIP. The arrows indicated where protein species with molecular weights between 75-100kDa and 37-50kDa immunoprecipitated with the anti-CHIP antibody. H.C. (heavy chain), L.C. (light chain)

ACKNOWLEDGEMENTS

Yeast two-hybrid screen was performed by ProteinLinks, Incorporated (San Diego, California) under the direction of Marie Ma, Ph.D.

Figure 4-1: Yeast Two-Hybrid Screen Results

Yeast two-hybrid results from full-length human CHIP cDNA screened against an adult brain cDNA library yielded 19 positive results. Clones that grow faster or are bluer than others in x-Gal/Gal plate suggest possible stronger interactions. Control interacting proteins are Ras/Raf.



Proteinlinks, Inc.

Table 4-1: Interacting Sequence Percent Matching

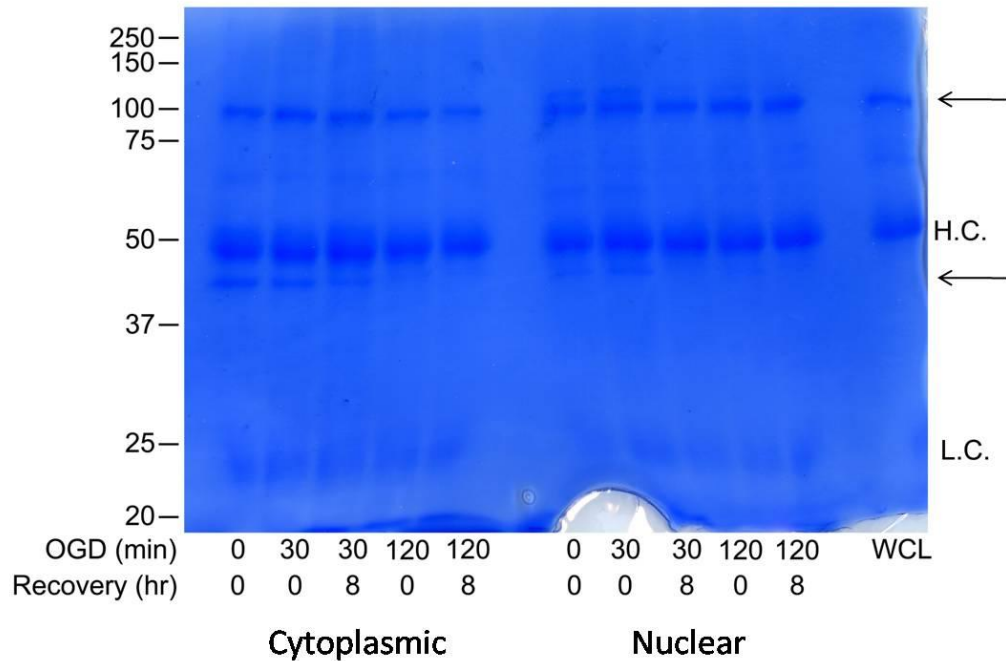
Summary of the 19 verified interacting clones from the yeast two-hybrid screen of bait

STUB1 (NM_005861) against Human Adult brain library (ProteinLinks, Inc., San Diego, CA).

Interacting Partner	Accession #	Query Start	Query End	Match Start	Match End	Query Coverage (%)
1	NM_006597.3	90	742	1603	2255	59
2	NM_006597.3	92	739	1610	2257	58
3	NM_006597.3	93	740	1610	2257	58
4	NM_006597.3	89	1098	659	1669	90
5	NM_006597.3	94	1100	1064	2077	91
6	NM_006597.3	91	404	1712	2025	28
7	NM_006597.3	90	1100	1108	2124	91
8	NM_001017963.1	105	814	2358	3071	69
9	NM_006597.3	92	1091	659	1661	90
10	NM_006597.3	96	533	1643	2081	39
11	NM_006597.3	95	886	1108	1900	89
12	NM_006597.3	83	1066	1064	2054	92
14	NM_006597.3	94	1643	531	2081	60
15	NM_006597.3	100	1643	713	2255	57
16	NM_006597.3	95	742	1610	2257	58
17	NM_006597.3	92	408	1712	2028	46
18	NM_006597.3	91	676	1670	2255	53
19	NM_001017963.1	90	1089	2012	3014	90
20	NM_006597.3	95	532	1643	2081	47

Figure 4-2: Coomassie Staining for CHIP-bound Complexes

Cytoplasmic and nuclear fractions from cultures subjected to OGD with or without recovery and whole cell lysate (WCL) were immunoprecipitated with a polyclonal antibody against CHIP. The arrows indicated where protein species with molecular weights between 75-100kDa and 37-50kDa immunoprecipitated with the anti-CHIP antibody. H.C. (heavy chain), L.C. (light chain)



CONCLUSIONS

The series of experiments presented in this thesis examined CHIP localization in the brain at baseline and following stress. Our models used temporal dissection and correlation with the development of pathology, in this case, delayed cell death. These data are the first to show a response by CHIP in the acute phase of injury. Previous reports have connected CHIP in chronic neurodegeneration after pathology has already developed (e.g. tau aggregates), while this observation starts a new page in understanding the role of CHIP following acute stresses and how acute phase responses correlate to functional endpoints.

Potential mechanisms of acute CHIP-related protection

Ischemic Tolerance

Insights into the protective capacity of the early stress response have come from studies of ischemic tolerance. In these studies the application of a sub-lethal stress has been shown to confer later protection against a subsequent ischemic challenge. Theories of ischemic tolerance have suggested that it may be due to an increase in the capacity of the stress response machinery. Burda *et al.* (2003) described two mechanisms essential for the acquisition of ischemic tolerance.⁴² The first is a significant reduction in translation inhibition after sub-lethal stress. A key factor after this type of stress is that both vulnerable and non-vulnerable neurons had the same response. For what Burda *et al.* (2003) described as full tolerance, reduced protein synthesis inhibition was followed by a second process, recovery of protein synthesis regulation.⁴² For recovery, the interval between the pre-conditioning ischemia and the lethal ischemia was critical because the recovery of

protein processing depends on the synthesis of protective proteins (i.e. HSPs) which prevented the delayed cell death of vulnerable neurons. After short durations (<5 minutes) of ischemia, *in vivo* protein synthesis regulation recovered in as little as 12 hours. Longer durations of 5-10 min required up to 2 days for adequate recovery.^{42,125} Once the stress response has been initiated, enhanced mechanisms for protein processing may remain for up to seven days. Beyond that the benefits of the sub-lethal stress are lost.

In our studies, depletion of CHIP correlated with the development of delayed cell death. During recovery following OGD, the decrease in nuclear CHIP occurred within a 24 hour window. Previous studies of ischemic pre-conditioning (PC) established the recovery interval between PC and the lethal stress that resulted in reduced injury following the lethal stress had a similar time frame of 12 hours to 2 days.^{42,106} Our results showed that CHIP is still present in the nucleus after 12 hours following a mild stress, whereas following the more severe stress, nuclear CHIP was significantly decreased after only 4 hours.

To better understand early mechanisms in stroke injury, a more thorough understanding of normal cellular processes and the failure of these processes is required. Protein processing is a fundamental function of cells that is rapidly compromised during stroke. The ability to retain this function may be one of the most important determinants of cell survival. The HSPs are a large part of that system. In healthy cells proteins are synthesized and then must be folded into their functional conformations to allow maximal activity. The ability to generate functional proteins is highly dependent on calcium (maintained by the sarco(endo)plasmic reticulum calcium ATPase (SERCA)), ATP (mitochondria) and a host of other proteins that chaperone newly synthesized proteins through each phase of the folding process.^{72,77} These chaperones include HSC70 and HSP70 and folding co-chaperones like HSP40. If any portion of this system fails, the proteins will not fold properly and there is a progressive loss of cellular function.⁷⁷ To keep

this system running smoothly, misfolded proteins must be re-folded or removed from the cell.⁷²

Anatomical and Cellular Location of CHIP

In the past, studies of CHIP in acute stress have been limited to myocardial ischemia experiments *in vivo* and heat stress experiments *in vitro*. These reports illustrated the importance of CHIP in the stress response since animals and cells lacking CHIP were more prone to injury following the stress.^{81,91,102} Neither group of experiments looked in the brain or in neurons. Our immunohistochemical mapping studies showed that CHIP was abundant in most neurons in mice and rats *in vivo*. A similar pattern was seen *in vitro* in primary cultures of dissociated cortical/hippocampal neurons, albeit with a slightly higher basal level of nuclear CHIP. This observation suggests that cultured neurons may partially depend on the stress response for survival.

Variability in the CHIP staining intensity *in vivo* suggested that different cell types and brain regions use this protein differently. The most intense staining was in the choroid plexus and ependymal cells (Figure 1-2E) and by comparison, the least intense staining was observed in the cerebellum (Figure 1-2D). Dickey *et al.* (2007) has suggested that CHIP levels may correlate with the number of proteins synthesized and the metabolic demands (“work”) of the cells.¹⁰⁸ Within the brain parenchyma, CHIP has a preferential localization to neurons. Beyond neurons the abundance of CHIP in the choroid plexus and ependymal cells may be due to the active secretory nature of these cells. A role for stress proteins in the initiation of immune responses has been suggested.^{49,164,165} So it is also possible that the location of these cells at the interface of the blood-CSF barrier and the CSF-parenchyma interface may expose these cells to more stress. The perinuclear intensity of CHIP alone suggests that it is poised for rapid translocation into the nucleus following stress.

Protein Processing

Ischemic stress is the result of the loss of blood flow *in vivo* which correlates to the loss of oxygen and glucose *in vitro*. The cellular response to ischemic stress involves a complex sequence of events designed to promote cell survival. One of the earliest steps is the suspension of on-going protein transcription and translation in favor of the synthesis of proteins necessary for survival.⁸ How this transition takes place is largely unknown, but provides the opportunity for early stress response processes to begin with the full support of the cells energy-and protein-producing machinery. Another key response to stress is that of the HSPs. HSPs, which normally fold proteins, will work in concert with the UPS via the ubiquitin ligases to facilitate protein degradation in response to stress. Specificity of the UPS has been attributed to the ligases⁶⁹, though their relevance in the early stages of the stress response has yet to be fully investigated. CHIP is one such ATP-dependent ubiquitin ligase.^{81,82} Under ischemic conditions, mitochondria depolarize, ATP is progressively limited and calcium levels destabilize within cells.⁹ Under these conditions protein folding is compromised and aggregates of the misfolded proteins and protein folding machinery will accumulate.¹⁰⁷ Cell viability is highly dependent on removal of the misfolded proteins as the accumulation of unfolded proteins can quickly lead to irreversible aggregation and additional functional decline. Folding intermediates may be partially functional but that functionality is likely largely dependent on the energy state of the cell.¹⁶⁶ In instances where energy is depleted, such as ischemia, there is a temporal gradient of functional loss. The cell gradually loses the ability to complete the folding process in contrast to cell stressors such as heat shock in which proteins are overtly damaged.

Following stress protein processing mechanisms prioritize protein degradation over protein folding.^{66,72} Both misfolded proteins as well as proteins that are not critical to the

cell's response to stress are ubiquitinated and eventually degraded.⁷² In a parallel effort to maintain cellular homeostasis, the synthesis of many other proteins is suppressed to allow the synthesis prioritization of chaperones and other proteins that help to manage the stress response.^{43,66,167,168} Survival of tissue with partial availability of metabolic substrates and oxygen will depend in part on the efficiency with which the cell regains homeostasis.⁴³ The restoration of ATP production and the improved efficiency of ER function is necessary to regain homeostasis.^{43,169} The efficiency of the ER is improved, in part, by the availability of rapidly synthesized stress proteins, the heat shock proteins, during the initial stages of ischemia.⁶⁷

In vitro experimentation has shown that if presented with an abundance of misfolded protein, CHIP will degrade that misfolded protein.^{85,157,170} This is an oversimplified view of what happens *in vivo*, however. Protein folding is a highly regulated process where decisions to re-fold or direct target protein for the UPS are made depending on energy status, availability of chaperones and other factors.⁷² The key decision whether to re-fold or degrade a given protein is a very important determination in stroke research and will take a great deal of further research to fully appreciate/understand.

Ubiquitin-Proteasome System

Ubiquitin ligases like CHIP provided specificity for targeting of particular proteins, but there is no clear protein recognition by CHIP alone. Possible targets for CHIP have been identified and degradation by CHIP is possible^{82,101,112,138,156,170-172}, however the extent to which these are selective *in vivo* targets of CHIP is largely undetermined. In each case a protein complex is necessary to achieve functional activity.^{80,81,86} Conformation of the molecular chaperone complex in folding and degradation is critical.^{80,85,173} A major challenge for the field is to determine how these complexes identify target proteins for chaperoning,

folding, or degradation. These studies will require multi-protein conformational analyses and studies of protein complex interactions.¹⁷⁴ A better understanding of the conditions under which the selection is made will reveal the early events that promote cell survival following stress.

Ubiquitin-proteasome system (UPS) regulation in the nucleus would be consistent with the early increase in the co-chaperones that we observed following heat stress. Before CHIP levels decreased, HSP70 levels peaked in both the cytoplasm and nucleus (Figure 2-3). The timing--10 minutes--suggests a very early window of activity for HSP70. Interestingly, CHIP and HSC70 levels are not elevated at this point suggesting that CHIP and HSF1 do not drive the initial transcriptional response of HSPs. The peak in CHIP and HSC70 comes minutes later--at 15 minutes. After 15 minutes however, a divergence takes place: CHIP levels continue to decrease while HSC70 and HSP70 levels remain constant or slightly increase. The majority of these cells at longer durations of HS ultimately die. Cells that are resistant to ischemic stress, like immortalized cells, have high levels of CHIP at baseline. Reports from KB cells, demonstrated elevated levels of CHIP were maintained throughout 24 hours of recovery following HS.¹²⁹ However, immortalized cells are known to survive HS, reinforcing the view that maintaining CHIP levels may be important to cell survival.

Recently a new concept for the role of CHIP in the nucleus has been introduced in the literature; a role which indicates a greater complexity of CHIP functionality than previously realized. It has been suggested that CHIP may function as both an intrinsic chaperone and temporal regulator of the stress response. This hypothesis is consistent with both the limited acute stress data, as well as the more extensive chronic neurodegeneration data⁹⁸⁻¹⁰¹ which has reported greater pathology in the absence of CHIP.¹⁷⁵ Additionally, during the course of biological aging increased protein aggregation occurred in the absence

of CHIP. In our experiments, hippocampal slice cultures lacking CHIP demonstrated larger areas of cell death following OGD compared to heterozygous or wild-type cultures. The mechanism of CHIP-related protection is currently unknown but these results suggest it may offer protection at two levels: 1) ubiquitin-proteasome regulation, possibly more important in chronic neurodegeneration, due to the progressive build-up of protein aggregates over time, or 2) nuclear CHIP, likely important in the response to acute stress, when normal protein synthesis is suspended and response mechanisms operate with limited metabolic resources.

Nuclear localization and CHIP following stress

Following stress, molecular chaperone and nuclear import components will accumulate in the nucleus.¹⁷⁶⁻¹⁷⁸ The import of proteins into the nucleus is most likely to occur through the nuclear pore complex. Small proteins (<5kDa) can passively diffuse through the pore whereas larger proteins have to be actively transported through the pore. Active transport is accomplished through a two-protein subunit complex, importin- α and importin- β . The latter is the physical import mediator, while importin- α binds to the cargo protein. Importin- α binds to the nuclear localization sequence (NLS) expressed by the cargo protein the importin- α will complex with importin- β at the nuclear pore in preparation for active transport of the cargo protein into the nucleus.¹⁷⁹ Cleavage of RanGTP (a small guanosine triphosphatase) provides energy for the successive docking of importin- β through the pore and dissociation of the cargo protein from the importins once in the nucleus.

CHIP is likely actively transported through the nuclear pore using the above mechanisms via its two NLSs in an energy-dependent process. The NLSs on CHIP are likely direct targets for recognition by importin- α for translocation into the nucleus. The

presence of strong perinuclear staining in our studies suggests that CHIP is readily available for import.

While mechanisms have been identified as to how CHIP is transported into the nucleus, its export may be less clear. Nuclear export in general is also RanGTP-dependent and limited under conditions of energy depletion. Nuclear export is mediated through leucine rich regions of a protein termed the nuclear export sequence. No such sequence has been reported for CHIP. CHIP might be actively exported through its NLS when importin- α is exported. Together impaired nuclear import is the most parsimonious explanation for the decrease in CHIP during recovery from OGD. Loss of ATP may an important role in the eventual loss of nuclear CHIP since the nuclear translocation is energy dependent. However, a surprising observation was the rapid loss of nuclear CHIP but not HSPs which appeared to co-migrate with CHIP. This would suggest that CHIP turnover in the nucleus is relatively rapid and must be continuously replenished to maintain function. Since total CHIP does not decrease it is reasonable to conclude that CHIP may be actively exported from the nucleus. To better understand these processes more studies of CHIP import and export are needed.

To study impaired import and export of CHIP, one would need to more closely examine the mechanisms that drive these two processes. Blocking nuclear import and export, generally at the nuclear pore complex, would be deleterious to the cell and necessitates a CHIP-specific approach. If CHIP is translocated through the interaction of one or both of its NLSs and importin- α , differences in localization might be observed if one or the other sequence is removed. Additional mutations could be used to determine if other functional regions are required.

Furthermore, our data support the hypothesis that nuclear localization of CHIP is important for cell survival following stress. To directly test this hypothesis in our culture and

stress models, expressing a gene construct engineered to express CHIP with a mutated nuclear localization sequences would theoretically allow CHIP to function but not translocate. A reporter gene, such as GFP, could also be included to directly verify there was no CHIP translocation. If nuclear localization of CHIP is important to cell survival following stress, we would expect an increase in cell death at shorter durations.

One of the important questions regarding the stress response is if active components of the response might be rate-limiting. The rapid loss of nuclear CHIP and the lack of new synthesis seen in our experiments suggest that CHIP availability may be a limiting factor. Without ATP, CHIP may decrease under extended ischemic conditions because it is consumed by protein degradation aggregates.¹⁰⁶ HSC70 has previously been shown to increase in insoluble aggregates with the same time course as CHIP.^{106,120} HSP70 only appeared in insoluble aggregates after 24 hours of recovery.¹²⁰ In our experiments, when CHIP levels decreased HSC70 levels also decreased, but HSP70 levels remained constant or increased. If there is a cellular preference under acute stress to prioritize nuclear processes, sustained nuclear presence of CHIP could be the result of having the energy to import and maintain CHIP. Furthermore, CHIP may be trapped and left to aggregate in the nucleus due to the lack of energy available for either export or normal functioning.

Rate-limiting potential of CHIP

As suggested by Kaufman, protein folding is not a pathway but rather a landscape.¹¹⁸ In this model, the intermediates in protein folding are active contributors to cellular processes (stress response, signaling, etc.) and are not traps of proteins that are unable to function (intermediate complexes as dysfunctional aggregates). Accelerated folding is facilitated through an elaborate system of chaperones.¹¹⁸ This model is based in the idea of unfolded protein as a client of chaperones and not a director. Thus, mediators of this

process (i.e. CHIP) may have functionally different roles depending on localization, cellular environment, and protein availability.

Figure 5-1 summarizes the potential functions of CHIP following acute stress.

Proteins enter into the system by a failure to maintain a functional conformation. The ubiquitinating complex consisting of chaperones, ubiquitin-conjugating enzymes, and CHIP will bind to unfolded or misfolded proteins to facilitate ubiquitination (1). Subsequent steps are influenced by the availability of ATP. This is a pivotal point in the cell stress response when the cell will either attempt to continue folding proteins or prioritize degradation. Failure at this level will lead to protein aggregation. In the presence of ATP, ubiquitinated proteins are degraded by the proteasome and freed ubiquitin is recycled. However, in the absence of ATP, the ubiquitinating complex and unfolded/misfolded proteins will form irreversible aggregates. The second function describes the function of nuclear CHIP. CHIP is part of the complex that regulates HSF1 activation (2). Under non-stress conditions, CHIP binds to the HSC70-HSF complex and in cooperation with HSP90 and HSP70 keeps HSF1 inactive. Following stress the CHIP-HSC70 complex holds HSF1 in an active, trimerized conformation. The trimeric complex translocates to the nucleus where HSF1 induces transcription of HSP70.

Our data illustrating a granular appearance of CHIP immunoreactivity in stressed cells suggests that nuclear translocation of CHIP may further augment the accumulation of aggregates over time. The absence of evidence for new CHIP synthesis in the protein synthesis inhibition experiments suggests that there may be a limited pool of CHIP available for these functions. Following stress, we show that the availability of CHIP in the cytoplasm is determined in part by the loss of CHIP due to its translocation of into the nucleus. The balance between degradation, translation, and protein aggregation is a function of the energy needed to support each of these functions. However, the decrease in nuclear CHIP

in our experiments was not balanced by an increase in cytosolic CHIP suggesting limited recognition by our antibodies or deposition into the insoluble fraction.

The current dogma about CHIP is that it is the lack of CHIP that negatively impacts the ability of cells to survive stress. These studies recognize that the significant relevance of the viability observations lies in the function of CHIP under stressful conditions. Our data suggest that the decrease in nuclear CHIP is a pivotal event in the stress response of the cell. However, our data also suggest that 1) an early peak in HSP70, 2) a divergence of CHIP and HSC70 localization and levels, or 3) some combination of both are parallel events and are key observations of the stress response. Additionally, the available energy will determine whether CHIP stores are exhausted through nuclear localization and trapped by aggregate formation, or if CHIP is reverted to its pre-stress status and is able to resume its normal activity upon restoration of ATP.

Unlike HSPs, CHIP synthesis is not rapidly induced in response to stress. This raises several questions regarding the utilization of CHIP. Is CHIP efficiently recycled? Is it rapidly lost/degraded? In our studies CHIP was neither synthesized following stress nor was CHIP degraded. If the functions of CHIP are split between HSF1 activation and mediating ubiquitination there may not be a sufficient amount of CHIP available to accommodate the needs of both functions following long durations of stress. Under severe stress conditions, translocation to the nucleus as an HSF1 chaperone or for an as yet unknown function precedes a significant decrease in nuclear CHIP at the earliest points in recovery. The possibility that this loss of CHIP immunoreactivity on Western blot is the result of aggregates is supported by studies of ischemic preconditioning.^{106,125}

As has been suggested by the Hu lab¹²⁵ among others¹⁰⁸, CHIP is lost following ischemia. CHIP may not be degraded, but the presence of protein aggregates *in vivo* following as little as 7 minutes¹²⁵ suggests that CHIP is being consumed by the system,

which may appear to be functioning, but is clearly under duress. We observed decreased nuclear CHIP following severe durations of OGD, but saw constant and elevated nuclear CHIP levels following mild durations of OGD. Cells that are destined to survive some of the ischemic stresses are morphologically no different from normal cells at this point, yet pathology exists. Even in cells that survive, aggregates are present.^{96,105} The relevance of these early aggregates, and the relevance of the relative decrease in nuclear CHIP to cellular pathology remain unresolved. However, these data do suggest that the availability of CHIP may be a crucial variable for survival and that treatments designed to preserve or restore these homeostatic processes may have substantial therapeutic utility.

Final considerations for future experiments

A clear gap in the story of CHIP thus far is the exact identification of CHIP's nuclear targets. Our experiments suggest that the nuclear translocation of CHIP increases rapidly to "prepare" the cell to deal with the stress. However, CHIP's ability to function in a productive way decreases rapidly with continued stress (heat shock, in particular). Although the chaperone functions of CHIP are well described, the mechanisms underlying recognition of target proteins is not clear; our yeast two-hybrid data, for example, showed only two domains constitutively interacted with CHIP and both correspond to known chaperone binding sites. The lack of strong direct interaction with other proteins suggests a significant dependence on the CHIP-chaperone molecular complex. Such complex interactions could contribute to the diversity of ubiquitin ligases that confer specificity. This would also explain the difficulty of demonstrating specific protein interactions by conventional methods in primary cultures.

Participation of CHIP in transcriptional regulation has been illustrated in studies where loss of CHIP prevents the synthesis of protective stress-associated proteins as

identified previously through interactions with HSF1.¹⁰⁴ Loss of CHIP would also interfere in the degradation of unfolded proteins and the repair of the cell.^{91,102,135,170} The loss of CHIP under ischemic conditions suggests that the protein is rapidly exhausted during prolonged stress and may be rate-limiting. Thus, we believe if CHIP is not quickly stabilized or restored to normal function the cell will not be able to engage in the recovery process, effectively shifting the balance toward cell death. Treatments designed to increase the availability of CHIP in recovery through new synthesis or prevention of sequestration in aggregates have therapeutic potential but will require a better understanding of CHIP regulation.

The treatment window could also be well before an actual ischemic event as suggested by the relationship between our results and ischemic pre-conditioning. If sub-cellular activity has shifted to increasing nuclear CHIP as our experiments suggest, producing HSP70 *in vivo*⁶⁷ or developing small aggregate deposits within cells as previous reports have shown¹²⁵, the results of a subsequent ischemic event may depend on the readiness of the cell to respond and treatments that elevate CHIP and chaperone availability may therefore offer therapeutic alternatives for increasing cells survival as well as prophylaxis in at-risk conditions.

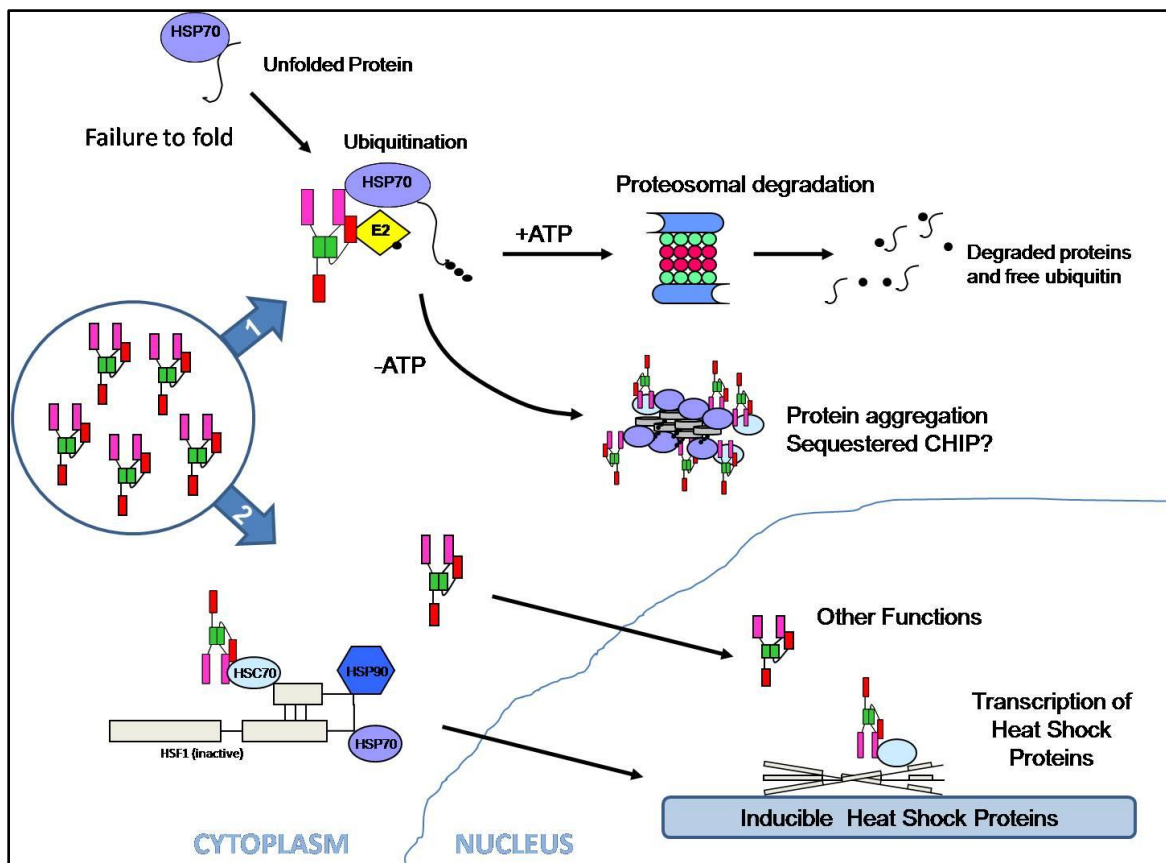
Summary

Changes in the sub-cellular localization of CHIP are key events in the survival of cells following stress. Increased nuclear localization of CHIP preceded the appearance of delayed cell death and was independent of new synthesis. Decreased nuclear CHIP correlated with delayed cell death. Cells were more sensitive to OGD in the absence of CHIP. Our finding of acute changes in sub-cellular localization of CHIP in response to

cellular stress suggests that changes that occur shortly after exposure to stress ultimately impact on whether or not a cell has the capacity and capability to recover.

Figure 5-1: Potential functions of CHIP following acute stress.

A limited pool of available CHIP is involved in two main cellular functions following stress. 1) Unfolded protein bound to chaperones that fail to fold are joined in a complex with CHIP and ubiquitin-conjugating enzymes. In the presence of ATP, ubiquitinated proteins are degraded by the proteasome and free ubiquitin is recycled. In the absence of ATP irreversible protein aggregates will form containing unfolded proteins, molecular chaperones, CHIP and ubiquitin. 2) The CHIP-HSC70 complex activates HSF1. The three proteins translocate to the nucleus where HSF1 induces transcription of HSP70. Because CHIP contains a nuclear localization sequence CHIP has the potential to localize to the nucleus independently for a yet unknown function.



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